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Behavioural Interpretations of the HPLC Peaks Derived from Egg-Pod Foam Extracts of the Desert Locust *Schistocerca gregaria* (Forskål)

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Author's contribution

This work was carried out by the author MSI who designed the study, performed the statistical analysis, and wrote the protocol and the first draft of the manuscript. He also managed the literature searches.

Research Article

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ABSTRACT

Aims: The present study was aimed at assessing the bioactivity of the HPLC fractions and peaks from egg-pod materials of the crowd-reared desert locust *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) that elicited gregarious behaviour in the first-instar hatchlings derived from treated eggs of solitary-reared insects.
Study Design: In a series of stepwise experiments using crude extracts of the egg-pod materials in different solvents, filtered foam-extracts, HPLC foam-extracts and their fractions, sub-fractions and peaks, bioactivities of the egg-pod foam of the crowd-reared locusts have been assessed in terms of behavioural gregarization in the hatchlings from treated solitary eggs.
Place and Duration of Study: The initial experiments were carried out in the Departments of Zoology and Chemistry, University of Oxford, UK, during January 2002 and May 2003. Further analyses, manuscript writing and updated interpretations were performed in the Laboratory of Genetics and Molecular Biology, Department of Zoology, University of Rajshahi, Bangladesh, during September 2012 and February 2013.
Methodology: Beginning with egg-pod wash and foam-extracts in ethanol, hexane and water, step-wise bioactivities of the filtered foam-extracts, HPLC foam-extracts, HPLC fractions, sub-fractions and their peaks were assayed in terms of behavioural gregarization

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of the hatchlings from treated solitary eggs. Behavioural assays and multiple logistic regressions (LR) analysis were used to determine the behavioural phase state in terms of median *Probability (solitary)* values of individual experimental hatchlings.
Results: It has clearly been demonstrated that filtered foam-extracts at the concentrations of 0.1 foam-plug equivalent (FPE) and their HPLC reconstitutes at 0.2-0.4 FPEs significantly shifted the behavioural phase of the treated solitary hatchlings towards gregariousness. Further fractionations of the HPLC foam-extract at 0.4 FPE reconstitutes into four fractions (Fraction 1-Fraction 4), four sub-fractions (Fraction 3.1-Fraction 3.4), three sub-fractions of Fraction 3.2 (Fraction 3.2.1-Fraction 3.2.3) and five peaks (peak 1-peak 5) of the sub-fraction 3.2.2 revealed that peak 3 contained substances that induced the maximum behavioural gregarization in the hatchlings from treated solitary eggs.
Conclusion: HPLC reconstitutes of the aqueous egg-pod foam-extracts of the crowd-reared *S. gregaria* are capable of shifting the behavioural phase of the treated solitary hatchlings towards gregariousness. Further fractionations of the HPLC foam-extracts into sub-fraction 3.2.2 and five peaks revealed that peak 3 contained bioactive substances that elicited maximum gregarizing behaviour in the hatchlings from treated solitary eggs. The results deserve further investigation with regard to determining the precise nature of the causal factor(s) that escaped NMR's detection thresholds in an earlier study.

Keywords: Egg-pod foam; bioactive substance; desert locust; Schistocerca gregaria; behaviour; HPLC.

1. INTRODUCTION

Locusts exhibit an extreme example of density-dependent phase polymorphism where crowding stimulates individuals to change from the shy and cryptically coloured, *solitaria* phase into the swarm-forming and conspicuously coloured, *gregaria* phase [1-3]. This is a key feature to the biology of locusts and is central to their occasional yet catastrophic impact on human beings. Phase change involves a suite of characters, including behaviour, development, physiology, morphology and colour [4]. Behaviour is the first character to change in response to crowding, occurring in a matter of hours. It has therefore been argued that a fully integrated study of behavioural phase change provides a powerful tool for understanding both the mechanisms of phase change and locust population dynamics, both of which offer possibilities for improved management and control of locust plagues [5].

Logistic regression (LR) is one of the most powerful statistical procedures [6] that test against such binary response variables as found in solitary *versus* gregarious (crowd-reared) hatchlings. Behaviour ascertains its sensitivity for phase predictions as LR model based on behaviour has previously been shown to be the best predicting power of the hatchling behavioural measure [7-9]. Behavioural changes in locusts not only take place within the lifetime of an individual, but also accumulate over generations [2,4]. Phase transition in these insects includes rapid behavioural change, occurring in a matter of hours while other characteristics change more gradually [5,10]. It has previously been demonstrated that the mother influences the phase-state of the developing offspring through a 'gregarizing factor' added to the foam that surrounds the eggs at laying [7,11-12]. Initial analysis of the factor indicated that it is a small (<3 kDa), hydrophilic substance produced by the mother at the time of oviposition, and that it triggers development of gregarious characteristics in the hatchlings if eggs are treated within the first day after laying, but not thereafter [13-14]. A later report provided evidence that the female accessory glands, being the main source of

egg-pod foam production, are associated with the gregarization process in the desert locusts [9]. The ideas of pheromonal, air-borne and/or chemical influences on locust gregarization have been advocated by a number of studies [15-18] which appear to be contradictory to the findings that a very polar gregarizing factor is likely to be present in the egg-pod material of *S. gregaria* [9,14]. Subsequent work on the egg-pod foam chemistry of the gregarious locusts led to the detection of an alkylated L-dopa analogue that when applied to solitary eggs elicited gregarious behaviour in the resulting hatchlings [19].

In contrast to the above findings, however, Tanaka and associates emphasized factors other than the proposed 'gregarization factor' responsible for phase-related changes in the desert locusts. First, a dark-colour-inducing factor was found to be a heat-stable neuropeptide, and extractable in methanol or saline [20]. Then the presence of a pheromonal factor on the colour of the hatchlings was doubted [21]. Subsequently, the phase-dependent differences in body size and colour of the hatchlings were claimed to be pre-determined in the ovary [22], and the amount of egg yolk or the availability of yolk material determined the body colouration of hatchlings [23], thus they argued that no foam factor was involved in this phenomenon [24]. Parental density was found to have no significant influence on locomotor activity in the progeny but it appeared to affect the degree of response to crowding in the progeny [25] and antennal tactile stimuli during 2-6 days before egg-laying elicited gregarious characteristics like larger eggs that yielded dark-coloured hatchlings in solitary females [26]. Parental rearing density was later reported to influence indirectly the locomotor activity in the progeny 0-2 days after hatching by affecting their body size as eggs or hatchlings [27]. Recently, environmental factors such as substrate colour of the habitat was found to affect green-brown polyphenism, black patterning and background body colour of the solitarious nymphs in *S. gregaria* [28]. On the other hand, however, accumulating evidence now-a-days suggest that epigenetic modifications play a key role in regulating gene expression in various insects [29]. Thus serotonin mediated behavioural gregarization leading to swarm formation [30] and epigenetic control of gene expression through DNA methylation in locust phase polyphenism have been implicated [31-33].

In this study behavioural interpretation of the hatchlings from individual eggs of solitary-reared *S. gregaria* after treatments of the high performance liquid chromatography (HPLC) fractions and peaks derived from the gregarious egg-pod foam extracts have been presented. Here step-wise experiments were performed, following one after another, where each successive experiment shared an overlapping, key treatment group with the previous one that has independently collected data for that key, overlapping treatment group. The statistical tests within each experiment were then focused on the one overlapping treatment group. In this way, experiments proceeded hierarchically from crude extracts of the egg pod material (egg pod washings and foam extracts) in ethanol, hexane and water to filtered, aqueous foam extracts to HPLC reconstitutes to fractionations and peaks. Because statistical analyses tested hypotheses within each experiment, this increased their power through having fewer multiple comparisons to have to correct for. The different fractionation methods used in the present investigation are justifiable because they were used to help separate better fractions and/or peaks that were found to be 'bioactive' in the previous steps. The reason for re-emphasizing the behavioural aspect of the locust phase polyphenism is obvious from several studies reported earlier [5, 7-10]. In this report, behavioural interpretations of the fractions and/or peaks that were predicted to be beneath the nuclear magnetic resonance (NMR) spectroscopic detection range in the previous study [19] are assessed further with a view that this might be helpful in elucidating the mechanism(s) underlying polyphenism in locusts. This forms the key objective of the present report.

2. MATERIALS AND METHODS

2.1 The Insects

First-instar hatchlings (=nymphs or hoppers) of *S. gregaria* from second-generation solitary-reared parents and from crowd-reared parents permanently maintained at density of 500-1000 insects per rearing bin (56 cm × 76 cm × 60 cm) were used. Treated solitary eggs were incubated in a constant temperature room at 30º±1ºC and 70-75% uncontrolled relative humidity under 12h: 12h light: dark photo regime. One day-old hatchlings from crowd-reared parents, collected from the locusts maintained under almost identical conditions as solitary-reared ones, were used either for building model or as a group response during observations [7,11]. The test hatchlings were fed and maintained either alone from the time of eclosion or in a crowd until used in the behavioural assays on day 1.

2.2 Insects Used to Build a Logistic Regression Model

For constructing LR model (see Section 2.8 below), 50 one day-old hatchlings from 4 solitary egg-pods and 68 hatchlings of similar age from an undetermined number of gregarious egg-pods from breeding bins were used. These insects were then used to compute the behavioural phase status in terms of median *Probability (solitary)* of individual solitary hatchlings from different treatment groups.

2.3 Egg-pod Washings and Crude Foam-Extracts in Different Solvents

Four freshly-laid egg-pods (≤5 h-old and collected between 0830 and 1330 hrs) from crowd-reared females were thoroughly washed in each of 2000 µL ethanol, hexane or distilled water (i.e. 500 µL solvent per egg-pod) and filtered using Millex® syringe-driven filter units (Millipore Corp., USA). Filter paper circles (4.25 cm; Whatman) each with an M-shaped crease in it and placed in small containers (25 mL) each provided with a pad of cotton wool and moistened with distilled water, were treated with 50 µL of the egg-pod washings *ca*. 5 min before placing individual eggs (≤2 h-old) from solitary-reared mothers on the groove in the treated filter paper. Each of the treated filter papers and the test eggs were covered with another non-folded, flat filter paper of the same size. The containers were sealed and incubated in the controlled temperature room at 30º±1º C until eclosion of the eggs. Foam-plugs from the same egg-pods used for egg-pod washings were collected, cleaned with a paint brush, homogenized in ethanol, hexane or distilled water using a mortar and pestle and filtered using Millex® filters as mentioned earlier. The crude homogenates were prepared at a concentration of one foam-plug per 500 µL of the solvent. Filter papers in small containers were treated with the foam-extracts and individual solitary eggs were placed in the same way as that described for egg-pod washings. In all cases, the egg-pod washings and the foam-extracts were prepared immediately before use.

2.4 HPLC Protocol

Samples of the filtered foam-extracts (200-1000 µL) were loaded onto a Varian HPLC system (USA) consisting of three main units: (1) a Varian pump comprising a solvent delivery system; (2) a Rainin sampler consisting of an automatic sample injector, having an oven at 30ºC and tray at 4ºC; and (3) an analyzer having a UV detector. The units were connected to a PC and operated with the help of a software called ProStar 330. Column conditions were as follows: (a) 4.6 mm × 25.0 cm analytical cartridge composed of 5 µm

ODS-2 Spherisorb® particles (Waters, USA); (b) 0.1% formic acid in HPLC water as solvent A; (c) acetonitrile (BDH, UK) as solvent B; (d) 1.0 mL per min flow rate; (e) 190-350 nm absorption units (AU) as detector range (λ); and (f) three methods: (i) H_20 gradient, where initial proportions of solvent A and solvent B were 95% and 5%, at 18.0 min 32% and 68% and from 18.1 min until the end of the run (22.0 min) were 0% and 100%, respectively (Figs. 2, 3, 4); (ii) Fraction 3-5, starting with 95% A and 5% B, at 12.0 min the ratio of the solvents becomes 70% and 30%, changing to 100% B during 17.5 and 22.0 min (Fig. 5); and (iii) Isocratic 3-3, where initial proportions are 92% A and 8% B, reaching to 72% and 28% respectively at 18.2 min, leading to 100% B from 19.0 to 24.0 min (Fig. 6, chromatograms 1, 2). The HPLC whole runs or desired fractions were collected manually in 50 mL round-bottomed flasks. Solvents were evaporated using vacuum rotary evaporators (Rotavapor, Switzerland) installed in the Department of Chemistry, Oxford University, under 30-60 mbar pressure and at 32º-36ºC on a water bath, leaving only about 5-10 µL solvent leftovers, which finally were reconstituted in 200-500 µL HPLC water as required.

2.5 Filtered Foam-Extracts and Their HPLC Reconstitutes

Filtered foam-extracts of the gregarious egg-pods were prepared by transferring the required number of foam-plugs into a porcelain homogenizer, in which 500 µL of HPLC water per foam-plug was added and homogenized using a porcelain mortar, thus giving a concentration of 0.1 foam-plug equivalent (FPE) of the extract. Vivaspin 0.5 mL polyethersulfone membrane micro-filters (Vivascience AG, Germany) were used to filter the extracts in a centrifuge at 15,000 rpm for *ca*. 30 min. Given that the micro-filters impregnated with glycerin might interact with the active component(s) of the extracts and thus could interfere with the filtration process, the micro-filters were rinsed three times with HPLC water immediately before use. The extracts were prepared before use and they were used to treat filter paper circles in which individual solitary eggs were placed in small containers for incubation as described earlier for crude foam-extracts. HPLC whole runs were collected, reconstituted in HPLC water after evaporation (see HPLC protocol above) and each was used to treat solitary eggs. Because a minimum of 4 foam-plugs extracted in 2000 µL of HPLC water and 50 µL used to treat individual solitary egg (i.e. 4 ÷ 2000 × 50) was found to be effective for gregarizing solitary hatchlings, it gave a concentration of 0.1 FPE. A protocol was maintained for treating solitary eggs with different concentrations of HPLC reconstitutes where the experimental eggs received treatments at concentrations of 0.1, 0.2, 0.3 and 0.4 FPEs per filter paper (Table 1). H_20 gradient method was used for this experiment.

Table 1. Protocol for preparing different concentrations of HPLC reconstitutes

Treatment groups	HPLC injections (µL)	HPLC reconstitutes (µL)	Final concentrations*
HPLC foam-extract (0.1)	500	500	0.1
HPLC foam-extract (0.2)	1000	500	0.2
HPLC foam-extract (0.3)	750	250	0.3
HPLC foam-extract (0.4)	1000	250	0.4

**Foam-plug equivalents (FPEs)*

2.6 Fractionations of HPLC Reconstitutes

Initially the HPLC whole run at 0.4 FPE was divided into four fractions (Fraction 1- Fraction 4) and elute of each fraction was then used for treating solitary eggs as before. Filtered foam-extract at 0.1 was maintained as control. H_20 gradient method was also used for fractionations. Fraction 3 was then fractionated into four sub-fractions (Fraction 3.1- Fraction 3.4) where Fraction 3 was maintained as control. Here also H_20 gradient method was used for the sub-fractionations. Then Fraction 3.2 was further divided into three sub-fractions (Fraction 3.2.1-Fraction 3.2.3) where Fraction 3.2 was used as a control treatment. For getting a better separation of the sub-fraction of our interest, Fraction 3-5 method was employed. Then Isocratic 3-3 method was used to fractionate Fraction 3.2.2 into five individual peaks (peak 1-peak 5) where Fraction 3.2.2 was used as a control. Finally, peak 3 from the sub-fraction F3.2.2 was separated using Isocratic 3-3 method for evaluating its bioactivity.

2.7 Sensitivity of the Treated Eggs to HPLC Solvent Leftovers

Sensitivity of the treated solitary eggs to HPLC solvent leftovers and the resulting hatching rates of the eggs in each treatment groups were determined.

2.8 Behavioural Assays and LR Analysis

As described earlier [11], a rectangular arena (35.5 cm × 15 cm × 10 cm) was used to investigate the behavioural response of individual hatchlings from different treatment groups. From a range of behavioural elements consisting of locomotory and non-locomotory movements, and avoidance and/or shock reactions, a total of 14 parameters were derived and included for analysis [7,11,34]. The LR analysis was used to predict a binary dependent variable from a set of independent variables. Using the behavioural phase of the experimental hatchlings as a dependable variable and behavioural parameters as independent variables, the predicted probability that a test insect belonged to the fully solitary-reared group, i.e. *Probability (solitary)*, was estimated. The LR model was derived from two extreme treatment groups, assigning P=1.0 for the hatchlings that originated from solitary-reared parents (n=50) and P=0.0 for those derived from crowd-reared parents (n=68) mentioned above.

2.9 Statistical Analyses

LR analyses of the behavioural data were made using SPSS version 16.0 for Windows. Median *Probability (solitary)* values, being better represented than the mean values of a population that do not show normal distribution [35], were used to construct histograms to show the effect of an overlapping, key treatment compared to a group of treatments in each experiment. The experimental data were further analyzed using one-tailed Dunnett's post-hoc tests where normalized ranked *Probability (solitary)* values were subjected to one-way analysis of variance [10,36].

3. RESULTS AND DISCUSSION

3.1 Insects in the LR Model

Probability (solitary) values for all experimental hatchlings were computed by using multiple LR analysis. Initially a model was built on the basis of the extreme categories of 50 solitary reared *versus* 68 crowd reared insects. All three methods of LR analyses *viz*., Enter, Backward step-wise and Forward step-wise, were considered, but the Enter method gave the most parsimonious result and classified 86.00% of the solitary reared and 91.18% of the crowd reared insects, giving an overall of 88.98% cases, correctly. The model showed a perfect goodness of fit (χ^2 = 102.88 at 6 df, P<0.001) where six variables contributed significantly to the building of the model (Table 2). The model was then used to compute the behavioural phase status of all other insects from different treatment groups.

Table 2. Behavioural parameters retained in the logistic regression model (Enter method) derived from two extreme groups of insects (fully solitary-reared *versus* fully gregarious) used for computing *Probability (solitary)* values for individual test insects from different groups (see text for further details)

Variables in the model	Coefficient β	Significance of change in the log likelihood ratios	Partial correlation coefficient (R)
X-distance	-0.74	0.0538	-0.10
Walking speed	-4.28	0.0003	-0.27
Treatment time middle	0.01	0.0005	0.25
Climbing time-fraction	-8.98	0.0008	-0.24
Defaecation time-fraction	58.99	0.0112	0.17
Swaying frequency	-68.41	0.0144	-0.16
Constant	2.87	0.0054	-

3.2 Egg-pod Washings and Crude Foam-Extracts in Different Solvents

Median *Probability (solitary)* values are plotted against different treatment groups to show the behavioural responses of the first-instar hatchlings of *S. gregaria* to egg pod washings and foam extracts in ethanol, hexane and water along with solitary- and crowd-reared insects used in the LR model (Fig. 1). One way ANOVA ($F_{9, 164}$ = 7.77; P<0.001) with Dunnett's one tailed post hoc tests show that solitary eggs receiving treatments from egg pod washings or foam extracts either in ethanol (P<0.001) or water (P<0.001) yielded hatchlings that behaved significantly gregariously compared to the untreated solitary hatchlings.

3.3 HPLC Reconstitutes of the Foam Extracts at 0.1-0.4

Data from this experiment (Fig. 2) revealed that the filtered foam-extract at 0.1 FPE (P<0.001) and HPLC foam-extracts at 0.2, 0.3 and 0.4 FPEs (P=0.021, P=0.022 and P<0.001, respectively) were effective in eliciting behavioural gregarization ($F_{5, 201}$ =12.63; P<0.001) in the treated solitary hatchlings.

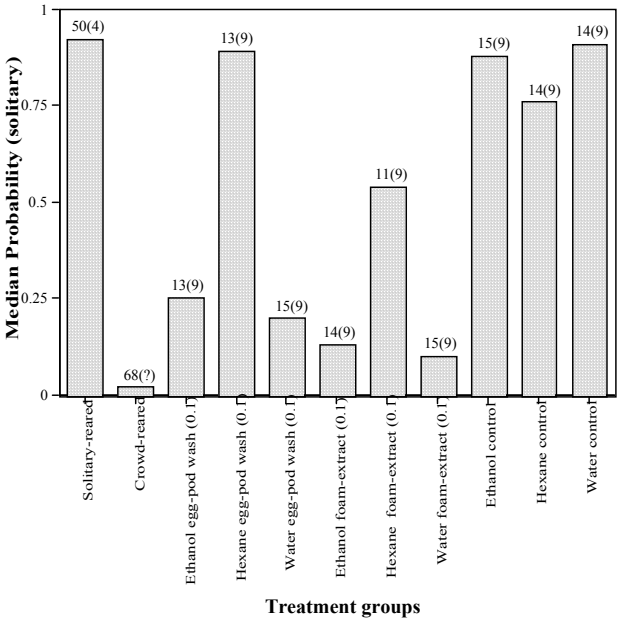


Fig. 1. Model insects and crude extracts of the egg-pod materials in different solvents; Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged

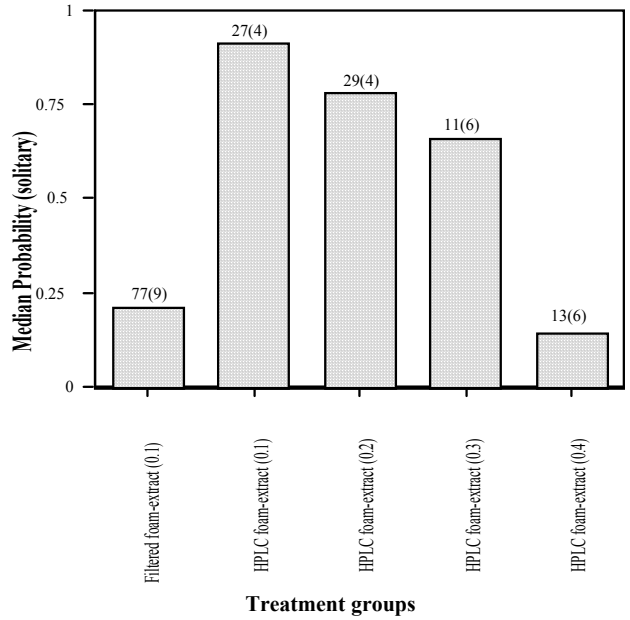


Fig. 2. Bioactivities of filtered foam-extract (0.1) and HPLC foam-extracts at different concentrations (0.1-0.4); Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged

3.4 Fractionations of HPLC Foam-Extract (0.4)

Frequency histogram of the median *Probability (solitary)* values against filtered foam-extract at 0.1, HPLC foam-extract at 0.4 and HPLC fractions 1-4 are presented in Fig. 3. Filtered foam-extract ($P<0.001$), HPLC foam-extract ($P=0.002$) and the fraction 3 ($P<0.001$) each induced significant gregarizing effect ($F_{6, 129}= 5.96$; $P<0.001$). Behavioural data shown in Fig. 4 demonstrate that fraction 3 ($P<0.001$) and sub-fraction 3.2 ($P<0.001$) had significant effect on shifting behavioural phase status of the hatchlings towards gregarization ($F_{5, 131} =7.50$; $P<0.001$). Moreover, median *Probability (solitary)* values plotted against four treatment groups (Fig. 5) reveal that sub-fraction 3.2 ($P<0.001$) and sub-fraction 3.2.2 ($P<0.001$) were capable of eliciting significant gregarious behaviour ($F_{4, 132} =7.62$; $P<0.001$). Finally, when the sub-fraction 3.2.2 was split into five peaks (Chromatogram 1), peak 3 (Fig. 6; $P<0.001$; Chromatogram 2) resulted in significant gregarization in comparison with the solitary-reared insects ($F_{7, 196} =1\ 3.45$; $P<0.001$). Data for the filtered foam-extract (0.1) and HPLC foam-extract (0.4) are shown here for comparison (Fig. 6).

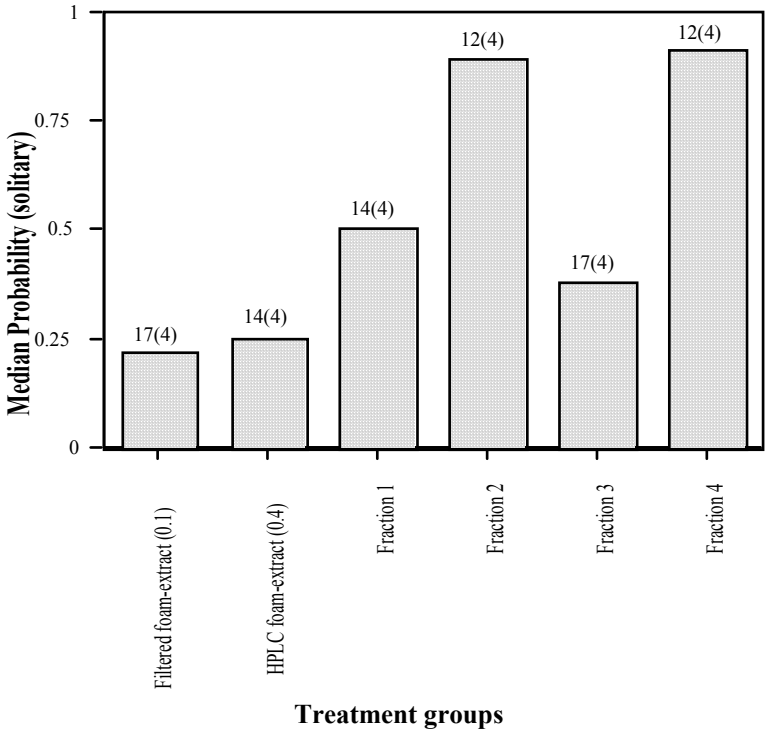


Fig. 3. Bioactivities of filtered foam-extract (0.1), HPLC foam-extracts (0.4) and HPLC fractions (Fraction 1-Fraction 4); Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged

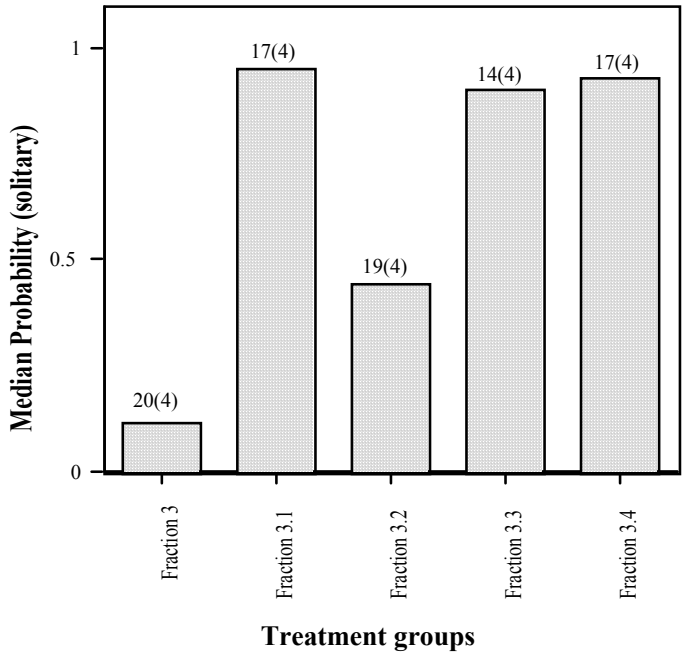


Fig. 4. Bioactivities of fraction 3 and its sub-fractions (3.1-3.4); Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged

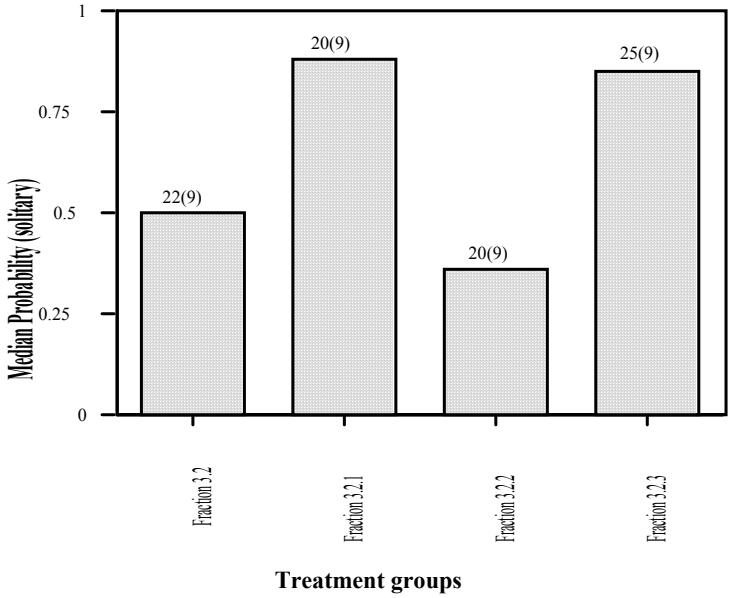
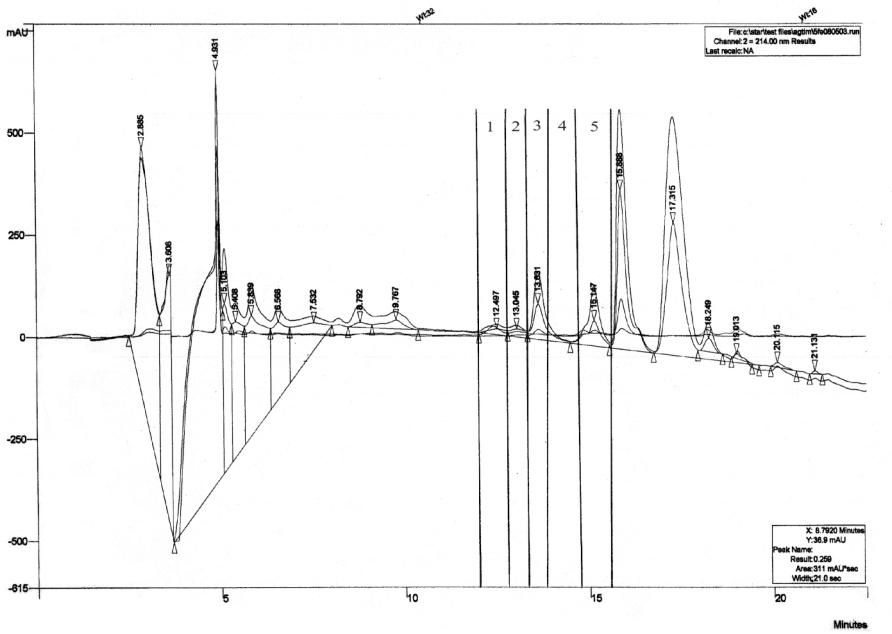


Fig. 5. Bioactivities of the fraction 3.2 and its sub-fractions (3.2.1-3.2.3); Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged



Chromatogram 1. Five HPLC peaks (1-5) derived from the sub-fraction 3.2.2 shown in Fig. 5

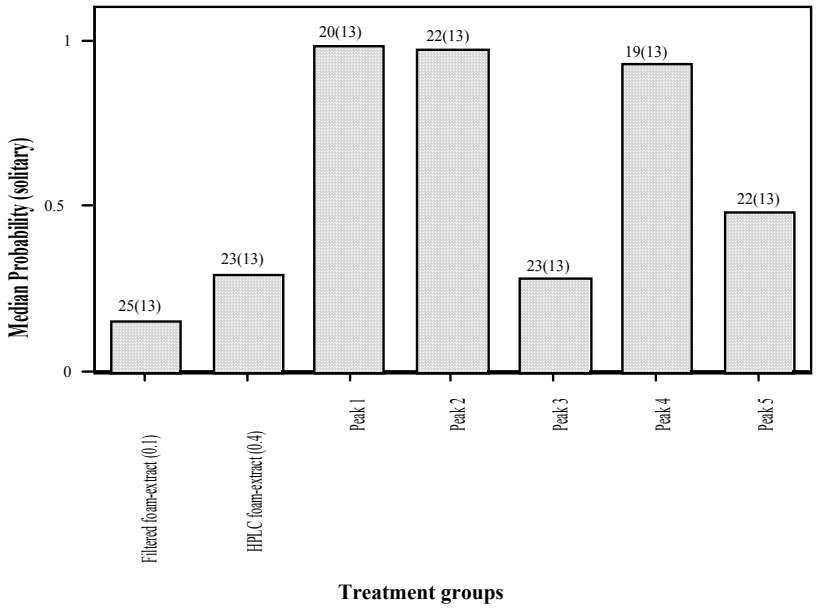
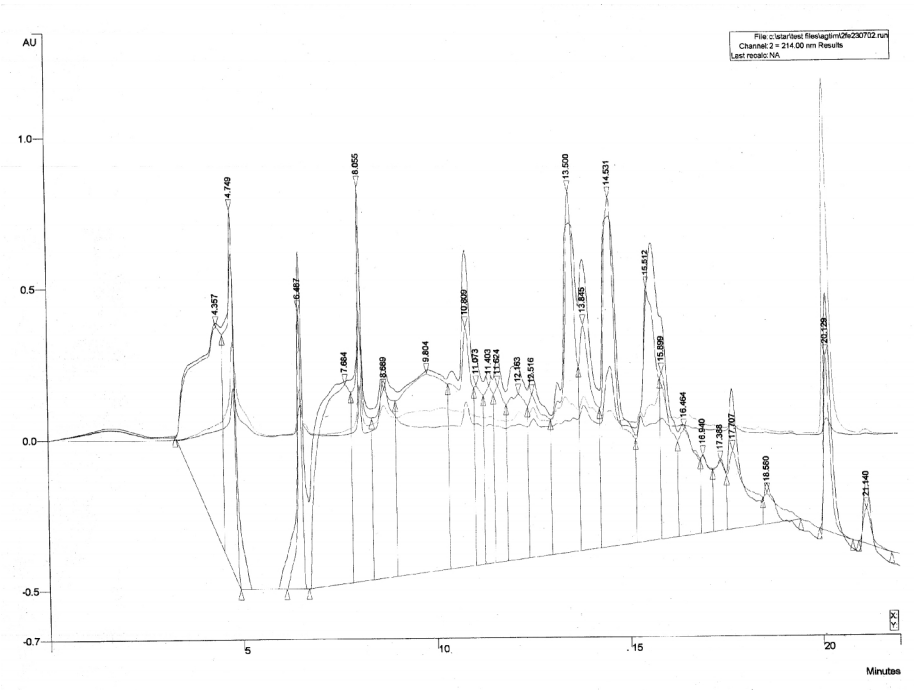


Fig. 6. Bioactivity of five HPLC peaks (1-5) derived from the sub-fraction 3.2.2 compared to the filtered foam-extract (0.1) and HPLC foam-extract (0.4); Figures on each bar indicate the number of solitary hatchlings tested; those in parentheses are the number of egg-pods from which they emerged



Chromatogram 2. HPLC peaks from peak 3 of the sub-fraction 3.2.2 (Fig 6, peak 3) that elicited the maximum gregarizing behaviour in the hatchlings from treated solitary eggs in *S. gregaria*

3.5 Sensitivity of the Treated Solitary Eggs to HPLC Solvent Leftovers

Compared to the control eggs (97.3% hatching), HPLC solvents evaporated up to a thin film (5-10 µL) and then reconstituted in requisite amount of HPLC water resulted in 77.5% hatching of the treated eggs; those evaporated to 1000 µL had 50.0% and those from the non-evaporated whole ran produced 57.1% hatching, indicating that HPLC solvents evaporated until 5-10 µL leftover gave a better hatching of the solitary eggs treated with the HPLC reconstitutes. The overall rates of hatching of the solitary eggs exposed to egg-pod washings; and foam-extracts in different solvents, HPLC concentrates (0.2-0.4) and their fractions were 76.5%, 64.4% and 53.0%, respectively. Subsequent treatment groups, *viz*. sub-fraction F3.2, sub-fraction F3.2.2 and five peaks resulted in 78.4%, 64.5% and 78.6% egg hatchings, respectively. The results suggest that HPLC reconstitutes of the foam-extracts have some negative impact on the hatching rates of the treated eggs.

The present results clearly demonstrated that both egg-pod washings and crude foam-extracts (0.1) in ethanol and water, but not in hexane, significantly shifted the behavioural phase of the hatchlings emerging from treated eggs of solitary-reared *S. gregaria*. In the same way, filtered foam-extracts (0.1), HPLC foam-extracts (0.2-0.4) and their selective fractions and peaks were found to have elicited gregarious behaviour in hatchlings that originated from treated solitary eggs. The findings are consistent with that of a previous study where crowd-reared females of *S. gregaria* with ligatured accessory glands (AGs) from lateral oviducts produced hatchlings of solitarious behaviour, thus implicating the AGs in the production, release or activation of the gregarizing factor in the egg pod material [9]. HPLC

fractionations and peaks of the present study also correspond to those reported earlier [9] where the higher concentrations of peaks I and 4 were indicative of solitariousness. In contrast to the earlier finding in which a positive correlation between egg-pod washings and hatchling colour but not behaviour was found [9], however, the present results demonstrated a significant gregarizing effect of egg-pod washings on solitary hatchling behaviour. This discrepancy might be due to the reason that the 'behavioural phase state' rather than the 'colour scores' of the treated hatchlings was the focal point in the present investigation.

The basic difference between the present experiment and the previous one [19] needs to be clarified here. Firstly, in contrast to the previous pooled data the present experiment deals with the individual solitary eggs that underwent treatments only of bioactive HPLC fractions and/or peak(s) derived from the crowd-reared egg-pod foam extracts in each logical step. Secondly, the HPLC fractionation methods in the final round of the experiment differed significantly. For achieving the bioactive fraction (F3.2.1.2) and peak (x), the previous study fractionated the sub-fraction F3.2 using Method 2 while the present work fractionated the sub-fraction F3.2.2 into five peaks and eventually to the most bioactive peak 3 using Isocratic 3-3 method. Since HPLC methods are principally based on the solvent proportions and run times, it is conceivable that Method 2 [19] and the present Isocratic 3-3 method would have separated different 'candidates' responsible for behavioural gregarization in the treated solitary hatchlings under two studies. Thirdly and most importantly, the present bioactive HPLC fraction F3.2.2 and peaks, but not fraction F3.2.1, might contain some eluting compound(s) whose concentration(s) was perhaps beneath the NMR's detection thresholds of the previous study. Lastly, out of five peaks of the fraction F3.2.2, peak 3 was particularly noticeable for its pronounced bioactivity. Although not a major difference, the present work designated the behavioural gregarization in terms of *Probabilty (solitary)* as done in earlier reports [7,11,14] rather than $P_{gregarious}$ [19], which is actually $1\text{-}P_{solitarious}$. In addition, sensitivities of the treated solitary eggs to the HPLC solvent left-overs and their consequences on the hatchling rates in different treatment groups are reported here for the first time. The information might be of vital importance for the future workers in this field.

The so called 'aggregation pheromone' [37-38] was found to have no gregarizing activity on nymphs or adults [12]. The gregarizing factor that was demonstrated earlier [19] and is shown here is also unlikely to be the same material described as a 'releaser pheromone' [15], or a 'primer gregarization signal' [17-18]. This is because the former is a saline and/or water extractable, very polar substance while the latter were claimed to be non polar, C-8 unsaturated ketones. Moreover, the present assay used to determine behavioural phase state of individual hatchlings is quite different from that used by the aforesaid authors [15,17-18].

The works of Tanaka and associates are not beyond controversy. Earlier on a heat-stable neuropeptide, extractable in methanol or saline, was reported to be a dark-colour-inducing factor [20]. Then it was demonstrated that the amount of egg yolk or the availability of yolk material determined the body colouration of hatchlings [23]. Later on, antennal tactile stimuli during 2-6 days before egg-laying were found to elicit larger eggs that yielded dark-coloured hatchlings in solitary females [26], and environmental factors such as substrate colour was found to affect green-brown polyphenism, black patterning and background body colour of solitarious nymphs [28], thus suggesting the lack of consistency in their claims.

Epigenetic modifications play a key role in regulating gene expression and therefore appeared to be critical to the development, regulation and maintenance of the normal cells [29]. Epigenetic mechanism such as DNA-methylation is reported to be involved in the

regulation of phenotypic plasticity and phase polyphenism in locusts [30-33], in early developmental stages in *Drosophila* [39], and caste differentiation and learning memory in honey bees [40-41]. So, depending on the present findings it could be hypothesized that the female locusts produce a causal factor(s) that influences the phase status of their hatchlings, and which presumably acts epigenetically by regulating embryonic gene expression. Moreover, by virtue of its derivation from the female reproductive tract at the time of oviposition and its intimate contact with the eggs, it is very likely that the egg-pod foam could provide an ideal vehicle for the exposure of eggs to the proposed gregarizing factor(s). The results of treating eggs from solitary-reared females with HPLC separated bioactive fractions and peaks provide the direct evidence that egg-pod foam of *S. gregaria* contains a gregarizing factor(s) that influences the development of the solitary locust eggs to the genesis of hatchlings with characteristic behaviour of the gregarious phase locusts.

The next logical step would be to investigate developmental genetics of the locusts either using pure substances or foam extracts. Three key questions that need to be addressed are: (a) how does the maternal gregarizing factor(s) cause the developmental shift from solitary to gregarious phase? (b) What is the time-course of action of the factor on the developing embryos? And (c) when are the embryos sensitive to the factor? Obviously, these fundamental questions can only be addressed when the exact chemical nature and/or structure of the factor is known in detail. Behavioural assays of the hatchlings emerging from treated eggs, accompanied by measurement of changes in a suite of diagnostic characters from egg-laying to hatching, for example, phase-specific organogenesis, isozymes, and haemolymph pigmentation techniques [42-43] could be evaluated. Apart from these, polyacrylamide gel electrophoresis (PAGE), differential displays and other such current molecular techniques as polymerase chain reaction (PCR) could be utilized to unravel the precise nature of the gregarizing factor whose concentration might have escaped the NMR's detection thresholds in the previous study [19].

4. CONCLUSION

HPLC reconstitutes of the aqueous egg-pod foam-extracts of the crowd-reared *S. gregaria* are capable of shifting the behavioural phase of the treated solitary hatchlings towards gregariousness. Further fractionations of the HPLC foam-extracts into sub-fraction 3.2.2 and five peaks revealed that peak 3 contained bioactive substance(s) that induced the maximum behavioural gregarization in the hatchlings from treated solitary eggs. The results deserve further investigation with regard to determining the precise nature of the causal factor(s) that escaped NMR's detection thresholds in an earlier study.

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COMPETING INTERESTS

The author has declared that no competing interests exist.

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