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Developmental- and food-dependent *foraging* transcript levels in the desert locust

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Abstract Drastic changes in the environment during a lifetime require developmental and physiological flexibility to ensure animal survival. Desert locusts, *Schistocerca gregaria*, live in an extremely changeable environment, which alternates between periods of rainfall and abundant food and periods of drought and starvation. In order to survive, locusts display an extreme form of phenotypic plasticity that allows them to rapidly cope with these changing conditions by converting from a cryptic solitary phase to a swarming, voracious gregarious phase. To accomplish this, locusts possess different conserved mediators of phenotypic plasticity. Recently, attention has been drawn to the possible roles of protein kinases in this process. In addition to cyclic AMP-dependent protein kinase (PKA), also cyclic GMP-dependent protein kinase (PKG), which was shown to be involved in changes of food-related behavior in a variety of insects, has been associated with locust phenotypic plasticity. In this article, we study the transcript levels of the *S. gregaria* orthologue of the *foraging* gene that encodes a PKG in different food-related, developmental and crowding conditions. Transcript levels of the *S. gregaria foraging* orthologue are highest in different parts of the gut and differ between isolated and crowd-reared locusts. They change when the availability of food is altered, display a distinct pattern with higher levels after a moult and decrease with age during postembryonic development.

Key words cyclic GMP-dependent protein kinase, feeding, neuronal plasticity, nutritional state, *Schistocerca gregaria*

Introduction

The desert locust, *Schistocerca gregaria*, displays an extreme form of phenotypic polyphenism. Locust phase transition is an example of phenotypic plasticity where one genome can result in very different phenotypes de-

pending on the environmental conditions. Indeed, under certain natural or laboratory conditions, solitary or isolated reared locusts will undergo a behavioral, morphological, and physiological change toward the gregarious or crowded reared phase (Despland & Simpson, 2000; Rogers *et al.*, 2003; Simpson *et al.*, 1998). A complete transition from one extreme phase to another takes more than one generation, but the first changes in behavior are already detectable after a few hours of crowding (Pener & Yerushalmi, 1998; Simpson *et al.*, 2001; Verlinden *et al.*, 2009). Solitary locusts avoid other locusts, while gregarious ones will group together and form enormous swarms that threaten the economy and health in the affected areas. These swarms are usually formed when, after conditions when rainfall and food are abundant and

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locusts can reproduce in large numbers; food conditions (presence and quality) change drastically and the locusts are forced to gather in patches where the food is still present. Moreover, swarm formation and migration are partially driven by the search for new food sources in order to survive (Al-Ajlan, 2007; Despland & Simpson, 2006; Raubenheimer & Simpson, 2003).

The *foraging* gene that encodes for a cyclic GMP-dependent protein kinase (PKG) has been shown to be implicated in foraging or food-seeking behavior in a variety of insects. It was first discovered in the fruit fly, *Drosophila melanogaster*, where a naturally occurring allelic variation in the *foraging* (*Dmfor*) gene is responsible for a behavioral polymorphism regulating foraging or food searching behavior (de Belle et al., 1989; Sokolowski et al., 1997). Rovers that have the dominant *for^R* allele, have higher *Dmfor* mRNA levels and higher PKG activity in their heads, while sitters have the *for^S* allele and have lower mRNA levels and PKG activity.

In the presence of food, both larvae and adults of the rover phenotype will display larger moving traits within one food patch or between different patches as compared to sitters (Osborne et al., 1997; Pereira & Sokolowski, 1993). Under different natural conditions, there will be a selection for one of these two alleles depending on the distribution of food and population density (Sokolowski, 1980; Sokolowski et al., 1997). The differences between *for^R* and *for^S* larvae under natural conditions are due to how PKG affects their food acquisition, intake and nutrient absorption (Kaun et al., 2007b, 2008; Kent et al., 2009), energy homeostasis, (Kaun & Sokolowski, 2009) and their different responses to food deprivation (Kaun et al., 2008). Besides a role in food-related behavior, *Dmfor* also has a clear role in responsiveness and habituation to different food and olfactory stimuli (Engel et al., 2000; Scheiner et al., 2004; Shaver et al., 1998), memory acquisition and retention (Kaun et al., 2007a; Mery et al., 2007), and pattern recognition (Wang et al., 2008).

In eusocial insects, differential PKG levels and/or enzymatic activities are correlated with caste determination and worker activity according to the nutritional needs of the colony (Ben Shahr et al., 2002; Heylen et al., 2008; Ingram et al., 2005, 2011; Kodaira et al., 2009; Lucas & Sokolowski, 2009; Tobback et al., 2008, 2011).

It is clear that the *foraging* gene is involved in the regulation of food-related behaviors in insects, although the specific mechanism seems to vary in different species. Given the fact that there is a large difference in general behavior of solitary and gregarious locusts and that phase transition is partially driven by food-related factors (presence and quality), this gene can be of interest in the desert locust. Moreover, PKG is a part of the Nitric

Oxide (NO)/cGMP pathway, which has been associated with neuronal plasticity (Hofmann, 2005; Kleppisch & Feil, 2009; Ott et al., 2007; Schlossmann et al., 2003). NO is synthesized throughout the entire locust's nervous system (Elphick et al., 1996; Kurylas et al., 2005; Ott & Burrows, 1998). The precursor of NO is arginine, and during NO synthesis by Nitric Oxide Synthase (NOS), citrulline is the main by-product. In the brain and thoracic ganglia of crowded reared locusts, higher citrulline titres were found than in isolated reared ones. Crowding of isolated reared locusts increased citrulline levels in the brain, but not in the thoracic ganglia. When crowded reared locusts were isolated, arginine levels declined in the first 24 h, but rose again when the isolation period increased (Rogers et al., 2004). These results suggest that NO synthesis is phase-dependent, and consequently so may be the activation of PKG, but no causal link with the initial, density-dependent, behavioral switch in the gregarization process has been established (Ott et al., 2012).

In this study, we have examined possible links between the transcript levels of the *S. gregaria foraging* orthologue (*Sgfor*) and different food-related and developmental conditions. A previous study already reported the presence of a PKG in the locust brain and revealed that the enzymatic activity of PKG was higher in crowded reared locusts than in isolated reared individuals (Lucas et al., 2010). These findings were the first to suggest the existence of phase-dependent differences in PKG activity or presence in locusts and they are complementary to several results that are now presented in this article.

Materials and methods

Animal rearing

S. gregaria used in this study were reared under crowded conditions, representing the gregarious phase and isolated conditions, representing the solitary phase. Crowded animals were kept in cages of 38 cm × 38 cm × 38 cm with 100–200 individuals per cage. Mature females were allowed to deposit eggs in pots containing a mixture of sand, peat, and water (7 : 3 : 1). The isolated animals were kept individually in transparent containers of 8.5 cm × 7.5 cm × 14 cm in a separate room. For the first generation of isolated animals, the eggs of crowded females were washed and separated. The next generations (>40 with occasional introduction of new individuals from the crowded reared colony to limit genetic drift) were obtained by using eggs of individually reared females that separated after hatching (Hoste et al., 2002). All animals

Table 1 Primer sequences.

Gene	Forward primer	Reverse primer
For RACE 1	GCT GCA GCA TGG ACG AAA	TCC AAG AGA CCA GTA GTC AGC ACT T
For RACE 2		ATA ATT CTC CTC CAA GGC AAG
<i>For</i>	GCT GCA GCA TGG ACG AAA	TCC AAG AGA CCA GTA GTC AGC ACT T
<i>β-actin</i>	AAT TAC CAT TGG TAA CGA GCG ATT	TGC TTC CAT ACC CAG GAA TGA
<i>Eflα</i>	GAT GCT CCA GGC CAC AGA GA	TGC ACA GTC GGC CTG TGA T
<i>CG13220</i>	TGT TCA GTT TTG GCT CTG TTC TGA	ACT GTT CTC CGG CAG AAT GC
<i>GAPDH</i>	GTC TGA TGA CAA CAG TGC AT	GTC CAT CAC GCC ACA ACT TTC
<i>Rp49</i>	CGC TAC AAG AAG CTT AAG AGG TCA T	CCT ACG GCG CAC TCT GTT G
<i>Tubulin</i>	TGA CAA TGA GGC CAT CTA TG	CGC AAA GAT GCT GTG ATT GA
<i>Ubiquitin</i>	GAC TTT GAG GTG TGG CGT AG	GGA TCA CAA ACA CAG AAC GA

For RACE Fw1 and Rv1/2 were used in 5' and 3' RACE. *β-actin*, *CG13220*, *Eflα* (*elongation factor 1α*), *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenase*), *Rp49* (*ribosomal protein 49*), *tubulin* and *ubiquitin* are qRT PCR primers for housekeeping gene (HKG) amplification used to determine the best HKG by means of the GeNorm program. *For* primers were used for the gene of interest in the qRT PCR.

were kept at a controlled temperature of 32°C, a relative humidity between 40% and 60% and a photoperiod of 14 h. They were fed daily with fresh cabbage and dried oat flakes *ad libitum*.

Cloning of the foraging orthologue of Schistocerca gregaria

The partial *Sgfor* open reading frame (ORF) was cloned and sequenced using methods and degenerate primers as described, with cDNA derived from neuronal tissues as a template (Tobback *et al.*, 2008, 2011). The gene specific primers used in 3' and 5' RACE (Marathon cDNA amplification Kit, Clontech, Mountain View, California, USA) for subsequent attempts to elongate the sequence are provided in Table 1. A multiple sequence alignment using ClustalOmega was performed with the available amino acid sequences of the *S. gregaria* PKG isoforms, as reported in this article (SgFOR-T) (HM363020) and by Lucas and colleagues (Lucas *et al.*, 2010) (SgFOR-L) (ACV89935.1), *Apis mellifera* (Linnaeus, 1758) AmFOR (NP_001011581.1) and *D. melanogaster* DmFOR (NP_477487.1) (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Sample collection

Tissues of adult crowded and isolated reared 10-day-old males and females were pooled in six groups (3 male and 3 female groups; 40, 10, and 10 animals per group for both males and females) for the tissue distribution study.

For the developmental samples, 10 brains (5♀ and 5♂, not including optic lobes) were dissected per sample and

this for each of the five nymphal stages and the adult stage 2 days after their molt. Four independent sample series were obtained for both the gregarious and solitary developmental series. To monitor transcript levels during the fourth nymphal, fifth nymphal, and adult stage, heads were collected at different time points during those stages, all mouthparts and antennae were removed.

For the starvation experiment, adult crowded reared locusts were synchronized after their final moult. At the age of 6 days, all food was taken out of the cage and no more was added. Every 3 days, the heads of six animals from each condition were collected and all mouthparts and antennae were removed. After 6 days of starvation, half of the animals were fed normally again for the remaining period, whereas the others were starved further. Controls were fed normally throughout the complete experiment.

All samples were dissected in tubes containing MagNA Lyser Green Beads Roche Diagnostics Belgium (Vilvoorde, Belgium), snap frozen in liquid nitrogen and stored at −80°C until further processing. After homogenization using the MagNA Lyser instrument (Roche), total RNA was extracted using the Lipid Tissue Mini Kit including a DNase treatment to exclude potential genomic DNA contamination (both Qiagen). First strand cDNA synthesis was then performed using SuperScript III Reverse Transcriptase and random hexamer primers (Invitrogen, Life technologies Europe B.V., Gent, Belgium), the reaction was diluted tenfold prior to use in qRT PCR.

Quantitative real time PCR (qRT PCR)

All samples were quantified using the Fast SYBR® Green master mix in the StepOnePlus detection system

(both Applied Biosystems) with the gene specific primers from Table 1 (Fig. S1) as described in Verlinden *et al.*, 2010. The PCR conditions were 50°C for 2 min, 95°C for 10 min, 40× (95°C for 15 s, 60°C for 1 min). For every experiment, we have first determined which housekeeping genes were the most stable in the relevant samples. Based on commonly used housekeeping genes in other insects, *β-actin*, *CG13220*, *elongation factor 1 alpha (Eflα)*, *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, *ribosomal protein 49 (Rp49)*, *tubulin*, and *ubiquitin* were tested. The combination of housekeeping genes for this normalization was determined using geNorm (van Hiel *et al.*, 2009; Vandesompele *et al.*, 2002). *Sgfor* quantities were normalized to *β-actin* and *GAPDH* for the tissue distribution and developmental samples (all stages). In the qRT PCR of the starvation experiment and developmental samples from the last three stages (both experiments done on heads), samples were normalized to *β-actin* and *Eflα* to account for the variability in initial mRNA concentrations and differences in reverse transcriptase efficiency. Relative standard curves for the endogenous controls and the FOR transcripts were generated with serial (10×) dilutions of a brain cDNA sample. Since the efficiency of all used primers were comparable, we could use the delta Ct method to analyze our data.

We performed statistical analysis by means of SPSS (v17.0, SPSS Inc., Chicago, Illinois), using the Mann–Whitney *U*-test for comparing two independent groups. A level of $P < 0.05$ was considered significant.

Results

Cloning of the foraging orthologue of Schistocerca gregaria

A partial sequence was obtained using degenerated primers based on conserved regions of insect PKG orthologues. Using 3' RACE, the C-terminal region of PKG was reached, however we were not able to complete the N-terminus using 5' RACE. The obtained sequence was submitted to NCBI GenBank (GenBank Acc. no. HM363020). When the desert locust EST-database became available (Badisco *et al.*, 2011), a PKG sequence was found with a BLAST search but contained no additional sequence information.

The *in silico* translated partial amino acid sequence (636 AA) was aligned with FOR sequences from other insects using ClustalOmega (Fig. 1). When comparing the sequence obtained with RACE in this study (SgFOR-T) to the other available SgFOR sequence (SgFOR-L) (Lucas *et al.*, 2010), first of all it is clear that the SgFOR-

T sequence is longer at both the N- (168 AA) and C-terminal end (67 AA) (Fig. 1). The majority of the sequence is identical, nevertheless one region shows dissimilarity (amino acids 346–383 in the SgFOR-T sequence). It should also be remarked that this region contained the sequence used to design one of the degenerated primers in our study because it is a conserved region in all other known insect PKGs (Fw1 designed using: the RQQQHIM stretch). When analyzing the sequence on the nucleotide level (Fig. S1), it becomes clear that the discrepancy between the sequences is caused by a frameshift in the SgFOR-L sequence (nucleotides 560–660 SgFOR-L sequence) and consequently that they are probably the same *for* sequence instead of two genes, alleles, or splice variants.

Quantification of Sgfor transcript levels in adult solitary and gregarious tissues and during development

Sgfor transcript levels were detected in all tissues comprised in the adult tissue distribution analysis. Since no differences in transcript levels were observed between the two sexes, the data are presented in one bar. The highest levels were found in the different parts of the locust gut and the salivary glands. The optic lobes ($P = 0.036$), salivary glands ($P = 0.012$), foregut ($P = 0.045$), midgut ($P = 0.046$), hindgut ($P = 0.030$), gonads ($P = 0.046$), and flight muscles ($P = 0.015$) show significantly higher *Sgfor* transcript levels in crowded reared compared to isolated reared animals (Fig. 2).

When comparing *Sgfor* mRNA levels in brains of locusts throughout development, expression is highest in the first larval stage and decreases until the fourth larval stage. In the first four larval stages, the *Sgfor* transcript levels are significantly higher in gregarious compared to solitary brains [Fig. 3, $P = 0.014$ (L1), 0.016 (L2), 0.034 (L3), 0.046 (L4), 0.712 (L5) and 0.243 (Adult)]. Also, transcript levels were studied at different time points in complete heads from the fourth nymphal stage on. In Figure 4, it is clear that transcript levels of *Sgfor* are high in the days around the moult with a peak at the first day of the new stage. A similar pattern can be observed of transcript levels in complete heads during the fifth nymphal and adult stage.

The effect of starvation on Sgfor transcript levels

The first 6 days of food deprivation, *Sgfor* transcript levels were not affected by the nutritional status. However, after 9 days of food deprivation, *Sgfor* levels in the locust

SgFOR-L	1	-----
SgFOR-T	1	-----
AmFOR	1	-----MGTLERELQELLRVKDEKIVEIE
DmFOR	1	MQSLRISGCTPSGTGGSATPSPVGLVDPNFIVSNYVAASPQEERFIQLIQAKELKIQEMQ
SgFOR-L	1	-----
SgFOR-T	1	-----SSINSTVGLS-----LRGGRHHGPGV-----
AmFOR	23	ALLCRRDAEIQELRSHLDKFLQCASIKLA-----FEPRNP GP GS
DmFOR	61	RAIQFKDNEIIEELKSHLDKFSVFPFSR--SAAGCAGTGGASGSGAGSSGGS GPGTATGAT
SgFOR-L	1	-----
SgFOR-T	22	---VLRKTQRAQGISAEPQ---SPSA---TADFFTPVKDDRSRELKAAAILDNDFMKNLEM
AmFOR	63	---K-----GPRDFPRSHRFKS---LPRCQLSNKRRDRSRELKAAAILDNDFMKNLEL
DmFOR	121	RKSGQNFQRQRAIGISAEPQSESSLLLEHVSFPKYDKDRSRELKAAAILDNDFMKNLDL
SgFOR-L	1	-----
SgFOR-T	74	AQIREIVDCMPVEYAAGSLIIKEGDVGSIVYVMEGRVEVSRENKYLSTLAAKGVHGL
AmFOR	109	TQIRDRGLHVSCSFSAGSTIIREGDVGSIYVMEEGVEVSRDGKYLSTLAPGKVLGEL
DmFOR	181	TQIREIVDCMPVKYPAKNLIIKEGDVGSIVYVMEGRVEVSREGKYLSTLSAKVLGEL
SgFOR-L	1	-----RTGLSRQAEYTDFLKSVPIFKNLPEET
SgFOR-T	134	AILYNCKRTATIKAAATDCKLWAIERQCFQTIMMRTGLSRQAEYTDFLKSVPIFKNLPEET
AmFOR	169	AILYNCKRTATITAAATDCQLWAIERQCFQTIMMRTGLSRQAEYTDFLKSVPIFKNLPEET
DmFOR	241	AILYNCKRTATITATTECNLWAIERQCFQTIMMRTGLSRQAEYTDFLKSVPIFKNLPEET
SgFOR-L	28	LTKISDVLEETYYNEGDIIRQGARGDTFFIISKGVKVTMKQPNLEEKYVRTLHKGDF
SgFOR-T	194	LTKISDVLEETYYNEGDIIRQGARGDTFFIISKGVKVTMKQPNLEEKYVRTLHKGDF
AmFOR	229	LTKISDVLEETYYNEGDIIRQGARGDTFFIISKGVKVTMKQPNLEEKYVRTLHKGDF
DmFOR	301	LTKISDVLEETYYNEGDIIRQGARGDTFFIISKGVKVTMKQPNLEEKYVRTLHKGDF
SgFOR-L	88	FGEKALQGDDLRTANIIASDPEGVTCCLVIDRET FNQLISSLDEIRTRYKDEGEV-----
SgFOR-T	254	FGEKALQGDDLRTANIIASDPEGVTCCLVIDRET FNQLISSLDEIRTRYKDEGEV-----
AmFOR	289	FGEKALQGDDLRTANIIADDEPGVTCCLVIDRET FNQLISSLDEIRTRYKDESSSSVEGWEN
DmFOR	361	FGEKALQGDDLRTANIIICESADGVSCCLVIDRET FNQLISNLDEIKHRYDDEGAM-----
SgFOR-L	142	---RRRIIDEEFRDVKLSDLRVLATLGVGGFGRVELVQIAGDLLRSFCFETNEEKSDCGTQ
SgFOR-T	308	---RRRIIDEEFRDVKLSDLRVLATLGVGGFGRVELVQIAGDPLRSFALKQMKKSQIVETR
AmFOR	349	RATIPENEEFRDLRIQDLRLATLGVGGFGRVELVQIAGDSSRSFALKQMKKAQIVETR
DmFOR	415	--ERRKINEEFRDINLIDLRVLATLGVGGFGRVELVQIAGDSSRSFALKQMKKSQIVETR
SgFOR-L	199	ATAALLSXXXXXXRPTATLIVKLYKTFKDRKYLMLMESCLGGELWTLIRDKGHFDDGTT
SgFOR-T	365	QQQHIMSEKEIMGEANCNFIVKLYKTFKDRKYLMLMESCLGGELWTLIRDKGHFDDGTT
AmFOR	409	QQQHIMSEKRIIMGEACDFIVKLYKTFKDRKYLMLMESCLGGELWTLIRDKGHFDDGTT
DmFOR	473	QQQHIMSEKEIMGEANCQFIVKLYKTFKDRKYLMLMESCLGGELWTLIRDKGHFDDGTT
SgFOR-L	259	RFYTACVVEAFDYLHSRNIIYRDLKPENLLLDNAGYVKLVDFGFAGKQLHGKRTWTFCGT
SgFOR-T	425	RFYTACVVEAFDYLHSRNIIYRDLKPENLLLDNAGYVKLVDFGFAGKQLHGKRTWTFCGT
AmFOR	469	RFYTACVVEAFDYLHSRNIIYRDLKPENLLLDNAGYVKLVDFGFAGKQLHGKRTWTFCGT
DmFOR	533	RFYTACVVEAFDYLHSRNIIYRDLKPENLLLDNAGYVKLVDFGFAGKQLHGKRTWTFCGT
SgFOR-L	319	PEYVAPEVILNRGHDISADYWSLGLVMFELLTGTPPFTGADPMKTYNIIILKGIDAIIFPR
SgFOR-T	485	PEYVAPEVILNRGHDISADYWSLGLVMFELLTGTPPFTGADPMKTYNIIILKGIDAIIFPR
AmFOR	529	PEYVAPEVILNRGHDISADYWSLGLVMFELLTGTPPFTGADPMKTYNIIILKGIDAIIFPR
DmFOR	593	PEYVAPEVILNRGHDISADYWSLGLVMFELLTGTPPFTGADPMKTYNIIILKGIDAIIFPR
SgFOR-L	379	TITRNAMALIKKLCRDNPAPERLG-----
SgFOR-T	545	TITRNAMALIKKLCRDNPAPERLGQKGGISEIQHKWFDGFNWEGLRNLTLTPPIVFPVR
AmFOR	589	SITRNAMALIKKLCRDNPAPERLGQKGGISEIQHKWFDGFNWEGLRNLTLTPPIVFPVR
DmFOR	653	NITRNAMALIKKLCRDNPAPERLGQKGGISEIQHKWFDGFNWEGLRNLTLTPPIVFPVR
SgFOR-L		-----
SgFOR-T	605	NVTDTNFDAYPPDSGPPDDLTGWDAF
AmFOR	649	NATDTTNFDAYPPDSGPPDDLTGWDAF
DmFOR	713	SVVDTTNFDAYPPDSGPPDDLTGWDAF

Fig. 1 Comparison between the amino acid sequences of cGMP dependent protein kinases. PKG from *Schistocerca gregaria* as sequenced by Tobback (SgFOR-T) and Lucas (SgFOR-L), PKG from *Apis mellifera* (AmFOR) and *Drosophila melanogaster* (DmFOR) in a CLUSTAL Omega alignment. The amino-acid position is indicated on the left. Identical residues are shown as white letters against black, whereas conservatively substituted residues are shaded. Dashes indicate gaps that were introduced to maximize homologies.

Fig. 2 qRT PCR analysis of *Sgfor* transcript levels in different tissues of adult desert locusts. Bars represent the mean level of *Sgfor* mRNA relative to β -actin and GAPDH mRNA levels with the standard error of mean and $n = 6$ pools of samples from 40 (1 male pool, 1 female pool) or 10 (2 male pools and 2 female pools) animals. Significant differences are indicated with an asterisk for $P < 0.05$ (Mann–Whitney). Opt Lob: Optical lobes; SOG: Suboesophageal ganglion; TG: Thoracic ganglion; CC: Corpora cardiaca; Sal gl: Salivary gland; Hg: Hindgut; Mg: Midgut; Fg: Foregut; Gon: Gonads; Fatb: Fat body; Musc: Flight muscle.

Fig. 3 qRT PCR analysis of *Sgfor* transcript levels in desert locust brains during development. Bars represent the mean level of *Sgfor* mRNA relative to β -actin and GAPDH mRNA levels with the standard error of mean. Note that $n = 4$ pools of samples from ten animals. Significant differences are indicated with an asterisk for $P < 0.05$ (Mann–Whitney).

head were significantly lower compared to the control animals and they remained low during the entire experiment ($P = 0.036$). There were no significant increases or decreases in the control animals. When starved animals were allowed to eat again, *Sgfor* transcript levels increased significantly when compared to both the control and the starved locusts (Fig. 5, $P = 0.028$ and 0.012 , respectively).

Fig. 4 qRT PCR analysis of *Sgfor* transcript levels in desert locust heads during the last three stages. Bars represent the mean levels of *Sgfor* mRNA relative to β -actin and *Ef1 α* mRNA levels with the standard error of mean. For each time point, transcript levels were measured for $n = 6$ samples of individual animals.

Fig. 5 qRT PCR analysis of *Sgfor* transcript levels in heads after starvation of gregarious desert locusts. Starvation starts at day 6 of adulthood. From day 12 of adulthood on, some starved animals are fed again. Bars represent the mean level of *Sgfor* mRNA relative to β -actin and *Ef1 α* mRNA levels with the standard error of mean. Significant differences between one time point as compared to the controls are indicated with an asterisk for $P < 0.05$ (Mann–Whitney). Per time point/condition, $n = 6$ samples of individual animals.

Discussion

We successfully cloned and sequenced a large part of the *Sgfor* open reading frame (ORF). *In silico* analysis revealed that this locust PKG sequence shows high similarity to other insect PKGs (Fig. 1). The current sequence data indicate that the *SgFor-L* cDNA sequence, as reported by Lucas *et al.* (2010), probably contained an error that caused a frameshift in the deduced amino acid sequence (Fig. S1). With the exception of this frameshift, both sequences are virtually identical (Fig. 1 and Fig. S1).

However, this does not exclude the possibility that there may be multiple *Sgfor* transcripts in the desert locust as there are in the fruit fly (Kalderon & Rubin, 1989).

Both the tissue distribution (Fig. 2) and the starvation experiment (Fig. 5) may point at a similar food-related role and regulation of PKG in the desert locust as reported in the fruit fly or social insects (Ben Shahr *et al.*, 2002; Heylen *et al.*, 2008; Ingram *et al.*, 2005, 2011; Kaun & Sokolowski, 2009; Kent *et al.*, 2009; Kodaira *et al.*, 2009; Lucas *et al.*, 2010; Lucas & Sokolowski, 2009; Osborne *et al.*, 1997; Sokolowski *et al.*, 1997; Tobback *et al.*, 2008, 2011). *Sgfor* transcript levels throughout the locust body were indeed highest in tissues related to feeding, namely the different parts of the gut and the salivary glands (Fig. 2). Moreover, when adult locusts were deprived of food, *Sgfor* transcript levels were drastically decreased in the head after 9 days of starvation. When adult animals that were first deprived of food for 6 days were fed normally again, *Sgfor* levels in the head showed a significant rise compared to controls that were fed continuously and also compared to starved animals (Fig. 5). Therefore, our findings suggest that also in the desert locust PKG levels are influenced by the nutritional state.

When studying the *Sgfor* transcript levels in the brain during postembryonic development, it was immediately clear that transcript levels were the highest in the first larval stages, both in crowded and in isolated reared animals. Then, transcript levels decreased until the fourth larval stage. During the first four stages, transcript levels were significantly higher in gregarious brains than in solitary ones (Fig. 3). Interestingly, a decrease of transcript levels of the foraging gene with increasing age was also observed in both bumblebee nurses and foragers (Tobback *et al.*, 2011). These higher *Sgfor* transcript levels early in postembryonic development may be correlated with the recruitment of molecules and mechanisms for *de novo* synapse formation during development (Bailey & Kandel, 2008), however a more detailed study both at mRNA and protein level would be useful to clarify this phenomenon.

PKG transcript levels also appeared to display a cyclic pattern during the moulting cycle. A peak in *Sgfor* levels was observed in the head of fourth, fifth larval, and adult locusts 1 day after moulting (Fig. 4). After this peak the transcript levels declined, to rise again when approaching the end of each stage. Before each moult, the hopper ceases to feed, reduces movement, and selects a site for moulting. Consecutive the hopper inflates its gut, the old cuticle splits, and the different body parts emerge. The newly moulted soft and wet hopper/adult will leave its old cuticle and the insect will expand itself to stretch the new cuticle after which it will harden and darken and the

gut will deflate (after which the locust can start feeding again) (as reviewed by Pener & Simpson, 2009). Moulting thus influences the nutritional state of locusts, which may affect the *Sgfor* transcript levels.

Higher *Sgfor* transcript levels were measured in crowded compared to isolated reared locusts (Figs. 2 and 3). This finding is complementary to the finding that adult brains of crowded reared locusts have higher PKG enzymatic activity (Lucas *et al.*, 2010). Thus, although the difference in transcript levels of *Sgfor* between adult crowded or isolated reared locusts in this study was not significant, the higher PKG enzymatic activity was. This may be the consequence of already higher PKG protein levels present in crowded reared locusts, which on their turn may be the consequence of the higher transcript levels in the larval stages. On the other hand also other factors like turnover rate and differences in the activity per protein may be at play. Direct measurements of the PKG protein levels and enzymatic activity throughout development may give a more conclusive answer. The discrepancy between the *Sgfor* transcript levels of isolated and crowded reared locusts observed in the optical lobes, the different parts of the gut and the flight muscles may be due to the very different lifestyles of the two locust phases with dissimilar food availability and interaction with conspecifics. Whereas solitary locusts need sensitive low-level perception, gregarious locusts need to be able to integrate a multitude of signals caused by living as generalist foragers in dense and highly mobile swarms (Ott & Rogers, 2010).

This study provides data pointing to a role in food-related processes, development, and phase polyphenism for the *foraging* gene homologue in the desert locust *Schistocerca gregaria*. The presented data, though giving an indication, are not sufficient to establish the role of PKG in these three physiological processes. Our data provide important information that will hopefully stimulate further research, which may lead to firm conclusions. If the role of PKG in these processes is as important as we expect, new opportunities for pest insect control will arise.

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Disclosure

All the authors declare no conflict of interest, financial or otherwise, that might potentially bias this work.

References

- Al-Ajlan, A.M. (2007) Relationship between desert locust, *Schistocerca gregaria* (Forskål), infestation, environmental factors and control measures in Gazan and Makkah Regions, Saudi Arabia. *Pakistan Journal of Biological Sciences*, 10, 3507–3515.
- Badisco, L., Huybrechts, J., Simonet, G., Verlinden, H., Marchal, E., Huybrechts, R., Schoofs, L., De Loof, A. and Vanden Broeck, J. (2011) Transcriptome analysis of the desert locust central nervous system: production and annotation of a *Schistocerca gregaria* EST database. *PLoS ONE*, 6, e17274.
- Bailey, C.H. and Kandel, E.R. (2008) Synaptic remodeling, synaptic growth and the storage of long-term memory in *Aplysia*. *Progress in Brain Research*, 169, 179–198.
- Ben Shahr, Y., Robichon, A., Sokolowski, M.B. and Robinson, G.E. (2002) Influence of gene action across different time scales on behavior. *Science*, 296, 741–744.
- de Belle, J.S., Hilliker, A.J. and Sokolowski, M.B. (1989) Genetic localization of *foraging* (*for*): a major gene for larval behavior in *Drosophila melanogaster*. *Genetics*, 123, 157–163.
- Despland, E. and Simpson, S.J. (2000) The role of food distribution and nutritional quality in behavioral phase change in the desert locust. *Animal Behavior*, 59, 643–652.
- Despland, E. and Simpson, S.J. (2006) Resource distribution mediates synchronization of physiological rhythms in locust groups. *Proceedings of the Royal Society: Biological Science*, 273, 1517–1522.
- Elphick, M., Williams, L. and Shea, M. (1996) New features of the locust optic lobe: evidence of a role for nitric oxide in insect vision. *Journal of Experimental Biology*, 199, 2395–2407.
- Engel, J.E., Xie, X.J., Sokolowski, M.B. and Wu, C.F. (2000) A cGMP-dependent protein kinase gene, *foraging*, modifies habituation-like response decrement of the giant fiber escape circuit in *Drosophila*. *Learning & Memory*, 7, 341–352.
- Heylen, K., Gobin, B., Billen, J., Hu, T.T., Arckens, L. and Huybrechts, R. (2008) *Amfor* expression in the honeybee brain: a trigger mechanism for nurse-forager transition. *Journal of Insect Physiology*, 54, 1400–1403.
- Hofmann, F. (2005) The biology of cyclic GMP-dependent protein kinases. *Journal of Biological Chemistry*, 280, 1–4.
- Hoste, B., Luyten, L., Claeys, I., Clynen, E., Rahman, M.M., De Loof, A. and Breuer, M. (2002) An improved breeding method for solitary locusts. *Entomologia Experimentalis et Applicata*, 104, 281–288.
- Ingram, K.K., Kleeman, L. and Peteru, S. (2011) Differential regulation of the *foraging* gene associated with task behaviors in harvester ants. *BMC Ecology*, 11, 19.
- Ingram, K.K., Oefner, P. and Gordon, D.M. (2005) Task-specific expression of the *foraging* gene in harvester ants. *Molecular Ecology*, 14, 813–818.
- Kalderon, D. and Rubin, G.M. (1989) cGMP-dependent protein kinase genes in *Drosophila*. *Journal of Biological Chemistry*, 264, 10738–10748.
- Kaun, K.R., Chakabarty-Chatterjee, M. and Sokolowski, M.B. (2008) Natural variation in plasticity of glucose homeostasis and food intake. *Journal of Experimental Biology*, 211, 3160–3166.
- Kaun, K.R., Hendel, T., Gerber, B. and Sokolowski, M.B. (2007a) Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. *Learning & Memory*, 14, 342–349.
- Kaun, K.R., Riedl, C.A.L., Chakabarty-Chatterjee, M., Belay, A.T., Douglas, S.J., Gibbs, A.G. and Sokolowski, M.B. (2007b) Natural variation in food acquisition mediated via a *Drosophila* cGMP-dependent protein kinase. *Journal of Experimental Biology*, 210, 3547–3558.
- Kaun, K.R. and Sokolowski, M.B. (2009) cGMP-dependent protein kinase: linking foraging to energy homeostasis. *Genome*, 52, 1–7.
- Kent, C.F., Daskalchuk, T., Cook, L., Sokolowski, M.B. and Greenspan, R.J. (2009) The *Drosophila foraging* gene mediates adult plasticity and gene-environment interactions in behavior, metabolites, and gene expression in response to food deprivation. *PLoS Genetics*, 5, e1000609.
- Kleppisch, T. and Feil, R. (2009) cGMP signalling in the mammalian brain: role in synaptic plasticity and behavior. *Handbook of Experimental Pharmacology*, 549–579.
- Kodaira, Y., Ohtsuki, H., Yokoyama, J. and Kawata, M. (2009) Size-dependent *foraging* gene expression and behavioral caste differentiation in *Bombus ignitus*. *BMC Research Notes*, 2, 184.
- Kurylas, A.E., Ott, S.R., Schachtner, J., Elphick, M.R., Williams, L. and Homberg, U. (2005) Localization of nitric oxide synthase in the central complex and surrounding midbrain neuropils of the locust *Schistocerca gregaria*. *Journal of Comparative Neurology*, 484, 206–223.
- Lucas, C., Kornfein, R., Chakabarty-Chatterjee, M., Schonfeld, J., Geva, N., Sokolowski, M.B. and Ayali, A. (2010) The

- locust *foraging* gene. *Archives of Insect Biochemistry and Physiology*, 74, 52–66.
- Lucas, C. and Sokolowski, M.B. (2009) Molecular basis for changes in behavioral state in ant social behaviors. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 6351–6356.
- Mery, F., Belay, A.T., So, A.K.C., Sokolowski, M.B. and Kawecki, T.J. (2007) Natural polymorphism affecting learning and memory in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 13051–13055.
- Osborne, K.A., Robichon, A., Burgess, E., Butland, S., Shaw, R.A., Coulthard, A., Pereira, H.S., Greenspan, R.J. and Sokolowski, M.B. (1997) Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science*, 277, 834–836.
- Ott, S.R. and Burrows, M. (1998) Nitric oxide synthase in the thoracic ganglia of the locust: distribution in the neuropiles and morphology of neurones. *Journal of Comparative Neurology*, 395, 217–230.
- Ott, S.R., Philippides, A., Elphick, M.R. and O'Shea, M. (2007) Enhanced fidelity of diffusive nitric oxide signalling by the spatial segregation of source and target neurones in the memory centre of an insect brain. *European Journal of Neuroscience*, 25, 181–190.
- Ott, S.R. and Rogers, S.M. (2010) Gregarious desert locusts have substantially larger brains with altered proportions compared with the solitary phase. *Proceedings of the Royal Society: Biological Science*, 277, 3087–3096.
- Ott, S.R., Verlinden, H., Rogers, S.M., Brighton, C.H., Quah, P.S., Vleugels, R.K., Verdonck, R. and Vandenbroeck, J. (2012) Critical role for protein kinase A in the acquisition of gregarious behavior in the desert locust. *Proceedings of the National Academy of Sciences of the United States of America*, 109, E381–E387.
- Pener, M.P. and Simpson, S.J. (2009) Locust phase polyphenism: an update. *Advances in Insect Physiology*, 36, 1–272.
- Pener, M.P. and Yerushalmi, Y. (1998) The physiology of locust phase polymorphism: an update. *Journal of Insect Physiology*, 44, 365–377.
- Pereira, H.S. and Sokolowski, M.B. (1993) Mutations in the larval *foraging* gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 5044–5046.
- Raubenheimer, D. and Simpson, S.J. (2003) Nutrient balancing in grasshoppers: behavioral and physiological correlates of dietary breadth. *Journal of Experimental Biology*, 206, 1669–1681.
- Rogers, S.M., Matheson, T., Despland, E., Dodgson, T., Burrows, M. and Simpson, S.J. (2003) Mechanosensory-induced behavioral gregarization in the desert locust *Schistocerca gregaria*. *Journal of Experimental Biology*, 206, 3991–4002.
- Rogers, S.M., Matheson, T., Sasaki, K., Kendrick, K., Simpson, S.J. and Burrows, M. (2004) Substantial changes in central nervous system neurotransmitters and neuromodulators accompany phase change in the locust. *Journal of Experimental Biology*, 207, 3603–3617.
- Scheiner, R., Sokolowski, M.B. and Erber, J. (2004) Activity of cGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learning & Memory*, 11, 303–311.
- Schlossmann, J., Feil, R. and Hofmann, F. (2003) Signaling through NO and cGMP-dependent protein kinases. *Annals of Medicine*, 35, 21–27.
- Shaver, S.A., Varnam, C.J., Hilliker, A.J. and Sokolowski, M.B. (1998) The *foraging* gene affects adult but not larval olfactory-related behavior in *Drosophila melanogaster*. *Behavioral Brain Research*, 95, 23–29.
- Simpson, S.J., Bouaichi, A. and Roessingh, P. (1998) Effects of sensory stimuli on the behavioral phase state of the desert locust, *Schistocerca gregaria*. *Journal of Insect Physiology*, 44, 883–893.
- Simpson, S.J., Despland, E., Hagele, B.F. and Dodgson, T. (2001) Gregarious behavior in desert locusts is evoked by touching their back legs. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 3895–3897.
- Sokolowski, M.B. (1980) Foraging strategies of *Drosophila melanogaster*—a chromosomal analysis. *Behavior Genetics*, 10, 291–302.
- Sokolowski, M.B., Pereira, H.S. and Hughes, K. (1997) Evolution of foraging behavior in *Drosophila* by density-dependent selection. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 7373–7377.
- Tobback, J., Heylen, K., Gobin, B., Wenseleers, T., Billen, J., Arckens, L. and Huybrechts, R. (2008) Cloning and expression of PKG, a candidate foraging regulating gene in *Vespula vulgaris*. *Animal Biology*, 58, 341–351.
- Tobback, J., Mommaerts, V., Vandersmissen, H.P., Smagghe, G. and Huybrechts, R. (2011) Age- and task-dependent *foraging* gene expression in the bumblebee *Bombus terrestris*. *Archives of Insect Biochemistry and Physiology*, 76, 30–42.
- van Hiel, M.B., van Wielendaele, P., Temmerman, L., van Soest, S., Vuerinckx, K., Huybrechts, R., Vanden Broeck, J. and Simonet, G. (2009) Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. *BMC Molecular Biology*, 10, 56.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric

- averaging of multiple internal control genes. *Genome Biology*, 3, RESEARCH0034.
- Verlinden, H., Badisco, L., Marchal, E., van Wielendaele, P. and Vanden Broeck, J. (2009) Endocrinology of reproduction and phase transition in locusts. *General and Comparative Endocrinology*, 162, 79–92.
- Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Tobback, J., Pfluger, H.J., Blenau, W. and Vanden Broeck, J. (2010) The cloning, phylogenetic relationship and distribution pattern of two new putative GPCR-type octopamine receptors in the desert locust (*Schistocerca gregaria*). *Journal of Insect Physiology*, 56, 868–875.
- Wang, Z.P., Pan, Y.F., Li, W.Z., Jiang, H.Q., Chatzimanolis, L., Chang, J.H., Gong, Z.F. and Liu, L. (2008) Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learning & Memory*, 15, 133–142.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Fig. S1 Comparison between the nucleotide sequences of cGMP dependent protein kinases. PKG from *Schistocerca gregaria* as sequenced by Tobback *et al.* (this study) (FOR-T) and Lucas *et al.* (2010) (FOR-L) in a CLUSTAL Omega alignment. The nucleotide position is indicated on the right. qRT PCR primers are indicated as white letters against black in the FOR-T sequence, whereas RACE primers are shaded in the FOR-L sequence.