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The role of the neuropeptide [His^7]-corazonin on phase-related characteristics in the Central American locust

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ABSTRACT

Density-dependent phase polyphenism in locusts is one of the most extreme forms of phenotypic plasticity. Locusts exist along the continuum between two density-dependent phenotypes that differ in nymphal coloration, behavior, morphology, physiology, and reproduction among others. Nymphs of the solitarious phase, found in low population densities, are usually green, relatively inactive, and avoid each other, while gregarious nymphs, found in high density, exhibit a very obvious yellow/orange background with black patterning, and are highly active and attracted to each other. The multifunctional neuropeptide [His^7]-corazonin has been shown to strongly affect black coloration and several other phase-related characteristics in at least two locust species, even though no effect on phase-related behavioral traits has been found. In this study, we investigate the role of [His^7]-corazonin in the Central American locust *Schistocerca piceifrons* (Walker), which evolved density-dependent phase polyphenism independently from the two previously studied locust species. After successfully knocking down the transcript encoding [His^7]-corazonin (*CRZ*) using RNA interference, we show that such a knockdown influences both color and morphometrics in this species, but does not influence phase-related behavioral traits. Our results suggest that the role of [His^7]-corazonin is conserved in different locust species. Finally, our study represents the first controlled study of behavioral solitarization in *S. piceifrons*.

1. Introduction

Density-dependent phase polyphenism in locusts, also known as locust phase polyphenism, is one of the most intriguing examples of coordinated phenotypic plasticity (West-Eberhard, 2003). Locusts exist along a continuum between two distinct density-dependent phenotypes called the solitarious phase and the gregarious phase, which are respectively found under low and high population densities (Uvarov, 1966). Individuals of different phases can be morphologically and behaviorally so different from one another that they were in some cases considered to be two different species before Sir Boris Uvarov discovered the existence of phase (Uvarov, 1921). Density-dependent phase polyphenism is all-encompassing and influences almost every aspect of the locusts' life, including but not limited to color, behavior, morphology, reproduction, physiology, molecular biology, and life history traits (Cullen et al., 2017; Pener, 1991; Pener and Simpson, 2009; Pener and Yerushalmi, 1998; Uvarov, 1966, 1977). Of these, the behavioral differences between both phases are highly relevant in making locusts such devastating pests. Gregarious individuals are highly active and are attracted to each other (Ellis, 1963; Gray et al., 2009; Guo et al., 2011; Pocco et al., 2019; Roessingh et al., 1993; Simpson et al., 1999; Uvarov, 1966), eventually forming massive migrating swarms that can cause tremendous economic damages and food security challenges (Cullen et al., 2017; Pener and Simpson, 2009; Simpson et al., 1999). In contrast, solitarious locusts tend to avoid each other and are rather inactive overall (Cullen et al., 2017; Ellis, 1963; Gray et al., 2009; Guo et al., 2011; Hoste et al., 2002b; Pener and Simpson, 2009; Pocco et al., 2019; Roessingh et al., 1993; Simpson et al., 1999; Uvarov, 1966). Surprisingly, the behavior of these locusts can change very quickly, depending on the local population densities. For instance, a solitarious nymph of the desert locust (*Schistocerca gregaria*) can completely transform behaviorally to a gregarious nymph in less than four hours when local population densities increase (Roessingh and Simpson, 1994; Rogers et al., 2014). In contrast, the reverse process of solitarization in *S. gregaria* is much slower and takes more than 96 h to complete (Roessingh and Simpson, 1994). Both solitarization and gregarization might be highly species-specific, as in the migratory locust *Locusta migratoria* solitarization occurs very quickly, within a few hours, while

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gregarization is a much slower process that might take even longer than the duration of a complete nymphal instar (Guo et al., 2011, 2013, 2015; Ma et al., 2011, 2015).

Changes in nymphal coloration are the most visible aspect of locust phase polyphenism, and in fact, it is one of the most frequently used traits for determining the phase status by locust control officers in the field (Steedman, 1990). Solitarious nymphs are generally green, but can exhibit different colors due to humidity-dependent homochromy, making them blend in easily their environment (Pener and Simpson, 2009; Tanaka et al., 2012; Uvarov, 1966). In contrast, their gregarious counterparts often exhibit an obvious yellow or orange background coloration with black patterning (Pener and Simpson, 2009; Uvarov, 1966). The adaptive function of gregarious coloration is still debated, but it most likely serves as aposematic coloration (Despland and Simpson, 2005a, 2005b; Simpson and Sword, 2009; Sword, 1999, 2001; Sword and Simpson, 2000; Wei et al., 2019). There is strong evidence that color and behavior may not be directly correlated, at least because the behavioral changes occur at a much faster rate than the changes in color. As such, it is possible to have gregarious-looking nymphs that are behaviorally solitarious, and solitarious-looking nymphs that are behaviorally gregarious. As a result, sole reliance on nymphal coloration to determine phase status is flawed, but it is nonetheless a practical tool for a quick assessment in the field. Because of this practical utility, much attention has been paid to the mechanisms underlying the development of black patterns in gregarious nymphs (Tanaka, 1993; Tanaka and Pener, 1994; Tanaka and Yagi, 1997; Tawfik et al., 1999).

It has long been known that implantation of the corpora cardiaca (CC) from gregarious nymphs into solitarious nymphs induced black pigmentation (Pener, 1991), but the identification of the specific darkening factor was difficult due to the lack of a proper bioassay. Hasegawa and Tanaka (1994) discovered an albino mutant of *L. migratoria* from Okinawa caused by the deficiency of a peptide present in the central nervous system and the CC, and implantation of the CC from a normal locust into these albinos induced obvious darkening (Tanaka, 1993; Tanaka and Pener, 1994; Tanaka and Yagi, 1997). These findings suggested that the albino mutant of *L. migratoria* could be used to identify the specific peptide(s) that control black pigmentation. Tawfik et al. (1999) used high performance liquid chromatography (HPLC) and the albino strain of *L. migratoria* as an assay to identify a dark-color-inducing neurohormone (DCIN) in *L. migratoria* and *S. gregaria*. The neuropeptide consisted of 11 amino acids and was shown to be identical to [His^7]-corazonin (Crz), which was previously isolated from the CC of *Schistocerca americana* without a known function (Veenstra, 1991). Crz seemed to be highly conserved across insects, as the implantation of brain-CC complexes from 47 species from 10 different insect orders induced darkening in the albino strain of *L. migratoria* (Tanaka, 2000a). In addition to the work done in locusts, [His^7]-corazonin has been studied in various other insects, establishing it as a multifunctional neuropeptide that can influence heart rate, stress response, reproduction, molting, pigment migration, and metabolism (Boerjan et al., 2010; Kapan et al., 2012; McClure and Heberlein, 2013; Pener and Simpson, 2009; Siangcham et al., 2013; Tayler et al., 2012; Veenstra, 1989). In grasshoppers, it was shown early on that the darkening effect represented a highly conserved characteristic of the neuropeptide, as injections of [His^7]-corazonin induced darkening in a large number of species in the suborder Caelifera (Tanaka, 2000a, 2004; Yerushalmi and Pener, 2001). In addition, Crz affects size, morphometric ratios (Hoste et al., 2002b; Maeno et al., 2004; Sugahara et al., 2015, 2016; Tanaka et al., 2002) and the number of antennal sensilla (Maeno and Tanaka, 2004; Yamamoto-Kihara et al., 2004) in both *L. migratoria* and *S. gregaria*. Similar to the black coloration, these characteristics are known to differ between solitarious and gregarious individuals (Dirsh, 1953; Greenwood and Chapman, 1984; Heifetz et al., 1994; Ochieng et al., 1998; Pener and Simpson, 2009; Uvarov, 1966). These findings collectively suggested that Crz had a gregarizing nature for certain phenotypic traits in locusts. This notion was further confirmed by knockdowns of the transcript coding for [His^7]-corazonin (*CRZ*) in gregarious nymphs of both *S. gregaria* and *L. migratoria*, which resulted in a complete loss of black patterning and more solitarious morphometric values (Sugahara et al., 2015, 2016). However, Crz cannot be the predominant regulator of locust phase polyphenism, as injections of this neuropeptide did not significantly alter other phase-related characteristics including the duration of nymphal development (Maeno et al., 2004; Tanaka et al., 2002), reproduction (Boerjan et al., 2010), offspring characteristics (Hamouda and Tanaka, 2016), and phase-related behavior (Hoste et al., 2003, 2002a, 2002b).

Until now, the effect of [His^7]-corazonin on locust phase polyphenism has been investigated in only two locust species, *L. migratoria* and *S. gregaria*. However, locust phase polyphenism has evolved independently several times (Song, 2011; Song et al., 2017), and differences in the proximate mechanisms of phase change in different locust species suggest that their molecular underpinnings might also differ (Cullen et al., 2017, 2010; Guo et al., 2011; Pener and Simpson, 2009; Roessingh et al., 1998; Roessingh and Simpson, 1994; Rogers et al., 2003). The genus *Schistocerca* represents a natural experiment of the evolution of locust phase polyphenism, making it a particularly attractive study system for evaluating the conservation of its regulatory mechanisms. The genus contains three different locust species, of which *S. gregaria*, the only species with an Old World distribution, is basal to the rest of the genus (Song et al., 2017). The two other locusts, the South American locust (*Schistocerca cancellata*) and the Central American locust (*Schistocerca piceifrons*), are found in two different clades than the desert locust (Song et al., 2017). They are thought to have descended from a *gregaria*-like ancestor and to have subsequently lost and convergently regained their locust characteristics (Song et al., 2017). Thus, the three species in this genus essentially represent three independent origins of locust phase polyphenism, even though some degree of conservation of their molecular machinery can be expected.

In this study, we characterized the effect of [His^7]-corazonin on phase-related traits in the Central American locust, a major pest species that regularly swarms in Mexico and Central America. It exhibits clear locust phase polyphenism in the field with both phases exhibiting the expected behavioral traits of a locust, even though its behavior has never been compared to *S. gregaria* or indeed studied in detail in a laboratory environment (Barrientos Lozano et al., 1992; Bredo, 1963; Cullen et al., 2017; Harvey, 1983). As in other locust species, the ratio between hind femur length and head width (F/C ratio) is a reliable predictor of phase change in *S. piceifrons* (Harvey, 1983; Hunter-Jones, 1967). Additionally, crowd-reared males in this species are larger than their isolated-reared counterparts, but no differences in size were found for females (Hunter-Jones, 1967). Finally, in terms of nymphal coloration, solitarious nymphs of *S. piceifrons* are green or tan, while gregarious nymphs have a peach-red background with black patterns (Harvey, 1983). Although *S. piceifrons* is congeneric to *S. gregaria*, the gregarious nymphal coloration is quite different between the two species (Harvey, 1981), and the physiological mechanisms of locust phase polyphenism in *S. piceifrons* are largely unknown, unlike the well-studied *S. gregaria*. Based on the conservation of Crz-induced changes in *S. gregaria* and *L. migratoria* (Hoste et al., 2002b; Maeno and Tanaka, 2004; Maeno et al., 2004; Sugahara et al., 2015, 2016; Tanaka, 2000b; Tanaka et al., 2002; Tawfik et al., 1999; Yamamoto-Kihara et al., 2004) and the high level of conservation of the darkening effect of Crz across Caelifera (Tanaka, 2000a, 2004; Yerushalmi and Pener, 2001), we expected that a knockdown of *CRZ* mRNA expression using RNA interference (RNAi) in *S. piceifrons* would result in similar changes as observed in other locust species. Nonetheless, we have chosen to revisit the hypothesis that [His^7]-corazonin does not influence behavior in locusts for multiple reasons. First, previous behavioral studies on the effect of [His^7]-corazonin on behavior predate the development of RNAi, limiting experiments to testing the effect of injections of Crz or comparing strains that lack functional [His^7]-corazonin-signaling to wild-type strains (Hoste et al., 2003, 2002a, 2002b). Second, the effect of *CRZ* on the process of

solitarization has, to our knowledge, never been considered. Finally, corazonin was recently shown to control caste identity and to directly influence the expression of behavioral traits associated with the different castes, including hunting behavior, in the ant *Harpegnathos saltator* (Gospocic et al., 2017), indicating that the neuropeptide has the potential to impact phenotypically plastic behavioral traits. Thus, we hypothesized that a knockdown of *CRZ* mRNA expression in crowd-reared nymphs would result in (1) a complete loss of black patterning, (2) more solitarious morphometric values, and finally (3) altered gregarious behavior, a faster solitarization, or both.

2. Materials and methods

2.1. Study insects

Specimens of *S. piceifrons* were originally collected from an outbreak population in Yucatan, Mexico in September 2015 and imported under a USDA permit (USDA APHIS PPQ P526P-15-03851). Locusts were maintained at a USDA-approved quarantine facility of the Department of Entomology at Texas A&M University. They were kept at 30 °C with a 12 h light cycle, and were fed daily Romaine lettuce and wheat bran. They were maintained at high rearing densities, with over 200 individuals in a cage (40.6 × 34.3 × 52 cm).

2.2. Production of dsRNA

The gene-specific portions of the primers used for production of dsRNA for [His^7]-corazonin (ds*CRZ*) were originally designed by Sugahara et al. (2015) for use in *S. gregaria*, while the gene-specific portion of the primers for the Green Fluorescent Protein (ds*GFP*) were previously described by Qu et al. (2012). A T7 promotor sequence (taatacgactcactatagggaga) was added to the 5′ end of each of these gene-specific portions (Table 1). As template for ds*CRZ* production, we used cDNA from *S. piceifrons* which was generated as previously described (Foquet and Song, 2020; Wang et al., 2020). Briefly, RNA was extracted from crowd-reared last instar nymphs using a Trizol-chloroform extraction, followed by clean-up with a RNeasy mini kit (Qiagen) using an on-column DNAse treatment with a RNase-free DNAse set (Qiagen). RNA concentrations were measured with a Denovix DS-11 spectrophotometer; 260/280 and 260/230 values were above 2 for all used samples. RNA was diluted to a concentration of 100 ng/µl and subsequently used to produce cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's guidelines. A GFP-expressing plasmid DNA (Altogen biosystems) was used as template for ds*GFP* production.

We subsequently set up a two-step PCR reaction with Platinum *Taq* polymerase (ThermoFisher Scientific) following manufacturer's guidelines with the following thermal cycling protocol: 2 min at 94 °C, 10 cycles of: (1) 30 s at 94 °C, (2) 30 s at 60 °C, (3) 1:30 min at 72 °C, followed by 25 cycles of: (1) 30 s at 94 °C, (2) 30 s at 69 °C, (3) 1:30 min at 72 °C, ending with 2 additional minutes at 72 °C. 5 µL of PCR product was run on a 1% agarose gel with gelred (Biotum) and a 100 bp-ladder to confirm primer specificity and product length. The amplified fragment was confirmed as T7-*CRZ* by Sanger sequencing. The amplicon was used as a template for a second PCR reaction, using the following thermal cycling protocol: 2 min at 94 °C, 29 cycles of: (1) 30 s at 94 °C, (2) 30 s at 69 °C, (3) 1:30 min at 72 °C, and finally 2 min at 72 °C. The resulting PCR product was cleaned and concentrated using a Monarch PCR & DNA cleanup kit (New England Biolabs) following the manufacturer's guidelines. dsRNA was generated with a MEGAscript RNAi kit (ThermoFisher scientific) following the manufacturer's guidelines. dsRNA was diluted to 20 ng/µL in locust saline (1 L: 8.766 g NaCl; 0.188 g CaCl2; 0.746 g KCl; 0.407 g MgCl2; 0.336 g NaHCO3; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) for injection.

2.3. Testing dsRNA knockdown efficiency

To test knockdown efficiency of the generated ds*CRZ*, four females of the last nymphal instar were injected with 6 µL of either ds*CRZ* or ds*GFP* with a Hamilton syringe (700 series, 705RN, 50 µL, Sigma-Aldrich) with a 22 s-gauge needle in the thorax through the second abdominal segment. Three days later, heads were snap frozen and preserved at −80 °C. RNA extraction and cDNA production were performed as described above. The resulting cDNA were used in a reverse transcriptase quantitative PCR (qPCR). Actin 5C (*ACT5C*) and ribosomal protein L5 (*RibL5*) were selected as reference genes, using primers described in Foquet and Song (2020). The mRNA sequence for *CRZ* was obtained from the *S. piceifrons* transcriptome generated by Foquet and Song (2020) (Genbank accession number MT756614). Based on this sequence, qPCR primers were designed using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012), with standard settings modified to allow for an optimal melting temperature of 60 °C and an amplicon length of 150–200 bp. All qPCR primers used in this study can be found in Table 1. The qPCR was performed as before in Foquet and Song (2020). In short, for each qPCR reaction, 5 µL of cDNA was added to 10 µL of SYBR green PCR mastermix qPCR (ThermoFisher Scientific) and 5 µL of primers at a final primer concentration of 500 nM. Each reaction was run in duplicate on a 96-well plate with a CFX connect real time system (Bio-Rad). The thermal cycling profile consisted of: 3 min at 95 °C, 40 cycles of (1) 15 s at 95 °C and (2) 45 s at 60 °C, and a melting curve from 65 °C to 95 °C. C_q values were exported from the Bio-Rad CFX manager using the default threshold, and were subsequently used to calculate relative expression with the $\Delta\Delta C_q$-method (Livak and Schmittgen, 2001).

2.4. Experimental design

After successfully generating ds*CRZ* that could efficiently knock down *CRZ* expression in *S. piceifrons*, we devised an experiment to test the effect of *CRZ* knockdown on gregarious behavior, solitarization, color, and morphometric ratios. We injected a total of 120 crowd-reared individuals with 6 µL of dsRNA constructs (20 ng μl^{-1}) in the thorax either with dsCRZ as a treatment or with ds*GFP* as a control within a day after molting to their fourth nymphal instar. Each injected individual was marked with a ceramic marker on the underside of the thorax, allowing for an easier identification of both conditions. For the duration of the experiment, 20 individuals of the same condition were kept together in a small screen-mesh cage (13.34 × 13.34 × 22.85 cm, Insect

Table 1
Primer sequences used in this study.

Gene	dsRNA primers	qPCR primers	E (%)
GFP	F: taatacgactcactatagggagaACGTAAACGGCCACAAGTTCAGC R: taatacgactcactatagggagaGAGGGTCTTCTGCTGGTAGTGGTCG		
CRZ	F: taatacgactcactatagggagaATGATGCGTCCGTGGGTGAGCGTGGTGCTG R: taatacgactcactatagggagaAGATCAGCGTGTCAGTCGAGAAGTGAAGCG	F: TGACGAGGACATGTGATGCC R: AGCGTGTCAGTCGAGAAGTG	100.5
Act5C		F: AACTTTCAACACCCCAGCCA R: AACGCCATCACCAGAATCCA	102.1
RIBL5		F: TCGGCTGCACAGAAGTTACC R: AGCTCCAGTAGTTGTGCGGA	98.86

Lore™). Booster injections of dsRNA were given within a day of molting to the fifth (penultimate) and sixth (ultimate) nymphal instar.

To study the effect of *CRZ* knockdown on both gregarious behavior and behavioral solitarization, we performed three sequential behavioral assays that varied in the time after isolation. Isolation treatment consisted of placing a single locust in separate plastic cages (10.16 × 10.16 × 25.4 cm) with only one transparent side and connected to a charcoal-filtered airflow, so that they were physically, visually and chemically isolated. The series of behavioral assays started three days after an individual molted to the ultimate nymphal instar. The first behavioral assay was performed on crowd-reared individuals taken from the small screen-mesh cage. Immediately after each assay, the tested individual was moved into isolation. A second behavioral assay was performed after one hour of isolation treatment, and the third behavioral assay was performed after 48 h of isolation treatment. Even though the time-course of solitarization in *S. piceifrons* is currently unknown, this experimental setup might inform us about the effect of the *CRZ* knockdown on both short-term changes (one hour of isolation) and more long-term changes (two days of isolation). After the final behavioral assay, each individual was frozen at −20 °C for subsequent analyses of color and morphometric ratios.

2.5. Behavioral assays

We randomly selected 30 individuals per group for our behavioral assays. Behavioral responses were quantified using a method previously described in Kilpatrick et al. (2019). In short, we used a rectangular behavioral arena (57 × 31 × 11 cm) developed by Roessingh et al. (1993) and modified by Gotham and Song (2013), which contained a stimulus chamber with 30 last instar nymphs on one side, and an empty non-stimulus chamber on the other side. A test subject was introduced in a central hole in the arena and was video-tracked for 12 min at 30 frames/second using a Basler acA1300 – 60gc camera (1280 × 1024) and EthoVision XT 12 (Noldus Information Technology Inc., Leesburg, VA) software. The arena was divided into three equal zones: a stimulus zone (the third adjacent to the stimulus chamber), a non-stimulus zone (the third adjacent to the non-stimulus chamber), and a neutral zone (the central third). We also designated all four walls (top, bottom, left, right) as a wall zone to measure climbing activity. Optimal detection settings were chosen by EthoVision. For each behavioral assay, tracks were manually inspected and corrected where necessary, after which they were smoothened using a 'Minimum Distance Moved' filter of 0.2 cm. Our final set of variables consisted of seven variables, of which the first three are activity-related and the last four are attraction-related: 'moved distance', 'frequency of direction changes' (the amount of times the individual made a small rotation of 90°), 'time spent on walls', 'time spent in stimulus zone', 'time spent in neutral zone', 'time spent in non-stimulus zone', and 'time spent on stimulus wall'. Each variable was analyzed separately using a linear mixed-effects (lme) model, with 'injection' as a between-subject factor and 'time since isolation' as a within-subject factor, using the R package nlme (Pinheiro et al., 2019) in R version 3.6.2 (R Core Team, 2017). Post hoc comparisons between different timepoints were made using the 'emmeans' package with Tukey's adjusted p values (Lenth, 2020). Results of the two injection treatments were pooled for these post hoc tests.

2.6. Analysis of morphology and black patterns

We quantified the effect of *CRZ* knockdown on black patterning for 30 individuals for each group. After frozen individuals were thawed, high-resolution digital images of the lateral side of the pronotum and the hind femur were captured for each test subject, using a LK Imaging System (Visionary Digital) with SpyderCHECKR 24 software for color correction (Datacolor, Lawrenceville, NJ). The R package patternize (Van Belleghem et al., 2018) in R version 3.6.2 (R Core Team 2017) was used to quantify and analyze the extent of black patterns on both tissues, as previously described in Kilpatrick et al. (2019). Landmarks were manually placed on each image using the polygon tool in ImageJ (Schneider et al., 2012), and images were aligned using these landmarks with the PatLanRGB-function of patternize. To detect black color, a set RGB-value of (0,0,0) with a cutoff of 0.15 was used, and heat maps of the black patterns were generated separately for each tissue and both experimental conditions. Percentages of black patterns, exported by patternize, were statistically compared with a student t-test in R 3.6.2 (R Core Team, 2017).

To analyze the effect of *CRZ* knockdown on morphometric ratios, we measured 20 males and 20 females for each group, with the exception of ds*GFP*-injected males for which only 16 individuals were available. Five linear dimensions were measured from each individual: the length of the pronotum from a dorsal view, the height of the pronotum from a lateral view, the length of the hind femur (F), the maximum width of the head (C) and the length of the wing pad. In addition, we calculated the F/C ratio, which is a reliable predictor of phase in locusts (Dirsh, 1953; Uvarov, 1966). A Mitutoyo CD-6″ CS digital caliper was used to make three independent measurements in mm for each structure, after which the average was used for all subsequent analyses. As locusts are known to have sexual size dimorphism (e.g. Kilpatrick et al., 2019; Stower et al., 1960), all statistical analyses were performed separately for males and females with a student t-test in R 3.6.2 (R Core Team, 2017).

3. Results

3.1. [His^7]-corazonin knockdown reduced black patterns in the Central American locust

The injection of ds*CRZ* successfully reduced *CRZ* mRNA expression by 99.7% in head tissue within three days (Fig. 1). This efficient knockdown enabled us to investigate the effect of *CRZ* on the coloration, morphology, and behavior of *S. piceifrons* nymphs. The test subjects were reared in a high-density condition prior to the experiment, and thus they had a typical gregarious coloration when they were injected at their fourth instar stage. We found that the knockdown of *CRZ* led to a complete loss of the nymphal coloration present in the gregarious control animals. Specifically, we found that the extensive black patterns normally found in the frontal area of the head, the dorsal and lateral portion of the pronotum, the wing pads, and the hind femur of the gregarious nymphs completely disappeared (Figs. 2 and 3). However, small black dots found all over the gregarious nymphs did not disappear with the knockdown of *CRZ*, but rather simply faded in light brown. The color of antennae changed from black to light brown, and the patterns on the dorsal and lateral portion of the abdomen and the bands below the eyes changed from black to light brown (Fig. 2). Furthermore, the background coloration changed from peach-red coloration to light yellow (Fig. 2). For both pronotum and hind femur, the extent of black patterning was reduced by more than 99% (Fig. 3). Even though these color data were obtained two molting cycles after the initial injection, the loss of coloration mostly occurred during the first molt after ds*CRZ* injection (personal observations).

3.2. [His^7]-corazonin knockdown had a structure-specific and sex-specific influence on the body size

Of the five linear dimensions measured, we only found statistically significant differences between ds*CRZ*-injected and ds*GFP*-injected individuals in 'head width' ($t_{df=35} = 2.1093$, $p = 0.04214$) and 'femur length' ($t_{df=36} = 2.1643$, $p = 0.03715$) of females (Fig. 4). In both measurements, the individuals injected with ds*CRZ* had larger body parts. We observed a similar trend in the 'pronotum length', although not statistically significant ($t_{df=37} = 1.7879$, $p = 0.08199$). For males, none of the measurements was statistically significant although we observed a trend towards longer wing pads in the individuals injected with ds*CRZ* ($t_{df=34} = 1.9397$, $p = 0.06075$). There was no statistical

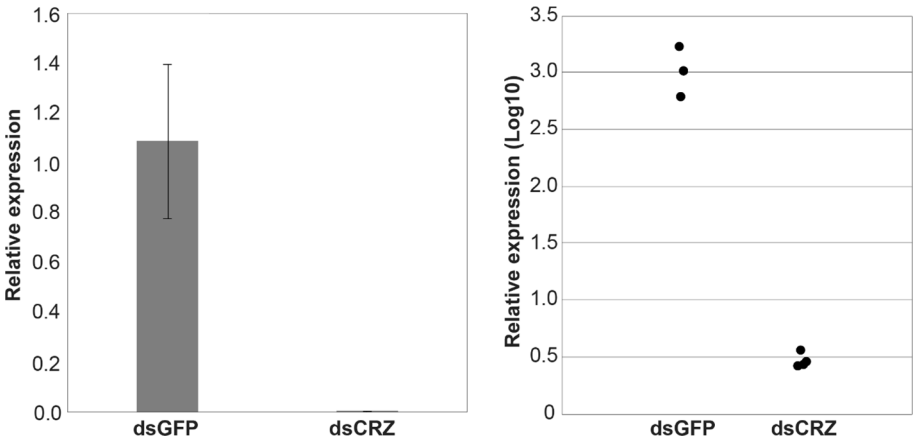

Fig. 1. Knockdown of *CRZ* is highly efficient. Relative expression of *CRZ* in female last instar nymphs of *S. piceifrons*, three days after injection of dsRNA targeting either *CRZ* (ds*CRZ*) or *GFP* (ds*GFP*). Relative expression was calculated using the $\Delta\Delta C_q$-method, and is shown both in raw expression level (left) and on a logarithmic (Log10) scale (right).

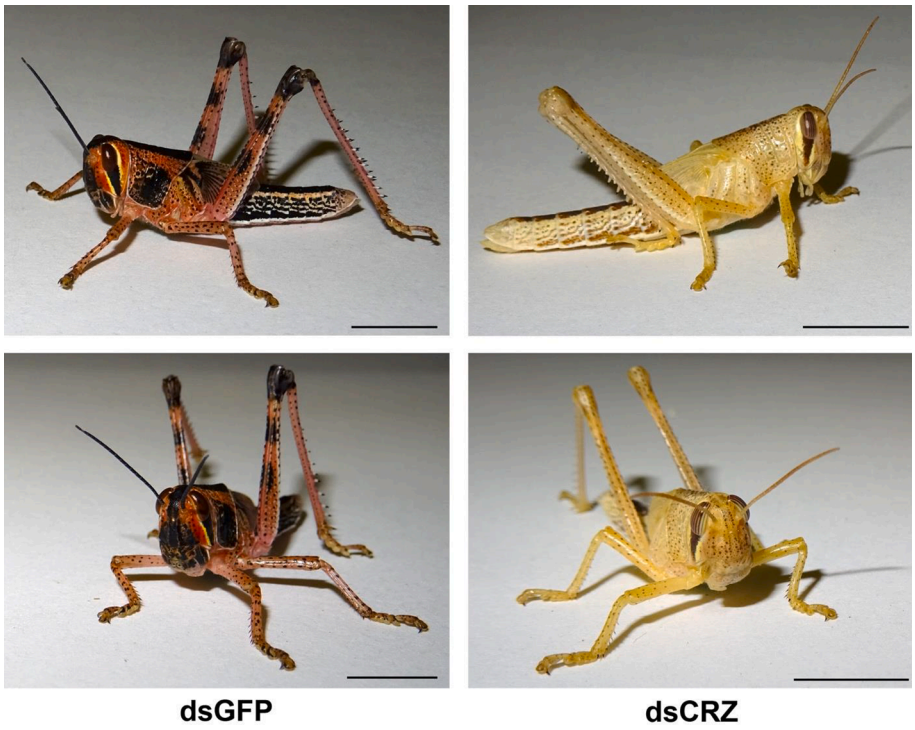

Fig. 2. *CRZ* influences both black patterning and background coloration. This panel shows the effect of the *CRZ* knockdown on both black patterning and background coloration in *S. piceifrons*. Nymphs were injected with either ds*CRZ* or ds*GFP* immediately after molting to the fourth nymphal instar, pictures were taken in the sixth nymphal instar. (Scale bar: 10 mm).

difference in F/C ratios in both males and females (Fig. 5).

3.3. Phase-related behavior was not influenced by [His^7]-Corazonin knockdown

We were unable to find any statistically significant effects of ds*CRZ* injection on any of the measured behavioral variables, nor any interaction effect between injection and time since isolation, using a linear mixed-effects model (Fig. 4, Table 2). However, for four of the measured behavioral variables, there was a significant effect of time since injection, suggesting that at least some solitarization indeed happened in *S. piceifrons* within 48 h (Fig. 4, Table 2). We further evaluated the differences between timepoints with a post hoc-test, using Tukey's adjusted *p* values. For the variables 'moved distance' and 'frequency of direction changes', these changes were highly significant even after only one hour of the isolation treatment ($p < 0.001$ for both comparisons). Between one hour and 48 h of the isolation treatment, the moved distance decreased significantly more ($p = 0.0200$), while for the amount of direction changes a similar trend was observed ($p = 0.0678$). The behavioral changes were less obvious for both 'time spent in the stimulus zone' and 'time spent in the non-stimulus zone', for which the only statistically significant differences were observed between crowd-reared nymphs and nymphs that were isolated for 48 h (respectively $p = 0.0482$ and $p = 0.0122$).

4. Discussion

In this study, we demonstrate that the functions of [His^7]-corazonin

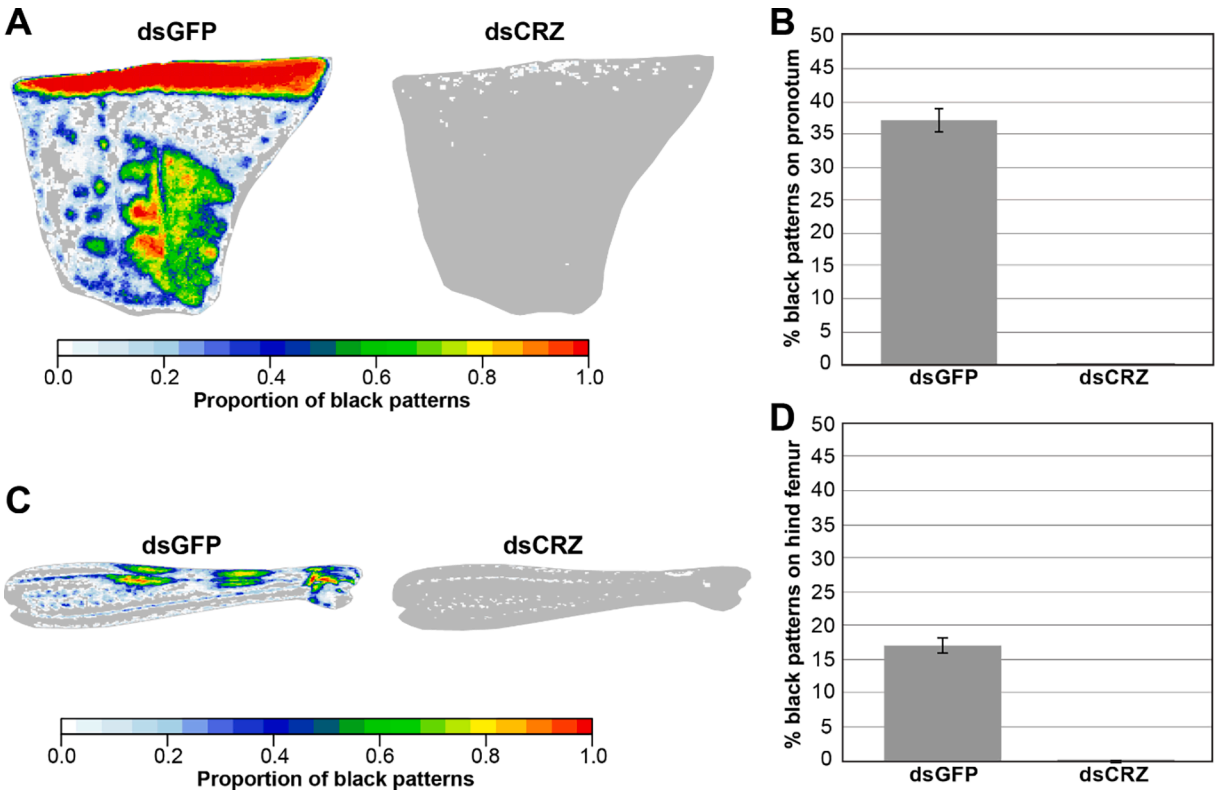

Fig. 3. Knockdown of *CRZ* almost completely eliminates black patterning. The left panels contain heat maps, generated with Patternize, that show the differences in black patterns between locusts injected with ds*CRZ* and ds*GFP* in the pronotum and the hind femur. On the right side, the percentage of black patterning for both tissues is shown in a bar chart, with error bars showing the standard error of mean.

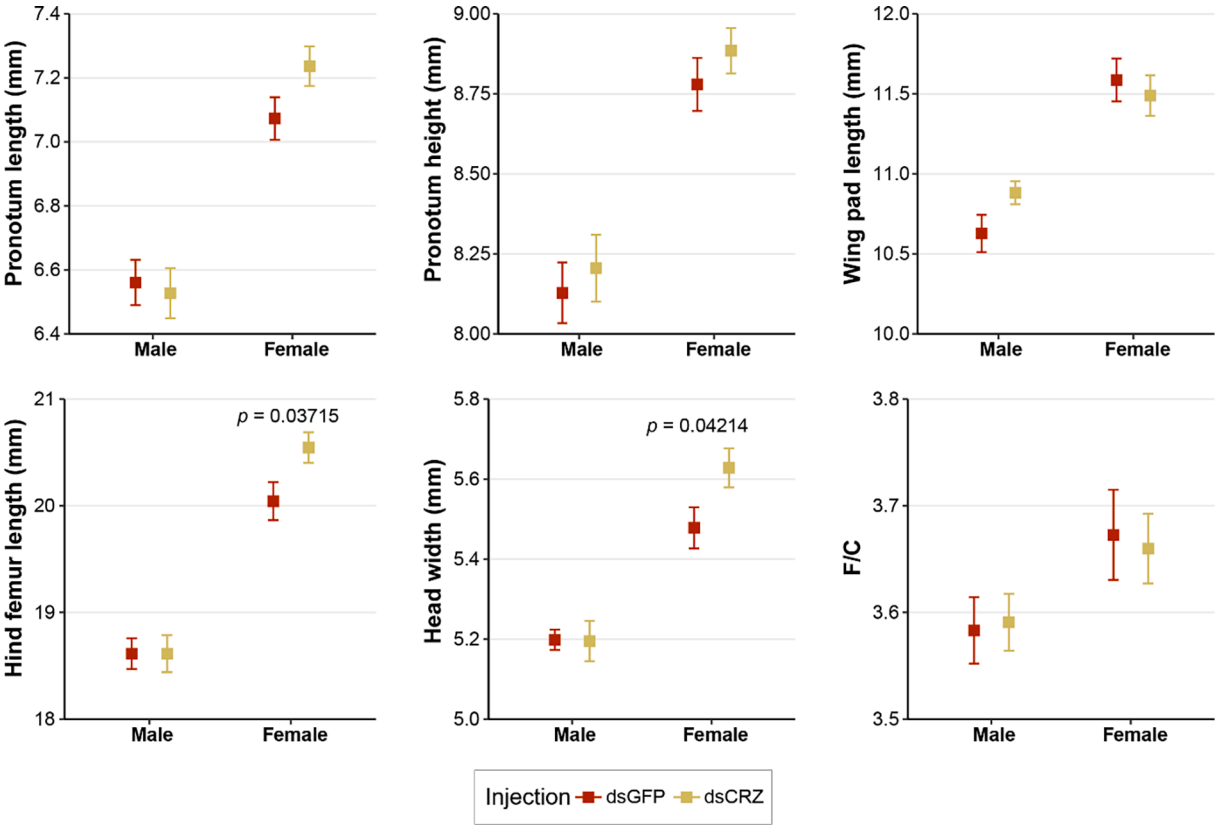

Fig. 4. Knockdown of *CRZ* influences body size. Shown are five linear dimensions and one morphometric ratio for males and females injected with either ds*GFP* or ds*CRZ*. Error bars represent standard error of mean.

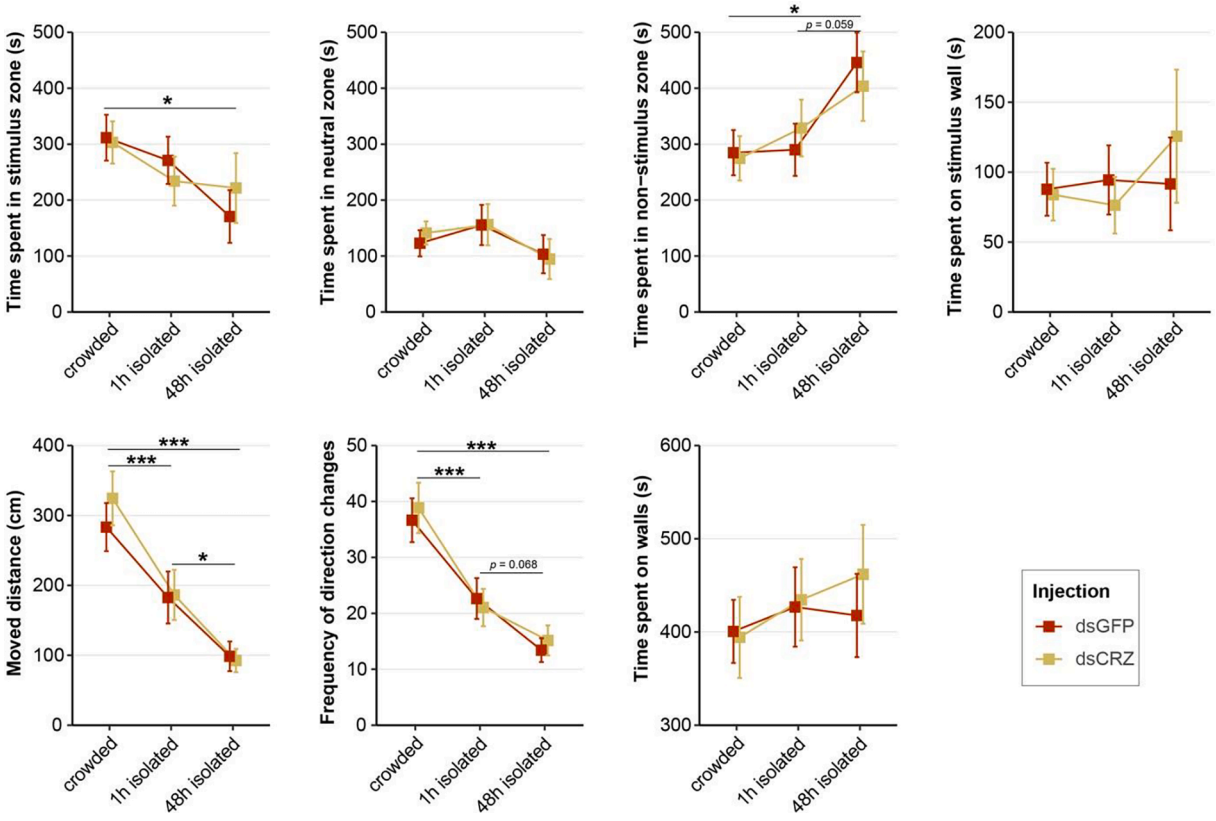

Fig. 5. Knockdown of *CRZ* does not influence locust behavior. Shown are the average values for four attraction-related and three activity-related behavioral variables for locusts injected with ds*GFP* or ds*CRZ*. Error bars represent the standard error of mean. $^{*}p < 0.05$; $^{***}p < 0.001$.

Table 2
F and *p*-values associated with linear mixed-effects model used to test effect of injection and amount of hours spent in isolation on different behavioral variables.

	Time spent in stimulus zone		Time spent in neutral zone		Time spent in non-stimulus zone		Time spent on stimulus wall	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(intercept)	193.737	<0.0001	81.795	<0.0001	285.093	<0.0001	70.386	<0.0001
Injection	0.0004	0.9838	0.0799	0.7785	0.0228	0.8806	0.0030	0.9566
Hours.isolated	3.2162	**0.0441**	1.5699	0.2129	4.9758	**0.0086**	0.3232	0.7245
Hours.Isolated: Injection	0.4558	0.6352	0.0662	0.9360	0.3415	0.7115	0.4610	0.6319
	Moved distance		Frequency of direction changes		Time spent on walls			
	F	*p*	*F*	*p*	*F*	*p*		
(intercept)	141.506	<0.0001	207.673	<0.0001	439.710	<0.0001		
Injection	0.4189	0.5200	0.1185	0.7319	0.0980	0.7553		
Hours.isolated	24.6680	**<0.0001**	28.737	**<0.0001**	0.5539	0.5764		
Hours.Isolated: Injection	0.2814	0.7552	0.2333	0.7923	0.2152	0.8067		

in *S. piceifrons* bear high similarities with what has been previously found in *S. gregaria* and *L. migratoria*. We have shown that a knockdown of *CRZ* expression in crowd-reared nymphs resulted in a loss of nymphal gregarious coloration, and that it additionally altered their morphological dimensions towards more solitarious values. Finally, we have shown that the *CRZ* knockdown did not have any effect on gregarious behavior or behavioral solitarization.

The complete loss of black patterning after the *CRZ* knockdown in our study agrees well with earlier studies in *S. gregaria* and *L. migratoria* (Pener and Simpson, 2009; Sugahara et al., 2015, 2016; Tanaka, 2006; Tanaka et al., 2016). However, the effect of Crz on the background coloration of locusts is less established. In our study, the knockdown of *CRZ* led to a complete loss of the orange background coloration in *S. piceifrons* (Fig. 2). Similarly, in *S. gregaria* a similar knockdown of *CRZ* expression led to a reduction of the yellow background coloration (Sugahara et al., 2015). However, while Tawfik et al. (1999) were able to induce changes in background coloration by injecting Crz in solitarious *S. gregaria*, other studies in the same species reported that nymphs kept their green background coloration after increasing Crz levels, even though the extent of black patterning significantly increased (Maeno et al., 2004; Tanaka, 2001; Tanaka and Yagi, 1997). Similarly, Tanaka (2004) reported that the injection of Crz in penultimate instar light colored nymphs of *S. americana* did not alter background coloration, even though the figures in his paper showed that some of the injected nymphs had a red background coloration and all of them had a slightly red head that was absent in the control. It is currently not clear what is the cause of these inconsistencies, but our data suggest a potential involvement of *CRZ* in the regulation of the gregarious background coloration. Supporting this notion, *CRZ* was recently shown to regulate the expression of genes suggested to alter background coloration in *S. gregaria*, even though their exact function has yet to be established (Sugahara et al., 2018). Interestingly, a recent study in *L. migratoria*

showed that a knockdown on β-carotene binding protein (β-CBP) also results in a loss of black patterning, but individuals retain a green coloration after the loss of this protein (Yang et al., 2019). However, both in our study, in *S. gregaria*, and in *L. migratoria*, a reduction of *CRZ* expression levels leads to tan rather than green individuals (Sugahara et al., 2015, 2016). It is currently unclear whether CRZ influences black coloration through carotenes, the suggested pathway by Yang et al. (2019), or through melanization. An increase in melanization could lead to both black patterning and a yellow/orange background coloration (Pener and Simpson, 2009; Uvarov, 1966), and individuals lacking these melanins would indeed be expected to be white rather than green. Clearly, more studies are needed to test whether the changes in background coloration and black patterning upon knockdown of *CRZ* are caused by the same pathway, or by two independent pathways that are both influenced by Crz.

In addition to its influence on color, we have shown that the knockdown of *CRZ* significantly increased two out of five tested morphological dimensions in females, suggesting the knockdown of *CRZ* might influence the size of the locusts. Interestingly, an earlier study in *S. piceifrons* only found significant differences in size between isolated-reared and crowd-reared males, but not in females (Hunter-Jones, 1967). Nonetheless, the results obtained in this study for females might still represent an actual phase-related effect. Indeed, in both *S. gregaria* and *S. cancellata*, isolated-reared females are significantly larger than crowd-reared females (Pener and Simpson, 2009; Pocco et al., 2019; Uvarov, 1966). Nonetheless, the absence of an effect of ds*CRZ*-injection on the F/C ratio in *S. piceifrons*, and the lack of significant morphological differences in males suggest that the effect of [His^7]-corazonin on morphology in *S. piceifrons* might be reduced compared to *S. gregaria* and *L. migratoria* (Hoste et al., 2002b; Maeno et al., 2004; Sugahara et al., 2015, 2016; Tanaka et al., 2002). Additionally, it is currently not clear whether the observed differences in female size in *S. piceifrons* are truly phase-related, or whether they are influenced by another, yet unknown, function of the CRZ gene. As our measurements were performed on nymphs and not on adults, as is traditionally done in locust research (but see Kilpatrick et al., 2019; Pocco et al., 2019), the study subjects went through only two molting cycles after the first ds*CRZ* injection. In earlier studies (Hoste et al., 2002b; Maeno et al., 2004; Sugahara et al., 2015, 2016; Tanaka et al., 2002), locusts went through at least three molting cycles after the levels of [His^7]-corazonin were altered, and this discrepancy might explain the reduced response observed in our study. As body dimensions in insects only change when they molt, it is noteworthy that the knockdown of *CRZ* already significantly altered body dimensions in females after just two molting cycles.

At the behavioral level, we were unable to observe any effect of the *CRZ* knockdown. Our study represents the first time that the effect of reduced expression of *CRZ* was studied within the same genetic background. Additionally, we tested for the first time whether altering the levels of [His^7]-corazonin influences the solitarization of gregarious nymphs. Our results showed that there was no significant effect of the *CRZ* knockdown on gregarious behavior or solitarization, which is in agreement with earlier studies in *S. gregaria* and *L. migratoria* (Hoste et al., 2003, 2002a, 2002b). However, there was a clear effect of time since injection on four of the tested behavioral variables. The time course of behavioral solitarization was studied in *S. gregaria* by Roessingh and Simpson (1994), allowing for a partial comparison between these two congeneric species that represent independent evolutionary origins of density-dependent phase polyphenism (Song et al., 2017). In the desert locust, solitarization of crowd-reared locusts seems to occur in two phases: a quick early phase in the first one to four hours, after which the *P*sol, the probability of a locust to be solitarious, remains intermediate between the extreme solitarious and gregarious ends of the behavioral spectrum for at least 96 h (Roessingh and Simpson, 1994). Even though there is currently no behavioral data for fully solitarious individuals of *S. piceifrons*, this species seems to have a quick initial solitarization followed by a slower phase in the following 48 h, similar to *S. gregaria*. This is especially the case when focusing on activity-related variables like the 'moved distance' and 'frequency of direction changes', which both exhibit the largest decrease within the first hour of isolation. However, the times spent in the stimulus zone and in the non-stimulus zone seemed to follow a slightly different time course, with only a small, non-significant change towards more solitarious values after one hour but a larger change after 48 h. Even though these results represent an interesting first step towards understanding behavioral solitarization in this species, a more in-depth study, including more timepoints and a comparison to isolated-reared nymphs is required.

Schistocerca piceifrons represents the third locust species in which the effect of [His^7]-corazonin has been investigated. All three locust species in which [His^7]-corazonin has been studied, represent independent evolutionary origins of density-dependent phase polyphenism (Cullen et al., 2017; Song et al., 2017). Nonetheless, the functions of [His^7]-corazonin share a lot of similarities between all three species, suggesting that they are either largely conserved, or that this neuropeptide represents an intriguing case of parallel evolution. Even though the darkening effect of [His^7]-corazonin seems to be conserved in both locusts and non-swarming grasshoppers within the suborder Caelifera (Tanaka, 2000a, 2004; Yerushalmi and Pener, 2001), it is currently unknown whether this is also the case for the other traits it influences. An in-depth investigation on the effects of Crz on morphology and even on the antennal sensilla in non-swarming grasshoppers would be very informative about the evolutionary mechanisms underlying the functions of this intriguing neuropeptide, but can also more broadly inform on the evolution of density-dependent phase polyphenism.

CRediT authorship contribution statement

Bert Foquet: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Hojun Song:** Conceptualization, Writing - original draft, Visualization, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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