

Swarm formation in the desert locust *Schistocerca gregaria*: isolation and NMR analysis of the primary maternal gregarizing agent

Gabriel A. Miller^{1,2}, M. Saiful Islam^{2,3}, Timothy D. W. Claridge⁴, Tim Dodgson¹ and Stephen J. Simpson^{1,2,*}

¹School of Biological Sciences, The University of Sydney, Heydon-Laurence Building A08, NSW 2006, Australia, ²Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, UK, ³Department of Zoology, University of Rajshahi, Rajshahi 6205, Bangladesh and ⁴Department of Chemistry, University of Oxford, South Parks Road, Oxford, OX1 3QH, UK

*Author for correspondence (e-mail: stephen.simpson@bio.usyd.edu.au)

Accepted 12 November 2007

SUMMARY

Depending on their rearing density, female desert locusts *Schistocerca gregaria* epigenetically endow their offspring with differing phenotypes. To identify the chemical basis for such maternal transmission, we compared solitary and gregarious locust egg pod foam using high performance liquid chromatography (HPLC). We revealed causal relationships between foam chemistry and hatchling phenotype (phase state) by iteratively applying HPLC fractions from gregarious locust egg foam extracts to solitary eggs and assessing resulting hatchlings with a behavioural bioassay involving logistic regression. Selection and application of increasingly specific HPLC fractions allowed us to isolate compounds with gregarizing properties. Hatchling gregarization was triggered only by certain fractions and was dose dependent. In a final series of experiments, we characterized the most specific gregarizing fraction by nuclear magnetic resonance (NMR) spectroscopy. Here we present tentative structural features of the primary locust maternal gregarizing agent, which appears to be an alkylated L-dopa analogue. In addition, we propose a mechanism for phase-dependent regulation of this compound's activity.

Key words: gregarizing factor, L-dopa, locust phase, change, maternal effect, NMR spectroscopy, phenotypic plasticity.

INTRODUCTION

Polyphenism affords organisms robustness against environmental fluctuation and promotes survival in dynamic habitats (Jablonka and Lamb, 2005; West-Eberhard, 2003). Locusts express extreme polyphenism (Pener, 1991; Pener and Yerushalmi, 1998; Simpson and Sword, 2007; Uvarov, 1966); when subjected to high population densities, these insects undergo changes in behaviour (Bouaichi et al., 1995; Islam, 1996; Roessingh et al., 1993; Rogers et al., 2003; Simpson et al., 1999), morphology (Dirsh, 1953) and colouration (Faure, 1932; Islam et al., 1994b), as well as in reproductive features (Schmidt and Albutz, 1999) and other characteristics which reinforce further crowding and facilitate swarm formation (Clynen et al., 2006; Pener, 1991; Pener and Yerushalmi, 1998; Simpson and Sword, 2007; Uvarov, 1966). In addition to changing between extreme phases in response to crowding during their own lifetime, locusts transmit information about their phase state to their progeny (Hunter-Jones, 1958; Islam et al., 1994a; Islam et al., 1994b): gregarious adults give rise to gregarious offspring, strongly influencing the dynamics of locust plagues. This process is epigenetic, being transgenerational, inducible and persistent for some duration in the absence of inducing stimuli (Jablonka and Lamb, 2002; Jablonka and Lamb, 2005; Jablonka et al., 2002).

Epigenetic transfer of phase state depends upon low molecular mass, water-soluble chemicals within foam secreted by the reproductive accessory glands at the time of oviposition (Hägele et al., 2000; McCaffery et al., 1998; Rahman et al., 2002; Simpson et al., 1999; Simpson and Miller, 2007). Although attempts to correlate egg foam chemistry with behaviour have been made (Malual et al., 2001) and some preliminary inquiries provided

promising results (Islam, 1997; McCaffery et al., 1998), bioactive foam components have yet to be identified (Simpson and Miller, 2007). Here we present the first direct evidence for a specific gregarizing compound and provide data about its structure and possible phase-dependent regulation.

MATERIALS AND METHODS

Insects

Eggs were collected from either solitary or gregarious *Schistocerca gregaria* Forskål mothers maintained on seedling wheat and dried wheat germ beneath heat lamps in a room kept at 30–33°C. [We will use the term 'solitary' rather than the ambiguous 'solitary' (Uvarov, 1966); p. 332.] Solitary mothers were second- or third-generation isolates reared in the absence of conspecific stimuli, excepting a brief mating period, in glass-fronted metal cages (10 cm×10 cm×25 cm) with upper and lower compartments separated by 5 mm mesh grids. Gregarious adults were housed at a density of 500–1000 insects per rearing bin (56 cm×76 cm×60 cm); females had continuous access to an oviposition box (27 cm×19 cm×12 cm) containing moist peat. Eggs for the study were collected in plastic cylinders (3.5 cm diameter×8 cm high) filled with moistened sand (10 g silver sand:1 ml water) placed either in the gregarious rearing bins, where they temporarily replaced the peat box, or in the lower compartments of the solitary chambers beneath holes in the mesh grids. Incubation took place in a room maintained at 30–32°C and 70–75% RH under a 12 h:12 h L:D cycle. Following eclosion, test hatchlings were either reared alone (solitary individuals) or in a group (gregarious individuals) until subjected to behavioural assay on the day of hatching.

Logistic regression behavioural assay

Fifty-one hatchlings (<24-h old) reared in isolation from four solitary egg pods and 68 hatchlings (<24-h old) from the gregarious culture (from an indeterminate number of egg pods) were used to construct the logistic regression model. The protocol for assaying individuals during model construction was identical to that used in later test assays on treatment group hatchlings of unknown behavioural phase. Hatchlings were placed singly into an arena (35.5 cm×15 cm×10 cm) and their behavioural responses to a stimulus group of 50–70 conspecifics behind a perforated plastic end wall were recorded for 10 min (Islam et al., 1994a; Islam et al., 1994b). Logistic regression analysis, as previously discussed (Simpson et al., 1999) is appropriate to predict a binary outcome such as solitary or gregarious behavioural phase from a set of independent variables. Behavioural variables, encapsulating both locomotory and non-locomotory movements and position in the arena relative to the stimulus group, were recorded for each individual [see Islam et al. (Islam et al., 1994b) for a list of variables]. These served as independent variables from which logistic regression assigned each test insect a value between zero and one representing the probability of being considered behaviourally gregarious (note that this differs from previous papers describing $P_{\text{solitary}}=1-P_{\text{gregarious}}$).

Treatment of solitary eggs with gregarious egg pod

washes, gregarious crude foam extracts and solvent controls

Between 08:30 and 13:30 h, 12 egg pods (<5-h old) were collected from crowd-reared females and separated into the foam plug and the remaining egg mass (from which encasing foam was removed). The eggs were washed in distilled water, ethanol or hexane (four pods per solvent, 2 ml each solvent). Resulting solutions were filtered by Millex syringe-driven units (Millipore Corp., Billerica, MA, USA) and 50 µl aliquots were applied to 4.25 cm fluted filter paper (Whatman, Maidstone, UK) pre-moistened with 5 µl distilled water in 25 ml plastic containers. Water-moistened cotton wool was positioned beneath each filter paper to maintain humidity. Within 5 min, individual eggs (<2-h old) from solitary mothers were

sandwiched between the treated filter paper and another untreated filter paper of the same size. The eggs were then sealed within these plastic containers and incubated at 30–32°C until eclosion. Egg foam from the 12 gregarious pods was also collected, cleaned of sand with a paintbrush, and homogenized using a pestle and mortar in ethanol, distilled water or hexane at a concentration of four plugs per 2 ml solvent. These crude foam extracts were syringe-filtered and applied to filter paper identically to egg pod washes. Analogous treatments for solvent controls (in which ethanol, distilled water or hexane alone were placed on fluted filter paper beneath eggs) were also performed. Egg washes, foam extracts and solvent controls were all prepared and deposited onto filter paper immediately before eggs were added. A total of 41 and 40 hatchlings from nine solitary pods were used for egg pod wash and foam extract treatments, respectively, and 43 hatchlings from the same pods were used as solvent treatment controls.

HPLC analysis

Extracts were prepared by homogenization of foam plugs in a porcelain mortar and pestle using 0.5 ml HPLC water (BDH, London, UK) per foam plug. Each run was conducted using freshly isolated egg foam. Homogenates were subjected to centrifugal filtration (10 000 g for 30 min) by triple-rinsed polyethersulfone membranes (3 kDa molecular mass cut-off; Vivascience AG, Hannover, Germany). Filtered extracts (500–1000 µl) were loaded onto a HPLC system (Varian Inc., Palo Alto, USA) composed of three components: a Varian 9012Q solvent delivery system, a Rainin Dynamax AI 200 automatic sample injector, and a Varian Prostar 330 photodiode array UV detector (with range set to 190–350 nm). The arrangement was controlled by a computer running Star Workstation v5.3. We utilized a 5 µm Spherisorb ODS-2 25 cm×4.6 mm (Waters, Milford, MA, USA) separation column and a flow rate of 1 ml min⁻¹. Two solvents were chosen: (A) 0.1% formic acid in H₂O and (B) acetonitrile (BDH). Method 1 was used for separation of samples related to the dose-dependence trial (Fig. 1B) and for primary and secondary separation runs (Fig. 2A,B,F,G): $t=0.0$ min, A:B=95:5; $t=18.0$ min,

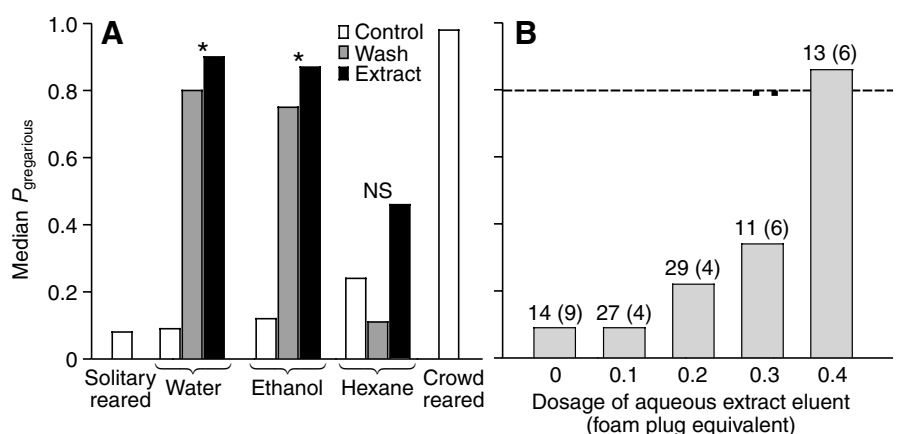


Fig. 1. Preferential solubility in polar solvents and dose-dependent behavioural effects of egg foam bioactive components. (A) Effects on hatchling phase state (represented by median $P_{\text{gregarious}}$ values) when water, ethanol or hexane are applied to eggs are compared to the effects of the same solvents applied to eggs following their use for either egg-washing or extraction of egg foam compounds. Asterisks indicate significant changes relative to control (see Table 2). Solitary-reared hatchlings (egg pods in parentheses): $N=50$ (4). Water: control, $N=14$ (9); wash, $N=15$ (9); extract, $N=15$ (9). Ethanol: control, $N=15$ (9); wash, $N=13$ (9); extract, $N=14$ (9). Hexane: control, $N=14$ (9); wash, $N=13$ (9); extract, $N=11$ (9). (B) Varying doses of whole extract HPLC eluent applied to eggs reveals a significant dose-dependent effect ($P=0.002$, one-tailed Jonckheere–Terpstra test). The median $P_{\text{gregarious}}$ value for crude (unprocessed by HPLC) foam extract at a concentration of 0.1 foam plug equivalent is shown as a broken line. Individuals per treatment are shown above bars with number of contributing egg pods in parentheses.

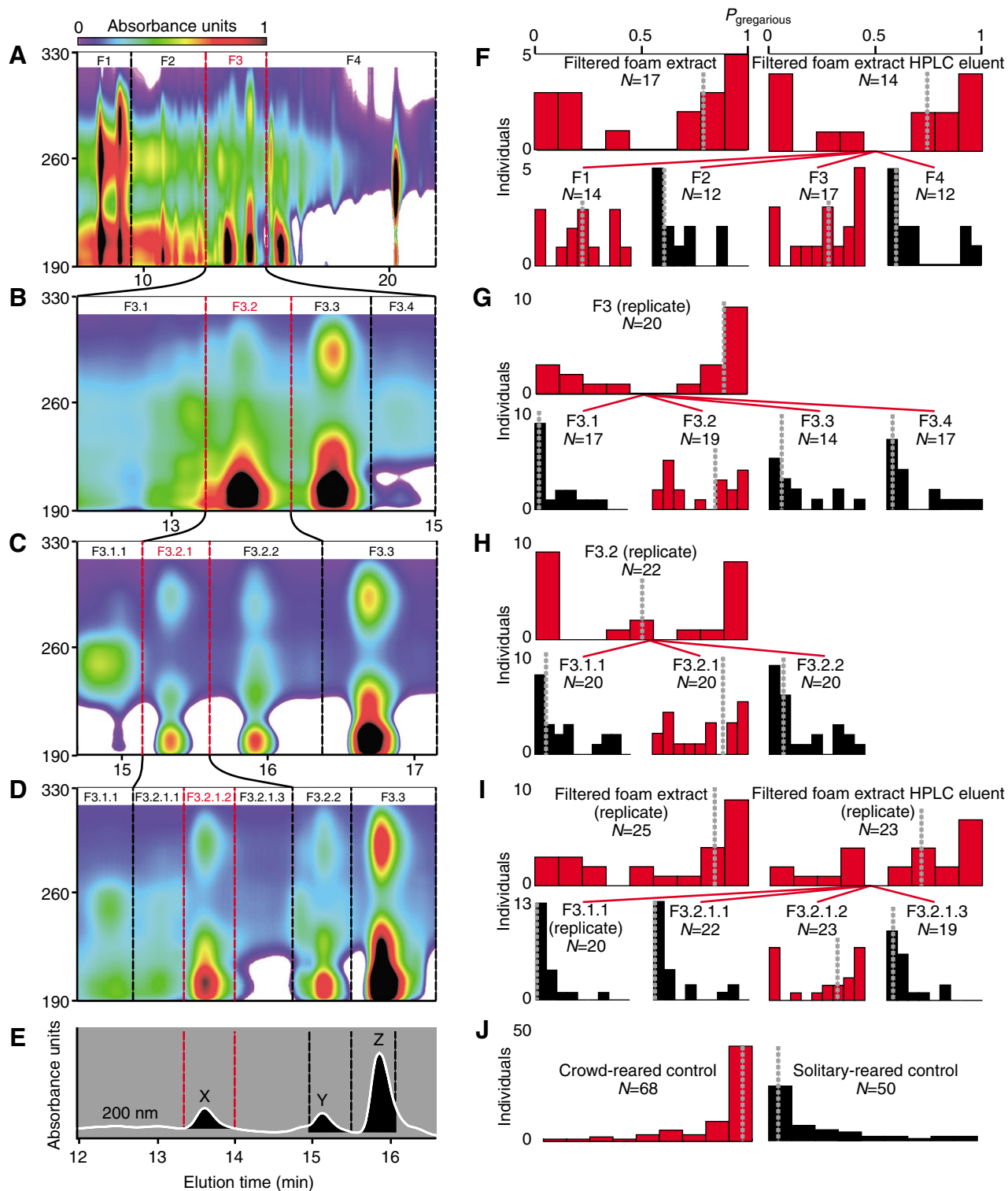


Fig. 2. Iterative HPLC separation (A–E) and behavioural bioassay results (F–J) of egg foam compounds. Eggs treated with HPLC fractions flanked by broken red lines give rise to predominately gregariously behaving hatchlings (corresponding red histograms; median $P_{gregarious}=0.5$). In the coarsest (i.e. primary) separation, all egg foam compounds were parsed into four fractions (A; F1–F4) and tested in a logistic regression behavioural bioassay (see text) for gregarizing properties (F). In the secondary run, components of fraction 3 were collected individually (B) and tested for bioactivity (G). The tertiary (C,H) and quaternary (D,I) runs were analogous with still greater specificity. Peak labelling is hierarchical and reflects peak origin in terms of previous fractions. The top row of histograms for each trial (F–I) shows the effect of known gregarizing compounds, sometimes reiterating findings of previous runs, applied to eggs as positive controls. Histogram bin widths=0.11 $P_{gregarious}$ units. Median $P_{gregarious}$ values are indicated by dotted grey lines. Peak areas (in black, E), representing F3.2.1.2 (relabelled peak X for clarity) and close analogue peaks Y and Z, were recorded for later use (see Fig. 4). Control behavioural classifications of known crowd- and solitary-reared individuals are provided (J). Insects from F were randomly drawn from four egg pods, (G) from four egg pods, (H) from nine egg pods, (I) from 13 egg pods and (J) (solitary-reared) from four egg pods.

A:B=32:68; $t=18.1$ – 22.0 min, A:B=0:100. Method 2 was used for the tertiary separation run (Fig. 2C,E): $t=0.0$ min, A:B=95:5; $t=12.0$ min, A:B=70:30; $t=17.5$ – 22.0 min, A:B=0:100. Method 3 was used for the quaternary separation run (Fig. 2D,I): $t=0.0$ min, A:B=92:8; $t=18.2$ min, A:B=72:28; $t=19$ – 24.0 min, A:B=0:100. Eluent collections were made either of entire runs (Fig. 1B) or fractions thereof (Fig. 2, all treatments excepting some controls) into 50 ml round-bottom flasks. Solvents were removed by rotary evaporation (Rotavapor, Switzerland) under 3–6 kPa at 32–36°C until 5–10 μ l solvent remained; samples were then reconstituted to a total of 250–500 μ l in HPLC water for treatment of eggs. Peak integration was performed using PolyView2000 (v5.3; Varian Inc.) on a total of 69 gregarious and 11 solitary samples. Outliers (greater than 1.5 times the interquartile range beyond upper or lower hinges) were excluded from statistical analysis.

Treatment of eggs by high performance liquid chromatography gregarious foam extracts

HPLC eluent (see below for HPLC protocols) was applied to solitary eggs in 50 μ l aliquots as described above for crude foam treatments; however, in contrast to the above treatments, each aliquot represented one of four different concentrations: (1) 0.1 foam plug equivalents (FPE) per egg (500 μ l injection, 500 μ l eluent reconstitute); (2) 0.2 FPE per egg (1000 μ l injection, 500 μ l eluent reconstitute); (3) 0.3 FPE per egg (750 μ l injection, 250 μ l eluent reconstitute); and (4) 0.4 FPE per egg (1000 μ l injection, 250 μ l eluent reconstitute). All subfraction treatments were at 0.4 FPE.

Nuclear magnetic resonance analysis

Samples for NMR were prepared identically to above, with several modifications to increase yield. Fifty gregarious egg pods were separated from sand cylinders and homogenized in 20 ml HPLC water. The resulting sand slurry was transferred to a Buchner funnel fitted with a Whatman no. 4 analytical-grade filter paper wetted with 0.5 ml HPLC water and attached to a vacuum flask. Sand was rinsed with a second volume of 20 ml, and the resultant ~40 ml extract was centrifuged at 30 000 g for 5 min. The supernatant was then centrifugally filtered through triple-rinsed polyethersulfone membranes as previously described. Samples were flash-frozen in liquid nitrogen and subjected to freeze-drying until solvents were removed (~2 days). This sample was then resuspended in 100 μ l HPLC water and separated by HPLC method 3. The gregarizing fraction (F3.2.1.2, Fig. 2D,I) was then collected, flash-frozen, and freeze-dried. This entire procedure was repeated with 100 egg pods, generating a replicate sample with more material. These two dried, purified fractions were dissolved in 7 μ l D_2O ; 6 μ l were placed into a 1 mm diameter NMR tube by centrifugation (the remainder was subjected to high-resolution analysis on a Bruker ApexQ FT-ICR-MS with Daltonics DataAnalysis 3.3, Billerica, MA, USA). Samples were analysed by 1H NMR spectroscopy on a Bruker AVANCE DRX500 NMR spectrometer equipped with a 1 mm $^1H/^{13}C/^{15}N$ TXI microprobe regulated at 25°C. There was insufficient material for the collection of 2D 1H - ^{13}C heteronuclear correlation spectra or NOE spectra.

Statistical analysis

All data were analysed with SPSS 14 (SPSS Inc., Chicago, IL, USA). Dose-dependence data were analysed by the distribution-free Jonckheere-Terpstra test for categorically related groups. Other $P_{\text{gregarious}}$ values were rank normalized prior to application of ANOVA and Dunnett's *post-hoc* analyses (Conover and Iman, 1981). Peak areas for solitary and gregarious foam were examined by Mann-Whitney *U*-tests adjusted by the Šidák correction for multiple comparisons (Abdi, 2007).

RESULTS

Logistic regression

A logistic regression model was created based upon the behaviour of 51 hatchlings from solitary-reared and 68 hatchlings from crowd-reared cultures. Three methods of model creation (enter, backward step-wise, and forward step-wise) were considered. The 'enter' method provided the most parsimonious result, correctly classifying 86% of solitary-reared and 91% of crowd-reared insects ($\chi^2=102.88$ at 6 d.f.; $P<0.001$, Table 1), and was chosen for ascribing behavioural phase state to experimental and control insects in the study. Gregarious nymphs tended to walk faster, sway more, spend less time in the middle of the arena, end up closer to the stimulus group, spend more time climbing the walls, and defecate less than did solitary nymphs (Table 1).

Effects of dosage and solvents on egg foam efficacy

We applied extracts and washes created with different solvents to eggs and assayed the behaviour of resultant hatchlings (Fig. 1A, Table 2) using the above logistic regression model. Aqueous foam-extracts and egg-washes had significant gregarizing activity when applied to solitary eggs (Table 2). Ethanolic foam-extracts and egg-washes also significantly gregarized hatchlings from solitary eggs, but hexane treatments showed no significant effect on behavioural phase state (Table 2). When water and increasing concentrations of aqueous foam extract eluent were applied to solitary eggs, a highly significant dose-dependent response on hatchling behavioural phase was observed (Fig. 1B, one-tailed Jonckheere-Terpstra test, $P=0.002$). At a concentration of 0.4 FPE, the gregarizing effect of aqueous extract eluent was comparable to 0.1 FPE crude (pre-HPLC) extract.

Effect of HPLC fractions applied to eggs on hatchling behavioural phase

Some HPLC fractions applied to eggs affected their development; we identified these bioactive fractions and iteratively examined

Table 1. Variables retained in logistic regression model used to parameterise differences between hatchlings from solitary- and crowd-reared females

Variable	Coefficient β	R^*	Significance of likelihood ratio
Walking speed	4.2757	0.2653	0.0003
Swaying	68.4100	0.1575	0.0144
Time spent in middle arena	-0.0707	-0.2514	0.0005
X-distance	0.7448	0.1034	0.0538
Climbing time-fraction	8.9775	0.2400	0.0008
Defecation time-fraction	-58.9910	-0.1661	0.0112
Constant	-2.8739		0.0054

Model: $\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_k x_k$ with probability (gregarious) = $e^{\eta} / (1 + e^{\eta})$.

* R values can range from -1 to +1; positive values indicate that as the variable increases so does probability (gregarious).

Significance values indicate the extent to which each variable contributes to the model. Positive coefficients indicate positive correlation with gregarious classification. X-distance varies from +1 (nearest stimulus group) to -1 (farthest from stimulus group).

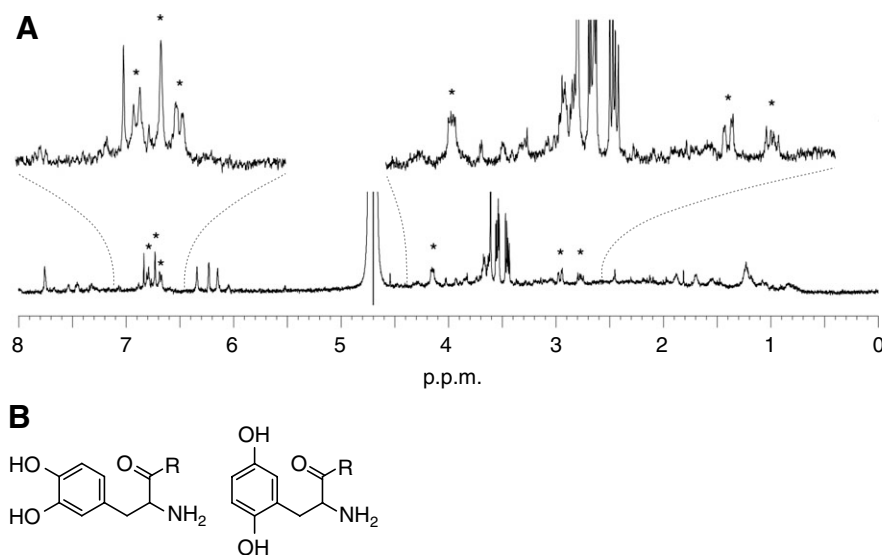


Fig. 3. Structural analysis. NMR spectroscopy (A) suggests the bioactive foam agent is an alkylated L-dopa analogue with either para- or ortho-substituted hydroxyl groups (B). The alkyl side chain (R) is probably an ethyl or propyl group. NMR data are summarized in the text as well as in Table 2.

increasingly specific subfractions (Fig. 2). The primary (Fig. 2A,F) run served to identify fraction 3 (F3) as bioactive [a bioactive fraction, in this context, is defined as one resulting in predominantly gregarious hatchlings (i.e. a set of hatchlings whose median $P_{\text{gregarious}} \geq 0.5$) when applied to eggs]. Filtered foam extract (FPE 0.1) and filtered extract HPLC eluent (FPE 0.4) were included as positive controls. Although fraction 1 (Fig. 2A,F) was marginally gregarizing (median $P_{\text{gregarious}} = 0.50$), further assays concentrated on F3 (median $P_{\text{gregarious}} = 0.62$). The behavioural phase state of hatchlings treated with F3 was not statistically distinguishable from phase distributions of either filtered extract or filtered extract HPLC eluent (Mann–Whitney U -tests, $P = 0.94$ and $P = 0.81$, respectively). A secondary (Fig. 2B,G) separation run confirmed bioactivity of F3, and subfractions 3.1, 3.2, 3.3 and 3.4 were further assayed for gregarizing potency. Fraction 3.2 (F3.2) was identified as the sole gregarizing portion of F3; this subfraction resulted in a hatchling phase distribution statistically indistinguishable from that induced by application of the complete F3 (Mann–Whitney U -test, $P = 0.18$). F3.2, when further separated by HPLC method 2, was revealed to consist of two very similar molecules (Fig. 2C), only one of which (F3.2.1, Fig. 2H) was bioactive. The hatchling phase distribution resulting from HPLC subfraction 3.2.1 was not statistically distinguishable from that of F3.2 (Mann–Whitney U -test, $P = 0.59$). In the final, quaternary separation run, the solvent gradient was adjusted to separate more finely F3.2.1 into three ‘sub-subfractions’. Only the second of these (F3.2.1.2, Fig. 2D) was bioactive, and the distribution of resulting hatchlings was not statistically distinguishable from either filtered foam extract or filtered foam extract HPLC eluent (Mann–Whitney U -tests, $P = 0.51$ and $P = 0.61$, respectively).

Table 2. Statistical analysis of solvent extracts and washes (ANOVA on rank-normalised data with Dunnett’s one-tailed *post-hoc* tests)

F values	P	Multiple comparisons	P
$F_{(2,41)} = 6.44$	0.004	Water control vs water egg-pod wash	0.006
		Water control vs water foam extract	0.002
$F_{(2,39)} = 8.30$	0.001	Ethanol control vs ethanol egg-pod wash	0.003
		Ethanol control vs ethanol foam extract	0.001
$F_{(2,35)} = 0.41$	0.670	Hexane control vs hexane egg-pod wash	0.326
		Hexane control vs hexane foam extract	0.379

Spectroscopic analysis

NMR analyses (Fig. 3A, Table 3) of two independent samples of F3.2.1.2 (Fig. 2D) yielded several consistent sets of resonances. Absorbances between 6.80 and 6.68 ppm (Fig. 3A, Table 3) are indicative of a dihydroxylated (trisubstituted) aromatic system. Chemical shifts accommodate the possibility of either para- or ortho-substituted hydroxylation (Fig. 3B) with coupling patterns (Table 3) indicating proton 1–2–4 relationships. Signals at 4.15, 2.96 and 2.77 ppm (Table 3) match the CH–CH₂ patterns typical for amino acids and are consistent with the shifts expected for L-dopa analogues (Fig. 3B). Broadened resonances in the region 2–0.6 ppm are consistent with a C2 or C3 alkyl sidechain.

Peak area analysis: a comparison of gregarious and solitary egg foam

Peak X (the bioactive peak) did not differ significantly in area between solitary and gregarious foam (Šidák-corrected Mann–Whitney, $P = 0.22$). However, peak Y was significantly elevated in solitary *versus* gregarious foam (Šidák-corrected Mann–Whitney, $P = 0.03$).

DISCUSSION

We present the first direct evidence for a specific chemical agent responsible for transmission of gregarious behaviour between locust generations. This set of experiments shows, for the first time, that phase-related behavioural effects can be elicited in a dose-dependent manner by application of specific egg foam components to developing solitary eggs. Our approach has been to isolate and bioassay successively more specific aqueous egg-foam-extract components; finally, by spectrometry, we characterized the most specific fraction. At each level of analysis (primary, secondary, tertiary and quaternary separation runs; Fig. 2A,B,C,D, respectively) we ‘magnified’ a region of interest while implementing a positive control for gregarization (application of a more general set of foam compounds). Such bootstrapping allows confidence in our conclusions; the bioactivity of filtered foam extracts, filtered foam extract HPLC eluents, fraction 3, and fraction 3.2 are all independently replicated.

Table 3. Summary of NMR data

Chemical shift δ (p.p.m.)	Coupling constant J (Hz)
6.80	8.5
6.73	Broadened singlet
6.68	8.5, ~1
4.15	4.5, 9.5
2.96	4.5, 16.5
2.77	9.5, 16.5

The presence of behaviourally gregarizing factors in polar (aqueous and ethanolic) but not organic (hexane) solvents (Fig. 1A) is consistent with and extends earlier studies (Islam, 1997; McCaffery et al., 1998) in which polar solvents were found to contain compounds affecting gregarious colouration. As recently reviewed (Simpson and Miller, 2007), findings such as these discount the notion of bioactive unsaturated ketones (Malual et al., 2001). The dose-dependent effect of HPLC eluent upon behavioural phase (Fig. 1B) further supports the presence of bioactive agent(s) in aqueous extracts. Whether or not the same compounds are involved in maternal transmission of colouration and behaviour is a fascinating question worthy of further study.

Since peaks X (F3.2.1.2) and Z (F3.3) share nearly identical UV spectra (Fig. 2C,D) and retention times (Fig. 2A–E), they may represent conformational isomers. Interestingly, in contrast to its highly bioactive (Fig. 2H,I) putative isomer, peak Z is not bioactive (Fig. 2B,G). Peaks X and Y (F3.2.2), which are similar in retention time (Fig. 2A–D) and UV absorbance (Fig. 2C,D), are posited to be related through metabolic or oxidative pathways.

Spectroscopic details (Fig. 3A–C) of the maternal gregarizing agent suggest that an alkylated L-dopa analogue (Fig. 3B) is responsible for transgenerational phase transmission. As an extremely versatile and widespread component of invertebrate biochemical systems, L-dopa can be synthesized from tyrosine by at least three distinct enzymatic pathways (Waite, 1992). In invertebrates alone, L-dopa and its metabolites are involved in processes as diverse as egg capsule formation (Smyth and Clegg, 1959), silk production (Kramer et al., 1989), immunity (Nappi et al., 1991) and predator defence (Prota et al., 1981). Since L-dopaminergic pathways are involved in both insect behavioural plasticity (Goldstein and Camhi, 1991; Kostowski et al., 1975) and learning (Zhang et al., 2007), perhaps it is not surprising that an L-dopa analogue is implicated in locust phase transmission.

Although differences in gene expression have been found between locust phases (Claeys et al., 2006; De Loof et al., 2006; Kang et al., 2004), our work is the first to identify and demonstrate bioactive egg foam components capable of causing such changes in gene expression. Using genetic arrays containing locust expressed sequence tags, the effects upon gene expression of compounds such as those in Fig. 3B could be assayed and compared to the extreme expression profiles of solitary and gregarious specimens. An EST library for *S. gregaria* is currently in preparation (De Loof et al., 2006).

How might peak X (the bioactive compound) be regulated, given its presence in equal amounts in solitary and gregarious foam (Fig. 4)? Differential sensitivity due to differential receptor expression, such as occurs in the tobacco hornworm (*Manduca sexta*) where ecdysterone sensitivity is regulated via variable receptor expression (Fujiwara et al., 1995), cannot apply to gregarization in the locust since solitary eggs are capable of responding to gregarizing cues (e.g. Figs 1, 2). Rather, our data (Fig. 4) are consistent with inhibition of the active compound X by

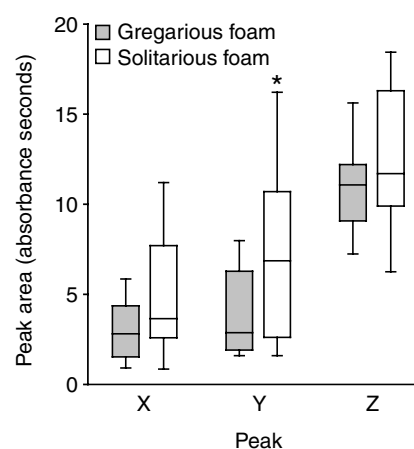


Fig. 4. Relative amounts of related peaks X, Y and Z (see Fig. 2D,E) in foam from solitary and gregarious locust egg pods. Peak Y is significantly elevated in solitary versus gregarious foam, whereas Peak X is unchanged (Šidák-corrected Mann–Whitney, $P=0.03$ and $P=0.22$, respectively). Boxplot whiskers represent 10th and 90th percentiles.

a second factor. We suggest peak Y may be the inhibitory factor, based on two observations: peak Y is found in significantly elevated (twice-median) concentrations in solitary versus gregarious egg foam (Fig. 2C), and peaks X and Y are likely to be close homologues since they elute at similar times and share nearly indistinguishable UV spectra (Fig. 2D). Owing to the molecular similarity of the two peaks, peak Y may competitively inhibit the action of peak X at the receptor level. Hence, gregarization is postulated to result from reduced levels of peak Y in the egg foam of behaviourally gregarized females. Such inhibitory effects are not uncommon in insect development; for example, juvenile hormone can inhibit certain ecdysteroid activity (Heming, 2003). Finally, although we show peak X is sufficient to elicit behavioural gregarization (Fig. 2I) and provide NMR data (Fig. 3) about the dominant molecule in this peak, we cannot exclude the possibility that gregarization is reliant upon a co-eluting compound whose concentration is beneath NMR detection thresholds.

Unfortunately we could not test for explicit inhibitory effects of peak Y or obtain conclusive mass spectral data because of unavailability of experimental *S. gregaria* in Australia. We suggest that investigation of the interactions of peaks X and Y are a matter of priority for those with access to *S. gregaria* in the laboratory. If our hypothesis is supported, future work might be directed toward blocking maternal gregarization (and consequently blocking perpetuation of locust swarms) by development of potent, specific inhibitors similar to peak Y.

The authors would like to thank Neil J. Oldham for extensive support and many helpful discussions. G.A.M. was funded by a National Science Foundation Graduate Research Fellowship (USA), an Oxford Clarendon Fellowship (UK) and a grant from the John Ray Trust (UK). M.S.I. would like to thank the Association of Commonwealth Universities (UK) for support and the University of Rajshahi (Bangladesh) for granting him sabbatical leave. S.J.S. is currently funded by an Australian Research Council Federation Fellowship; his work at Oxford was supported by the Biotechnology and Biological Sciences Research Council (UK). Technical assistance from Steve Roberts is gratefully acknowledged.

REFERENCES

- Abdi, H. (2007). Bonferroni and Sidak corrections for multiple comparisons. In *Encyclopedia of Measurement and Statistics* (ed. N. J. Salkind), pp. 103–107. Thousand Oaks, CA: Sage.

- Bouaichi, A., Roessingh, P. and Simpson, S. J. (1995). An analysis of the behavioral-effects of crowding and re-isolation on solitary-reared adult desert locusts (*Schistocerca gregaria*) and their offspring. *Physiol. Entomol.* **20**, 199-208.
- Claeys, I., Breugelmans, B., Simonet, G., Van Soest, S., Sas, F., De Loof, A. and Vanden Broeck, J. (2006). Neuroparsin transcripts as molecular markers in the process of desert locust (*Schistocerca gregaria*) phase transition. *Biochem. Biophys. Res. Commun.* **341**, 599-606.
- Clynen, E., Huybrechts, J., Verleyen, P., De Loof, A. and Schoofs, L. (2006). Annotation of novel neuropeptide precursors in the migratory locust based on transcript screening of a public EST database and mass spectrometry. *BMC Genomics* **7**, 15.
- Conover, W. J. and Iman, R. L. (1981). Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* **35**, 124-129.
- De Loof, A., Claeys, I., Simonet, G., Verleyen, P., Vandersmissen, T. I. M., Sas, F. and Huybrechts, J. (2006). Molecular markers of phase transition in locusts. *Insect Sci.* **13**, 3-12.
- Dirsh, V. M. (1953). Morphometric studies on phases of the desert locust (*Schistocerca gregaria* Forskal). *Antilocust Bull.* **16**, 1-34.
- Faure, J. C. (1932). The phases of locusts in South Africa. *Bull. Entomol. Res.* **23**, 293-405.
- Fujiwara, H., Jindra, M., Newitt, R., Palli, S. R., Hiruma, K. and Riddiford, L. M. (1995). Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. *Insect Biochem. Mol. Biol.* **25**, 845-856.
- Goldstein, R. S. and Camhi, J. M. (1991). Different effects of the biogenic amines dopamine, serotonin, and octopamine of the thoracic and abdominal portions of the escape circuit in the cockroach. *J. Comp. Physiol. A* **168**, 103-112.
- Hägele, B. F., Oag, V., Bouaichi, A., McCaffery, A. R. and Simpson, S. J. (2000). The role of female accessory glands in maternal inheritance of phase in the desert locust *Schistocerca gregaria*. *J. Insect Physiol.* **46**, 275-280.
- Heming, B. S. (2003). *Insect Development and Evolution*. Ithaca: Cornell University Press.
- Hunter-Jones, P. (1958). Laboratory studies on the inheritance of phase characters in locusts. *Antilocust Bull.* **29**, 1-32.
- Islam, M. S. (1996). Dynamics of behavioural phase change in the first-instar nymphs of the desert locust *Schistocerca gregaria* (Forsk.). *Pak. J. Zool.* **28**, 323-330.
- Islam, M. S. (1997). A preliminary report on the biochemical properties of a maternally-produced gregarizing factor in the desert locust *Schistocerca gregaria* (Forsk.). *J. Asiat. Soc. Bangladesh Sci.* **23**, 111-122.
- Islam, M. S., Roessingh, P., Simpson, S. J. and McCaffery, A. R. (1994a). Effects of population-density experienced by parents during mating and oviposition on the phase of hatching desert locusts, *Schistocerca gregaria*. *Proc. R. Soc. Lond. B Biol. Sci.* **257**, 93-98.
- Islam, M. S., Roessingh, P., Simpson, S. J. and McCaffery, A. R. (1994b). Parental effects on the behavior and coloration of nymphs of the desert locust *Schistocerca gregaria*. *J. Insect Physiol.* **40**, 173-181.
- Jablonska, E. and Lamb, M. J. (2002). The changing concept of epigenetics. *Ann. N. Y. Acad. Sci.* **981**, 82-96.
- Jablonska, E. and Lamb, M. J. (2005). *Evolution in Four Dimensions: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life*. Cambridge: MIT Press.
- Jablonska, E., Matzke, M., Thieffry, D. and Van Speybroeck, L. (2002). The genome in context: biologists and philosophers on epigenetics. *BioEssays* **24**, 392-394.
- Kang, L., Chen, X., Zhou, Y., Liu, B., Zheng, W., Li, R., Wang, J. and Yu, J. (2004). The analysis of large-scale gene expression correlated to the phase changes of the migratory locust. *Proc. Natl. Acad. Sci. USA* **101**, 17611-17615.
- Kostowski, W., Tarchalska, B. and Wanchowicz, B. (1975). Brain catecholamines, spontaneous bioelectrical activity and aggressive-behaviour in ants (*Formica rufa*). *Pharmacol. Biochem. Behav.* **3**, 337-342.
- Kramer, K. J., Bork, V., Schaefer, J., Morgan, T. D. and Hopkins, T. L. (1989). Solid state ¹³C NMR and chemical analyses of insect noncuticular sclerotized support structures: mantid oothecae and cocoon silks. *Insect Biochem.* **19**, 69-77.
- Malual, A. G., Hassanali, A., Torto, B., Assad, Y. O. and Njagi, P. G. (2001). The nature of the gregarizing signal responsible for maternal transfer of phase to the offspring in the desert locust *Schistocerca gregaria*. *J. Chem. Ecol.* **27**, 1423-1435.
- McCaffery, A. R., Simpson, S. J., Islam, M. S. and Roessingh, P. (1998). A gregarizing factor present in the egg pod foam of the desert locust *Schistocerca gregaria*. *J. Exp. Biol.* **201**, 347-363.
- Nappi, A. J., Carton, Y. and Frey, F. (1991). Parasite-induced enhancement of hemolymph tyrosinase activity in a selected immune reactive strain of *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* **18**, 159-168.
- Pener, M. P. (1991). Locust phase polymorphism and its endocrine relations. *Adv. Insect Physiol.* **23**, 1-79.
- Pener, M. P. and Yerushalmi, Y. (1998). The physiology of locust phase polymorphism: an update. *J. Insect Physiol.* **44**, 365-377.
- Prota, G., Ortonne, J. P., Voulot, C., Khatchadourian, C., Nardi, G. and Palumbo, A. (1981). Occurrence and properties of tyrosinase in the ejected ink of Cephalopods. *Comp. Biochem. Physiol. B* **68**, 415-419.
- Rahman, M. M., Hoste, B., De Loof, A. and Breuer, M. (2002). Developmental effect of egg pod foam in the desert locust *Schistocerca gregaria* (Caelifera: Acrididae). *Entomol. Gen.* **26**, 161-172.
- Roessingh, P., Simpson, S. J. and James, S. (1993). Analysis of phase-related changes in behavior of desert locust nymphs. *Proc. R. Soc. Lond. B Biol. Sci.* **252**, 43-49.
- Rogers, S. M., Matheson, T., Despland, E., Dodgson, T., Burrows, M. and Simpson, S. J. (2003). Mechanosensory-induced behavioural gregarization in the desert locust *Schistocerca gregaria*. *J. Exp. Biol.* **206**, 3991-4002.
- Schmidt, G. H. and Albutz, R. (1999). Identification of solitary and gregarious populations of the desert locust, *Schistocerca gregaria*, by experimental breeding (Caelifera: Acrididae). *Entomol. Gen.* **24**, 161-175.
- Simpson, S. J. and Miller, G. A. (2007). Maternal effects on phase characteristics in the desert locust, *Schistocerca gregaria*: a review of current understanding. *J. Insect Physiol.* **53**, 869-876.
- Simpson, S. J. and Sword, G. A. (2007). Phase polyphenism in locusts: mechanisms, population consequences, adaptive significance and evolution. In *Phenotypic Plasticity in Insects* (ed. T. Ananthakrishnan and D. Whitman), pp. 93-135. Enfield, NH: Science Publishers.
- Simpson, S. J., McCaffery, A. R. and Hägele, B. F. (1999). A behavioural analysis of phase change in the desert locust. *Biol. Rev. Camb. Philos. Soc.* **74**, 461-480.
- Smyth, J. D. and Clegg, J. A. (1959). Egg-shell formation in trematodes and cestodes. *Exp. Parasitol.* **8**, 286-323.
- Uvarov, B. P. (1966). *Grasshoppers and Locusts: A Handbook of General Acridology*, Vol. 1. London: Cambridge University Press.
- Waite, J. H. (1992). The DOPA ephemer: a recurrent motif in invertebrates. *Biol. Bull.* **183**, 178-184.
- West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. New York: Oxford University Press.
- Zhang, K., Guo, J. Z., Peng, Y. Q., Xi, W. and Guo, A. K. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. *Science* **316**, 1901-1904.