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Biochemical changes in developing embryos of *Schistocerca gregaria* (Orthoptera: Acrididae) induced by pheromone produced by ovipositing gregarious females

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Abstract. Trans-generational transfer of gregarious-phase traits in the desert locust *Schistocerca gregaria* (Forskål, 1775) is mediated by primer gregarizing pheromonal signals produced by ovipositing females that experience crowding. We monitored time-course proteomic events in eggs from solitary-reared locusts that had been exposed for 1, 3, 5, 7, 10 and 12 days to different levels of the sand-associated gregarizing signal originating from 0, 3, 5 or 10 ovipositions by crowd-reared females. Evidence for the phase transition was sought by comparing the protein patterns of embryos thus exposed with those from crowd-reared (gregarious) controls; this comparison was continued until the stage of the first instars. Expressed proteins were analysed by two-dimensional protein gel electrophoresis, and patterns from the different treatments within stages were compared by profile matching and χ^2 analyses. Eggs derived from crowd- and solitary-reared females showed essentially similar protein patterns at early stages of embryogenesis; however, mature stages (particularly, days 10 and 12) and hatchlings demonstrated significantly different patterns. Protein patterns of eggs from solitary-reared females that were incubated in sand contaminated with the pheromonal signal and of the hatchlings that emerged were similar to those derived from gregarious females and dependent on the level of the pheromone to which the embryos had been exposed. The results confirm the gregarizing effect of the signal and constitute a useful basis for unravelling the mechanism of the signalling cascades associated with gene expressions triggered by the pheromone.

Key words: *Schistocerca gregaria*, primer gregarizing pheromone, phase transition, 2D protein patterns

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Introduction

Schistocerca gregaria (Forskål, 1775) (Orthoptera: Acrididae) is characterized by a pronounced ability to transform reversibly between two extreme phases (solitaria and gregaria) that differ in physiology, biochemistry, behaviour and morphology (Uvarov, 1966; Applebaum *et al.*, 1997; Pener and Yerushalmi, 1998). Phase changes are predicated based on locust density; this may be promoted by a variety of environmental and biotic factors including improved rains and vegetation, habitat topologies, favourable microclimates, convergent winds, and patchy distribution of food plants and oviposition sites (Bouaïchi *et al.*, 1996). Crowding and uncrowding can lead to rapid changes (over hours or days) in some of the phase-related traits, such as aggregation behaviour (Roessingh and Simpson, 1994) and the production of mediating releaser pheromones (Deng *et al.*, 1996; Hassanali *et al.*, 2005*a*). Others, such as morphometrics, may take several generations (Pener, 1983). Gregarious characters may spread horizontally through the recruitment of solitary individuals into gregarizing groups (Njagi *et al.*, 1996; Hassanali *et al.*, 2005*b*) and accrue across generations through the eggs (Pener, 1991; Islam *et al.*, 1994*a*,*b*; Bouaïchi *et al.*, 1995). Two types of signals have been implicated in priming the embryonic development of eggs towards the gregarious phase: a water-soluble chemical within egg foams (probably an alkylated L-dopa) secreted by the reproductive accessory glands at the time of oviposition (Hägele *et al.*, 2000; Simpson and Miller, 2007; Miller *et al.*, 2008) and a blend of volatile C-8 unsaturated ketones, also produced by accessory glands, present in relatively large amounts in the eggs and the surrounding soil (Malual *et al.*, 2001). The role and relative contribution of the two signals in predisposing a phase shift in developing eggs remain yet to be determined.

In the study reported by Malual *et al.* (2001), the aggregative behaviour of hatchlings emerging from egg pods that had been incubated in sand previously used for consecutive ovipositions by gregarious females was compared with that of hatchlings emerging from sand that had been similarly exposed but subsequently washed with organic solvents or flushed with nitrogen gas. The first group of hatchlings showed a high level of aggregation similar to those derived from a crowd-reared locust colony. However, washing the sand with solvents or flushing with nitrogen led to a substantial loss of its gregarizing effect (Malual *et al.*, 2001). The results of the study indicate that exposure of locust eggs to the signal that diffuses into the soil during oviposition by gregarious females causes the up-grading or down-grading of specific genes which then trigger other changes associated with the transition of developing embryos to the gregarious phase. The objectives of the present study were to expose developing embryos derived from solitary-reared females to different levels of the signal and compare their time-course protein patterns by two-dimensional (2D) gel electrophoresis with those obtained from embryos derived from gregarious females.

Materials and methods

Experimental insects

The test insects used in the study were *S. gregaria* locusts in *solitaria* or *gregaria* forms from the insectary of the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The stock from which the locusts were derived was originally supplied by the Desert Locust Control Organization for Eastern Africa (DLCO-EA), Addis Ababa, Ethiopia, and had been periodically supplemented with collections from Port Sudan, Sudan.

Locust rearing and maintenance

The locusts were reared by the methods developed by Ochieng-Odero *et al.* (1994). Briefly, the locusts were fed on fresh wheat seedlings (*Triticum* spp.) and wheat bran with the *solitaria* and *gregaria* being kept in separate rooms at 28–37°C, with a photoperiod of 12 h light–12 h dark and relative humidity of 40–50%. Morphometry, pigmentation and/or the existence of an extra moult were used to distinguish generational changes in the phases of the locusts.

Exposure of oviposition substrate to primer gregarizing pheromone

The oviposition substrate was prepared by sieving builders' sand through a wire mesh (2 mm^2 mesh size) to obtain uniform fine granules devoid of debris. The sand was sequentially washed with hot water, methanol, acetone and dichloromethane to remove possible inorganic and organic contaminants. This was followed by heat sterilization at 205°C for 24 h, and cooling to 27°C. The sand was then moistened to approximately 15% distilled water by placing 15 ml of water in 100 g of sand (Norris, 1968). The sand was finally placed in standard aluminium oviposition cups (height 10 cm, internal diameter 4 cm) stationed at the front of the false floors of standard aluminium locust-rearing cages (Ochieng-Odero *et al.*, 1994) containing 100 gravid gregarious females per cage

(Saini *et al.*, 1995). Two cages were employed in this study. The cups had previously been sterilized by overnight soaking in 2% sodium hypochlorite detergent, washed with soap and water, and baked at 150°C for 2 h, before they were cooled to 27°C. A total of 15 oviposition cups were put in each cage. Each oviposition tube was retrieved from the respective cage 24 h later; the sand content was sieved through the wire mesh and the resultant egg pods restrained by the mesh were counted. It was assumed that each egg pod released a significant and equal quantity of the gregarizing pheromone (Malual *et al.*, 2001). Total pheromone dose in each tube was therefore considered to be directly proportional to the number of egg pods present. For the purposes of this study, each egg pod was considered to represent a pheromone dose as described previously (Malual *et al.*, 2001), and the dose present in the sieved sand was therefore empirically estimated.

Exposure of solitaria eggs to sand containing primer gregarizing pheromone

Sand containing the pheromone obtained as described above was placed in cages as also described above with solitarious gravid females. Four doses of the pheromone (from 0, 3, 5 and 10 days) were evaluated in five separate replicates. For each replicate, a control was prepared and handled in exactly the same way as the experimental material except that the sand was devoid of pheromone exposure. The tubes containing egg pods (1–3 per tube) were transferred into cages for instars' emergence 24 h later (Fig. 1). A total of 20 eggs were randomly sampled from each tube. In this process, care was taken to ensure that all the pods present were sampled. The sampling for each treatment and replicate was conducted at intervals of 1, 3, 5, 7, 10 and 12 days including the first instar. The samples were placed in 1.5 ml Eppendorf tubes and stored at −80°C for subsequent protein analyses. Therefore, there were four treatments × seven sampling intervals × five replicates giving rise to a total of 140 experimental units.

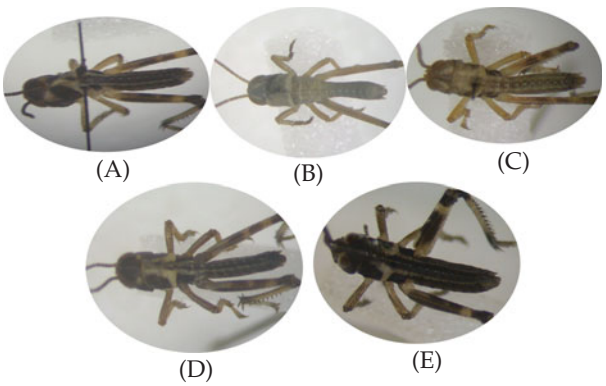

Fig. 1. Emergence of first instars from embryos: gregarious, solitary, unexposed and exposed to various pheromone doses. (A) Gregarious-phase first instar; (B) solitary phase unexposed to pheromone first instar; (C) solitary-phase instar exposed to three doses of pheromones; (D) solitary-phase instar exposed to five doses of pheromones; (E) solitary phase exposed to 10 doses of pheromones.

Protein extraction from embryos and first instar samples

Proteins were extracted from the eggs and first instar samples by the methods developed by Kiely and Riddiford (1985a,*b*). Briefly, three eggs or instars were homogenized at 4°C in 100 µl extraction buffer (6.25 mM Tris–HCl, pH 6.8, 1% Nonidet P-40 and 1 mM phenylmethylsulphonyl fluoride [Sigma Chemical Co., St Louis, MO, USA)]. The extracted protein was quantified by Bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) using Bovine serum albumin (Fraction V; Calbiochem, San Diego, CA, USA) as a standard.

Two-dimensional gel electrophoresis

The extracted protein from each individual replicate was separated into the respective peptide by the classical methods developed by O'Farrell (1975) and modified by Jones (1980). Briefly, individual samples were separated by isoelectric focusing (IEF) gels in the first dimension using a 4:1 ratio of pH 5–7 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and pH 3.5–10 (Amersham Biosciences AB, Uppsala, Sweden) ampholines. The extruded gels were placed in 1 ml of equilibrium soaking solution (O'Farrell *et al.*, 1977). The gels were allowed to equilibrate by gently shaking for 30 min at room temperature. For the second dimension, the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to methods developed by Laemmli (1970). In this respect, the samples were run on 8.5% polyacrylamide overlaid with 3% stacking gel. Individual peptide spots resolved were visualized through silver staining (O'Farrell *et al.*, 1977).

Data analysis

The protein expression profiles (number of spots and relative positions of proteins of the embryos and fledglings from different treatments) were captured by Compugen Z3 2D – PAGE Analysis System version 3.0 (Compugen Limited, Tel Aviv, Israel). The analysis generated information on the relative matching patterns of expressed proteins

(spots) between and among various treatments at different stages, including the first instars. The patterns were further compared within and between treatments using the χ^2 test, at $a = 0.05$ rejection criteria using the statistical program R (2.13.1). All the analyses were performed using the R (2.13.1) statistical software (R Development Core Team, 2011), while relying heavily on the epicalc package (Chongsuvivatwong, 2012).

Results

Temporal and stage-specific correlations between protein expression (spots) and the relative *gregaria* phenotype of solitarious *S. gregaria* locust embryos or instars exposed to various doses of gregarizing pheromones are summarized in Fig. 2A. Representative pictorial figures of the resultant first instars are also presented (Fig. 2B). Surprisingly, the largest gregarious phenotype (irrespective of dose) among the embryos was exhibited on day 1 of exposure. With the exception of the profile irregularity on day 3, the relative probabilities of gregarious matching protein spots were generally similar ($P > 0.05$) and dose independent in the early stages (days 1–7) of exposure, but assumed a dose-dependent disposition thereafter, in a general direct proportional manner. Within the dose-dependent period (days 10–12), the solitary eggs exposed to 10 pheromone doses exhibited phenotypes very similar to those from gregarious eggs, corroborated by the emergent black and green first instar from the dose, a *gregaria*-specific characteristic. The profile from the emergent first instars

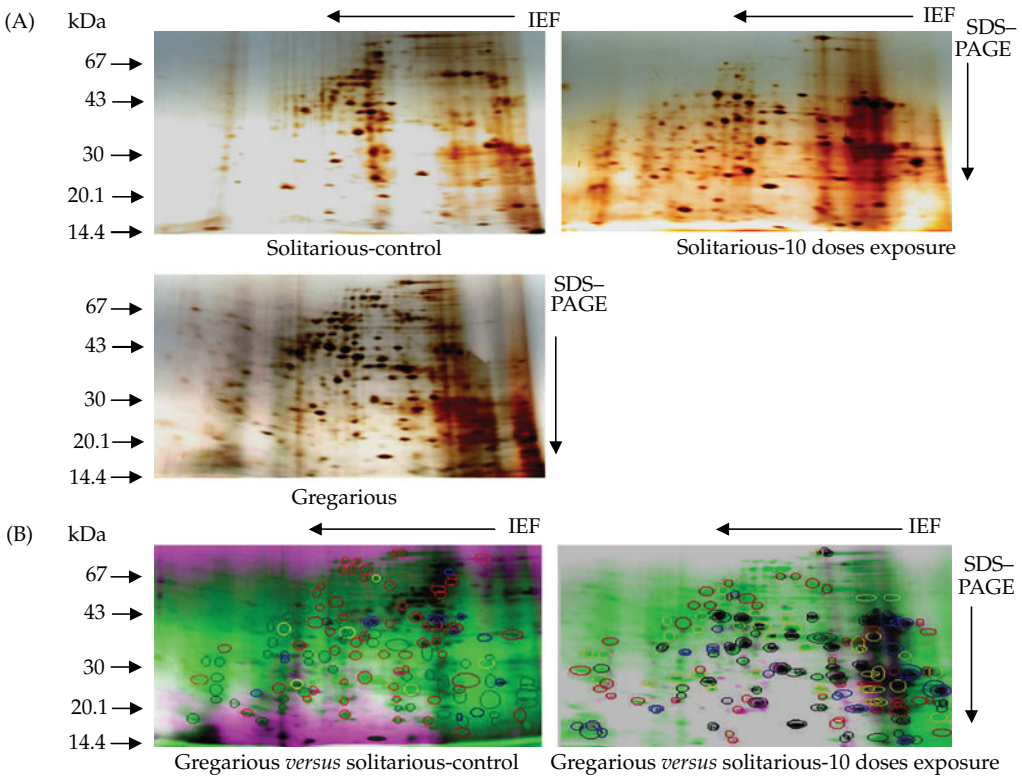

Fig. 2. 2D protein gel profiles of 12-day-old *S. gregaria* locust solitary-phase embryos exposed to various doses of gregarizing pheromones, gregarious-phase embryos, and various overlays between gregarious and solitarious phases. (A) Representative 2D-gel images of proteins from embryos of solitarious *Schistocerca gregaria* locusts not exposed to the gregarizing pheromone (solitarious-control), exposed to 10 doses of the pheromone (solitarious-10 dose exposure), and from a gregarious-phase embryo. The gels were obtained through 2D gel electrophoreses. (B) Match protein profile overlay of images obtained from gregarious-phase embryos and solitarious control or solitarious 10-dose exposure. Each overlay consists of images from gregarious phase (green) and solitarious control or solitarious exposure to 10 doses (purple). Red circles on green spots represent protein spots unique to gregarious profiles, while similar spots on purple spots represent the same on the solitarious phases. Spots common to gregarious and solitarious phase profiles are indicated in grey circles. Yellow and blue circles represent down- and up-regulated proteins in respect of the solitarious phase relative to the gregarious phase. The overlays were implemented through Compugen Z3 2D – PAGE Analysis System version 3.0 (Compugen Limited, Tel Aviv, Israel).

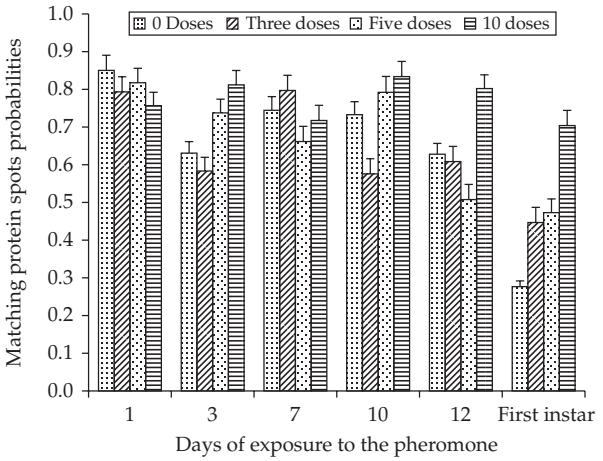

Fig. 3. Matching protein spot probabilities between gregarious and solitary *Schistocerca gregaria* embryos and instars exposed to various doses of the gregarizing pheromone.

exhibited a dose-dependent phenotypic response in tandem with the correlated embryo. This was manifested by the black and green colourations that increased in depth with an increasing dosage. As in the gregarious case, the profile of protein spots in various dosage treatments relative to the control varied widely in the earlier post-exposure period (days 3–7), before significantly departing in a dose-dependent manner, especially the 10-dose treatment (Fig. 3). A similar trend was observed among the emergent first instars.

The protein expression profiles of the 12-day-old solitarious embryos exposed to 10 doses of the pheromone (the embryonic stage with the most striking phenotype compared with the solitarious or gregarious controls) are represented in Fig. 4. Putative induction, repression or proteolytic processing of some proteins in the solitarious embryos, due to exposure to the gregarizing pheromone, is evident as the suggested presence or absence of some spots, or shifts in their positions in the 10-dose treatment, compared with the controls. Most proteins in the solitarious controls were basic whose molecular weight is between 43.0 and 94.0 kDa, whereas that of the remaining acidic proteins is between 14.4 and 20.1 kDa.

Discussion

The present study investigated protein expression in growing embryos of solitary-phase *S. gregaria* due to the gregarizing pheromone associated with gravid females, and in relation to the *solitaria*-to-*gregaria* phase transition in the locust. Effects of the primer gregarizing pheromone on the phase shift of embryos from solitary females were discernable in the mature stages of embryogenesis and in the first instars. This was particularly true at high pheromone titres. Such responses are accompanied by an interestingly wide fluctuation in protein expressions relative to gregarious or solitarious phenotypes in the initial duration of exposure of the embryos, before assuming a dose-dependent relationship towards the gregarious phase. The fluctuations may be attributed to specific protein titres related to the level of embryogenesis and/or to the degree of phase shift. The subsequent dose-dependent responses are in agreement with those of Malual *et al.* (2001) who established a significant shift towards the gregarious phase in a dose-dependent manner under similar circumstances. This suggests a putative functional (phenotypic) and direct relationship between the differentially expressed proteins and the shift in phenotype, a likely reflection of the changing phase status of the developing insects. Although a similar phenomenon has previously been putatively associated with some haemolymph proteins (Wedekind-Hirschberger *et al.*, 1999), the specific identifications of the predominantly basic proteins of low molecular weight, and the nature of the putative proteomic processing observed in the current study remain yet to be determined through a complex and detailed study to elucidate the roles of the proteins in *gregaria*- or *solitaria*-specific signal transduction at these stages. This will help to unravel the mechanism of the signalling cascades associated with specific gene expression triggered by the primer gregarizing pheromone.

In practical terms, there is an absence of discernable differences between the *solitaria* and *gregaria* embryos in the early stages of embryogenesis, suggesting that eggs derived from both

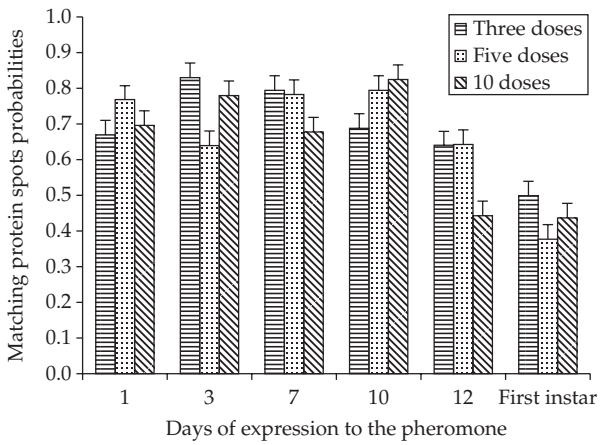

Fig. 4. Matching protein spot probabilities between *Schistocerca gregaria* solitary embryos and instars unexposed and exposed to various doses of the gregarizing pheromone.

phases are essentially similar, and that phase-related differentiation occurs on exposure to the high pheromone titres over duration. The implication of this in the field is that sustainable gregarization can occur only at high locust densities, resulting in dense communal oviposition. This would have two consequences. First, the developing eggs would be primed to produce gregarious hatchlings. Second, dense aggregation of egg pods from gregarizing or gregarious females would attract solitary females also to oviposit communally (Bashir *et al.*, 2000), thus ensuring a rapid spread of the gregarious character in the population. Since the study demonstrated the most similar protein expression patterns between the gregarious and the solitarious phases induced with 10 doses of the pheromone on day 10 or 12, and in hatchlings, the differentially expressed proteins (spots) can potentially be exploited in the development of molecular tools for the regulation of the phase transitions or of transgenic strains of the locust permanently deficient in the pest gregarious phenotype.

Conclusion

Dose-dependent effects of the primer gregarizing pheromone on the phase shift of embryos from solitary females find prominent expression in the mature stages of embryogenesis and in the first instars. Thus, effective trans-generational transfer of gregarious-phase characters as revealed by proteomic analysis occurs in the desert locust.

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