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Socially induced behavioural plasticity precedes pronounced epigenetic differentiation in the CNS of Desert Locusts

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Abstract

Desert locusts *(Schistocerca gregaria)* show a dramatic form of socially induced phenotypic plasticity known as phase polyphenism. In the absence of conspecifics, locusts occur in a shy and cryptic solitarious phase. Crowding with conspecifics drives a behavioural transformation towards gregariousness that occurs within hours and is followed by changes in physiology, colouration and morphology, resulting in the full gregarious phase syndrome. We analysed methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) to compare the effect of acute and chronic crowding on DNA methylation in the central nervous system. We find that rearing the offspring of gregarious-phase locusts in social isolation causes pronounced differentiation of the neuromethylome within an individual's life-time. Crowding isolation-reared locusts for a day, however, has no significant effect on their MS-AFLP fingerprint. The differentiation of the neuromethylome seen in long-term gregarious locusts is therefore unrelated to the acquisition and expression of gregarious behaviour, suggesting that it serves to consolidate long-term phase state.

Keywords: DNA methylation, phase change, *Schistocerca gregaria.*

Introduction

Modification of DNA by cytosine methylation is emerging as an important mechanism in tailoring behavioural phenotypes to environmental conditions, including the social environment [1–3]. However, rarely do we understand the role of DNA methylation in the chain of events from environmental signals to changes in behavioural phenotype [3]. Do changes in the neuromethylome

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effect behavioural change, or do they instead serve to consolidate changes that first arose through other mechanisms?

Phenotypic plasticity is particularly common in insects, a fact implicated in their evolutionary success [4]. A striking example is provided by phase polyphenism in locusts. Locusts are grasshoppers (Acrididae) that can transform between two extreme phenotypes known as the solitarious and gregarious phase, which differ profoundly in morphology, physiology, and most importantly, in behaviour [5]. The solitarious phase is cryptic and shy, and avoids other locusts; gregarious-phase locusts are active and mobile and seek out conspecifics, causing them to aggregate in swarms. Several distantly related grasshopper species show phase polyphenism, with Migratory Locusts *(Locusta migratoria)* and Desert Locusts *(Schistocerca gregaria)* being amongst the most extreme and economically relevant. The sole direct environmental driver of phase change is the presence or absence of conspecifics. Solitarious Desert Locusts acquire gregarious behaviour within a few hours of forced crowding [6, 7]. Behavioural solitarisation of long-term gregarious locusts is markedly slower, indicating a consolidation of the gregarious state with prolonged crowding. In Desert Locusts, phase state at hatching is additionally determined by transgenerational epigenetic inheritance [8].

Phase change provides a model for answering fundamental questions about the role of DNA methylation in socially induced behavioural plasticity. Although locusts have lost DNA (cytosine-5)-methyltransferase 3 (DNMT3) [9, 10], methylation occurs on 1.6–1.9% of all genomic cytosines and on over 3% in exons (in *S. gregaria;* [10, 11]). These rates are over tenfold higher than in honeybees, where DNMT3 is present and methylation is implicated in caste polyphenism [12, 13]. A reduced representation bisulphite sequencing study in Migratory Locusts identified about 90 differentially methylated genes in the brains of solitarious and gregarious nymphs [14] but the gigantic size of the as yet unsequenced Desert Locust genome remains challenging (8,550 Mb; [15]).

DNA methylation may serve three not mutually exclusive roles in phase polyphenism: it may (1) be part of the effector cascade that *initiates* behavioural change; (2) underpin the *consolidation* of gregarious behaviour with prolonged crowding within a lifetime; or (3) mediate *inheritance* of phase state across generations. To begin to distinguish between these possibilities, we analysed methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) to compare the neuromethylome of Desert Locusts with identical parental histories, but different individual social histories.

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Methods

Locust rearing and treatments

Desert Locusts (*Schistocerca gregaria* Forskål, 1775) were obtained from an inbred gregarious colony at Leicester. Solitarious-phase locusts were produced from this stock by transferring them within a day of hatching into individual cages and rearing them in visual, tactile and olfactory isolation [16]. All locusts were maintained on a diet of fresh seedling wheat and dry wheat germ under a 12:12 photoperiod.

All locusts were virgin adults sacrificed 17–21 days after the final moult. Long-term gregarious (LTG) locusts were removed from the colony as final larval instars, sexed, and set up as one all-male and one all-female cohort of 40 each in separate tanks ($40 \times 30 \times 25$ cm^3) in the controlled-environment room that also housed the solitarious locusts. Solitarious locusts were offspring from a single gregarious mother (first-generation solitarious, 1GS). There were three treatment groups of four males and four females each: (i) $n = 8$ 1GS locusts that never experienced crowding; (ii) $n = 8$ LTG locusts; and (iii) $n = 8$ behaviourally gregarised 1GS locusts. These were produced by placing four male and four female 1GS locusts in the tanks that housed the 40 LTG virgins of the respective sex for 24 h before sacrifice. Locusts were sacrificed by decapitation and immediate dissection under ice-cold saline. The brain (excluding the retinae) and the thoracic ganglia were dissected out and snap-frozen on dry ice.

MS-AFLP analysis

Differences in DNA methylation patterns were detected by MS-AFLP analysis in $n = 4$ independent samples per treatment group, for a total of $N = 12$ samples. Each sample comprised the pooled brains and thoracic ganglia from one arbitrarily chosen male and female within the same treatment group. DNA was extracted with the QIAamp DNA Micro Kit (QIAGEN) following the manufacturer's instructions. Full experimental details including primer sequences and PCR conditions are given in the Supplementary Material. Briefly, for each sample of genomic DNA, one 500 ng aliquot was digested with EcoRI and MspI and another with EcoRI and HpaII. The restriction-digested products were ligated with EcoRI and HpaII-MspI adaptors and PCR pre-amplified. The pre-amplified PCR products served as templates for selective PCR-amplifications with 12 different combinations of HpaII-MspI and EcoRI primers. Diluted PCR products were separated electrophoretically (120 V, 81 min at 55°C) on 9% poly(NAT) gels (Elchrom). Gels were stained with SYBR® Gold (Invitrogen). Bands were scored as either present or absent. The resulting matrix was analysed for differentiation between groups by principal coordinates analysis (PCoA) and by analysis of molecular variance (AMOVA) in the R package *msap* [17].

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Results

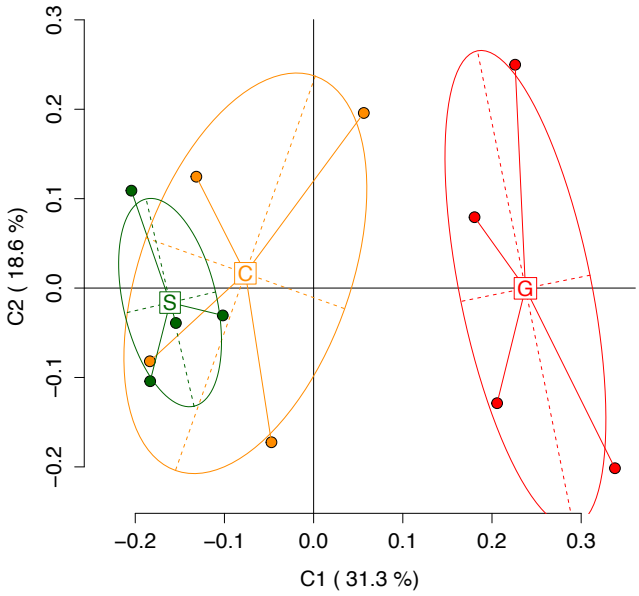

Figure 1. Principal Coordinate Analysis (PCoA) of epigenetic differentiation between uncrowded solitary-reared locusts (S), long-term gregarious locusts (G) and solitary-reared locusts crowded for 24 h (C), as identified by MS-AFLP. The first two coordinates (C1, C2) are shown with the percentage of variance explained by them. Group labels show the centroid for each group, points correspond to individual MS-AFLP samples, ellipses represent their average dispersion around the group centroids.

We scored 102 unique AFLP bands (loci); 99 were identified as methylation-susceptible based on different digestion patterns with HpaII and MspI, and 53 showed different banding patterns between individual samples (MS-polymorphic loci). The three treatment groups showed significant multi-locus differentiation in methylation pattern (AMOVA, $\phi_{ST} = 0.2086$, $p = 0.0114$). Figure 1 gives a simplified representation of the multi-locus differences between the samples. The two axes represent the first two principal coordinates, which together explained 49.9% of the total variation. The gregarious samples are set apart from both of the solitary-reared samples (uncrowded and 24 h crowded) by a shift along the first principal coordinate, indicating that the largest source of variance in multi-locus methylation is associated with long-term phase state.

Pair-wise comparisons identified significant epigenetic differentiation between crowd-reared locusts and locusts reared in social isolation ($\phi_{ST} = 0.3952$, $p = 0.0303$). These solitary-reared locusts were direct offspring of

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Table 1. Proportion of methylation-sensitive restriction band patterns found in the CNS of locusts of different phase state, and their corresponding methylation status; methylated cytosines are indicated in bold type.
[a] + and − indicate the presence and absence, respectively, of a band following digestion with HpaII or MspI.
[b] may indicate methylation of either outer or both cytosines on one strand.
[c] HPA− / MSP− was taken to indicate hypermethylation rather than absence of target due to a genetic mutation [17].

Banding pattern [a]	Methylation		solitary	24 h crowded	crowd-reared
HPA+ / MSP+	none:	5′-CCGG GGCC-5′	13.9%	16.7%	32.8%
HPA+ / MSP−	hemi: [b]	5′-**CC**GG GGCC-5′	10.1%	13.6%	15.7%
HPA− / MSP+	full internal:	5′-C**C**GG GG**C**C-5′	12.9%	12.6%	18.2%
HPA− / MSP−	hyper: [c]	5′-**CC**GG GG**CC**-5′	63.1%	57.1%	33.3%

long-term gregarious parents, demonstrating that social isolation causes pronounced alterations in the neuromethylome within a life-time. It would now be interesting to see whether isolation over multiple generations further deepens the epigenetic differences between the two phases.

Our key finding is that locusts that have been crowded for 24 h do not show the differentiated neuromethylome that is manifest in long-term gregarious locusts (Figure 1). One day of crowding is more than sufficient to establish gregarious behaviour [6, 7], yet the neural MS-AFLP fingerprint is still markedly different from that found in long-term gregarious locusts ($\phi_{ST} = 0.2084$, $p = 0.0272$). In fact, crowding for 24 h caused no significant epigenetic differentiation ($\phi_{ST} = -0.1135$, $p = 0.9446$). MS-AFLP resolves only a small random subset of CpG sites; clearly, our results do not preclude that changes in methylation are already occurring at some sites within the first 24 h of crowding. The differentiation of the neuromethylome that is manifest in the fully established gregarious phase must, however, develop only some time after the first 24 h of crowding. It cannot, therefore, underpin the transition to gregarious behaviour, nor can it be required for its expression. Instead, our findings suggest a role for neural DNA methylation in the *consolidation* of gregarious behaviour. Long-term gregarious locusts solitarise only partially when isolated for four days as final instar nymphs; but when long-term solitarious locusts are re-isolated after 24–48 h of crowding, they return to fully solitarious behaviour within 8 h. The resilience of gregarious behaviour to re-isolation thus increases with time spent in crowded conditions [16, 18]. Serotonin was previously found to mediate behavioural gregarisation within four hours [19]. That 24 h of crowding does not induce the major epigenetic changes that manifest after prolonged crowd-

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ing suggests that DNA methylation provides a consolidation mechanism by which neurochemically mediated rapid changes in behaviour become more stable with time. Differential DNA methylation may also underpin long-term phase differences in the CNS that are not directly responsible for gregarious behaviour but represent adaptations to the respective life styles.

The distinct methylation pattern of crowd-reared gregarious locusts were also apparent in the proportions of fully methylated and unmethylated loci (Table 1). The proportion of unmethylated loci was about twice as high (33%) than in either uncrowded (14%) or 24 h-crowded (17%) solitary-reared locusts. Conversely, the proportion of hypermethylated loci was about twice as high in uncrowded and 24 h-crowded solitary-reared locusts (63% and 57%) than in crowd-reared gregarious locusts (33%). This may indicate that the consolidation of gregariousness is associated with strong net demethylation. If so, experimentally shifting the methylation balance in the CNS towards hypermethylation may provide a means for reversing the consolidation of gregarious behaviour. At the group level, this would help the disbanding of locust aggregations, with possible implications for swarm management. In conclusion, our data demonstrate that phase change in the Desert Locust is associated with a major epigenetic shift in the neuromethylome which manifests only after the initial transition from solitarious to gregarious behaviour.

Authors' contributions

SRO and EBM conceived the study and designed the experiments. SRO carried out the animal treatments and dissections. HEA carried out all MS-AFLP bench-work and gel analyses and prepared the first draft. EBM and SRO performed statistical analyses. SRO wrote the final draft with input from HEA and EBM. All authors gave final approval for publication.

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