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Non-swarming grasshoppers exhibit density-dependent phenotypic plasticity reminiscent of swarming locusts

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ABSTRACT

Locusts are well known for exhibiting an extreme form of density-dependent phenotypic plasticity known as locust phase polyphenism. At low density, locust nymphs are cryptically colored and shy, but at high density they transform into conspicuously colored and gregarious individuals. Most of what we know about locust phase polyphenism come from the study of the desert locust *Schistocerca gregaria* (Forskål), which is a devastating pest species affecting many countries in North Africa and the Middle East. The desert locust belongs to the grasshopper genus *Schistocerca* Stål, which includes mostly non-swarming, sedentary species. Recent phylogenetic studies suggest that the desert locust is the earliest branching lineage within *Schistocerca*, which raises a possibility that the presence of density-dependent phenotypic plasticity may be a plesiomorphic trait for the whole genus. In order to test this idea, we have quantified the effect of rearing density in terms of the resulting behavior, color, and morphology in two non-swarming *Schistocerca* species native to Florida. When reared in both isolated and crowded conditions, the two non-swarming species, *Schistocerca americana* (Drury) and *Schistocerca serialis cubense* (Saussure) clearly exhibited plastic reaction norms in all traits measured, which were reminiscent of the desert locust. Specifically, we found that both species were more active and more attracted to each other when reared in a crowded condition than in isolation. They were mainly bright green in color when isolated, but developed strong black patterns and conspicuous background colors when crowded. We found a strong effect of rearing density in terms of size. There were also more mechanoreceptor hairs on the outer face of the hind femora in the crowded nymphs in both species. Although both species responded similarly, there were some clear species-specific differences in terms of color and behavior. Furthermore, we compare and contrast our findings with those on the desert locust and other relevant studies. We attribute the presence of density-dependent phenotypic plasticity in the non-swarming *Schistocerca* species to phylogenetic conservatism, but there may be a possible role of local adaptation in further shaping the ultimate expressions of plasticity.

1. Introduction

Density-dependent phenotypic plasticity is a defining feature of locusts (Pener and Simpson, 2009; Sword and Simpson, 2008). Locusts are grasshoppers that can form dense migrating swarms through a phenomenon known as locust phase polyphenism, in which cryptically colored, shy individuals (solitarious phase) can transform into conspicuously colored, gregarious individuals (gregarious phase) in response to increases in population density (Pener, 1983; Uvarov, 1966). In addition to color and behavioral changes, locusts exhibit morphological, reproductive, developmental, physiological, biochemical, molecular, and ecological changes in response to change in density (Applebaum et al., 1997; Hassanali et al., 2005; Kang et al., 2004; Pener, 1991; Pener and Simpson, 2009; Roessingh and Simpson, 1994; Simpson et al., 1999, 2002; Simpson and Miller, 2007; Sword and Simpson, 2008; Tanaka, 2001, 2006; Verlinden et al., 2009). One of the most well studied examples is the desert locust, *Schistocerca gregaria* (Forskål), which is the biblical plague locust recorded in ancient literatures that still affects many lives in Africa and the Middle East (Pener and Simpson, 2009). The desert locust has been studied in depth since Uvarov (1928) showed the existence of locust phase, but over the last two decades, tremendous advances have been made in understanding proximate mechanisms of locust phase polyphenism and swarm formation (Pener and Simpson, 2009). We now know that behavioral gregarization of solitarious locusts can be induced by a combination of sight and smell of gregarious locusts (Despland, 2001; Hägele and Simpson, 2000; Roessingh et al., 1998) or by a physical stimulation of mechanosensory receptors located on the outer surface of hind femora (Simpson et al., 2001). These two sensory pathways transmit signals to the thoracic

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central nervous system (Rogers et al., 2003, 2004), releasing serotonin [5-hydroxytryptamine (5-HT)], a conserved neuromodulator, which is shown to be responsible for the initial behavioral shift from the solitarious to gregarious phase (Anstey et al., 2009). A large number of phase-specific genes are expressed as a consequence (Badisco et al., 2011a,b), and at least one gene product cyclic Adenine Mono-Phosphate group (cAMP)-dependent protein kinase A (PKA), has been shown to play a critical role in initial behavioral gregarization (Ott et al., 2012). When scaled up to a population level, behavioral gregarization is a result of an interaction between local population increase and habitat structure (Bouaïchi et al., 1996; Collet et al., 1998; Despland, 2003; Despland et al., 2000). Once locusts are in the gregarious phase, they exhibit collective movement by aligning with other members of the group (Buhl et al., 2006) in the form of nymphal marching bands or adult swarms (Ellis, 1963; Kennedy, 1939; Uvarov, 1966). The resulting mass movement is partly driven by the risk of cannibalism (Bazazi et al., 2008).

The desert locust belongs to the genus *Schistocerca* Stål (Acrididae: Cyrtacanthacridinae), which contains about 50 species, widely distributed throughout the New World (Dirsh, 1974; Harvey, 1981; Song, 2004a). Within the genus, only four species are known to be swarming locusts (Harvey, 1981; Pener and Simpson, 2009; Song, 2011), and the majority of the species within *Schistocerca* are actually non-swarming, sedentary grasshoppers (Song, 2004b, 2005; Song and Wenzel, 2008). Recent phylogenetic studies based on molecular data suggest that *S. gregaria* is the earliest branching lineage within the genus (Lovejoy et al., 2006; Song et al., 2013), which points to a possibility that the presence of density-dependent phenotypic plasticity may be an ancestral trait for the genus. However, little is known about the extent of density-dependent phenotypic plasticity in non-swarming species in the genus *Schistocerca*. Therefore it is of great interest to investigate whether non-swarming species in the genus are capable of expressing density-dependent phenotypic plasticity by experimentally varying rearing density during nymphal development. In this study, we examine two non-swarming *Schistocerca* species, *Schistocerca americana* (Drury) and *Schistocerca serialis cubense* (Saussure), both of which natively occur in Florida and are morphologically similar to the desert locust, but not known to swarm in nature (Harvey, 1981). Specifically, we address the following questions: (i) Do non-swarming *Schistocerca* species express density-dependent phenotypic plasticity in behavior, color, and morphology? (ii) How similar or different are the density-dependent plastic responses between the non-swarming species and the desert locust? and (iii) Are there species-specific differences in the plastic responses between the non-swarming species? The main motivation behind this study is to explicitly quantify density-dependent reaction norms in the non-swarming *Schistocerca* species with the same rigor as done in the desert locust in order to establish a comparative framework for studying the evolution of density-dependent phenotypic plasticity in *Schistocerca*.

2. Materials and methods

2.1. Animals

We collected *S. americana* as nymphs from Brooksville, Pasco County, Florida, in September 2010 and reared them for three generations before the study. This particular population was found in relatively high density in disturbed hay fields. We also collected *S. serialis cubense* from Islamorada in the Florida Keys in January 2011. We found several populations of this species patchily distributed in the Florida Keys in low density. This species is known from the Caribbean and Hispaniola (Harvey, 1981) and our study represents the first report of its distribution in the US. We collected *S. serialis cubense* as adults and reared them for one generation before the study. For the remainder of this paper we refer *S. americana* as *americana* and *S. serialis cubense* as *cubense* for the sake of conciseness. Initially we conducted the experiment on *americana* with successful results and then repeated the experiment on *cubense*.

2.2. Experimental set-up

We reared *americana* and *cubense* from hatchling to last (6th) nymphal instar in two density conditions (isolated and crowded) in order to quantify potential expressions of density-dependent phenotypic plasticity. For the isolated treatment, we placed individual hatchlings in separate inverted plastic cups (11.9 cm diameter bottom to 8.64 cm, height 14.2 cm), which were covered with white paper to keep them physically and visually isolated from each other. We used 30 individuals from different egg pods hatched within the same week. For the crowded treatment, we reared approximately 200 nymphs in a small cage (73,899 cm^3) to stimulate a high-density condition.

The isolated and the crowded treatments were placed in two separate Percival environmental chambers. The grasshoppers in both treatments were reared at 16 h of light at 30 °C and 8 h of darkness at 25 °C and fed daily Romaine lettuce and wheat bran.

2.3. Quantification of phenotypic plasticity

When the nymphs of *americana* and *cubense* were reared in different density treatments and molted to the last nymphal instar, we quantified the effect of rearing density in terms of behavior, color, and morphology.

To quantify behavioral reaction norms, we used the behavioral assay arena designed by Roessingh et al. (1993), which was originally developed to study the behavior of the desert locust, *S. gregaria*. We constructed the arena according to the exact specifications ($57 \times 31 \times 11$ cm) described in Roessingh et al. (1993). Briefly, the arena had a stimulus chamber at each end, one simulating a low-density condition (no grasshoppers) and the other simulating a high-density condition (50 last nymphal instar reared in a crowded condition). A test subject (a grasshopper reared in either density condition) was introduced through a small hole in the center of the arena, and its behavior in response to the stimulus chambers was recorded using a video camera located directly above the arena for 5 min. We used the software EthoVision (Noldus) to video-track the behavior of the test subject.

The arena was split into crowded, neutral, and isolated zones according to their proximity to the appropriate stimulus chambers and the duration of time recorded that the test subject spent in each zone. We also recorded angular velocity (deg/s), distance moved (cm), duration spent in each zone(s), mean meander (deg/cm), duration(s) and frequency immobile, mobile, and highly mobile, turn angle (deg), mean velocity (cm/s), and transition between zones. The frequency of mobility was calculated as the percentage of pixels covering the subject changing over time. In that a subject was considered immobile, if less than 20% of the pixels had changed between timestamps and highly mobile if higher than 80% of the pixels had changed between timestamps. Between 20% and 80%, the subject was considered mobile, which usually indicated walking.

After we quantified behavior, we immediately placed the grasshoppers in a −80 °C freezer to preserve coloration and prevent decomposition. To quantify color reaction norms, we removed individuals from the freezer one at a time and thawed in order to capture a high-resolution digital image of each specimen using the BK+ Imaging System (Visionary Digital). To ensure consistency across all the images captured, we used identical lighting and

camera settings throughout the entire process of digital imaging. We photographed three images of each specimen: a dorsal view of the pronotum, a whole-body lateral view focused on the pronotum and wingpads, and a lateral view of the hind femur. From the captured images, we specifically measured two attributes of color changes that were expressed in response to rearing density: background color and black patterns. The background color refers to the baseline color of a specific body part. For several locust species, the baseline color of an isolated nymph is generally green and that of a crowded nymph is generally yellow, orange, or red (Song, 2005; Song and Wenzel, 2008). The black pattern represents a physiological response to dark-color inducing peptide or His^7-corazonine (Tanaka, 2006). For instance, an isolated nymph generally lacks any distinguishable black patterns, but a crowded nymph develops distinct black patches in the head, thorax, abdomen, and legs (Tanaka, 2001, 2006; Tanaka and Yagi, 1997; Tawfik et al., 1999). These two attributes are shown to be expressed independently from each other although they can change simultaneously in response to change in rearing density (Song, 2005; Song and Wenzel, 2008). To measure background color, we arbitrarily defined a square area (6.7×6.27 mm) above the first sulcus of the dorsal surface of the pronotum and another square area on the dorsal carinula of the hind femur. These squares were cropped and saved as individual image files in Adobe Photoshop CS5, and then the RGB component values of these squares were measured in ImageJ64 (Rasband, 1997–2012). To measure the amount of black patterns, we analyzed both dorsal and lateral views of the entire pronotum, a lateral view of the wing pad, and a lateral view of the hind femur. To do this, we converted each image from a stack to red, green, and blue channels. The red channel was used for measuring black patterns. We selected the area in the images depicting the structures of interest using the polygon selection tool in ImageJ64 and adjusted the threshold to convert the images into binary images to capture only the black patterns. The amount of pixels of the resulting threshold images in a given area of the structures was analyzed as a percent value. To measure the amount of black pattern within an area we subtracted the color pixel value from one, which gave the percent area of the selection that was covered with black pattern.

The high-resolution digital images were also used to quantify morphology. We used a ruler tool in Adobe Photoshop CS5 to calculate the length of the pronotum as well as the length of the hind femur. In addition to measuring the body size, we also counted the number of hairs located on the outer surface of the hind femur. At least in the desert locust, the locusts can detect local population density through these mechanoreceptors (Rogers et al., 2003; Simpson et al., 2001), and it has been shown that the number of hairs differ in the nymphs reared in two density conditions (Rogers et al., 2003). We counted the number of hairs by examining the hind femur under a stereomicroscope.

2.4. Statistics

The raw data for behavior and pronotum/femur length were log-transformed to obtain a normal distribution, and Gibbs test for outliers was used to identify and remove outliers skewing the data in any variable. Color data were standardized because of the difference in scales between color pixel values and black pattern percentage of a given area. To study the effect of species, rearing density, and sex on the overall behavior, color, and morphology, we used a parametric MANOVA based on Anderson (2001) and Sword (2003). All variables were standardized for MANOVA because of the differences in scales among the response variables. We then used ANOVA to test the effect of rearing density in each of the variables (behavior, color, and morphology) we measured in order to test how each contributed to the overall difference. All statistical analyses were performed using SPSSStatistics ver. 21 (IBM).

3. Results

3.1. Behavior

We found that in both *americana* and *cubense* the nymphs reared in a crowded condition tended to be more active and more attracted to the crowded stimulus than those reared in isolation. Behavior data were lost for 10 isolated *cubense* individuals due to experimental error, but we still recovered a strong enough signal to show the difference in behavior between treatments. When all the behavioral variables were analyzed simultaneously, we found a strong effect of density, indicating that rearing density had a major impact on resulting behaviors regardless of species (Table 1). However, we did not find any significant effect of species, suggesting that the density-dependent behavioral reaction norms were similarly expressed in both species. We also did not find any effect of an interaction between species and density. For *americana*, there were statistically significant differences between isolated and crowded treatments in terms of distance moved, heading, velocity, and time spent near the crowded stimulus (Table 2 and Fig. 1). For *cubense*, there were also similar differences between the treatments, specifically in terms of distance moved, heading, mobility, velocity, and time spent near the crowded stimulus (Table 2 and Fig. 1). Although both species responded similarly to rearing density, there were some noticeable differences between the two that we were able to observe. The isolated nymphs of *cubense* moved much less in the arena than the isolated *americana*. The crowded *cubense* moved around with a higher velocity than the crowded *americana*. In terms of the amount of time spent in the arena, both isolated and crowded *cubense* spent most of their time in the neutral zone near the entrance centered in the arena.

3.2. Color

It was visually apparent that both species responded differentially in terms of color and black patterns to change in rearing density (Fig. 2). Qualitatively, the isolated nymphs of both species were generally bright green with little or no black patterns. In *americana*, the crowded nymphs developed deep orange-red background color in the head, thorax, and legs with strong black patterns on the dorsal and lateral parts of the pronotum and abdominal tergites. Veins in the wing pads also developed distinct black patterns and the dorsal half of the hind femora developed two black patches. In *cubense*, the crowded nymphs developed a yellowish-tan background color throughout the body with some black patterns on the pronotum, wing pads, abdomen, and the hind femora. However, the amount of black pattern was less pronounced in *cubense* than in *americana*. There were significant effects of an interaction between species and density both for the background color and black patterns when analyzed simultaneously (Table 1), suggesting that each species responded differently to the change in rearing density. Statistically, we found that there were significant differences between the treatments in both species in terms of background color, when it was quantified using the RGB component values ranging from 0 to 255 for each color channel (Table 3). The background color in both the pronotum and the hind femora changed significantly in response to change in density (Fig. 3). Similarly, there was a significant difference between the treatments in both species in terms of the amount of black patterns (Table 4). The crowded nymphs developed a much higher percentage of black patterns in dorsal and lateral parts of the pronotum, wing pads, and the hind femora (Fig. 3).

Table 1
Parametric MANOVA table showing the effects of species, rearing density, and sex (in case of morphology) on the overall behavioral, color, and morphology of *americana* and *cubense*. ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.005$; $^{***}P \leqslant 0.0005$).

Variable		Source	*df*	SS	MS	*F*-ratio	*P*-value
Behavior		Species	1	0.427	0.427	2.742	0.101
($N = 110$)[a]		Density	1	2.379	2.379	15.260	0.000***
		Species × density	1	0.246	0.246	1.578	0.212
		Residual	106	16.522	0.156		
Color							
	Background	Species	1	0.501	0.501	1.572	0.212
	Color	Density	1	26.740	26.740	83.903	0.000***
	($N = 121$)[b]	Species × density	1	10.118	10.118	31.746	0.000***
		Residual	117	37.289	0.319		
	Black	Species	1	2.326	2.326	47.064	0.000***
	Patterns	Density	1	74.073	74.073	1499.083	0.000***
	($N = 121$)[b]	Species × density	1	3.048	3.048	61.687	0.000***
		Residual	117	5.781	0.049		
Morphology							
	Body Size	Species	1	3.002	3.002	5.888	0.017*
	($N = 122$)[c]	Density	1	7.319	7.319	14.355	0.000***
		Sex	1	42.236	42.236	82.837	0.000***
		Species × density	1	1.011	1.011	1.983	0.162
		Species × sex	1	4.338	4.338	8.508	0.004**
		Density × sex	1	2.529	2.529	4.960	0.028*
		Species × density × sex	1	2.041	2.041	4.004	0.48
		Residual	113	57.616	0.510		
	Hair[d]	Species	1	41.163	41.163	58.622	0.000***
	(N = 122)[c]	Density	1	15.046	15.046	21.428	0.000***
		Sex	1	0.384	0.384	0.546	0.462
		Species × density	1	3.065	3.065	4.366	0.040*
		Species × sex	1	0.007	0.007	0.009	0.924
		Density × sex	1	0.130	0.130	0.186	0.668
		Species × density × sex	1	0.273	0.273	0.389	0.535
		Residual	82	57.578	0.702		

[a] *americana* (30 isolated, 30 crowded); *cubense* (20 isolated, 30 crowded).
[b] *americana* (30 isolated, 30 crowded); *cubense* (31 isolated, 30 crowded).
[c] *americana* (19 isolated ♂, 11 isolated ♀, 16 crowded ♂, 14 crowded ♀); *cubense* (15 isolated ♂, 16 isolated ♀, 11 crowded ♂, 19 crowded ♀).
[d] The number of hairs was compared using ANOVA.

Table 2
ANOVA table showing the differences between isolated and crowded treatments in response to the eight behavioral parameters used for both species. The 10 data points in behavior for isolated *cubense* were lost and not included in the analysis. ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.005$; $^{***}P \leqslant 0.0005$).

americana ($N = 60$)	SS	MS	*F* (1, 58)	*P*-value
Log angular velocity (deg/s)	0.062	0.062	0.296	0.589
Log distance moved (cm)	0.649	0.0649	9.371	0.003**
Log heading (degrees)	4.581	4.581	21.665	0.000***
Log mobile (s)	0.017	0.017	0.054	0.817
Log velocity (cm/s)	0.408	0.408	8.492	0.005**
Log isolated zone (s)	0.208	0.208	2.640	0.110
Log neutral zone (s)	0.072	0.072	0.372	0.545
Log crowded zone (s)	1.006	1.006	15.426	0.000***
cubense ($N = 50$)	SS	MS	*F* (1, 48)	*P*-value
Log angular velocity (deg/s)	0.100	0.100	0.314	0.578
Log distance moved (cm)	0.577	0.577	14.035	0.000***
Log heading (deg)	2.232	2.232	11.810	0.001**
Log mobile (s)	3.145	3.145	6.009	0.018*
Log velocity (cm/s)	0.628	0.628	13.935	0.001**
Log isolated zone (s)	0.120	0.120	0.104	0.749
Log neutral zone (s)	0.037	0.037	0.078	0.782
Log crowded zone (s)	5.849	5.849	5.534	0.023*

3.3. Morphology

Overall, we found significant effects of species, density, sex, the interaction between species and sex, and the interaction between density and sex on the resulting size when two size variables were analyzed simultaneously (Table 1). We found that the rearing density had variable effects on the resulting size depending on the species and the sex (Fig. 4 and Table 5). In *americana*, the density had little impact on the size of males, but had a statistically significant effect on the size of females in which the crowded female nymphs had a smaller pronotum and shorter hind femora than the isolated ones. In *cubense*, however, there was no statistically significant effect of rearing density on the two measured variables regardless of sex, although in general isolated nymphs appeared to be larger than the crowded nymphs. We also found that there were more mechanoreceptor hairs on the outer face of the hind femora in the isolated nymphs in both sexes of *americana* and the females of *cubense* (Fig. 4 and Table 5). In *americana*, the number of hairs in the crowded nymphs was more than twice as many as in the isolated nymphs. In *cubense*, there was a less pronounced difference between the two treatments compared to *americana*. To test whether the number of hairs was simply correlated with the femur size, we normalized the data by dividing by the femur length. We found that crowding had a statistically significant influence in the development of mechanoreceptor hairs in *americana* and the female *cubense* and this pattern was not affected by the variation in size (Fig. 4).

4. Discussion

4.1. Behavioral plasticity in non-swarming Schistocerca

In this study, we clearly demonstrate that two non-swarming *Schistocerca* species express density-dependent phenotypic plasticity in behavior, color, and morphology. In terms of behavior, both *americana* and *cubense* were more active and more attracted to each other when reared in a crowded condition than in isolation. The observed behavioral patterns were reminiscent of how the

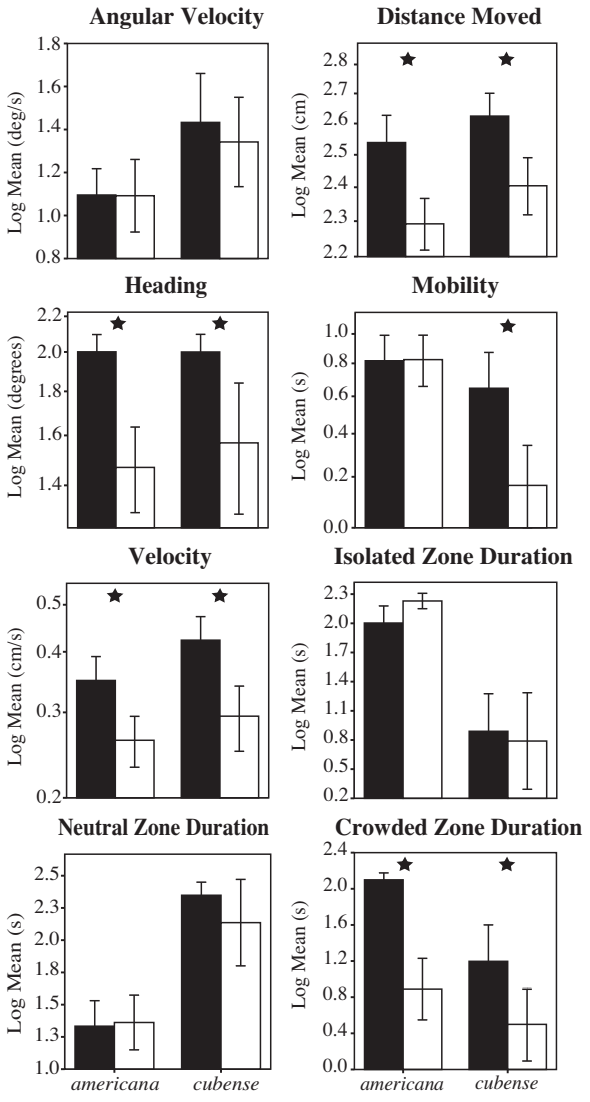

Fig. 1. Behavioral reaction norms of *americana* and *cubense* to rearing density conditions as quantified by eight behavioral parameters. Black bars represent the crowded condition and white bars represent the isolated condition for both species. Error bars show ±2 standard error. Stars represent statistical significance.

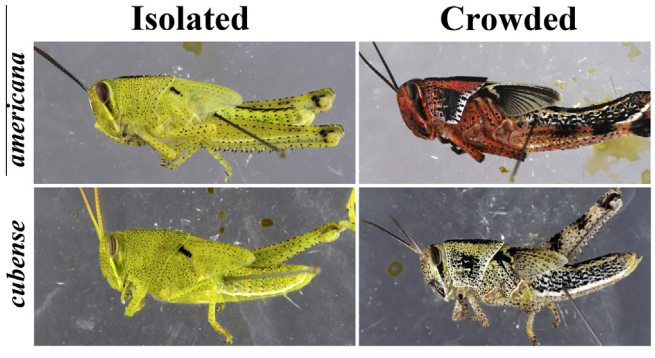

Fig. 2. Lateral views of last nymphal instar of *americana* (top) and *cubense* (bottom), reared in isolation (left) and in a crowded condition (right). This figure qualitatively shows the extreme density-dependent plastic reaction norm in nymphal coloration.

desert locust would behave in response to change in density (Simpson et al., 1999). Roessingh et al. (1993) experimentally demonstrated that the nymphs of the desert locust repelled each other when reared in isolation, but were attracted to each other when crowded. The presence of density-dependent behavioral plasticity

Table 3
ANOVA table showing the differences in color measured for areas of the body using RGB values between isolated and crowded density treatments for both species. (*$P \leqslant 0.05$; **$P \leqslant 0.005$; ***$P \leqslant 0.0005$).

americana ($N = 60$)	SS	MS	$F(1, 58)$	P-value
Log red pronotum	1.008	1.008	228.106	0.000***
Log red hind femur	0.233	0.233	130.943	0.000***
Log green pronotum	1.827	1.827	221.090	0.000***
Log green hind femur	0.722	0.722	196.962	0.000***
Log blue pronotum	0.960	0.960	71.379	0.000***
Log blue hind femur	0.669	0.699	96.804	0.000***
Log dorsal background color	1.259	1.259	205.119	0.000***
Log leg background color	0.472	0.472	107.753	0.000***
cubense ($N = 61$)	SS	MS	$F(1,59)$	P-value
Log red pronotum	0.002	0.002	2.873	0.095
Log red hind femur	0.038	0.038	33.250	0.000***
Log green pronotum	0.013	0.013	12.282	0.001**
Log green hind femur	0.211	0.211	69.670	0.000***
Log blue pronotum	1.078	1.078	11.998	0.001**
Log blue hind femur	1.529	1.529	7.111	0.010*
Log dorsal background color	0.004	0.004	2.440	0.124
Log leg background color	0.029	0.029	11.557	0.001**

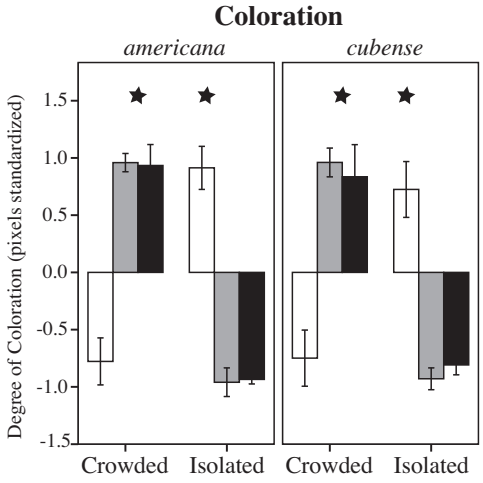

Fig. 3. Color reaction norms of *americana* and *cubense* to rearing density conditions as measured in background coloration and black patterns. The *y*-axis shows degree of coloration as measured by standardized pixel values. The white bars represent the degree of background coloration as measured in green in which positive values indicate brighter green and negative values indicate dark color. The grey and black bars represent the degree of black patterns on the dorsal side of the pronotum and the degree of black patterns on the wing pads, respectively. For both, positive values indicate more black patterns and negative values indicate less. Error bars show ±2 standard error. Stars represent statistical significance.

Table 4
ANOVA table showing the differences in the amount of black patterns in different body parts between isolated and crowded density treatments for both species. (*$P \leqslant 0.05$; **$P \leqslant 0.005$; ***$P \leqslant 0.0005$).

americana ($N = 60$)	SS	MS	$F(1, 58)$	P-value
Dorsal pronotum	2.243	2.243	670.369	0.000***
Lateral pronotum	2.695	2.695	1021.143	0.000***
Wing pad	3.345	3.345	397.058	0.000***
Hind femur	0.647	0.647	265.895	0.000***
cubense ($N = 61$)	SS	MS	$F(1, 59)$	P-value
Dorsal pronotum	1.572	1.572	580.465	0.000***
Lateral pronotum	1.295	1.295	439.539	0.000***
Wing pad	0.661	0.661	129.187	0.000***
Hind femur	0.598	0.598	429.031	0.000***

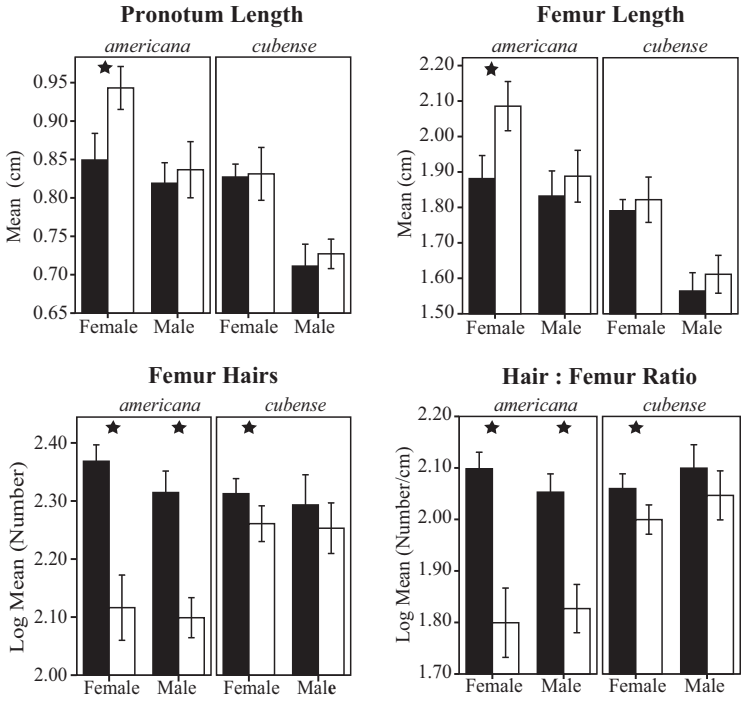

Fig. 4. Morphological reaction norms of *americana* and *cubense* to rearing density conditions as quantified by pronotum length, femur length, the number of femur hairs, and the ratio between the number of femur hairs and femur length. The measurements were further divided by sex because the two species are known to be sexually dimorphic in size. Black bars represent the crowded condition and white bars represent the isolated condition for both species. Error bars show ±2 standard error. Stars represent statistical significance.

Table 5
ANOVA table showing the differences in morphology in different body parts between isolated and crowded density treatments for both species. ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.005$; $^{***}P \leqslant 0.0005$).

Morphology	Source	*df*	SS	MS	*F*-ratio	*P*-value
americana[a]						
Pronotum length	Density	1	0.045	0.045	10.745	0.002**
(*N* = 60)	Sex	1	0.067	0.067	16.121	0.000***
	Sex × density	1	0.021	0.021	5.013	0.029*
	Residual	56	0.233	0.004		
Femur length	Density	1	0.240	0.240	12.526	0.001**
(*N* = 59)	Sex	1	0.216	0.216	11.249	0.001**
	Sex × density	1	0.078	0.078	4.053	0.049*
	Residual	55	1.054	0.019		
Log femur hairs	Density	1	0.637	0.637	127.768	0.000***
(*N* = 51)	Sex	1	0.015	0.015	2.962	0.092
	Sex × density	1	0.004	0.004	0.778	0.382
	Residual	47	0.234	0.005		
cubense[b]						
Pronotum length	Density	1	0.001	0.001	0.591	0.445
(*N* = 62)	Sex	1	0.180	0.180	71.300	0.000***
	Sex × density	1	0.001	0.001	0.208	0.650
	Residual	58	0.146	0.003		
Femur length	Density	1	0.025	0.025	3.793	0.056
(*N* = 62)	Sex	1	0.723	0.723	108.181	0.000***
	Sex × density	1	0.001	0.001	0.078	0.781
	Residual	58	0.388	0.007		
Log femur hairs	Density	1	0.031	0.031	6.178	0.016*
(*N* = 62)	Sex	1	0.003	0.003	0.540	0.465
	Sex × density	1	0.000	0.000	0.098	0.755
	Residual	58	0.295	0.005		

[a] *americana* (19 isolated ♂, 11 isolated ♀, 16 crowded ♂, 14 isolated ♀).
[b] *cubense* (15 isolated ♂, 16 isolated ♀, 11 crowded ♂, 19 crowded ♀).

had not been shown in other *Schistocerca* species until Sword (2003) used a similar method to measure behavioral plasticity in *americana*. He showed that the behavior of *americana* was only slightly affected by high rearing density, but the magnitude of the behavioral change was reduced compared to that of the desert locust. He used individuals collected from Texas and North Carolina for his experiments and found that there were population-level differences in behavior at least in the first instar, which became indistinguishable in the final instar. Of the several behavioral parameters that he measured, only climb time (proportion of time spent climbing the stimulus chamber walls during the assay) increased significantly in response to high density. Our findings, however, differ considerably from what Sword (2003) reported in that we found more substantial behavioral differences between two density treatments in *americana*. This difference may be due to population-level differences in genetic variation in behavior for differentially responding to density. Sword (2003) speculated that the density conditions experienced by the parental generation could have contributed to the expression of behavioral plasticity in the offspring. It has been well documented in the desert locust that crowding in parents have a major influence in promoting gregarious behavior in the offspring (Islam et al., 1994a,b; Simpson and Miller, 2007), and it is reasonable to suspect that there may be parental effects in the expression of behavioral plasticity in *americana* as well. The grasshoppers that Sword (2003) used were collected from low-density, non-outbreak populations in North Carolina and Texas. The grasshoppers used in our study, on the other hand, were collected from unusually dense populations in Pasco County, Florida. If the effects of parental density can be transmitted to the offspring in *americana* similar to what has been reported in the desert locust, it would be possible to suspect that the differences in density at the source populations might have contributed to the differences between Sword's (2003) study and ours.

We demonstrate the presence of density-dependent behavioral plasticity in *cubense* for the first time. Although both *americana* and *cubense* strongly responded to high rearing density, we noted that there were some species-specific differences not obviously captured by the behavioral assay. For *cubense*, the test individual

would remain stationary for a very long time when initially introduced to the arena and noticed that in general *cubense* was not as active as *americana*. In other words, the two species differ in behavior in magnitude, just as the behavior of the desert locust and *americana* differ in magnitude (Sword, 2003). It has been reported that the population of *americana* can build up to an outbreak proportion (Kuitert and Connin, 1952), but it is not clear whether the population of *cubense* can become dense in nature. Not much is known about the biology of *cubense*, but when we collected the individuals in the field, the local population density was not particularly high.

4.2. Color plasticity in non-swarming Schistocerca species

Within *Schistocerca*, a wide range of variation is known to exist across species in terms of their expression of density-dependent color plasticity (Song and Wenzel, 2008). At one extreme are the desert locust, the Central American locust (*Schistocerca piceifrons* (Walker)) and the South American locust (*Schistocerca cancellatau* (Serville)), all of which exhibit classic color polyphenism associated with locust phase polyphenism (Barrientos-Lozano, 2002; Bruch, 1939; Harvey, 1983; Hunter-Jones, 1967; Jago et al., 1982; Pener and Simpson, 2009; Waloff and Pedgley, 1986). At the other extreme are sedentary grasshopper species that do not appear to respond to change in density. For example, *Schistocerca pallens* (Thunberg) which is closely related to the South American locust, is reported to be unaffected by crowding in terms of nymphal coloration (Antoniou and Robinson, 1974). Not much is known about density-dependent color plasticity in other sedentary *Schistocerca* species, but for those that have been studied, their responses to density changes lie somewhere between the two extremes. For instance, the nymphs of *Schistocerca obscura* (Fabricius) and *Schistocerca flavofasciata* (De Geer) develop black patterns when crowded (Duck, 1944; Kevan, 1943), while the nymphs of *Schistocerca vaga* (Scudder) turn brown (Rowell and Cannis, 1971). A North American species, *Schistocerca lineata* Scudder, exhibits extreme population-level variation in the expression of color polyphenism. Sword (2002) showed that two populations of *S. lineata*, one feeding on palatable *Rubus* and another feeding on toxic *Ptelea*, change nymphal coloration in response to density, but differ in terms of degree of color change. Specifically, the nymphs that are associated with *Ptelea* develop striking black and yellow patterns when crowded, which represents a spectacular example of density-dependent aposematism (Sword, 1999).

In this study, we add two more species of *Schistocerca* that show density-dependent color plasticity. In both *americana* and *cubense*, we find drastic differences in background color and black patterns between the two rearing densities (Figs. 2 and 3). When isolated, the nymphs of both species stayed green or yellow with little or no black patterns, but they developed large areas of black patterns in the pronotum, wing pads, and hind femora when crowded. While both species clearly responded to high density, there were some noticeable species-specific differences in which the background color of *americana* was much more red than that of *cubense* (Fig. 2). This indicates that the two color patterns that are often associated with crowding (background color and black patterns) may evolve independently from each other, as suggested by Song and Wenzel (2008).

While we clearly demonstrate the existence of density-dependent color plasticity in *americana*, there was an earlier study that found a contradictory result, which we feel is important to discuss here. Tanaka (2004) examined the effect of environmental factors on nymphal coloration in *americana* and reported that low temperature (30 °C) was the most influential in inducing black patterns. In the present study, the temperature regime we used was 30 °C during the day (16 h) and 25 °C at night (8 h). As such our study is somewhat comparable to Tanaka's (2004) study, but we did not find much of black patterns developing in the isolated grasshoppers. What we did observe was that there was a large amount of variation in terms of black patterns and background coloration in the isolated nymphs. None of the isolated nymphs developed the full amount of black patterns that was typically observed in the crowded nymphs. However, the amount of black patterns in the isolated nymphs ranged from nearly none to a few speckles, to some noticeable patterns, on the abdomen and hind femora. Moreover, we did observe a large amount of variation in background color. While many nymphs were green, there were some that were yellow, tan, or even bright pink. Our isolated nymphs were initially from the egg pods laid by female reared in a crowded condition. If phase-related traits can be transmitted across generations in *americana*, as in the desert locust (Simpson and Miller, 2007), it is conceivable that some of these characteristics might have been transmitted to the offspring. In that sense, the presence of some black patterns in the isolated nymphs was not surprising, but rather expected. Although we did not compare among other temperature regimes, we did not observe any pattern that would suggest that low temperature was the main cause of inducing black patterns. In Tanaka's (2004) study the effect of density was tested by rearing the nymphs at different densities, including 1, 2, 5, and 30 individuals per cage. He did not find significant differences amongst different densities and concluded that crowding had only a moderate effect on inducing black patterns. Our study is different from Tanaka's (2004) study in that the crowded nymphs were reared in a much higher density (more than 200 individuals in a cage). We think that the different density treatments that Tanaka (2004) used were not sufficient enough to induce density-dependent color plasticity and our data strongly indicate that crowding in fact has a major role in inducing color change.

4.3. Morphological plasticity in non-swarming Schistocerca

In this study, we measured two linear variables (pronotum length and femur length) to test the effect of rearing densities in resulting size of the grasshoppers and found that there were species-specific and sex-specific responses. In the desert locust, solitarious individuals are larger than gregarious ones in females, but slightly smaller than gregarious ones in males (Uvarov, 1966). In the present study, we found that isolated female *americana* were larger than the crowded females, but did not find the same pattern in males. In *cubense*, there was no statistically significant effect of rearing density on the resulting size. However, when both variables were collectively analyzed, we did observe a strong effect of rearing density on size regardless of the species (Table 2). The effect of crowding and isolation on morphology is known to take several generations in the desert locust (Pener and Simpson, 2009). However, our findings, which were based on the effect of rearing density within the lifespan of the test subjects, suggest that even a short-term isolation could have a clear effect on the resulting size. Our findings represent the first demonstration that non-swarming *Schistocerca* species do exhibit density-dependent phenotypic plasticity in size.

In the desert locust, tactile stimulus alone can elicit a very strong shift in nymphal behavior from solitarious to gregarious phase (Roessingh et al., 1998) and Simpson et al. (2001) identified the mechanoreceptors on hind femora as the main site for tactile stimulation. Subsequently, Rogers et al. (2003) found that isolated nymphs of the desert locust had about 30% more mechanoreceptor hairs on the hind femora than the crowded nymphs, but also noted that they had similar or fewer hairs on other hind leg segments and other legs. In our study, we found an opposite pattern in which the crowded nymphs of both species had more mechanoreceptor hairs than the isolated ones (Fig. 4), about 50% for *americana* and 10% for

cubense. The difference was more pronounced in *americana*, which exhibited more dramatic changes in behavior, color, and size. These findings on morphology represent another novel discovery of density-dependent phenotypic plasticity in the sedentary *Schistocerca* species. It is curious why an opposite pattern is found in these non-swarming species. It is also unclear whether the mechanoreceptor hairs on the hind femora of *americana* and *cubense* are the main sites for sensing local population density as in the desert locust because the functional morphology has not yet been studied. We plan to pursue this line of research in the future.

4.4. Evolutionary implications

In this study, we report that two non-swarming *Schistocerca* species exhibit density-dependent phenotypic plasticity in behavior, color, and morphology, reminiscent of the desert locust. In the desert locust, several phase-related traits have been shown to be adaptive (Despland et al., 2000; Despland and Simpson, 2005a,b; Simpson and Sword, 2009; Sword, 2002; Sword et al., 2000). For example, density-dependent color change has been shown to be an effective anti-predatory strategy in the early stage of swarm formation (Sword and Simpson, 2000; Sword et al., 2000), especially when it is coupled with preferential feeding on toxic plants (Sword, 1999, 2002). Life-history traits that change in response to changes in density, including maturation period, longevity, and reproductive potential, are also shown to be adaptations against rapidly changing environments (Simpson and Sword, 2009). However, the two species in our study do not form swarms in nature. For *americana*, which can occasionally develop into ahigh population density to cause minor outbreaks (Kuitert and Connin, 1952), it is conceivable that some of the density-dependent reaction norms may be adaptive, but this idea has not been tested explicitly. Not much is known about the ecology of *cubense*, but our field observations suggest that they occur at low to medium density with little opportunities for population build-up, which suggests that the above adaptive explanations may not necessarily apply to this species. Whereas the desert locusts are found in places where the availability of resources fluctuates (Despland, 2003; Despland et al., 2004), *americana* and *cubense* in Florida find no shortage of vegetation or resources, and do not necessarily live in rapidly changing environments. Phenotypic plasticity is usually considered an adaptation to heterogeneous environmental conditions (Schlichting and Pigliucci, 1998), but it is difficult to imagine how density-dependent phenotypic plasticity might have evolved *de novo* in these two species given the environment that they are found. Can there be another explanation for why these species exhibit density-dependent phenotypic plasticity?

Perhaps, the answer lies in their evolutionary relationships with the desert locust. According to recent molecular phylogenetic studies (Lovejoy et al., 2006; Song et al., 2013), the desert locust is the earliest branching lineage within *Schistocerca*, which would indicate that the expression of density-dependent phenotypic plasticity is a plesiomorphic trait for the genus. As such, the two non-swarming *Schistocerca* species may be expressing density-dependent reaction norms because they are phylogenetically conserved (Song, 2005; Song and Wenzel, 2008). To be more explicit, it may be that these non-swarming species already have the genetic capacity to respond to different density conditions due to their shared ancestry (Lovejoy et al., 2006; Sword, 2003), but normally do not live in an environment in which the full manifestation of phenotypic plasticity is rare. However, when experimentally crowded, the 'hidden' density-dependent phenotypic plasticity can be revealed. While the presence of density-dependent phenotypic plasticity in non-swarming species can be explained by phylogenetic conservatism, it is curious that the expression of phenotypic plasticity in these species is actually somewhat different from that in the desert locust and also from each other. Especially, those traits that have a physiological basis such as color, size, and the number of hairs on the hind femora show distinctly different patterns compared to the desert locust. For example, it is not clear why crowded *cubense* nymphs develop black patterns, but without bright coloration in the background color, as in *americana* or in the desert locust. Song and Wenzel (2008) showed that individual reaction norms of locust phase polyphenism evolved separately from each other in several species of locusts, which suggests that these individual reaction norms, whether it is behavior, color, morphology, or any life history traits, have different evolutionary trajectories that can be shaped by either selection or drift (Song and Wenzel, 2008; Sword et al., 2000). It is also possible that these ancestral reaction norms may become adaptive in a completely different context, as shown in the case of *S. lineata*, which exhibits density-dependent aposematism (Sword, 1999). Thus, what we observe in *americana* and *cubense* may be the result of different evolutionary processes shaping the individual components of ancestral density-dependent phenotypic plasticity.

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