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Paternal epigenetic effects of population density on locust phase-related characteristics associated with heat-shock protein expression

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Abstract

Many species exhibit transgenerational plasticity by which environmental cues experienced by either parent can be transmitted to their offspring, resulting in phenotypic variants in offspring to match ancestral environments. However, the manner by which paternal experiences affect offspring plasticity through epigenetic inheritance in animals generally remains unclear. In this study, we examined the transgenerational effects of population density on phase-related traits in the migratory locust *Locusta migratoria*. Using an experimental design that explicitly controls genetic background, we found that the effects of crowd or isolation rearing on phase plasticity could be inherited to the offspring. The isolation of gregarious locusts resulted in reduced weight in offspring eggs and altered morphometric traits in hatchlings, whereas crowding of solitarious locusts exhibited opposite effects. The consequences of density changes were transmitted by both maternal and paternal inheritance, although the expression of paternal effects was not as pronounced as that of maternal effects. Prominent expression of heat-shock proteins (*Hsp*s), such as *Hsp90*, *Hsp70* and *Hsp20.6*, could be triggered by density changes. *Hsp*s were significantly upregulated upon crowding but downregulated upon isolation. The variation in parental *Hsp* expression was also transmitted to the offspring, in which the pattern of inheritance was consistent with that of phase characteristics. These results revealed a paternal effect on phase polyphenism and *Hsp* expression induced by population density, and defined a model system that could be used to study the paternal epigenetic inheritance of environmental changes.

Keywords: epigenetic inheritance, heat-shock protein, *Locusta migratoria*, maternal effect, paternal effect, transgenerational plasticity

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Introduction

The transgenerational inheritance of environmental information experienced by parents to their offspring is rapidly gaining the interest of ecologists and evolutionary biologists (Grossniklaus *et al.* 2013; Heard & Martienssen 2014). Transgenerational inheritance provides a mechanism that allows organisms to inform and pre-adapt their progeny to prevailing hostile environmental conditions. If widespread, transgenerational effects induced by ancestral heterogeneous environments have far-reaching implications on population dynamics, phenotypic plasticity and evolutionary changes (Feil & Fraga 2011; Heard & Martienssen 2014).

Our understanding of transgenerational effects has rapidly improved in recent years (Daxinger & Whitelaw

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2010). However, the majority of the described transgenerational environmental effects are attributed to only a few environmental factors, such as chemical pollutants (Anway *et al.* 2004), dietary components or nutrient availability (Carone *et al.* 2011), maternal behaviour (Weaver *et al.* 2004), and thermal stress (Seong *et al.* 2011). Population density has emerged as a prevalent environmental factor that comprehensively influences animal behaviour, physiology and population dynamics (Dantzer *et al.* 2013). A common example of maternal effects induced by changes in population density is provided by several locust species, including the desert locust *Schistocerca gregaria* and the migratory locust *Locusta migratoria* (Simpson & Miller 2007; Tanaka & Maeno 2010). These insects exhibit a remarkable feature of phenotypic plasticity called phase polyphenism, in which a continuum of trait variation between extreme gregarious and solitarious phases is affected by local population density (Uvarov 1966). Solitarious locusts develop at low density and are characterized by sedentary and repellent individuals with a green- or brown-coloured body. By contrast, gregarious locusts form at high density and are characterized by a dark colour, heavy eggs, high mobility, and conspecific attraction in nymphs and adults (Pener & Simpson 2009; Wang *et al.* 2012; Chen *et al.* 2015). Solitarious and gregarious locusts also differ in morphometric and behavioural characteristics in response to changes in population density (Uvarov 1966). Parental effects on these phase characteristics induced by changes in population density have been recorded in the desert locust (Simpson & Miller 2007; Tanaka & Maeno 2010) and the migratory locust (Chapuis *et al.* 2008; Harano *et al.* 2011). Parental effects accumulate across generations (Maeno & Tanaka 2009); hence, these effects are important in the production of locust swarms.

Despite the significant advances in our understanding of transgenerational effects, only few examples of paternal effects have been documented in animals (Carone *et al.* 2011; Rando 2012). Exclusive paternal effects of population density on phase-related traits are also rarely observed (Islam *et al.* 1994b). This lack of observation can be ascribed to the fact that maternal effects are greater and easier to detect than paternal effects, particularly during embryo development. Paternal effects on progeny characteristics of desert locusts are even considered negligible (Maeno & Tanaka 2010). Transgenerational effects, either by paternal or maternal inheritance, can involve epigenetic mechanisms by which transgenerational modification of the genome occurs without a corresponding change in DNA sequence (Seong *et al.* 2011). However, maternal effects are limited in terms of revealing transgenerational effects resulting from epigenetic inheritance. In addition, to contribute to nuclear and mitochondrial DNA and chromatin information, mothers can influence their offspring through several ways, such as supplying bioactive agents from the accessory glands or in the egg cytoplasm, or contributing nutrients and hormonal information during embryogenesis (Grossniklaus *et al.* 2013). Therefore, separating maternal epigenetic effects from indirect effects of ovarian environmental exposure and simple plastic response in offspring becomes difficult. By contrast, paternal effects avoid this issue because the contribution of the father to offspring is often limited to sperm alone (Carone *et al.* 2011; Rando 2012). Therefore, the epigenetic inheritance of phase-related characteristics should be explored by investigating the paternal effects.

Transgenerational effects can result in widespread gene expression changes; some of these changes are responsible for phenotypic variation in offspring. For example, heat-shock of *Drosophila* flies caused the upregulation of dozens of genes in their offspring (Seong *et al.* 2011). Hsps, including Hsp90, Hsp70 and small Hsps, are important molecular chaperones that play pivotal roles in developmental homeostasis, organism plasticity, stress resistance and environmental response (Rutherford *et al.* 2007; Chen & Wagner 2012). *Hsp* expression in the migratory locust can be promptly induced by density changes and is significantly upregulated in response to crowding (Wang *et al.* 2007; Chapuis *et al.* 2011). Thus, Hsps can function in inducing and maintaining the polyphenism phase. However, the extent to which variation in parental *Hsp* expression is maintained in the offspring and transgenerational effects are dependent on specific parental density factors (e.g. crowding or isolation) remains unknown. Changes in the expression of key regulatory genes, such as *Hsp*s, across generations should be tracked to determine the molecular mechanism underlying transgenerational inheritance.

The rigorous control of genetic variation deserves particular attention for the investigation of epigenetic inheritance of phase changes. One common method used to reveal transgenerational effects involves the direct comparison of the phase characteristics of the offspring produced by isolation- and crowd-reared locusts. However, the colonies of gregarious and solitarious locusts, developed either in the laboratory or in nature, can be genetically differentiated in terms of their density responses and degrees of parental effects (Chapuis *et al.* 2008; Berthier *et al.* 2010). Background genetic variation is commonly present among parental colonies and individuals, such as those with different rearing histories (Chen & Wagner 2012; Chandler *et al.* 2013). Segregating genetic background can cause confounding effects on mild phase changes in a context-dependent

manner (Berthier *et al.* 2010). Despite the importance of background genetic variation in exploiting epigenetic inheritance, the effects of such variation on transgenerational plasticity have been largely ignored.

In the current study, the effect of genetic factors on the inheritance of phase plasticity was minimized by constructing isofemale lines of solitarious and gregarious locusts and crowd- or isolation-reared locusts from the isogenic lines of the migratory locust. The paternal and maternal effects were demonstrated by comparing tightly controlled mating pairs. We initially investigated the transgenerational effects induced by population density changes on the phase characteristics of offspring eggs and hatchlings. To explore the possible molecular basis of transgenerational inheritance, we also determined the cross-generational changes in the gene expression levels of *Hsp90*, *Hsp70* and *Hsp20.6* that were induced by crowding and isolation. Our results revealed that the effects of phase plasticity induced by density changes were transferred to the offspring by prominent paternal effects. Interestingly, the variation in the expression profile of *Hsp* genes was also inherited from parents to offspring in a density-dependent manner. Our study provided direct evidence on the paternal epigenetic inheritance of phenotypic plasticity in locusts. Our results also revealed insights into the adaptive strategy of locusts to fit in their environment.

Materials and methods

Establishment of isofemale lines

Gregarious and solitarious lines (named *G* and *S*, respectively) were the offspring of one female (F0), which was caught in Hebei Province and reared for three consecutive generations (F1, F2 and F3) using standard methods (see detailed rearing method below, Fig. 1A). From the fourth generation (F3), solitarized lines were established from the *G* lines by isolated-rearing gregarious locusts from F3 new hatchlings in accordance with the standard methods. The resulting lines were labelled as G_{isol}. Similarly, gregarized lines were established from the F3 *S* lines by crowd-rearing solitarious locusts from new hatchlings in accordance with the standard methods. The resulting lines of locusts were labelled as S_{crowd} (Fig. 1A).

Husbandry of gregarious and solitarious locusts

Gregarious and solitarious locusts were maintained in accordance with the standard method of Ma *et al.* (2011) and Guo *et al.* (2011). In brief, gregarious locusts were cultured in a large cage (40 cm × 40 cm × 40 cm) from new hatchlings at a density of approximately 400

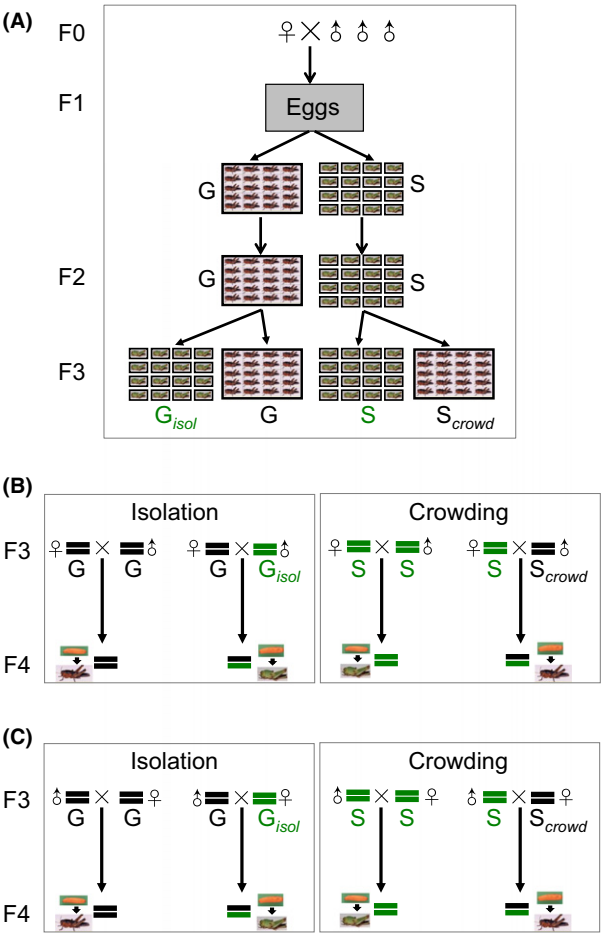

Fig. 1 Construction of the isofemale lines and mating procedures for the paternal and maternal effects. (A) Isogenization and construction of the four test lines (i.e. *G*, G_{isol}, *S* and S_{crowd}). (B) The mating procedures used in investigating the paternal effects of population density. For the effects of isolation, one *G* female was mated with two *G* males (control) or two G_{isol} males of the parental generation (F3). For the effects of crowding, one *S* female was mated with two *S* males (control) or two S_{crowd} males. (C) The mating procedures for the maternal effects of locust density. To study the effects of isolation, two *G* males were mated with one *G* female (control) or one G_{isol} female in F3. To study the effects of crowding, two *S* males were mated with one *S* female (control) or one S_{crowd} female. Seven matings for paternal effects and at least three matings for maternal effects were performed for the controls and treatments.

individuals per cage. All of the sides of each cage, except the floor and front door side, were covered with a mesh screen. Solitarious nymphs were cultured individually in white metal chambers (10 cm × 10 cm × 25 cm) that were covered with transparent boards at the front and back sides. The chambers were supplied with circulating fresh air. All lines were fed with fresh wheat seedlings and bran and maintained under a 14:10 h (L: D) photoperiod and 30 ± 2 °C.

Paternal effects

From the late fifth instar, female nymphs of *G* and S_{crowd} were separated from males and maintained in separate cages to maintain virginity. The individuals from *S* and G_{isol} were cultured in separate chambers before mating. When the eclosed adults reached 5-day-old, one female and two males were paired in a cage and allowed to mate. After the locusts were paired for 12 h, the female and males were labelled and returned to their original rearing container to minimize any pairing-induced effects and maintain their original phase states. After another 12 h, the female and males were mated again. The mating regime was repeated in the following days. The mating cages were placed in an air-ventilated room at 30 ± 2 °C and photoperiod of 14:10 h (L:D) without the presence of other locusts. Four mating regimes were established as follows to investigate the paternal effect: *G*♀ × *G*♂ (control) vs. *G*♀ × G_{isol}♂ for isolation effect, and *S*♀ × *S*♂ (control) vs. *S*♀ × S_{crowd}♂ for crowding effect (Fig. 1B). Seven matings of adults were established for each regime. Eggs were then collected from the female source cage and mating cage every day until the first five consecutive egg pods were obtained from each mating. Parthenogenetic reproduction was rarely observed in sexually mature females. The collected egg pods were kept in a plastic cup (diameter of 6 cm and height of 9 cm) filled with sterilized sands with 8% humidity and then maintained in an incubator at 30 ± 1 °C.

Maternal effects

To investigate the maternal effects on progeny traits, four mating regimes were established as follows: *G*♂ × *G*♀ (control) vs. *G*♂ × G_{isol}♀, and *S*♂ × *S*♀ (control) vs. *S*♂ × S_{crowd}♀ (Fig. 1C). The methods used for sample collection and maintenance were the same as in the experiment on paternal effects. At least three matings of adults were established for each regime.

Morphological traits of male adults

Morphometric ratios were adopted to characterize the morphological changes induced by the changes in the population density of parental adults (F3). The most widely used morphometric ratios are F/C (hind femur length/maximum head width) and E/F (tegmen length/hind femur length). The F/C ratio is lower, and the E/F ratio is higher in gregarious adults than those in solitarious adults of *L. migratoria* (Pener & Simpson 2009). The lengths of E, F and C in adults were measured using a digital automatic calliper (Model: CD-8″ CSX; Mitutoyo Co.) with a resolution of 0.01 mm. At least 20 males from each line (*G*, G_{isol}, *S* and S_{crowd}) were used in this experiment. The lengths of each sample were measured three times, and the average value was used as the final length.

Parental coloration

A pronounced trait characteristic of locust phase shift is the change in body coloration pattern. Gregarious adults exhibit grey to black patterns in the thorax pronotum and pleurite. By contrast, solitarious adults show a green pattern (green or brown coloration) (Pener & Simpson 2009). To show the effect of crowd and isolation rearing in parents, we recorded body coloration in 5-day-old male adults in the F3 generation. The number of individuals with either gregarious or solitarious coloration patterns was recorded. At least 20 individuals were examined in each of the four types of parental lines (*G*, G_{isol}, *S*, and S_{crowd}).

Weight of progeny eggs

Among the heritable traits characteristic of gregarious and solitarious phases, behavioural response was used to determine the effect of population density on the offspring (Islam *et al.* 1994a; Harano *et al.* 2011). However, the behavioural activity of nymphs is extremely labile and highly dependent on developmental and environmental cues, as well as the measurement method used (Islam *et al.* 1994b; Harano *et al.* 2011; Ma *et al.* 2011). The behavioural phase state can be reversed in minutes (Pener & Simpson 2009; Guo *et al.* 2011; Harano *et al.* 2011). Furthermore, the locomotor activities of hatchlings are highly dynamic, even as behavioural measurement proceeded (Harano *et al.* 2011, 2012). Therefore, we measured the egg weight and body morphometric traits (F/C) of the hatchlings, which are stable and heritable traits, instead of behavioural characteristics (Pener & Simpson 2009; Wang *et al.* 2012). Egg weight can be used to distinguish between locust phases because eggs laid by gregarious locusts are significantly heavier than those laid by solitarious ones in both the migratory locust and the desert locust (Tanaka & Maeno 2010; Wang *et al.* 2012). The body size and colour of hatchlings are correlated with egg size in the desert locust (Tanaka & Maeno 2010). Therefore, egg weight variation is a good indicator of the phase change in locusts. We measured the weight of 7-day-old eggs of the offspring. Eggs at both ends of the egg pod were discarded to minimize possible differences in the quality of eggs at different portions of the egg pod. Eggs collected from the half portion of the egg pod were carefully cleaned with a soft brush. At least 10 eggs from each egg pod were subjected to weight measurement using a digital

balancer (Model: AE240; Mettler). The eggs were then stored in liquid nitrogen to extract RNA and quantify gene expression. The eggs from the other half of the egg pod were incubated until hatchlings were observed.

Morphological traits of progeny hatchlings

Newly hatched (<4 h) nymphs were transferred to a gauze-covered transparent plastic cup (diameter, 6 cm; height, 9 cm) placed in an incubator at 30 ± 1 °C, 75% humidity and photoperiod of 14:10 (L:D). The hatchlings were maintained in the incubator for 24 h at a density of five individuals per cup before measurement to minimize the effect of the heterogeneous environment on hatchling traits. During incubation, hatchlings were fed with sufficient wheat seedlings and bran. Only the morphometric measures of F and C of the hatchlings were examined because wing development was not observed at that time. The 24-h-old hatchlings were anesthetized with CO_2 and mounted at the centre point of an operation pad under a microscope (version 3.3.0; Leica Application suite M205C). The microscope was set up with the same component and parameters: 10 × /23B eyepieces, 50%/50% trinocular tube, 0.63× image objective, 1× main objective, 7.8× amplification and 1 pix resolution. The first egg pod was laid by parents that were exposed to the pairing environment for the least amount of time, and eggs in the first egg pod exhibited phase characteristics that represented those in the following egg pods (see Results). Thus, we used only the first egg pod to determine gene expression in eggs and morphology in hatchlings. At least 10 hatchlings from seven mating sessions for paternal effect and 10 hatchlings from at least three mating sessions for maternal effect were subjected to measurement. Each sample was imaged three times, and the average value from the three images was used for analysis.

Quantification of Hsp gene expression

Hsps are a large family of proteins that can be classified according to their molecular weight (large Hsps, e.g. Hsp90 and Hsp70; and small Hsps, e.g. Hsp20.6) (Feder & Hofmann 1999). To demonstrate the effects of crowd and isolation rearing on *Hsp* gene expression in the parental generation, we collected the brain and hind femur from 5-day-old male adults in the F3 parental lines (G, G_{isol}, S and S_{crowd}). The dissected samples were immediately frozen in liquid nitrogen and stored until needed to prepare RNA. Five independent biological replicates were prepared with eight males in each replicate. To reveal the pattern of parental inheritance of gene expression, we examined the offspring egg stage instead of larval and adult stages because *Hsp* expression in eggs is least subject to the influence of local phase-related environments, particularly density changes compared with in the other stages. Eight eggs in the first egg pod from each mating line were collected for the four treatments, namely G, G_{isol}, S and S_{crowd}, and frozen in liquid nitrogen to prepare RNA. Gene expression was determined by quantitative real-time PCR using SYBR Green I kit (Roche, Switzerland) according to the manufacturer's instructions. PCR was performed using a LightCycler® 480 real-time PCR system. The PCR program was conducted at 95 °C for 2 min and then subjected to 40 cycles of 95 °C for 20 s, 58 °C for 20 s and 68 °C for 20 s. The ribosomal protein 49 gene (*Rp49*) was used as the internal control and standard (Chen *et al.* 2007). The relative expression of *Hsp* genes was quantified by the comparative cycle threshold method ($2^{-\Delta C_t}$ method) (Chen & Wagner 2012). The PCR primers of *Rp49* were CGTAAACCGAAGGGAATTGA (forward primer) and GAAGAAACTGCATGGGCAAT (reverse primer). The primers of *Hsp90*, *Hsp70* and *Hsp20.6* were obtained from a previous report (Wang *et al.* 2007). All of the reactions were performed in triplicate.

Statistics

The F/C and E/F values for the morphometric traits of adults and nymphs were log-transformed before statistical analysis was conducted. The effects of crowding/isolation treatment on the adult morphometric traits were evaluated using independent-sample *t*-test. Fisher's exact test was performed to compare the difference in the number of adults with different coloration patterns. For egg weight, the effects of crowding/isolation treatment and mating in each treatment on the mean value were analysed by nested one-way ANOVA, in which treatments were considered as fixed factors. The matings nested within treatments were used as a random variant. To demonstrate the specific effects on egg weight in the sequential egg pods, we subjected each egg pod to a separate one-way ANOVA, in which treatment was considered as a fixed factor. Given that phase-related characteristics of progeny vary significantly depending on egg pods in locusts (Maeno & Tanaka 2008), the egg weight and morphometric traits of nymphs used for the analysis were averaged for each egg pod. The two levels of the treatment factor represent $S♀ \times S♂$ vs. $S♀ \times S_{crowd}♂$ for the crowding effect and $G♀ \times G♂$ vs. $G♀ \times G_{isol}♂$ for the isolation effect in the paternal analysis. This method was also applicable in the maternal analysis. The effects on the morphometric traits of nymphs were evaluated by one-way ANOVA, in which treatment was considered as a fixed factor.

Independent *t*-test was performed to compare the differences in the expression of each *Hsp* gene between the two levels of treatments.

Results

Parental morphological and coloration changes induced by isolation and crowd rearing

We initially validated whether or not isolation and crowd rearing affect the two phase-related traits of parental adults, including the E/F and F/C values and coloration pattern (Fig. S1, Supporting information). The E/F and F/C values of males were significantly modified when the gregarious locusts were reared in isolation. Isolation rearing significantly decreased the E/F value (*t*-test: $P = 0.009$) but significantly increased the F/C value ($P = 0.002$) of the adult gregarious locusts. The E/F and F/C values of the solitarious locusts also significantly changed after they were subjected to crowd rearing ($P < 0.001$ for both E/F and F/C). However, the pattern of morphometric changes from crowd rearing was in contrast to that from isolation rearing (Fig. S1A, Supporting information).

Isolation rearing significantly reduced the black-backed individuals in males (Fisher's exact test: $P = 0.02$); thus, 30% of the isolation-reared adults exhibited green patterns. By contrast, crowd rearing significantly increased the proportion of adults with black/grey patterns from 70% to 100% ($P < 0.001$; Fig. S1B, Supporting information). These results indicated that isolation and crowd rearing in the parental generation altered the morphometric traits and coloration patterns of adult locusts.

Paternal and maternal effects of density changes on egg weight

To investigate the paternal effects elicited by density changes, we compared the eggs produced by a female that was mated with isolation- or crowd-reared males with the corresponding control locusts (eggs produced from mating with gregarious or solitarious males). The combined analysis of the average egg weight of the five egg pods showed that isolation rearing significantly decreased egg weight by 12%, which was significantly different from the control group (one-way nested ANOVA: $P < 0.001$; Fig. 2). The weights of the eggs from isolation-reared males were reduced by 17%, 12%, 9% and 15% in the first four consecutive egg pods; thus, a significant effect was observed compared with the control group (one-way ANOVA: $P < 0.001$, $P = 0.003$, $P = 0.002$, and $P < 0.001$, respectively). No significant change was observed in the fifth egg pod (Fig. S2, Supporting information). The weakened effect in the fifth egg pod may result from the presence of other mating locusts perceived by the female during continual pairing

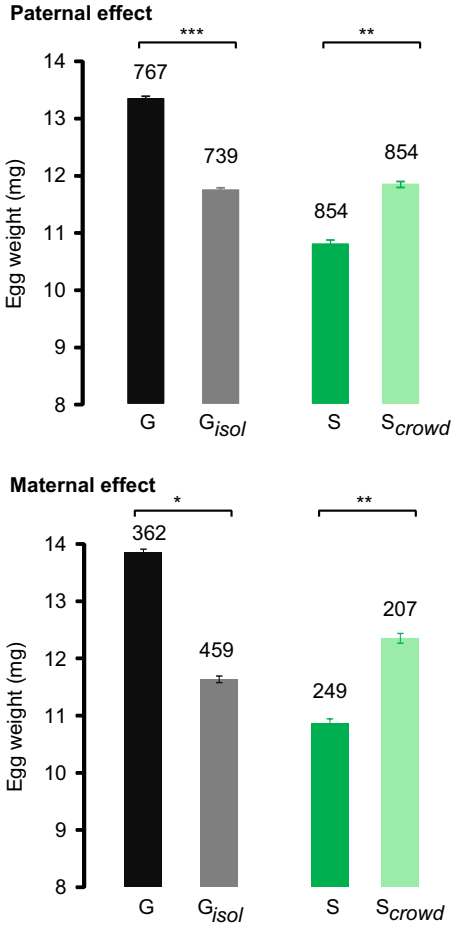

Fig. 2 Paternal and maternal effects of isolation and crowd rearing on offspring egg weight. Paternal effects: In the isolation treatment, *G* represents eggs produced from mating *G*♀ × *G*♂ (control), and G_{isol} indicates eggs from *G*♀ × G_{isol}♂. In the crowding treatment, *S* represents eggs produced from mating *S*♀ × *S*♂ (control), and S_{crowd} from mating *S*♀ × S_{crowd}♂. Eggs in each treatment were collected from seven replicate matings, which provided the first five consecutive egg pods for measurement. Maternal effects: The effects of isolation were determined by comparing the weights of the eggs produced from mating G♂ × G_{isol}♀ (denoted as G_{isol}) and *G*♂ × *G*♀ (control, denoted as *G*). The effects of crowding were determined by comparing *S*♂ × S_{crowd}♀ (denoted as S_{crowd}) and *S*♂ × *S*♀ (control, denoted as *S*). Eggs in each treatment were collected from three to four replicate matings. At least 10 eggs from each egg pod were weighed. The egg weight is the average of the five egg pods. The error bar represents 1 SE. The number above each column shows the total number of eggs examined. Asterisks indicate the significance level of one-way nested ANOVA, with treatment as the fixed factor and mating as the random factor: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Maeno & Tanaka 2010). By contrast, paternal crowd rearing significantly increased egg weight ($P = 0.006$), which was 10% different from that of the control group (Fig. 2). The weights of the eggs from crowd-reared males were increased by 13%, 11%, 9%, 9% and 4% in the five ovipositions. The increase in weight observed in the first four consecutive ovipositions was significant ($P = 0.015$, 0.038, 0.007, 0.019). No significant difference was observed in the fifth oviposition (Fig. S2, Supporting information). Different matings did not significantly affect paternal crowding and isolation.

To investigate the maternal effects, the weights of eggs in the first five egg pods produced by isolation- or crowd-reared females were compared with those of eggs from their respective control female groups. Isolation rearing significantly decreased the average progeny egg weight (one-way nested ANOVA: $P = 0.025$) by 16% (Fig. 2). The weights of the eggs in the first five consecutive egg pods deposited by isolation-reared females decreased by 15%, 18%, 13%, 13% and 18%, which were significantly different from those of the control group ($P = 0.011$, 0.036, 0.035, 0.002, 0.007, respectively; Fig. S2, Supporting information). By contrast, crowd rearing of solitarious locusts resulted in a significant increase in progeny egg weight ($P = 0.006$), with an average difference of 12% compared with the control group (Fig. 2). The weights of the eggs laid by crowd-reared females were increased by 19%, 9%, 8%, 16% and 20% in the first five consecutive ovipositions. All the increased weight values were significantly different from those in the control group ($P = 0.045$, 0.003, 0.001, 0.018, 0.016; Fig. S2, Supporting information). Different mating lines did not significantly affect maternal crowding and isolation. The maternal effects of isolation or crowd rearing on egg weight were more evident than the paternal effects (Figs 2 and S2, Supporting information).

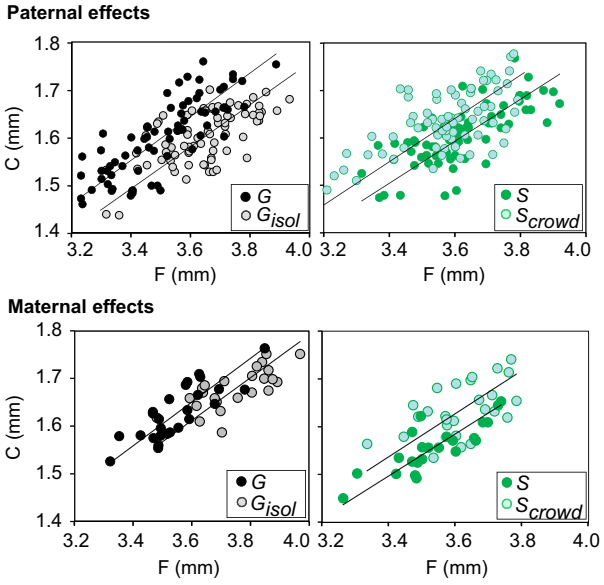

Fig. 3 Paternal and maternal effects of isolation and crowding on the morphometric traits of hatchlings. Morphometric trait F denotes the hind femur length, and C denotes the maximum head width. Paternal effects: In the isolation treatment, *G* represents the hatchlings produced from mating *G*♀ × *G*♂ (control), and G_{isol} represents the hatchlings produced from mating *G*♀ × G_{isol}♂. In the crowding treatment, *S* represents the hatchlings produced from mating *S*♀ × *S*♂ (control), and S_{crowd} represents the hatchlings produced from mating *S*♀ × S_{crowd}♂. Maternal effects: In the isolation treatment, *G* denotes hatchlings produced from mating *G*♂ × *G*♀ (control), and *G* represents the hatchlings produced from mating *G*♂ × G_{isol}♀. In the crowding treatment, *S* denotes *S*♂ × *S*♀ (control), and S_{crowd} denotes *S*♂ × S_{crowd}♀. F and C were measured from at least ten 24-h-old hatchlings from the first egg pod of at least three matings. The regression line between F and C is shown for each treatment.

Paternal and maternal effects on the morphometric traits of hatchlings

The morphometric traits of the hatchlings (Fig. 3) were examined to reveal any morphological variation inherited from parents. The results of the paternal effects showed that the F/C value significantly increased in the offspring of the isolation-reared locusts (one-way ANOVA: $P < 0.001$) but significantly decreased in the offspring of crowd-reared locusts ($P = 0.030$) compared with their respective control locusts. Similarly, maternal isolation significantly increased the F/C value of the hatchlings compared with the control group ($P = 0.008$). Crowding resulted in a marginally significant decrease in F/C ($P = 0.052$; Fig. 3). The difference in the F/C value in hatchlings between the treatment and control groups was similar to that observed in their parents (Fig. S1, Supporting information).

Hsp gene expression changes induced by isolation and crowd rearing of the parents

The expression levels of three *Hsp* genes, namely, *Hsp90*, *Hsp70* and *Hsp20.6*, in the hind femur and brain tissues of the parents were determined by quantitative real-time PCR. *Hsp90* expression significantly decreased by 1.5-fold in the hind femur after the locusts were reared in isolation (*t*-test: $P = 0.001$) compared with that of the control group. *Hsp90* expression did not significantly increase after crowd rearing was performed ($P = 0.06$), but a threefold difference in *Hsp90* expression was observed between solitarious and crowd-reared adults. *Hsp70* expression significantly decreased after the locusts were subjected to isolation rearing

($P = 0.015$), but this expression did not significantly change after the locusts were subjected to crowd rearing. *Hsp20.6* expression significantly decreased after isolation ($P = 0.004$) and significantly increased after crowding ($P = 0.018$; Fig. 4A). *Hsp90* expression in adult male brains decreased by 14 times after they were reared in isolation, but this expression doubled after crowd rearing (significant difference: $P = 0.027$ and $P = 0.005$, respectively). Similarly, *Hsp70* expression significantly decreased after the locusts were subjected to isolation rearing ($P = 0.003$) but significantly increased after these insects were subjected to crowd rearing ($P < 0.001$). However, brain *Hsp20.6* expression was not altered after the locusts were subjected to crowd and isolation rearing (Fig. S3, Supporting information).

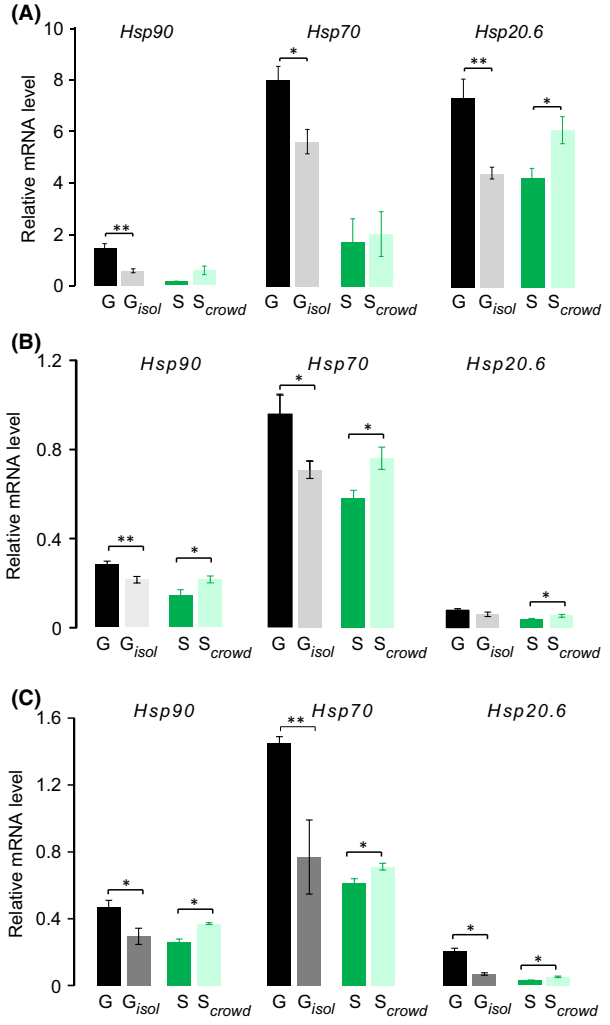

Fig. 4 Paternal and maternal effects of crowding and isolation on the gene expression levels of three *Hsp*s. (A) Changes in parental *Hsp90*, *Hsp70* and *Hsp20.6* expression levels in the hind femur of male adults after isolation and crowd rearing. *G* denotes gregarious 5-day-old males, and G_{isol} denotes isolation-reared males. *S* denotes solitarious 5-day-old males, and S_{crowd} denotes crowd-reared males. (B) Paternal effects of crowding and isolation on the expression of three *Hsp* genes in the eggs. Refer to the annotations on *G*, G_{isol}, *S* and S_{crowd} in Fig. 1. (C) Maternal effects of crowding and isolation on the expression of three *Hsp* genes in the eggs. Refer to the annotations on *G*, G_{isol}, *S*, and S_{crowd} in Fig. 1. Egg samples were collected from the first egg pod produced by at least three replicate matings. The error bars represent 1 SE. Asterisks indicate the significance level determined through independent-sample *t*-test: *, $P < 0.05$; **, $P < 0.01$.

Paternal and maternal effects on Hsp gene expression

The *Hsp* expression levels in the eggs were determined and compared between the offspring produced from the mating of isolation- or crowd-reared males and their corresponding controls (Fig. 4B) to determine whether or not the variation in expression is inherited from the fathers to their respective offspring. Paternal isolation significantly reduced *Hsp90* expression ($P = 0.005$) by 25% compared with the control group. In contrast to the isolation effects, crowding effects on eggs produced from mating with crowd-reared males showed that *Hsp90* expression increased significantly by 50% ($P = 0.027$). *Hsp70* expression significantly reduced by 20% in paternal isolation ($P = 0.043$) and significantly increased by 31% in paternal crowding ($P = 0.027$). *Hsp20.6* expression in the eggs was not affected by isolation rearing, but that from male parents who were subjected to crowd rearing significantly increased by 40% ($P = 0.007$). Thus, the changes in *Hsp* gene expression induced by paternal crowding or isolation were inherited by the offspring, and the profile of *Hsp* variation was the same as that of their fathers (Fig. 4A).

The variation in *Hsp* gene expression induced by maternal crowding or isolation was examined in the eggs (Fig. 4C). *Hsp90* expression was significantly reduced by 37% in eggs produced by isolation-reared females ($P = 0.035$) but significantly increased by 42% in eggs produced by crowd-reared females ($P = 0.01$). Isolation also resulted in a highly significant reduction of *Hsp70* expression by 47% ($P = 0.006$), whereas crowding yielded a 16% increase in *Hsp70* expression ($P = 0.043$). The expression levels of *Hsp20.6* in the eggs were reduced by 66% by isolation rearing ($P = 0.002$) and increased by 73% by crowd rearing of parental females (Fig. 4C).

Discussion

Transgenerational plasticity induced by population density change

In various species, only some environmental information can be transmitted to the offspring (Heard &

Martienssen 2014). Our results revealed that local population density (crowding or isolation) induced changes in phase-related characteristics and gene expression. The altered phase-related traits were passed on to the next generation, thereby predisposing the offspring to exhibit traits similar to their affected parents. For example, gregarious migratory locust eggs are usually larger and heavier than solitarious eggs (Wang *et al.* 2012; Chen *et al.* 2015). The eggs produced by crowd-reared solitarious parents became heavier than those produced by solitarious controls. The transgenerational effects on the phase characteristics of locusts were manifested not only in the embryonic stages but also in their hatchlings. The results of this study also demonstrated that population density changes induced an inherited pattern of variation in morphometric traits between parents and offspring. For example, F/C increased in response to isolation and decreased in response to crowding of the adult parents. F/C also increased in offspring hatchlings produced by the isolation-reared parents, and decreased in hatchlings produced by the crowd-reared parents. Combined results from the offspring eggs and young nymphs provided compelling evidence that parental density changes induced transgenerational phase plasticity.

Paternal epigenetic inheritance of phase plasticity

This study provided new insights into the inheritance of paternal phase plasticity, whereas previous studies demonstrated the prevalence of the maternal effects of population density on phase plasticity in locusts (Tanaka & Maeno 2006; Simpson & Miller 2007). Paternal inheritance represents an unconventional mode of information transfer involving epigenetic mechanisms, which needs to be carefully distinguished from the inheritance associated with classical genetic (DNA sequences) variations. As such, isogenized lines of gregarious and solitarious individuals were used in this study to minimize genetic variation between treated and control lines. Furthermore, chromosomal control was introduced to genetic background by mating with nontreated sister lines. Such experimental methods ensure a more specific parent-of-origin epigenetic effect on the phenotypic variations observed in offspring.

Our results indicated that the phase plasticity induced by population density can be transmitted by paternal and maternal inheritance. The inherited pattern of phase-related traits by paternal effect was similar to that of maternal effect, suggesting that paternal and maternal effects elicited by density changes shared the same mechanisms of epigenetic transmission in locusts. Paternal and maternal effects were clearly manifested in response to population density changes, but the extent of the respective responses differs. Maternal effects on egg characteristics were more distinct than paternal effects. Maternal and paternal effects on gene expression levels also differed (Fig. 4). Some nongenetic maternal factors, such as ovary environment or nutritional and hormonal factors in the glands, may enhance maternal effects (Boerjan *et al.* 2011; Grossniklaus *et al.* 2013). These transgenerational effects are believed to contribute to certain maternal nutrition and pheromone agents derived from reproductive accessory glands (Mccaffery & Simpson 1998). However, this study on paternal effects excluded the influence of nutritional and pheromone factors to a large degree, thereby providing direct evidence of the epigenetic regulation of transgenerational plasticity.

The paternal modulation of egg size induced by the rearing density could be regulated by genetic and nongenetic factors. Early egg size and weight might be affected by bioactive agents transferred from males to females at mating, which may change yolk deposition in the ovary (Rando 2012). However, the sizes of developing eggs increase rapidly with embryonic growth, which is largely decided by the programming of embryonic development that can be regulated by epigenetic mechanisms (Jacob & Moley 2005; Grandjean *et al.* 2009). For example, genomic imprinting results in parent-of-origin-dependent gene expression, which has been observed in mammals and insects (Ferguson-Smith 2011). In particular, the paternal imprinting of a genome is required for the normal development of extraembryonic membranes and trophoblast of embryos (Surani *et al.* 1984; Wilkins & Haig 2003). Thus, epigenomic modifications induced by density changes and possibly certain bioactive agents could play a major role in controlling the paternal inheritance of egg traits.

Inheritance of the variation in Hsp gene expression

Hsps may be responsible for the regulation of locust phase plasticity by orchestrating molecular and biochemical processes involved in density response. For example, Hsp90 is a chaperone for over 100 client proteins; most of these proteins are signal transducers and growth regulators (Rutherford *et al.* 2007; Ruden & Lu 2008). Hsp90 expression in *Drosophila* is associated with diverse phenotypes, including embryogenesis, adult fecundity, morphology, longevity and oogenesis (Rutherford *et al.* 2007; Chen & Wagner 2012). Hsp70 and small Hsps are also related to the morphological development and fecundity of insects (Chen *et al.* 2008; Takahashi *et al.* 2010). Parental crowd and isolation

rearing elicited great variation in *Hsp* expression in brain and femur muscle of adults, although the level of variation differed between tissues (Figs 4A and S3, Supporting information). In addition to the expression response of *Hsp*s to crowding and isolation, the *Hsp*s in the gregarious and solitarious phases were differentially expressed in various stages of eggs and nymphs (Wang *et al.* 2007; Chapuis *et al.* 2011). These findings indicated that the sustained upregulation or downregulation of *Hsp* expression related to population density was not transiently induced, but tightly controlled by developmental processes associated with locust phases.

Interestingly, the transgenerational transmission of changes in *Hsp* expression in response to density changes was observed in this study. Previous studies on plants (Smith 2013) and animals (Norouzitallab *et al.* 2014) also demonstrated the transgenerational inheritance of variation in *Hsp* expression induced by stressors. The variation in *Hsp* expression in progeny was visibly reduced relative to that in the parents, but still maintained at moderately high levels. For example, the constitutive expression of *Hsp90* in offspring eggs changed by 25–50% resulting from paternal effects, and 37–42% from maternal effects (Fig. 4). Given that Hsp90 is abundant in cells, the reduction in Hsp90 level by <50% in *Drosophila* is sufficient to cause dramatic consequences in development and fitness (Rutherford *et al.* 2007; Chen & Wagner 2012). Therefore, the moderate level of changes in *Hsp* expression in embryos could be consequential for the development of inherited phase-related traits in offspring. The maternal effects on *Hsp* gene expression were more pronounced than the paternal effects, and this pattern was similar to that of egg weight and morphometric traits (Fig. 4). These findings implied that the inherited variation in *Hsp* expression might be correlated with the phase-related phenotypic changes, but the specific functional roles of these *Hsp* genes need further investigations.

The inheritance of *Hsp* gene expression variation suggested that Hsps may be directly or indirectly involved in the epigenetic regulation and maintenance of transgenerational plasticity. Hsp90 supports diverse signal transducers and growth regulators that act as key nodes of several developmental pathways. Hsp90 is also associated with chromatin remodelling factors and histone H3 lysine-4 methyltransferase (Ruden & Lu 2008). Thus, Hsp90 can act as an environmentally sensitive chromatin remodelling regulator and cause a heritable change in the chromatin state to influence transcription (Sollars *et al.* 2003). Such features render Hsp90 a possible candidate regulator to explain the transgenerational epigenetic inheritance of phase plasticity. Local density stress can activate methyltransferases in the locust genome (Wang *et al.* 2014) and induce changes in methylation as an epigenetic mark in *Hsp* genes. The inheritance of *Hsp* expression variation may help maintain a stable conformation of their client proteins, whose properties are also modified in response to ancestral density changes. Under this scenario, Hsps continue to stabilize their client proteins to properly function in the offspring by the inheritance of their expression variation when density state persists; Hsps can readily revert and further adapt depending on the environmental density state.

Ecological and evolutionary implications

Population swarm or segregation is a natural phenomenon frequently encountered by many animals, including locusts. The changes in population density affect the survival and fitness of the ancestral generation, and these fitness-related traits can be transmitted to offspring. For example, exposing mothers to high density cues induces higher maternal glucocorticoid levels and increases offspring growth rates in a population of wild squirrels (Dantzer *et al.* 2013). In contrast to previous studies, our study showed that paternal experience could also significantly affect the complex traits of offspring, although paternal effects were milder than maternal factors. These findings suggested that the presence of paternal epigenetic factors influenced transgenerational phenotypic plasticity, which has been long ignored in ecological experimental design and analysis. Locusts, including the migratory locust and the desert locust, belong to a large group of insects that exhibit phase plasticity in response to density changes (Uvarov 1966; Pener & Simpson 2009). Therefore, the transgenerational inheritance of phenotypic plasticity induced by population density changes is widespread and evolutionarily conserved in many organisms.

Whether the parental environmental effects on offspring are adaptive remains unresolved (McCormick 2006). Crowding induces phase changes in the embryo and larvae with gregarious phase-like characteristics. As such, the progeny becomes well prepared for stressful conditions of the previous generation. The offspring readily aggregate and migrate if stressful conditions, such as food shortage, persist. Hsps strengthen stress resistance and robustness to cellular instability (Chen *et al.* 2007; Rutherford *et al.* 2007; Chen & Wagner 2012). In this study, crowding increased *Hsp* expression in the offspring, which acquired a molecular mechanism that enhances tolerance to crowding stress. Therefore, the transgenerational phase plasticity of the locusts provided an adaptive strategy that prepared offspring for ancient crowding stress in nature.

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B.C. and L.K. conceived the project and wrote the manuscript. B.C. and S.L. designed the experiments. B.C., Q.R., X.T. and X.Z. performed the experiments. B.C., Q.R., X.T. and S.L. analysed the data.

Data accessibility

Data for adult morphology and coloration, egg weight and larval morphometric traits, and data for gene expression are available from: doi:10.5061/dryad.88606.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Parental changes in the morphometric traits and coloration in response to population density.

Fig. S2 Paternal and maternal effects of isolated- or crowd-rearing on offspring egg weight.

Fig. S3 Expression changes of *Hsp90*, *Hsp70* and *Hsp20.6* in brain of male adults after isolating and crowding culture.