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Are color or high rearing density related to migratory polyphenism in the band-winged grasshopper, *Oedaleus asiaticus*?

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ABSTRACT

Locusts represent an impressive example of migratory polyphenism, with high densities triggering a switch from a solitarious, shorter dispersal range, and sometimes greenish phenotype to a gregarious and sometimes darker form exhibiting behavioral, morphological and physiological traits associated with long-distance migratory swarms. While such polyphenism has been well documented in *Locusta migratoria* and *Schistocerca gregaria*, the extent to which other grasshoppers exhibit this type of migratory polyphenism is unclear. Anecdotally, the Chinese grasshopper, *Oedaleus asiaticus*, forms migratory swarms comprised mostly of a darker, brown-colored morph, but also exhibits a non-migratory green-colored morph that predominates at low densities. In a population in Inner Mongolia not currently exhibiting migratory swarms, we found that while green and brown *O. asiaticus* are found concurrently across our sampled range, only brown grasshoppers were found in high densities. Differences between field-collected brown and green forms matched some but not key predictions associated with the hypothesis that the brown form is morphologically and physiologically specialized for gregarious migration. Controlling for body mass, brown forms had more massive thoraxes, abdomens and legs, and higher metabolic rates, but not more flight muscle or lipid stores. Further, the brown and green grasshoppers did not differ in gregarious behavior, and neither would fly in multiple lab and field trials. Lab or field-rearing at high densities for one-to-multiple juvenile instars caused grasshoppers to exhibit some morphological traits predicted to benefit migration (larger wings and a shift in relative mass from abdomen to thorax), but did not change color or induce flight behavior. One hypothesis to explain these data is that a migratory form of *O. asiaticus* is partially triggered by high field densities, but that existing ecological conditions blocked full expression of such traits (and outbreak swarms). Alternatively, color variation in this species may more tightly linked to other functions in this species such as crypsis or disease resistance, and mechanisms other than late-juvenile rearing density (e.g. genetic variation, maternal effects) may be more critical for promoting variation in color and/or migratory polyphenism.

1. Introduction

In a changing world, developmental plasticity (polyphenism) is critical to enable a single genome to produce alternative morphologies in response to environmental cues (reviewed in Via et al., 1995). One of the most common and fundamentally important alternative phenotypes in animals may be an increased migratory or dispersal capacity, often including increased flight muscles and wing size (Dingle, 1985; Harrison, 1980). One common cue that induces an increased migratory capacity in insects is higher population density (Applebaum and Heifetz, 1999). High population density may increase intraspecific competition and lead to deteriorating local food availability, providing an advantage for migratory behavior (Roffey and Popov, 1968; Uvarov, 1977). In this study, we tested for density-regulated or color-associated migratory characteristics in *Oedaleus asiaticus*, a common band-winged grasshopper from the Inner Mongolia grasslands of China.

Enhanced migratory capacity in macropterous (long-winged) insects is often associated with relatively large wing areas, well-developed flight muscles and increased long-distance flight endurance (Dingle, 1985; Rankin and Burchsted, 1992). Increased lipid stores are also observed, though these can require an increase in metabolic rate during flight due to both added mass and increased drag (Cenedella, 1971; Dudley, 1995). Such variation

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between migratory and non-migratory forms has been demonstrated in a diverse array of insects including the milkweed bug *Oncopeltus fasciatus* (Dingle, 1968), the African armyworm moth *Spodoptera exempta* (Parker and Gatehouse, 1985), and the grasshopper *Melanoplus sanguinipes* (McAnelly and Rankin, 1986). Migratory forms of insects often exhibit reduced or delayed reproduction, suggesting that investment in locomotory structures and energy stores has significant costs (Roff and Fairbairn, 2007). Therefore, there are likely to be strong ecological effects on fitness of migratory and non-migratory forms, as well as trade-offs in fitness between these forms, that maintain intra-specific variation in the plasticity of migratory phenotypes (Southwood, 1977).

High density often but not always enhances morphological and physiological characteristics associated with migratory capacity. For example, in both the planthopper *Nilaparvata lugens* (Morooka et al., 1988) and the lygaeid bug *Cavelerius saccharivorus* (Fujisaki, 1989), high density increases the frequency of macropterous as opposed to brachypterous adults. In addition, high density increases dark coloration in *N. lugens* and the body length and mesothorax development in *C. saccharivorus*. Larvae of the African armyworm *S. exempta* heighten food consumption, respiratory rate, and fat content in response to high density (reviewed in Applebaum and Heifetz, 1999). In contrast, no changes in flight behavior or morphology was observed in response to density in the noctuid, *Agrotis ipsilon* (Sappington and Showers, 1992).

Grasshoppers (family: Acrididae) are well known to exhibit phenotypes that vary continuously from gregarious and migratory to solitary and non-migratory (Uvarov, 1921, 1966, 1977). Locust phase polyphenism is best documented in *Locusta migratoria* and *Schistocerca gregaria* where it is directed primarily by larval density (Ellis, 1963; Ellis and Pearce, 1962), but development of phenotypes can also be influenced by other environmental cues and maternal factors (Miller et al., 2008). In *L. migratoria* and *S. americana*, migratory and non-migratory forms of these species can differ in color, behavior, physiology, and morphology during some developmental stages (reviewed in Pener and Simpson, 2009; Pener and Yerushalmi, 1998). Migratory locusts tend to have increased lipid stores for long-distance flight, elevated resting metabolic rate (perhaps due to the elevated maintenance cost of larger fight muscles) and a delayed onset of reproduction (Butler and Innes, 1936; Pener et al., 1997; reviewed in Pener and Yerushalmi, 1998). Newly emerged gregarious (migratory) female *S. gregaria* have relatively larger fat bodies (Schneider and Dorn, 1994). However, solitarious (non-migratory) locusts tend to be larger in body size due to an extra juvenile molt, and thus have an increased average wing length, thorax width, and hind legs by direct comparison (Uvarov, 1966). In *L. migratora* adults and *S. gregaria* juveniles, non-migratory, solitary forms tend to be green, blending in with the vegetation, while migratory forms tend to be conspicuous black or yellow (Pener and Simpson, 2009).

The scant comparative work available suggests that the magnitude of migratory polyphenism varies in grasshopper species, and that there is substantial variation in the degree to which behavior and morphology of grasshoppers is density-dependent (reviewed in Pener and Simpson, 2009). *S. americana*, a North American species that is closely related to *S. gregaria*, expresses locust-like density-dependent changes in behavior (Sword, 2003). Gaines (1989) demonstrated that short-winged adults of *Phoetaliotes nebrascensis* produced long-winged offspring when reared at high but not low densities. The northern Israel grasshopper, *Aiolopus thalassinus*, responds to high population density by increased activity level, hemolymph lipid and carbohydrates, metabolic rate, and food consumption, but without any changes in morphology or coloration (Heifetz and Applebaum, 1995). In sum, these studies suggest that the model documented for *S. gregaria* and *L. migratoria* in which high density promotes plastic formation of a migratory morph likely applies to a wide range of species, but that the suite of correlated traits varies interspecifically.

Key to enhancing migratory theory is understanding characteristics that are correlated with migratory phenotypes and the environmental triggers which may enhance outbreak and migratory phenotypes (Johnson, 1969). We tested the hypotheses that migratory characteristics of *O. asiaticus* are enhanced by population density and associated with color as documented in some other grasshopper species. We studied a population of *O. asiaticus* prone to frequent outbreaks that occasionally develop into migratory swarms in Inner Mongolia, as migrating swarms have had significant socioeconomic impacts throughout Asia for more than a century (Kang et al., 2007; Kang and Zhang, 1996). Anecdotally, these outbreaks and subsequent swarms are comprised predominantly of a brown-colored morph (S. Hao and L. Kang, pers. comm.; Jiang et al., 2003). Color phenotypes are continuous from green to brown in this species. Despite numerous attempts, we have been unable to rear *O. asiaticus* in the laboratory for multiple generations, so it is unknown whether color is genetically or maternally related. Anecdotal observations indicate that color can change at molt for many individuals, suggesting that color might be sensitive to density as in *S. gregaria* and *L. migratoria* (Pener and Simpson, 2009).

First, we surveyed field populations to document general patterns of density and color in field populations of *O. asiaticus*. Second, we collected green and brown females from the same field population and measured multiple morphological, physiological, and behavioral traits that have been shown to differ among migratory and non-migratory morphs of *L. migratoria* and *S. americana*. Third, we experimentally manipulated population density in the lab (during the final juvenile instar) and field (from the first instar) and recorded the degree of plasticity in migratory traits in females. We focused our studies on females as they are predicted to have the greatest degree of trade-offs between migratory capacity and reproduction, and thus, we would expect more detectable differences between migratory and non-migratory females as compared to males (oogenesis-flight syndrome; Johnson, 1969).

2. Materials and methods

2.1. Field site

Oedaleus asiaticus were collected from a field population near the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin, Inner Mongolia Autonomous region, China (43°38′N, 116°42′E). This station is a research facility of the Institute of Botany, Chinese Academy of Sciences (Bai et al., 2004; Wang et al., 2005). Inner Mongolia is representative of much of the Eurasian Steppe region floristically and ecologically (Li et al., 1988; Wu and Loucks, 1992). This typical steppe is characterized by dark chestnut soil with relatively homogeneous physiochemical properties and dominated by *Leymus chinensis*. The mean annual precipitation and temperature in the study area are 345 mm and 1.1 °C, respectively (Chen and Wang, 2000). *Oedaleus asiaticus* begins hatching from egg pods deposited in the ground in the beginning of June, goes through five juvenile instars and then molts into an adult in mid-July. Adults live for 4–9 weeks (Li et al., 1987).

2.2. Experiments

2.2.1. Field patterns in color

To document patterns of density and color in field populations, we surveyed two 750 m transects in two fields that were known to have high density of this species (Transect 1: 43°32′N, 116°32′E,

Fig. 1. Brown and green *Oedaleus asiaticus* females collected from the field population near the Inner Mongolia Grassland Ecosystem Research Station. Populations appear to have a bimodal distribution of brown and green phenotypes, but there is a continuum of grasshoppers expressing green to brown coloration. For our studies, we selected grasshoppers from either end of the color extremes.

July 8, 2009 and Transect 2: 43°37′N, 116°44′E, August 7, 2009. To reduce bias in selection of each sampling site, we sampled at regular intervals along each transect: 75 m (transect 1) and 250 m (transect 2). We used a global positioning unit (Garmin: GPSmap 60CSx; 6.4 m (±3 m) accuracy) to measure distance between sampling points. We collected grasshoppers using a standardized sweep net method of one sweep per step for at total of 10 sweeps at each sampling point. The three people involved in collecting were rotated to minimize collection bias.

Grasshoppers were classified as either green or brown. These green and brown grasshoppers were visually apparent (Fig. 1). While some *O. asitiaticus* are a mixture of both green and brown color components, there were no completely ambiguous grasshoppers collected in samples during the field survey for density patterns and thus we classified them based on their strongest color. For the following experiments, we selected only those at the extreme of either color scale.

2.2.2. Differences between field-caught green and brown females in adult morphology

If brown *O. asiaticus* are a migratory phenotype, we predicted that newly emerged adult female brown grasshoppers would have increased lipid stores, relative thorax, wing, and hind leg investment, and increased flight and hind leg muscle mass compared to green grasshoppers. To minimize the time grasshoppers spent in the lab but to ensure that we were comparing animals at the same developmental stage, we collected late 5th instar grasshoppers from the field (July 18, 2008). Grasshoppers were then kept in outdoor cages placed over *Leymus*-dominated plant communities at either 10 (green) or 30 (brown) individuals m^{-2} for approximately 1–2 days until the molt to adult occurred. The field density at that time was approximately 30 *O. asiaticus* m^{-2}, thus we kept brown grasshoppers at the same high density and reduced the density of green grasshoppers. Individuals were collected within 24 h of their molt to the adult stage, and were isolated for 2 days with *Leymus* grass in lab cages until sacrificed or used in behavioral trials. Grasshoppers were kept in cages for 2 days to ensure their cuticle was hardened prior to taking morphological measurements. One subset of animals was weighed to the nearest 1 mg and then frozen at −20 °C. Morphometrics, muscle, and lipid mass were all estimated for this first subset.

2.2.3. Density and color effects on gregarious adult female behavior

If *O. asiaticus* exhibit locust phase polymorphism similar to *L. migratoria* and *S. gregaria*, then we would expect high density to increase gregarious behavior (defined in this study as increased activity level and time spent near to a stimulus group). If brown *O. asiaticus* are a migratory phenotype, then we would further predict that brown animals would be more prone to exhibit gregarious behavior than green animals. Fifty green and 50 brown adult females were transported at approximately 25 grasshoppers per 60 cm × 30 cm × 30 cm cage to the Institute of Zoology, Chinese Academy of Sciences, Beijing for behavioral trials; the drive took about 9 h. Because the level of density can alter behavioral phase in *S. gregaria* within 4 h (Bouaichi et al., 1995), once at the institute, brown grasshoppers were kept at high density; while green grasshoppers were transferred to solitary chambers for 24 h before the behavioral testing. All grasshoppers were fed wheat seedlings and wheat bran, *ad libitum*, during those 24 h. We predicted that brown grasshoppers kept at high densities would be more prone to exhibit gregarious behavior than green forms kept at low densities. Note that this experimental design does not allow us to separate the effects of density and color on gregarious behavior.

2.2.4. Differences in field-caught green and brown juvenile females in gas exchange rates

Insect migratory phenotypes often have an elevated resting metabolic rate (Candy, 1985). This has been attributed, in part, to the metabolic cost for maintaining larger flight muscles (Rankin and Burchsted, 1992), the development of which can be detected during the final juvenile instar (Ready and Josephson, 2005). Therefore, based on our hypothesis that brown *O. asiaticus* exhibit traits that increase their migratory capacity, we predicted that brown forms would have higher rates of gas exchange in the terminal juvenile instar.

We collected mid-5th instar *O. asiaticus* from the field population (July 12, 2008). Grasshoppers were kept in indoor wire mesh cages with *Leymus* grass overnight; oxygen consumption and carbon dioxide production were then measured the following day between 10 AM and 4 PM using closed system respirometry (see technical methods for description). Pairs of one green and one brown grasshopper were matched for size so there was no difference in average mass between green and brown grasshoppers. To determine if there was a difference between green and brown grasshoppers in movement while in the metabolic chambers that could lead to differences in metabolic rate, we quantified movement patterns for seven green and seven black grasshoppers, counting all changes in location within the respirometer, as well as jumps.

2.2.5. Effects of color and density on adult flight capacity

We predicted that brown grasshoppers and grasshoppers reared at high density would have a greater flight capacity (long-distance flight endurance) as compared to green grasshoppers and those reared at low density. We tested flight capacity using two methods: a flight mill (see technical methods for description of mills) and outdoor free flying. For both, grasshoppers (males and females) were tested from 5 AM to 9 PM and at an

approximate temperature range of 20–38 °C. Because the grasshoppers tended not to fly when we tested them, we attempted flight trials using the flight mills with multiple protocols based on migratory patterns in other acridids (Farrow, 1990). In some cases, we attempted to induce flight indoors with a light focused on the top-center of the flight mill. We also tried a paper guide wrapped around the exterior, with the bottom portion painted black and the top portion painted white to create a visual horizon (Wilson, 1968). We attempted flight trials during both the day and night, and with the flight mills outdoors on either sunny or rainy days. In each case, grasshoppers tested included a minimum of five representatives from each of the following categories: 1, 5, and 10 days post-molt to adult, green, brown, male, female, field caught, and reared in field cages at high (approximately 20 adults m^3) or low (approximately 7 adults m^3) density. Since none of these variations resulted in more than 30 s of flight on the flight mill, we also tried a free-flying method. This approach involved either dropping a grasshopper from a 2-m height, or tossing it into the air and then measuring the distance it flew before landing. In a few cases, a grasshopper would fly up to 5 m. However, during most of the trials, the grasshopper would open its wings only to break its direct fall to the ground. In multiple cases, the grasshopper never opened its wings before landing on the ground. In sum, flight behavior was minimal for both color morphs and for grasshoppers reared at high and low density.

2.2.6. Effects of density on color, body mass, shape and metabolic rate

Based on our hypotheses that high population density enhances migratory characteristics and that brown grasshoppers are a migratory phenotype, we predicted that high density would increase the proportion of brown grasshoppers, increase the relative investment in thorax, wings, and hind legs, and increase metabolic rate.

2.2.6.1. Lab. We collected fourth-instar brown female nymphs from field populations and kept them in an outdoor arena for 1–2 days until they molted to the 5th instar. Nymphs were randomly assigned to low- (1 grasshopper $cage^{-1}$) or high- (8 grasshoppers $cage^{-1}$) density treatment groups ($n = 10$ cages per treatment). Cages (10 cm × 10 cm × 15 cm with 1 mm^2 cloth mesh) were kept in an incubator set to day and night conditions similar to field conditions: 14:10 L:D cycle, 27 °C:25 °C, 50% RH:40% RH. However, due to frequent power outages, the temperature of the incubator fluctuated in the range of 20–35 °C approximately every 3 days; all treatment groups were exposed to the same temperature fluctuations. Fresh *Leymus chinensis* grass was cut from the field plots every other day, secured with cotton in glass cylinders containing water, and presented *ad libitum*. Development time and daily mass gain were recorded over the entire 5th instar. Metabolic rate was measured over a 60-min period when grasshoppers were approximately 3/4 through the 5th instar. Animals were withheld food for 1 h prior to, and during, the gas exchange measurement. Within 4 h after molt to adult, we isolated treatment grasshoppers in a cage with no food available for 24 h. Adults were weighed, frozen at −20 °C and then dried (50 °C for 3 days)

2.2.6.2. Field cages. Cages (1 m^3) were constructed over *Leymus*-dominated plant communities (coordinates) using iron rod frames and fine 1 mm^2 cloth mesh covering. Brown male and female grasshoppers were collected from the field population at first instar (June 7, 2009) and transferred to either high (200 per cage; $n = 5$) or low (40 per cage; $n = 5$) density treatments. We selected the high-density treatment level to be slightly higher than the high-density field population from which they were collected (∼175 first instar *O. asiaticus* m^{-2}). We selected the low-density treatment level to ensure that sufficient animals would be available at the end of the experiment, knowing that there is a high mortality rate during the early instars. Because there were too few green grasshoppers available during the first instar, we collected additional brown and green grasshoppers at approximately mid-third instar (June 16–18) to fill additional treatment cages. At that time, the field density had decreased, as it does naturally due to high mortality in early instars, to approximately 80 *O. asiaticus* m^{-2}. We again selected a high treatment density that was slightly higher than that (100 per cage = high density; 20 per cage = low density). The number of cages for each treatment group was: green, low density (16; all started at 3rd instar), green, high density (6; all started at 3rd instar), brown, low density (25; 5 cages started at 1st instar, 20 cages started at 3rd instar), and brown, high density (8; 5 cages started at 1st instar, 3 cages started at 3rd instar) – 2820 grasshoppers in total. Average densities in field cages several weeks later when grasshoppers were in the 5th instar or adults and during our final cage assessment (July 8) were 7.5 (±0.4 SEM) and 21.7 (±2.8 SEM) for low and high density treatments, respectively. The population density in the field population at that time (mostly brown grasshoppers) was approximately 18 grasshoppers m^{-2}. This suggests that grasshoppers in our field cages suffered similar mortality rates as those in the field population. We surveyed field cages every 2 weeks and recorded the color and instar, but not the sex of each grasshopper in each cage

2.3. Technical methods

2.3.1. Morphology, lipid and muscle mass

Animals were weighed to the nearest 0.1 mg using a Mettler-Toledo AB204-S/Fact balance and then frozen. We measured pronutum and head maximum lateral width and hind femur length and maximum width (dorsal–ventral in approximately the center of the femur) to the nearest 0.01 mm (as described in detail in Dirsh, 1953) with a digital micrometer (Mitutoyo CD-6"BS, Japan). Animals were then dried at 50 °C for 3 days. Dissections occurred post-drying and consisted of gut removal, and separation of the head, wings, legs, thorax, and abdomen. Body parts were weighed to the nearest 0.001 mg using a Mettler Toledo MX5 microbalance. Flight and hind leg muscle mass were determined from the difference in dry masses of thorax and femur masses before and after dissolving all tissue with 1 mol L^{-1} NaOH (Marden, 1987). Abdominal lipid content was determined from the difference in mass of the abdomen before and after lipids were extracted by soaking for 24 h in 2 mL of a 2:1 (v/v) chloroform: methanol solution (similar to Kent and Rankin, 2001). Wings were relaxed using a weak vinegar solution, spread, pinned, and digitally scanned. Area of the hind wings was measured using Image J software (resolution = 79 pixels/cm; Rasband, 1997–2009).

2.3.2. Metabolic rate and behavior in chambers

Animals were placed in a 60-mL plastic syringe covered in tin foil to minimize visual disturbance. The syringe was flushed for 3 min with dry CO_2-free (Drierite, Ascarite) air at 100 mL min^{-1}, which lowered CO_2 levels within the syringe to below 0.1 ppm. The syringe was sealed and placed in a dark incubator (27 °C) for 60 min. Then the syringe was gently removed, and a 25-mL air bolus was ejected from the syringe into an air stream within plastic tubing. Air in the tubing was pulled at approximately 100 mL min^{-1} through a water scrubber (magnesium perchlorate), the CO_2 analyzer (LiCor model LI-6252), a CO_2 scrubber (Ascarite) the O_2 analyzer (Sable Systems Foxbox 2004 model) and then the pump (Sable Systems Foxbox). The analog output of the gas analyzers was digitized (Sable Systems UI2) and continuously recorded using Sable Systems Expedata and a laptop. To calculate the gas exchange rates, we integrated the area under the O_2 and CO_2 peaks produced by the 25-mL bolus injection and converted

this to mL CO_2 produced and O_2 consumed h^{-1} as described in Lighton (2008, pp. 31–40).

To record behavior in the metabolic chambers, grasshoppers were placed in the chambers within the 27 °C incubator in the same manner as for measuring gas exchange but without the foil covering. Pairs of green and brown grasshoppers were videotaped for 25 min. Time moving (jumping or walking) and number of jumps were recorded manually by an observer using a stop watch while watching the videos.

2.3.3. Gregarious behavior

Gregarious behavior was measured by a using a behavioral assay similar to Roessingh et al. (1993). The main difference in our assay was the size of each arena (22 cm long × 18 cm wide × 7 cm high as opposed to 57 cm long × 30 cm wide × 10 cm high). Four arenas and trials were filmed simultaneously. Each arena had three sides that were opaque and one that was clear with perforations to allow visual and chemical cues from the stimulus group. Stimulus groups of 20 conspecifics were placed behind the clear perforated partition. Grasshoppers being tested were pre-treated in dark solitary chambers for 2 min. Next, a grasshopper was gently placed in one of the corners furthest from the stimulus group and held in place by a plastic cylinder. The cylinder was then removed and grasshoppers were filmed from above for 10 min. Behavior was analyzed using the computer software Ethovision.

2.3.4. Flight mill

We constructed a flight mill similar to Schumacher et al. (1997). The axis consisted of two magnets placed in a plexiglass frame. A drinking straw was used as a flight arm and a pin pushed through this straw served as an axle held in place by the two magnets, thus minimizing friction. An infrared transmitter/receiver mounted to the frame registered every rotation of the mill. Grasshoppers were attached to the head of a second pin using dental gum. The pin was then pushed through one end of the straw at a 95° angle. The flight mills were tested using colony-reared *S. americana* (subfamily: Cyrtacanthacridinae; average female is 1700 mg) and field-caught *Trimerotropis pallidipenis* (subfamily: Oedipodinae; average female is 500 mg – the same as *O. asiaticus*) collected near Phoenix, Arizona. Most individuals of both species flew for a minimum of 3 min on these flight mills, so they were suitable for grasshoppers in this size range.

2.4. Statistics

Prior to analyses, all data were checked for the assumptions of parametric tests. If data did not meet these assumptions, they were either transformed to meet assumptions or non-parametric tests were performed. For all multivariate tests, to ensure that all response variables were evenly weighted, we standardized variables using a *Z*-score transformation (Figs. 3A and 6A; Gotelli and Ellison, 2004, p. 400). For all covariate analyses, we examined the assumption of slope homogeneity using homogeneity-of-slopes model MANCOVAs. In all cases, the categorical variable (e.g. color) by covariate (e.g. dry mass) interaction was not significant and therefore, we concluded that our assumption was met and proceeded with traditional MANCOVA models. Retrospective power analyses were conducted on non-significant results ($P > 0.05$) to assess likelihood of committing type II errors. We set $\alpha = 0.05$. The power analyses were conducted for a 'medium effect size' given as *d* (Student's *t*-test, Mann–Whitney *U*-test), *f* (ANOVA/ANCOVA) or f^2 (MANOVA) (Cohen, 1988). While there is no standard, and the usefulness of a retrospective power analysis is controversial (Thomas, 1997), a power of 0.8 is generally considered adequate to accept the null hypothesis, assuming $P > 0.05$ (Cohen, 1988). Initial analyses were performed using Statistica 9 (2009). Power analyses were performed using GPOWER (Erdfelder et al., 1996). Details of statistical analyses performed can be found in the results section and figure legends. Throughout, statistical significance was judged as $\alpha < 0.05$.

3. Results

3.1. Field patterns in color

Both green and brown *O. asiaticus* were found in all collection areas but green animals were never abundant while brown *O. asiaticus* were relatively abundant and their frequency increased as overall population density increased (Fig. 2).

3.2. Differences between field-caught green and brown females in adult morphology

In field populations, 2 days post-molt to adult, brown females were, on average, heavier than green (Fig. 3A). To determine if there was an overall shape difference in these two groups, we standardized all variables (Gotelli and Ellison, 2004, p. 400) and then conducted a multivariate analysis, testing the categorical factor of color (covariate = dry mass; dependent variables = wing, head, hind leg, abdomen, and thorax mass; wing area, femur length, femur max width, head max width and pronotum max width. We followed the MANCOVA by Tukey multiple comparisons tests and found that overall shape was different between the two color morphs (MANCOVA: $F_{(9,28)} = 2.9$, $P = 0.01$; covariate means: dry body mass (mg): 119). When controlling for body mass, brown *O. asiaticus* females had a significantly increased relative investment in hind legs and thorax, and a non-significantly reduced investment in wings and head (Fig. 3B and C).

We then tested for predicted higher levels of abdomen lipid, and thoracic and hind leg muscle masses in brown grasshoppers (covariate = dry mass; dependent variables = abdomen lipid, thorax, and hind leg muscle masses; prediction = brown > green). The masses of these features did not differ between green and brown grasshoppers (MANCOVA: $F_{(3,33)} = 0.49$, $P = 0.69$; covariate means: dry body mass (mg): 119). To further investigate differences, we followed the MANCOVA by separate ANCOVAs (categorical factor = color; covariate = dry mass; dependent variable = either lipid, thorax, or hind leg muscle mass; prediction = brown > green). All ANCOVAs were also non-significant with a Power = 0.33, $f = 0.25$, and covariate means (dry body mass mg) = 119: abdominal lipid mass ($F_{(1,36)} = 0.25$, $P = 0.6$), thoracic muscle mass

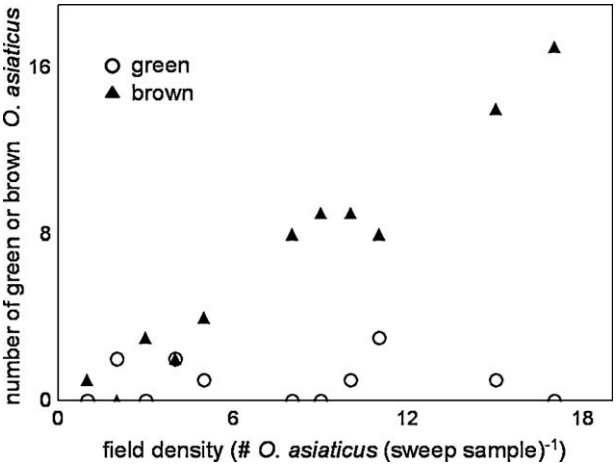

Fig. 2. Field surveys of male and female *O. asiaticus*. Brown and green *O. asiaticus* were found across all field densities surveyed; however only brown grasshoppers were found in high abundance (greater than three grasshoppers per sweep sample).

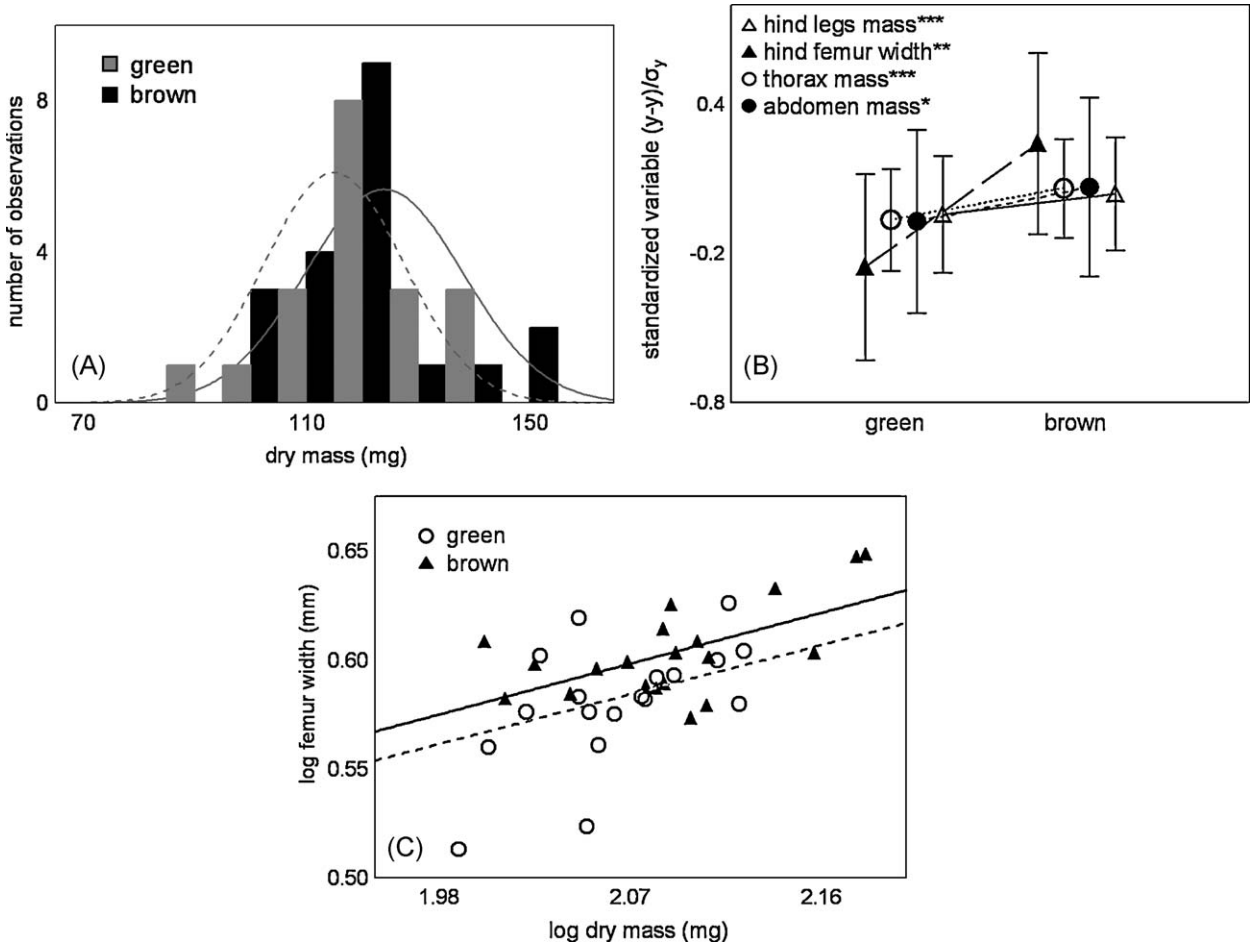

Fig. 3. Brown adult female *O. asiaticus* were heavier, on average, and had an increased relative investment in hind legs, thorax, and abdomen (from a MANCOVA with dry mass as a covariate). Green and brown female grasshoppers were field-caught in late 5th instar and caged until 2 days post-molt to adult. (A) Brown grasshoppers had a heavier dry body mass as compared to green (*t*-test: $t = 2.09$, $P = 0.04$). (B) Asterisks indicate significant differences from Tukey multiple comparisons tests ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Other variables included in the overall MANCOVA, but that resulted in non-significant differences from Tukey multiple comparisons tests (all within $0.05 < P < 0.3$) are wing area, wing mass, head mass, head width, femur length, and pronotum max width. With the exception of pronotum width, brown had lower means for these six variables (MANCOVA: $F_{(9,28)} = 2.9$, $P = 0.01$; covariate means: dry body mass (mg): 119). Error bars denote 95% confidence intervals. (C) Example regression of a variable from the MANOVA where significant differences were found (femur width).

($F_{(1,35)} = 0.9$, $P = 0.35$), hind legs muscle mass ($F_{(1,35)} = 0.7$, $P = 0.4$). Mean proportions of abdominal lipid, thoracic, and hind legs masses relative to total dry body mass ± SEM were as follows: abdominal lipids (green: 0.079 ± 0.004, brown: 0.075 ± 0.003), thoracic muscle (green: 0.25 ± 0.004, brown: 0.26 ± 0.005), and hind leg muscle (green: 0.127 ± 0.005, brown: 0.133 ± 0.005).

3.3. Density and color effects on gregarious adult female behavior

When all variables were standardized and included in the initial analysis (independent variable = color, dependent variables = average distance from stimulus group, total distance moved, average velocity, turn angle, angular velocity, and meander), there were no differences in gregarious behavior between green and brown adults (MANOVA: $F_{(6,100)} = 1.2$, $P = 0.3$, Power = 0.84, $f^2 = 0.15$). When tested separately, brown grasshoppers kept at high densities tended to have a higher mean angular velocity than green grasshoppers kept at low densities for 24 h (°/s; i.e. the amount of turning per unit time; t test: $t = 2.1$, $df = 94$, $P = 0.036$), and mean meander (°/cm; i.e. the amount of turning per unit distance; *t*-test: $t = 2.02$, $df = 94$, $P = 0.045$).

As another approach to testing for gregarious behavior, we compared the amount of time brown or green grasshoppers spent near the stimulus group of grasshoppers. We divided the arena into four equal-sized zones ranging from closest to furthest from the stimulus group, and compared the percentage of time spent in each zone (independent variable = color, dependent variables = percent time in zone 1, 2, 3, and 4; dependent variables were standardized prior to the analysis). We found no significant differences between brown and green grasshoppers in their tendency to spend time near other grasshoppers (MANOVA: $F_{(3,103)} = 0.9$, $P = 0.46$, Power = 0.89, $f^2 = 0.15$).

3.4. Differences in field-caught green and brown juvenile females in gas exchange rates

Field-caught brown mid-5th instar females had an increased rate of gas exchange as compared to green (Fig. 4; O_2 consumption: $t = -2.37$, $df = 34$, $P = 0.02$; and CO_2 production $t = -2.03$, $df = 34$, $P = 0.05$ from *t*-tests). The difference in gas exchange was more apparent in larger grasshoppers. For brown grasshoppers, both O_2 consumption and CO_2 production were positively correlated with body mass. There was no correlation between these variables in green grasshoppers, suggesting that mass specific metabolic rate decreases with increasing body size in green, but not in brown grasshoppers (Fig. 5A and B).

The differences in metabolic rates between brown and green grasshoppers were not explained by observable differences

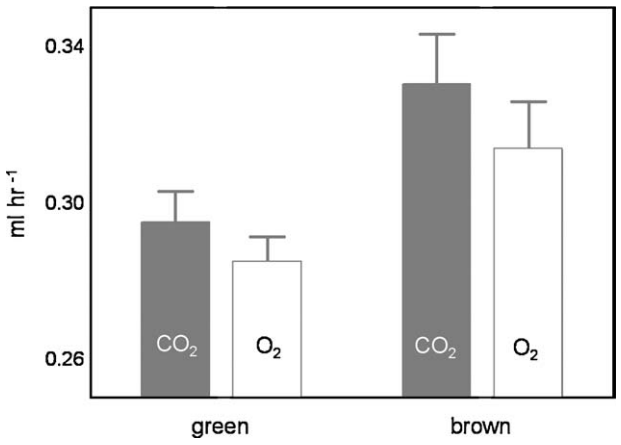

Fig. 4. Brown mid-5th instar female *O. asiaticus* had a higher rate of gas exchange than green. Brown grasshoppers had a higher CO_2 production (*t*-test: $t = -2.03$, $df = 34$, $P = 0.05$) and O_2 consumption (*t*-test: $t = -2.37$, $df = 34$, $P = 0.02$). Green and brown grasshoppers were matched for size so that there was no difference in the average mass between the groups.

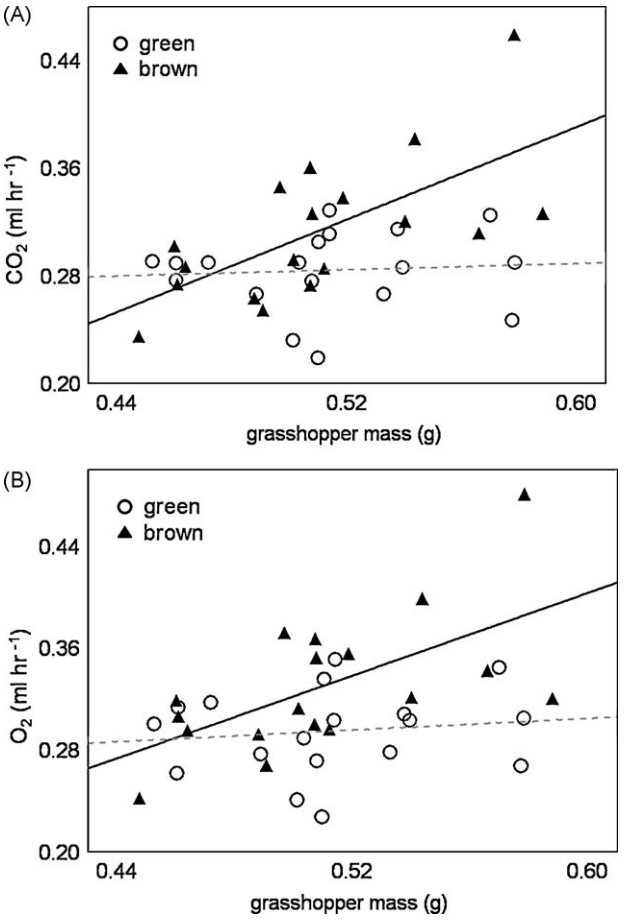

Fig. 5. Brown mid-5th instar female *O. asiaticus* had a higher rate of gas exchange than green at higher body masses. (Homogeneity-of-slopes model MANCOVA: $F_{(2,31)} = 3.6$, $P = 0.04$; covariate means: dry body mass (mg): 511). Brown grasshoppers exhibited a positive correlation between body mass and both (A) CO_2 production ($y = -0.1 + 0.86 \times x$; $R^2 = 0.44$; $P < 0.01$) and (B) O_2 consumption ($y = -0.08 + 0.8 \times x$; $R^2 = 0.37$; $P < 0.01$). Green grasshoppers did not exhibit any correlation between body mass and metabolic rate (CO_2 production: $R^2 < 0.01$; $P = 0.80$; O_2 consumption: $R^2 = 0.01$; $P = 0.60$).

in activity in the respirometry chambers. There were no significant differences between brown and green grasshoppers in the time spent moving in the respirometer (minutes grasshopper was mobile out of a total of 25 min: *t*-test: $t = -1.7$, $df = 12$, $P = 0.12$, Power = 0.14, $d = 0.5$; green 2.4 ± 1.1 SEM; brown 0.4 ± 0.34 SEM; average of all animals was 5.6% time spent moving). There was also no difference in the total number of jumps during 25 min (Mann–Whitney *U*-test: $df = 12$, $P = 0.5$, Power = 0.13, $d = 0.5$; green 1.3 ± 0.7 SEM; brown 0.8 ± 0.8 SEM; average for all animals was 0.03 jumps per minute).

3.5. Effects of color and density on adult flight capacity

There were no differences in flight capacity between any of the groups tested, and *O. asiaticus* individuals generally did not fly at all regardless of how tested.

3.6. Effects of density on color, body mass, shape and metabolic rate

3.6.1.1. Lab

We used the same variables and statistical procedure as for the field-caught grasshoppers, predicting that color morphs would differ in overall shape (categorical factor = density; covariate = dry mass; dependent variables = wing, head, hind leg, abdomen, and thorax mass; wing area, femur length, femur max width, head max width and pronotum max width). Grasshoppers reared at high density during the 5th instar had a different shape as adults than those reared at low density (MANCOVA: $F_{(10,5)} = 4.9$, $P = 0.046$; covariate means: dry body mass (mg): 89). We followed the MANCOVA by Tukey multiple comparisons tests and found that grasshoppers reared at high density had a significantly increased relative investment in hind legs and wings and a non-significantly reduced investment in head and abdomen (Fig. 6A and B).

There was no difference in mass between the two groups (*t*-test: $t = 0.07$, $df = 15$, $P = 0.94$, Power = 0.16, $d = 0.5$; low density 88.79 ± 4 SEM, high density 88.44 ± 2.4 SEM) and no evidence of color change over that time period. There were no differences in rate of gas exchange between the two groups (covariate = body mass; prediction = high density > low density): CO_2 production (ANCOVA: $F_{(1,16)} = 0.0008$, $P = 0.97$; covariate means: dry body mass (mg): 47; Power = 0.17, $f = 0.25$) and O_2 consumption (ANCOVA: $F_{(1,16)} = 0.0001$, $P = 0.99$; covariate means: dry body mass (mg): 47; Power = 0.17, $f = 0.25$).

3.6.1.2. Field cages

While many grasshoppers (10–20%) did change from one color to the other, density had no effect on the average color of grasshoppers. For the grasshoppers that were collected as brown at first instar, there was no difference in the proportion of brown grasshoppers per cage between high (0.93 ± 0.04 SEM) and low (0.86 ± 0.05 SEM) density treatments, though the power was relatively low (Mann–Whitney *U*-test: $df = 6$, $P = 0.23$, Power = 0.09, $d = 0.5$). However, for the grasshoppers that were collected as brown at third instar, there was a non-significant tendency for a density effect opposite to the predicted one, as the proportion of brown in high density cages (0.84 ± 0.08 SEM) was less than the proportion in low density cages (0.93 ± 0.02 SEM; Mann–Whitney *U*-test: $df = 31$, $P = 0.30$, Power = 0.24, $d = 0.5$). For grasshoppers collected as green at the third instar, there was a non-significant tendency for high density to increase the proportion of brown; again the test had relatively low power (high densities: proportion brown: 0.36 ± 0.04 SEM; low densities: proportion brown: 0.26 ± 0.04 SEM; Mann–Whitney *U*-test: $df = 53$, $P = 0.32$, Power = 0.19, $d = 0.5$)

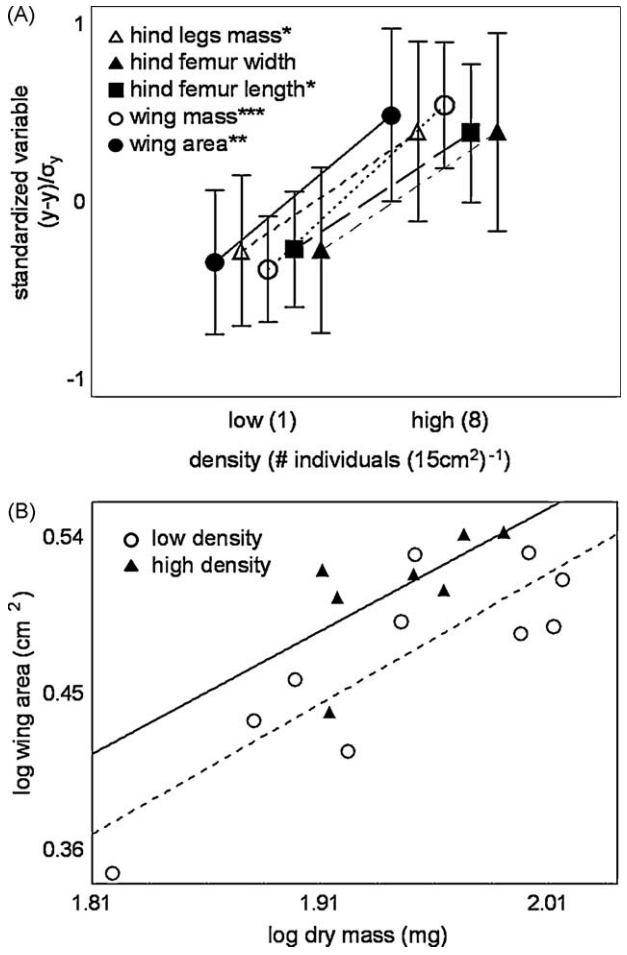

Fig. 6. Grasshoppers reared at high density during the 5th (final) juvenile instar had an increased relative investment in hind legs and wings 1 day post-molt to adult (MANCOVA with dry mass as a covariate). All grasshoppers were collected as and retained brown coloration. (A) Asterisks indicate significant differences from Tukey multiple comparisons tests (no asterisk: $P \leq 0.08$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Other variables included in the overall MANCOVA where high density grasshoppers had higher mean values, but that resulted in non-significant differences ($P < 0.6$) from Tukey multiple comparisons tests were thorax mass, pronotum max width, and head max width. Two remaining variables included in the MANCOVA that resulted in non-significant differences ($0.05 < P < 0.3$) where high density grasshoppers had lower mean values were head mass and abdomen mass (MANCOVA: $F_{(10,5)} = 4.9$, $P = 0.046$; covariate means at a dry body mass of 89 mg). Error bars denote 95% confidence intervals. (B) Example regression of a variable from the MANOVA where significant differences were found (wing area).

4. Discussion

As in the much better studied African plague locusts, grasshoppers with different colors differed in size and shape, and rearing at different densities even for one juvenile instar, induced changes in shape. Most of the significant differences supported the hypothesis that high-density and brown color were associated with improved migratory capacity. There was evidence for larger wings, thorax and legs, and higher metabolic rates being associated with high density rearing or brown color. However, key differences most commonly associated with migratory polyphenism (differences in flight behavior, gregariousness, flight muscle, lipid stores) were not correlated with color or high-density rearing. At present, it is unclear whether these negative results are due to ecological conditions that suppressed migration, our experimental design, or interspecific variation.

4.1. Experimental design issues

Experiments with the African plague locusts have clearly demonstrated that maternal and early-instar effects are important for development of color and migratory polyphenism (refs). Experiments with other grasshoppers and insects have also demonstrated that genetically based variation can contribute to variation in color and physiological and morphological traits related to migration (refs). Tests for maternal or genetically based effects require laboratory rearing, and unfortunately, as yet this has not been accomplished with *O. asiaticus*, despite significant efforts (juveniles die during lab-rearing, possibly because key dietary requirements are missing). Our experiments have necessarily focused on animals collected from the field in the midst of their juvenile development. The possibility of maternal, early-instar, or genetic effects on color or migratory polyphenism must be considered in interpretation of all of the results presented here.

4.2. Brown, but not green, grasshoppers are found in high abundance in the field

Our surveys showed that green *O. asiaticus* are generally present at low density wherever *O. asiaticus* are found and are rarely, if ever, found in high abundance (i.e. in our sampling, we never collected more than two green at any given coordinate). Conversely, brown *O. asiaticus* are found in both high and low abundance. These field observations suggest are consistent with prior anecdotal reports that migratory swarms are largely comprised by the brown phenotype. However, it is important to point out that our experiments do not allow determination of how the differences in field abundances arise. For example, it is possible that regions with high concentrations of brown grasshoppers result from density effects on color, nutritional effects on color, or color-associated genetically based differences in gregariousness.

4.3. Weak connection between color variation and migratory capacity

Brown and green *O. asiaticus* collected from the field had some significant differences in morphology and physiology, but these differences were not clearly linked to migratory polyphenism. Some of the predictions based on the hypothesis that brown morphs are migratory were met; brown grasshoppers had an increased investment in thorax and legs (Fig. 3B and C), and increased metabolic rates (Fig. 4). Larger hind legs may enhance jumping into flight (Katz and Gosline, 1992) and a larger thorax can allow for increased flight muscles. The higher metabolic rates in brown grasshoppers was not related to greater activity in the respirometer, and resulted from higher rates in larger animals (Fig. 5), suggesting that heavier animals might have a higher proportion of metabolically active tissues.

In contrast to these supportive findings, key predictions of the hypothesis that brown grasshoppers have a greater migratory capacity were not met. Thoracic and leg muscle masses and abdominal lipid stores did not differ between green and brown morphs. Mean values were very similar for flight muscle mass and lipid stores, suggesting that the lack of statistical significance was not due to lack of power. Wing size also was not larger in brown grasshoppers (in fact, the mean wing area was greater in the green morphs in the MANCOVA), although wing size does not always correlate with migratory capacity (reviewed in Rankin and Burchsted, 1992). Finally, we found no evidence that brown grasshoppers were more prone to fly; in fact, neither morph could be induced to fly.

There was also no evidence that brown morphs were more gregarious, though this conclusion should be taken with caution. We measured gregarious behavior for green and brown grasshoppers collected from the same population, driven to Beijing for 9 h at relatively high densities, and then subsequently kept in solitary confinement or high density, respectively, for 24 h. While 4 h is sufficient to induce gregarization in solitary-reared locusts, up to 24 h may be necessary to reduce gregarization in a crowd-reared locusts (*S. gregaria*; Roessingh et al., 1993). Conceivably a longer time may be required to eliminate gregarious behavior in this species. If that was the case, we would not be able to detect evidence of gregarious behavior as we have no baseline behavior for solitary-reared *O. asiaticus*. Testing *O. asiaticus* that have been reared for the entirety of their juvenile development at either high density or in solitary cages would provide a more definitive answer to this question.

In sum, the green-brown phenotypes from this field population had some differences in morphology and metabolic rates, but these did not seem to be linked with migratory polyphenism. Thus, our comparisons of field-collected green and brown forms did not support the hypothesis that brown *O. asiaticus* represent a gregarious, migratory form of this grasshopper analogous to those documented for *S. gregaria* or *L. migratoria*. However, it is important to note that color, migratory behavior, morphology and physiology exist on a continuum. It is possible that ecological conditions (e.g. good local forage) precluded development of fully functional migratory *O. asiaticus*, and that under such conditions a clearer correlation between color and migratory phenotype might be observed.

4.4. Effects of rearing density

Rearing density did significantly affect the shape of *O. asiaticus*, and the significant differences observed were generally consistent with the hypothesis that high densities promote formation of a migratory form. As predicted by this hypothesis, females reared at high density during the 5th instar had an increased investment in wings and hind legs, and a decreased investment in head and abdomen. The greater wing mass and area per body mass will result in a lower wing loading and could translate into increased flight capacity (Rankin and Burchsted, 1992). However, no *O. asiaticus* would fly for long distances, regardless of rearing density. Also, since we did not measure flight or leg muscle mass in this experiment, and the comparison between color morphs indicated that changes in thorax and leg dimensions do not correspond to differences in flight and leg muscle masses of the morphs, the conclusion that the observed changes in morphology actually improve flight performance must be considered provisional. Further, we found no direct evidence for an effect of density on color suggesting that color, shape, and migration are not necessarily linked in this species. However, high-density rearing induced larger hind legs as found for the brown forms, suggesting that these differences in field-caught brown and green forms could be caused by rearing density. It is possible that longer juvenile rearing at high density, or multi-generational rearing at high density is necessary to elicit a full migratory phenotype that exhibits flight behavior. Alternatively, other ecological conditions may be critical for triggering migratory development and behavior in this species.

The population studied was in the Xilin River basin region where *Oedaleus* have exhibited swarming in other years (S. Hao and L. Kang, unpublished). Thus, this grasshopper can and will fly under some circumstances. However, during the 2 years of this study, the populations of *O. asiaticus* were not swarming, despite exhibiting densities exceeding those previously documented for swarming *O. asiaticus* (Jiang et al., 2003). Perhaps deteriorating local forage conditions are required in addition to high densities to induce swarming and migration in *O. asiaticus*.

4.5. Potential functional significance of color variation

While we cannot completely reject the hypothesis that color variation is linked with migratory polyphenism in *O. asiaticus*, our results do suggest that other hypotheses for the significance of color variation should be considered. For example, color may affect likelihood of predation with green forms being more cryptic in more lush microhabitats and brown forms more cryptic in dryer or open habitats (Chapman and Joern, 1990). As an alternative to crypsis, the brown color may be aposematic, reducing deaths to predation in large groups, as suggested for some locusts (Sword et al., 2000). Brown coloration may be related to melanization and resistance to diseases, cannabalism or parasites (Schmid-Hempel, 2005), with a trade-off of increased energetic costs (consistent with the higher metabolic rates in brown forms, Fig. 4).

4.6. Potential mechanisms causing color variation

Grasshoppers commonly exhibit three types of color polyphenism: phase color-polyphenism, green-brown color polyphenism, and homochromy where an individual matches the color of the underlying background (described in Pener and Simpson, 2009). These color polyphenisms typically have different underlying mechanisms and some locusts, including *L. migratoria*, exhibit all three hierarchically (e.g. in the absence of the mechanistic triggers for phase and green–brown polyphenism, they exhibit the third type, homochromy).

When crowded, *L. migratoria* exhibit phase color-polyphenism, developing dark, contrasting colors by way of the neurohormone [His^7]-corazonin (Tawfik et al., 1999). In the absence of crowding (i.e. low density), green–brown polyphenism can be regulated by humidity. High humidity, found in wetter seasons or microhabitats, directs development into a cryptic green morph. In the absence of high humidity, many grasshoppers exhibit homochromy and can have a tan or yellow to dark brown coloration depending on dominant environment color (Chapman and Joern, 1990). The high concentrations of brown *O. asiaticus* documented in Fig. 2 occurred in fields subject to heavy livestock grazing (Kang and Chen, 1995; A. Cease et al., personal observation), and these heavily grazed fields have a significantly reduced ground cover and soil water content (Zhao et al., 2007).

Alternatively, brown coloration could be caused by better nutrition, which was supported by the larger size of brown forms (Fig. 3A) and the correlation of brown forms with higher field abundances (Fig. 2). Further experiments will be necessary to distinguish these various hypotheses concerning the mechanism and significance of color variation in this species.

4.7. Summary and future directions

Our results show that neither brown color nor high density rearing for one-to-several instars is associated with expression of a clear migratory phenotype, consistent with the lack of migratory swarms observed for *O. asiaticus* during the 2 years of this study. Our results support the general understanding that the type and degree of migratory polyphenism can exist along a continuum in grasshopper species (reviewed in Pener and Simpson, 2009). The lack of flight behavior and migratory swarm formation despite high field densities suggests the importance of other ecological factors in determining migration in this species. Definitive tests of hypotheses regarding the mechanisms and functional significance

of color migratory polyphenism in this species will require development of laboratory rearing protocols. In addition to enhancing migratory theory, understanding characteristics that are correlated with migratory phenotypes and the environmental triggers that may enhance outbreak and migratory phenotypes is key to developing sustainable management strategies for pestivorous and swarm-forming grasshoppers.

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