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Phase related differences in female's accessory glands and oocytes proteins in the desert locust, *Schistocerca gregaria*

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ABSTRACT

Molecular content of the female's accessory glands and oocytes were studied in crowded (gregarious phase) and isolated (solitary phase) Schistocerca gregaria adults using electrophoretic, chromatographic and immunological techniques. Quantitative differences were found in the presence of a proteic band between 28 and 38 KDa and a peptide with a retention time of 37.20 min in the accessory glands. Their presence in much higher concentrations in the crowd-reared (gregarious) phase than in the isolated-reared (solitarious) one suggests a role in phase polyphenism. This result was confirmed by western blot assay; an antiserum of crowd-reared female's accessory glands was raised against the gregarious and solitarious extract of female's accessory glands shows a supplementary proteic band between 28 and 38 KDa in gregarious phase. An additional western blot analysis was successfully done in gregarious and solitary oocytes shows the same supplementary band between 28 and 38 KDa in gregarious oocytes, absent in the solitary ones. This concordance in our results shows that there was a gregarizing factor transmitted from the gregarious S. gregaria mothers to their progeny.

Keywords: *Schistocerca gregaria*, phase polyphenism, female accessory glands, oocytes.

INTRODUCTION

The desert locust, *Schistocerca gregaria*, demonstrates an extreme form of density dependent polyphenism known as phase change. Local population density induces the expression of graded changes in a suite of traits that include colouration, morphometry, anatomy, egg mass, food selection, nutritional physiology, reproductive physiology, metabolism, neurophysiology, endocrine physiology, molecular biology, immune responses, longevity and pheromone production [1-2-3-4-5-6-7-8-9-10]. The extreme forms are termed solitaria (the solitarious phase) and gregaria (the gregarious phase). 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 [11-12-13]: 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 [14-15-16]. Epigenetic transfer of phase state depends upon low molecular mass, water-soluble chemicals; it is an alkylated L-dopa analogue present in foam surrounding gregarious eggs [17-18-19-3-20-21]. Here we present preliminary information for the specific gregarizing suspected protein in female's gregarious accessory glands and provide data about their transmission to the progeny.

MATERIALS AND METHOD

Locusts

S. gregaria culture was maintained in our laboratory for many consecutive generations either under crowded or under isolated coditions, as already described by [22]. The cages were heated and illuminated continuously (L13:L11) by incandescent electric bulbs. Temperatures were between 30°C and 36°C, with some daily fluctuations. Data on the morphometrics and colour confirmed considerable phase differences between the crowded and isolated locusts [23-24].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Nu PAGE)

Accessory glands and oocytes were dissected in a Ringer's solution (8.77 g/L NaCl, 0.19g/L CaCl2, 0.75 g/L KCl, 0.41 g/L MgCl2, 0.34 g/L NaHCO3, 30.81 g/L sucrose, and 1.89g/L trehalose, pH 7.2) and transferred to a 0.5-ml Eppendorf tube on ice by means of a stainless steel insect needle. About 100 accessory glands and 50 oocytes from each phase were homogenised in ice-cold extraction buffer (0.1 M Tris-HCl (pH 8.5) containing 0.25 M NaCl, 0.1% Triton-X 100). After centrifugation at 2000g to remove insoluble material, the supernatant was analyzed for protein content [25]. The protein concentrations of different samples on each gel were kept identical in order to enable direct comparisons between the samples. Samples were assessed by SDS-PAGE [26] using a precast NuPAGE Novex 4–12% Bis-Tris gel, MES buffer and the protein electrophoresis system (all Invitrogen). A protein ladder (SeeBlue Prestained standard, Invitrogen) was run in parallel with an affinity purified protein sample. Following electrophoresis, the gel was stained in Coomassie Brilliant Blue solution (overnight) and proteins were visualized after destaining in a methanol acetic acid solution.

Capillary high pressure liquid chromatography (CapLC) analysis:

Accessory glands from 25 day females adults reared under crowded and isolated conditions were dissected in a Ringer's solution (8.77 g/L NaCl, 0.19g/L CaCl2, 0.75 g/L KCl, 0.41 g/L MgCl2, 0.34 g/L NaHCO3, 30.81 g/L sucrose, and 1.89g/L trehalose, pH 7.2) and immediately placed in an ice-cold methanol/water/acetic acid (90:9:1) solution. The accessory glands were homogenized, sonicated and centrifuged for 30 min (10. 000 g; 4°C). The supernatant was dissolved in 200 ml of an extraction medium containing methanol, MilliQ water and acetic acid (90:9:1v/v/v). The samples were then centrifuged at 13.000 rpm for 5 min at 4 ◦C. The supernatant was removed and combined with 400ml of 0.1% aqueous trifluoroacetic acid (TFA). The methanol was evaporated in a speedvac. The aqueous solution was then extracted with 200ml of n-hexane to remove the bulk of lipids. The remaining traces of solvents were evaporated in vacuo. The watery layer was again diluted with 0.1% TFA, filtered through a Millipore PVDF filter (0.45mm pore size) and used for HPLC analysis. After reconstitution of the sample in 15 µl acetonitrile/water (4:96) solution, 10 µl was injected by the CapLC Autosampler (Waters Associates) onto the Symmetry C-18 column (5 µm, 0.32 mm×150 mm). Column conditions were as follows: solvent A: milli-Q water with 0.1% trifluoroacetic acid, solvent B: acetonitrile (HPLC gradient grade, Riedel-de Haën, Germany) with 0.1%

trifluoroacetic acid. Gradient elution was performed using a linear gradient from 15% B up to 70% B in 90 min at a constant flow rate of 5 µl/min. The eluting peptides were detected with the CapLC Photo Diode Array detector (Waters Associates) within an absorbance area from 200 to 400 nm and with a resolution of 1.2 nm and a sampling rate of 1 spectrum per second. A chromatogram was generated as a function of the highest absorbance during the chromatographic run (100%).

Preparation of female's accessory glands antisera

The rabbit immunization was performed by a combination of intravenous, subcutaneous and intramuscular injections of gregarious accessory glands extract with Freund's adjuvant. Protocol for the immunization of the rabbit was shown in Table 1.

Table 1. Protocol for the immunization of the rabbit

Day	Combination of antigen	Injection method
1 2 3	1 ml antigen	intravenous
10 17 24 31	0,5 ml antigen + 0,2 ml incomplete Freund's adjuvant	subcutaneous
	Swab	
52	0,5 ml antigen + 0,2 ml incomplete Freund's adjuvant	intramuscular
59	Blood collection	

The rabbit was immunized. Antiserum was gained by separation of the serum from the whole blood.

Western Blots Immunostaining

For Western blot analysis, proteins from SDS–PAGE of gregarious and solitarious accessory glands and oocytes were transferred onto a nitrocellulose membrane at 4 °C for 2 h. The membrane was rinsed briefly with washing buffer (PBST: 0.3% Tween 20 in ml PBS) and blocked in blocking buffer (5% non-fat milk and 0.1% NaN3 in 1ml PBS) at room temperature for 1 h and rinsed briefly with washing buffer. After the removal of blocking buffer, the membrane was incubated in PBST containing the anti-gregarious accessory glands antibody at 1:20,000 dilution for 2 h at room temperature. After incubation in primary antibody, the membrane was briefly rinsed and washed thrice in washing buffer (1ml PBS) for 20 min each and was further incubated in secondary antibody (i.e. goat antirabbit IgG-alkaline phosphatase (AP) conjugated (Invitrogen,USA) at 1:5000 dilution at room temperature for 1 h. The membrane was then washed thrice with PBST for 10 min each. Signals were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) in alkaline phosphate detection buffer (0.1 M Tris, pH 9.5, and 0.1 M NaCl) and color development was terminated with running tap water.

RESULTS

NuPAGE analysis of crowd- and isolated-reared locust's

In the target to know whether there is a proteic female factor inducing the gregarization in progeny, Nu-PAGE of accessory gland's and oocyte's extracts of gregarious and solitarious females was run. Figure 1 shows that there was a differential band between the two phases. This band was between 28 and 38 KDa and was gregarious phase specific.

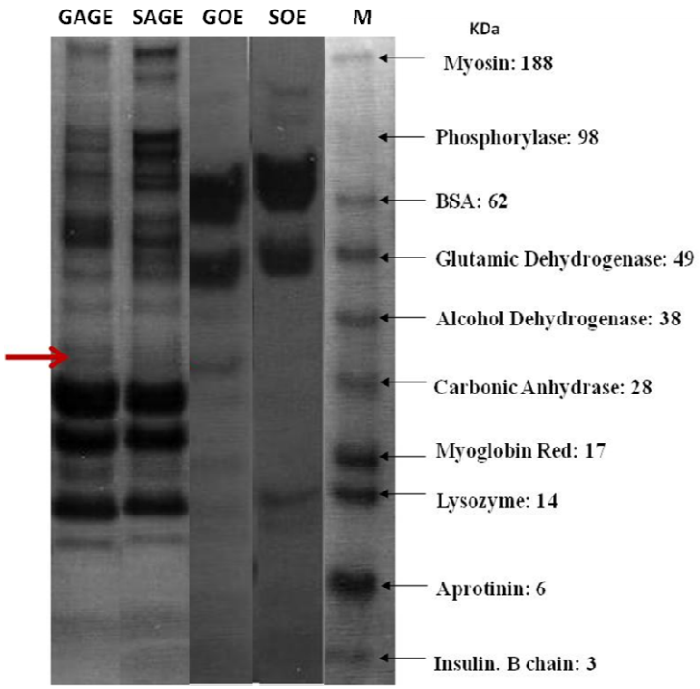

Fig.1. NuPAGE profiles of accessory gland's and oocyte's extracts of females adults from crowd-reared (gregarious) and isolated-reared (solitarious) *S. gregaria*. The red arrow shows the differential proteic band. GAGE: Gregarious Accessory Gland's Extract; SAGE: Solitarious Accessory Gland's Extract; GOE; Gregarious Oocyte's Extract; SOE: Solitarious Oocyte's Extract; M: Marker.

HPLC profiles of crowd- and isolated-reared locusts

The extracts of accessory glands samples of females *S. gregaria* were analyzed by CapLC System. The chromatographic pattern of locusts reared under the different conditions revealed some striking differences (Fig. 2). Most remarkable was a peak with a retention time of 37 min that was found to be quite dominant in the accessory glands of most of the crowd-reared adults. The peak was much lower when the locusts had been reared solitarily.

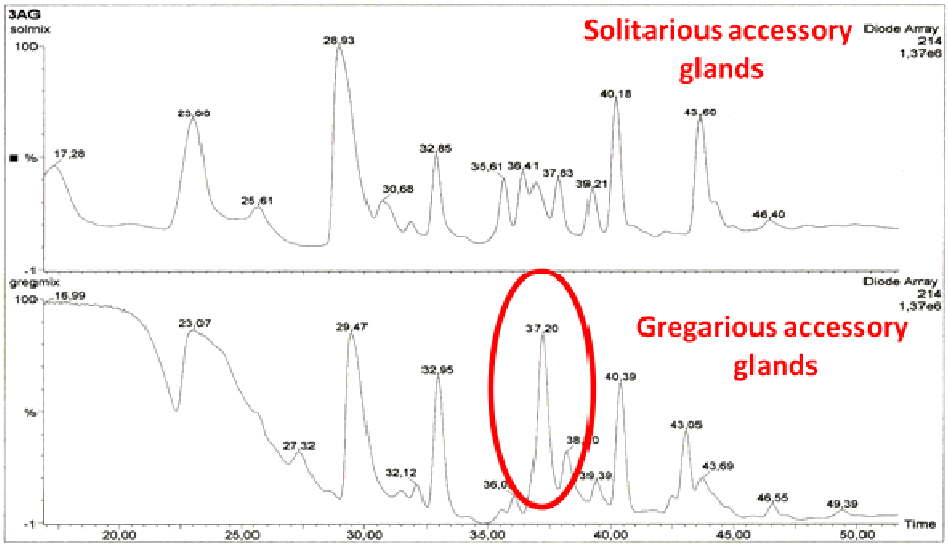

Fig.2. HPLC profiles of accessory gland's extract of females adults from crowd-reared (gregarious) and isolated-reared (solitarious) *S. gregaria*. The red circle shows the differential peak.

Western Blots Immunostaining of female's accessory glands and oocytes

Western blotting profiles of gregarious and solitarious accessory gland's and oocyte's extract with gregarious accessory gland's antisera show the presence of a supplementary proteic band in gregarious accessory gland between 28 and 38 KDa as shown in Fig. 3. The difference in molecular factors was in accordance with the previous NuPAGE analysis (Fig.1).This additional band may contain a factor responsible for the transmission of gregarizing characteristics to the progeny [22-27]. The gregarious accessory gland's antiserum detected a 28-38 KDa protein in gregarious oocytes antigen (Fig.3.). This protein was not detected in solitarious oocytes. Polyclonal antibodies of gregarious accessory glands detected band only in antigen from gregarious oocytes.

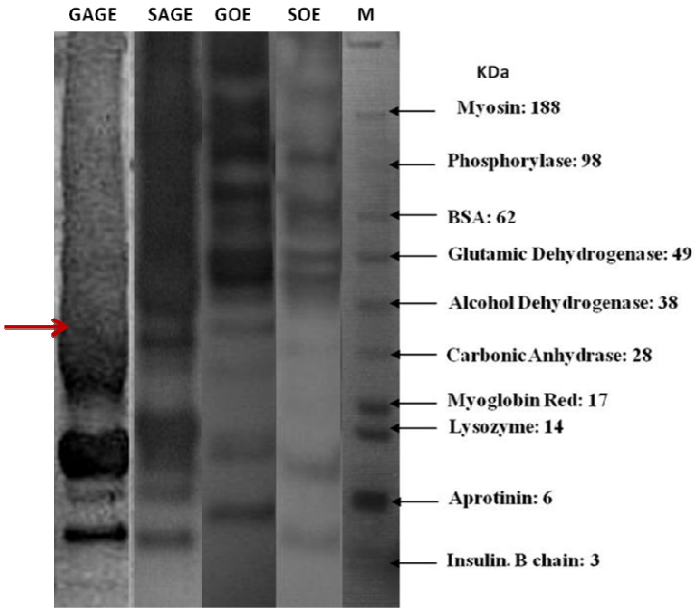

Fig.3. Western blots detecting the 28-38KDa protein from gregarious accessory gland's and oocyte's extracts. The red arrow shows the differential gregarious protein suspected to be transferred from gregarious accessory glands to oocytes. GAGE: Gregarious Accessory Gland's Extract; SAGE: Solitarious Accessory Gland's Extract; GOE; Gregarious Oocyte's Extract; SOE: Solitarious Oocyte's Extract; M: Marker.

DISCUSSION

Recent developments in mass spectrometry, new genomic databases such as the *Locusta migratoria* EST library and rapid advances in bioinformatical techniques have provided new opportunities to seek peptides and proteins associated with phase polyphenism. One of the potential molecular markers of phase transition that was already found is a 6080 Da peptide in *S. gregaria*, provisionally designated as 'phase-related peptide' (PRP) [28]. It was discovered by comparing the peptidome of the haemolymph of solitarious and gregarious *S. gregaria*. This peptide is a potential molecular marker of phase transition, because PRP is present in higher concentrations in the gregarious phase than in the solitarious one. Our peptide, eluted at 37.20 min also was present in higher concentrations in the gregarious female's accessory glands than in the solitarious one and may be possible to constitute another form of the PRP transferred from the hemolymph to the accessory glands. The 28-38 KDa protein is present only in oocytes from gregarious locusts compared to those of solitarious locusts. It seems that the protein present in gregarious accessory glands was transferred to oocytes which have the same weight. Therefore, it has been hypothesised that this protein might represent an additional maternal factor that may play a role in the determination of the phase state of the offspring.

Phase state not only changes within an individual's lifetime but also transmits epigenetically between generations. Present understanding of transgenerational phase control stems from early studies in which hatchlings coloration was shown to depend on parental rearing density; crowded parents produce darker hatchlings characteristic for the gregarious phase, while solitarious locusts produce light green hatchlings. These observations have since been extended: many offspring features, including morphometry, behaviour, hatchling mass and development time, are related to parental rearing density. Therefore, our 28-38 KDa protein may be possible to play a role in the apparition of gregarious characters in progeny. Supplementary experiments will be necessary to confirm our hypothesis.

CONCLUSION

The accessory glands of the ovaries play a role in the determination and transmission of parental phase characteristics. Our study shows the presence of additional peptide with a retention time of 37, 20 min in gregarious accessory gland's extract compared with solitarious females. The difference in physiological composition of the female's accessory glands and their specificity were also visible by western blots of the extract of both phases'accessory glands. Here, we are certainly convinced by the presence of gregarizing factor in gregarious accessory glands. It seems by western blot immunostaining that there is an immunospecificity between gregarious accessory glands and the 28-38 KDa protein gregarious oocytes. This specificity between gregarious accessory glands and gregarious oocytes suggests the transmission of gregarious factor from accessory glands to the progeny. The absence of this protein immunospecificity in solitarious oocytes could be explained by the fact that there are protease inhibitors in solitarious accessory glands which block the synthesis of such gregarizing proteins. It remains to be investigated, whether the relatively high concentrations of the 37, 20 min peptide that had been found in accessory glands of gregarious females may play a role in the determination of the phase–state of the offspring and whether the 28-38 KDa protein found in gregarious oocytes is responsible for the apparition of gregarious phase in offspring.

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