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Supporting Online Material for

**Serotonin Mediates Behavioral Gregarization Underlying Swarm
Formation in Desert Locusts**

Michael L. Anstey, Stephen M. Rogers,* Swidbert R. Ott, Malcolm Burrows,
Stephen J. Simpson

*To whom correspondence should be addressed. E-mail: smr34@cam.ac.uk

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Underlying Swarm Formation in Desert Locusts**

Michael L. Anstey*, Stephen M. Rogers*†, Swidbert R. Ott, Malcolm Burrows and
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* These Authors contributed equally to this work.

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Materials and Methods

Animals and husbandry

Experiments were performed on final nymphal-instar desert locusts, *Schistocerca gregaria* (Forskål) taken from the gregarious and solitary colonies in the Department of Zoology at the University of Oxford, UK. Gregarious phase locusts were reared in large plastic bins (56 cm × 76 cm × 60 cm) containing 450–1000 insects per bin over many generations. Solitary phase locusts were generated from this long-term gregarious stock by rearing them in isolation for two or three generations (*SI*). The colonies were maintained under a 12:12 light/dark photo regime at $30 \pm 2^\circ\text{C}$ and on a diet of fresh greenhouse-grown wheat seedlings and wheat germ.

Behavioral assay and logistic regression model

Experiments were performed in a rectangular Perspex arena (57 cm × 30 cm × 10 cm) with opaque walls and clear top. The side walls of the arena were coated with Fluon (Whitford Plastics Ltd. UK) to prevent climbing. The disposable paper floor was marked with a Cartesian grid. Stimulus chambers, backlit so that they were brighter than the central area, were located at each end of the arena fronted by perforated transparent plastic partitions. One randomly selected chamber contained 20 fifth-instar long-term gregarious locust nymphs from the crowded colony, and the other chamber was left empty. Prior to observation, the test insect was placed in a modified opaque 20 ml plastic syringe with a wide neck and cap for 10 min. It was then introduced into the arena through a 2 cm hole in the center of the arena floor and observed for 500 s. Behavior was

recorded in real time using an event recorder (*SI*). Eighteen different behaviors were recorded, expressed as a mixture of time-delimited behaviors and categorical events.

To quantify the behavioral state of an experimental animal (i.e., of solitary locusts that had received gregarizing treatment and / or pharmacological manipulations), we developed a binary logistic regression model of phase state in SPSS v14.0 (SPSS Inc., 2005) The model was generated by comparing 100 second or third generation solitary-reared locusts with 100 long-term gregarious insects. Behavioral covariates were added using a forward stepwise approach until no further improvement was gained by adding further behaviors. The parameters of the model were adjusted until an optimal difference between these two groups of known phase was achieved, according to the logistic equation:

$$P_{greg} = e^{\eta} / (1 + e^{\eta}), \text{ where } \eta = \beta_0 + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \dots + \beta_k \cdot X_k$$

where X_1 to X_k are behavioral covariates, β_1 to β_k are coefficient weightings associated with the covariates as optimized by the model and β_0 is a constant. P_{greg} is the probability that a locust should be considered a member of the crowd-reared population and therefore ranges from 1 to 0, where a value of 1 means that the individual displayed behaviors that were indistinguishable from those produced by the crowd-reared group and 0 means that the individual displayed behaviors that were indistinguishable from those produced by the solitary-reared group.

The most robust indicators of phase state retained in the model were: walking speed, proportion of time spent motionless, time spent near the stimulus group, and time spent

grooming (Table S1), which correctly classified 91% and 89% of known solitary and gregarious locusts, respectively. The overall significance of the model was <0.001 (Omnibus test of model coefficients, Chi-square =173.349, 4df) and the significance levels of the individual model parameters are given in Table S1.

Having built the model using animals of known phase state it was possible to quantify behavior of locusts subjected to different treatments and therefore of unknown phase state by generating P_{greg} based on their performance in the arena. Intermediate values indicated a transitional or ambiguous behavioral state. Nevertheless, binary logistic regression models of locust behavior characteristically yield skewed distributions of behavioral state. This is in part a reflection of the model classification algorithm but also of underlying biological processes. Gregarization once initiated is a rapid process that can be difficult to catch in transit based on a single brief period of observation. This can lead to the appearance of somewhat bimodal behavioral patterns. A snapshot of behavior will include high proportions both of individuals that have already made the transition and of those yet to begin to change, purely through a process of stochastic variation. This necessitated the use of non-parametric statistics to compare treatments, or the use of rank normal transformation to render the data suitable for parametric analysis.

Treatments used to induce behavioral gregarization

Solitary locusts were gregarized through one of four methods. The duration of treatment was between 30 min and 4 h depending on the particular experiment.

1) *Forced crowding with gregarious locusts.* Up to 15 solitary locusts were crowded together with 30 12-h food-deprived gregarious locusts in a 15 cm × 19 cm × 13 cm cage

with a metal perch and without food. Control insects were individually transferred to similar small cages containing no food and no other locusts.

2) *Exposure to visual and olfactory stimuli from gregarious locusts.* Individual solitary locusts were placed in clear plastic pint glass beakers with double-layered aluminum mesh covering the top. The beakers were placed in a rearing cage containing 450–1000 fifth-instar locust nymphs in the gregarious colony room. Test insects therefore received visual and olfactory stimuli from the gregarious locusts but no physical contact.

3) *Mechanosensory stimulation of the left hind femur.* Individual solitary locusts were placed into clear plastic cages (8 cm × 6 cm × 10 cm) that had mesh wire at both ends through which a fine paintbrush (Synthetic Humbrol no. 2) was used to stroke the outside surface of the left hind femur (S2). The femur was repeatedly stimulated for 5 s in each minute.

4) *Electrical stimulation of metathoracic nerve 5.* The methodology was designed to simulate stroking the hind femur with a paintbrush (S3). In brief, the left metathoracic nerve 5 received a 5 s pulse train (5–8 V) every minute via a pair of 50 μm steel wire electrodes driven by a stimulus generator (Master 8, A.M.P.I., Jerusalem, Israel). Each 5 s pulse train consisted of bursts of 10 ms square pulses at 50 Hz organized into blocks of 200 ms separated by 200 ms intervals. Following treatment the animal was given 10 minutes of recovery time.

Quantification of serotonin in nervous tissue extracts

Serotonin (5-hydroxytryptamine, 5HT) was quantified in extracts of thoracic ganglia by reverse-phase high performance liquid chromatography (HPLC) and electrochemical

detection (S4). After behavioral observation, each locust was immediately submerged in liquid nitrogen for approximately one minute. The frozen locust was placed on an ice-cooled block. The thoracic ganglia were dissected out and placed in a 100 µl micro-homogenizer (Glass Precision Engineering Limited, Leighton Buzzard, UK) containing 50 µl of ice-cold 0.15 M perchloric acid and homogenized for 3 min on ice. The homogenate was transferred to 1.5 ml Eppendorf tubes (Eppendorf International, Hamburg, Germany) using a Hamilton syringe (Hamilton Company, Reno, NV, USA) and centrifuged at 17500 g for 30 min at room temperature. The supernatant was transferred to a second Eppendorf tube and stored at -80°C until HPLC analysis.

A cooled auto-injector (CMA/200) was used to load 10 µl samples of supernatant into an isocratic HPLC system comprising a M480 pump (Gynkotec, Germering, Germany; flow rate 200 µl / minute; 0.085 M sodium acetate buffer), a C18 reversed phase column heated at 35°C (Phenomenex, 3 µm Sphere Clone, 15 cm length × 2.0 mm inner diameter; Torrance, California, USA), and a BAS 6 mm Unjet cell at +0.65 V for detection (Waters M469, Milford, MA, USA; see S5). For integration and results handling, a Gynkosoftware (Dionex, Sunnyvale, CA, USA) integration package was employed. Serotonin was quantified by reference to external standards and the detection limits were 0.05–0.25 pg per sample.

Serotonin receptor antagonist injections

Two different serotonin receptor antagonists, ketanserin and methiothepin, were administered as a cocktail to attempt to cover the range of possible pharmacological types of 5HT receptors present in locusts. Ketanserin is a selective antagonist of vertebrate

5HT₂ receptors (*S6*), but has also been used successfully to block the action of serotonin in several insect species including locusts (*S7–S10*). Methiothepin is a non-selective 5HT_{1,5,6} receptor antagonist in vertebrates and displays high affinity to most invertebrate 5HT receptors (*S11*). It has been used in a number of invertebrate studies (*S12, S13*), including insects (*S14*). The antagonist solution contained 0.1 mM ketanserin (Sigma-Aldrich, Poole, UK), 0.1 mM methiothepin (Sigma-Aldrich) and 0.5% Fast Green (Sigma-Aldrich; a dye used to confirm that the drugs successfully entered the ganglia) in standard locust saline. Control insects received injections of 0.5% Fast Green in saline. Locusts were restrained ventral side up in Plasticine™ and a small flap was cut in the thoracic cuticle to expose the meso- and meta-thoracic ganglia. The preparation was then cooled on ice for 20 min. The injection solutions were loaded into glass microelectrodes and injected directly into the meso- and metathoracic ganglia by 500 ms pressure pulses (15 psi) using a pneumatic picopump (World Precision Instruments Ltd., Stevenage, Hertfordshire, UK). Each ganglion received three 54 ± 29.1 μ l (mean \pm SD) injections. Following injections, the cuticle was replaced and the animals allowed 30 minutes recovery time before being given a gregarizing treatment: either the left hind femur was mechanically stimulated, or they were presented with the sight and smell of gregarious locusts for 1 h as detailed above. The surgery and injections had no apparent negative effects on the locusts and all insects were still alive and active 24 h after the treatment.

AMTP injections

Repeated systemic injection with α -methyltryptophan (AMTP; Sigma-Aldrich), a competitive inhibitor of 5HT synthesis, has previously been used to deplete 5HT stores in insects (*S15–S17*). Solitarious locusts received injections of 40 μ l standard locust saline

containing 0.1 mM AMTP or without (controls) 120 h, 72 h, 24 h and 1 h before having their hind legs mechanically stimulated for 2 h. Injections were made into the thoracic haemocoel by inserting a microsyringe (Hamilton) ventrally between the second and third abdominal segments and maneuvering the syringe needle until the tip was in the thoracic cavity.

Topical application of serotonin

Locusts were restrained ventral side up in Plasticine™ and the meso- and metathoracic ganglia exposed. A fragment of filter paper soaked in 2% protease solution (Sigma-Aldrich, type XIV) in saline was applied to the ventral surface of the ganglia for 10 min to facilitate the access of 5HT to the CNS, and then rinsed off with saline. The exposed ganglia were surrounded with a barrier of petroleum jelly and drops of 5HT solution (1 mM in saline, Sigma-Aldrich) or saline only (control) were added until a pool of the solution formed. The preparation was left for 2 h and fresh solution was added every 30 minutes or when needed. Afterwards the cuticle was replaced and secured using wax. The locust was removed from the Plasticine™ and allowed 10 minutes recovery before having its behavioral phase state assayed.

Injections of serotonin receptor agonists

Targeted injections of a 5HT receptor agonist cocktail were used to activate the serotonergic pathways in the thoracic ganglia. α -Methylserotonin has been used previously to activate serotonin receptors in locusts (S18) and has a high affinity for the *Drosophila* 5HT_{2Dro} receptor (S19). 5-carboxamidotryptamine (5CT) is a non-selective 5HT_{1,5,7} receptor agonist in vertebrates (S20) and displays a high binding affinity to many

invertebrate serotonin receptors (*S11*, *S12*, *S14*). Locusts were injected directly into the meso- and metathoracic ganglia with 1 mM α -methylserotonin (Sigma-Aldrich), 1 mM 5CT (Sigma-Aldrich) and 0.5% Fast Green (Sigma-Aldrich) in standard locust saline using the method described above for receptor antagonists. Control insects received injections of 0.5% Fast Green in saline. Following injections locusts were returned to their rearing cage for 1 h before being behaviorally assayed. The agonists were applied in a single set of injections, to avoid the potential trauma involved in making repeated intraganglionic penetrations. This contrasted with the application of serotonin, which was applied externally as a continuously replenished pool. Since we did not know how long the agonists persist after injection in the central nervous system we elected to assay the locusts after a briefer period of just 1 h. The surgery and injections had no apparent negative effects on the locusts and all were still alive 24 hours after the treatment.

Injections of 5HTP

The intermediary product 5-hydroxytryptophan (5HTP) is the rate-limiting factor in the synthesis of 5HT (*S21*) and application of exogenous 5HTP has been shown to cause increased 5HT synthesis in the central nervous systems of insects (*S22*). Locusts were injected into the thoracic haemocoel (using the method described above for AMTP) with 40 μ l of either 10 mM 5HTP (Sigma-Aldrich) in locust saline or a saline control.

Following the injection, the animals were returned alone to their rearing cages and allowed to recover for 30 minutes. Locusts were then given either 30 more minutes in the rearing cage with no stimulation or 30 minutes crowding with gregarious locusts.

Supporting Data

Time course of behavioral gregarization through the two sensory pathways

Solitarious locusts acquire fully gregarious behavior within 4 h of crowding (S23). This can be driven through activation of either the cephalic (sight and smell of other locusts) or the thoracic (mechanosensory stimulation of a hind leg) pathways. We analyzed whether the rate of acquisition of gregarious behaviors within this 4 h window differed when locusts were stimulated through different sensory modalities. We subjected solitarious locusts for 0 h (controls), 1 h, 2 h or 4 h to one of four different gregarizing treatments: (1) crowding with gregarious locusts; (2) sight and smell of gregarious locusts; (3) mechanosensory stimulation of a hind femur; and (4) electrical stimulation of hind leg nerve 5, designed to mimic the input elicited by mechanosensory stimulation. Immediately following the gregarizing treatment, the behavioral phase state of each locust was determined. There was a rapid acquisition of gregarious behavioral characteristics with a significant increase in P_{greg} to between 0.7 and 0.8 for all treatments after only 1 h treatment and no further significant increase after 2 h (ANOVA of rank normal transformed data, effect of time $F_{3, 257} = 34.05$, $P < 0.0001$, Bonferroni post-hoc test). There were no differences between treatments ($F_{3, 257} = 0.166$, $P < 0.919$, interaction between time and treatment $F_{9, 257} = 0.756$, $P < 0.657$).

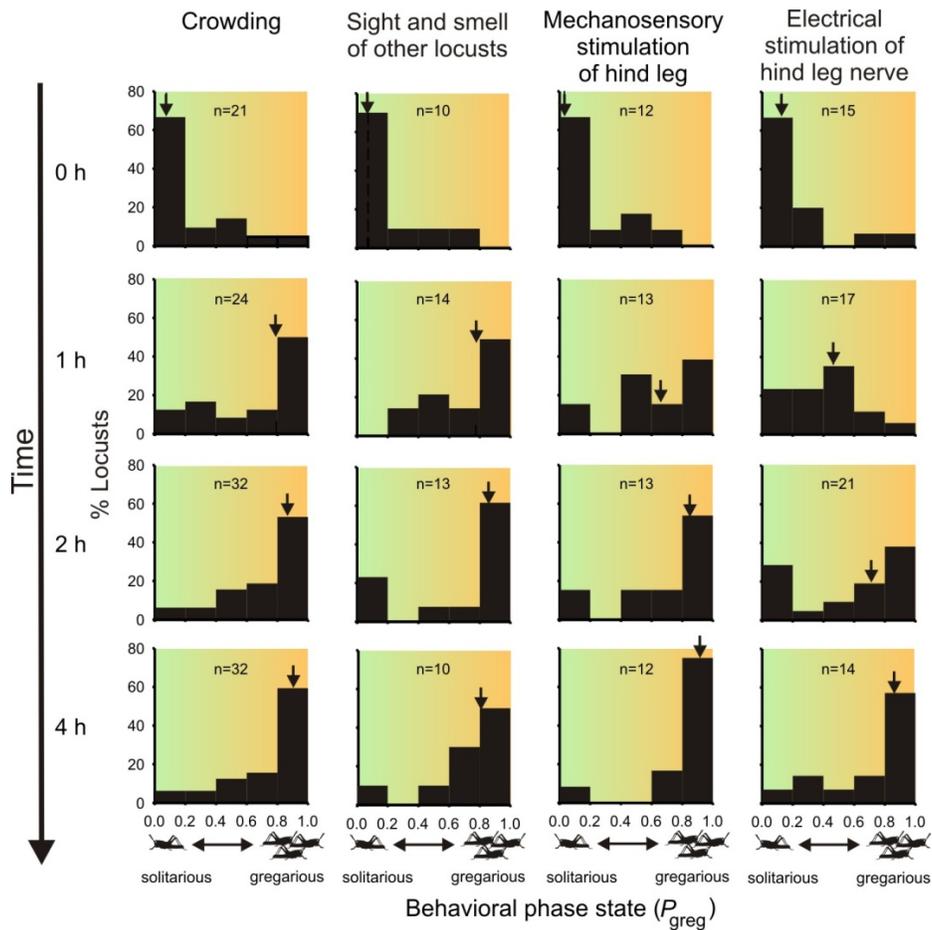


Figure S1. Behavioral gregarization in solitary locusts subjected for 0 h, 1 h, 2 h or 4 h to one of four different treatments (see text above for details). Each graph shows the percentage of locusts exhibiting different behavioral phase states (P_{greg}). Arrows indicate the median P_{greg} .

	Model						Model if term removed		
	β	Standard error	Wald	df	Sig.	Exp(β)	Model Log Likelihood	Change in -2 Log Likelihood	Sig. of the Change
Walking speed	1.942	0.429	20.484	1	<0.000	0.143	-66.872	29.834	<0.000
Time at stimulus group*	0.005	0.002	4.901	1	0.027	0.995	-54.561	5.212	0.022
Proportion of time motionless	-8.872	1.742	25.948	1	<0.000	7132.89	-73.421	42.932	<0.000
Time spent grooming	597.5	114.076	27.433	1	<0.000	0.000	-85.398	66.886	<0.000
Constant	3.151	1.263	6.226	1	0.013	0.043			

*calculated as time spent in the 25% area of the arena closest to the stimulus group of locusts.

Table S1. Behavioral variables retained in the best-fitting logistic regression model obtained from observations of 100 solitary-reared and 100 crowd-reared final-instar locust nymphs. Negative coefficients (β) indicate that solitary-reared individuals performed higher levels of the respective behavior compared to crowd-reared individuals. The significance of change in log likelihood ratio indicates the extent to which inclusion of the respective behavioral covariate improves the fit of the model.

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