The Sequence of *Acheta* Adipokinetic Hormone and the Variation in Corpus cardiacum Content and Hyperlipaemic Response with Age

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The principle neuropeptide separated by reversed-phase liquid chromatography RP-HPLC from extracts of the corpora cardica of *Acheta domesticus* showed strong adipokinetic activity when injected into *Acheta*. The N-terminal pyroglutamate of the peptide was removed by enzymatic digestion, and the remaining peptide sequenced. The structure is identical to the peptide Grb-AKH previously described from the corpus cardiacum (CC) of *Gryllus bimaculatus* (pGlu–Val–Asn–Phe–Ser–Thr–Gly–Trp–NH_2). The ED_{50} was (0.8 pmol) and saturation was achieved with injection of 2 pmol of synthetic Grb-AKH. The time to maximum hyperlipaemic response was 90–120 min. The response of the fat body to injected synthetic Grb-AKH doubled in 4 days during the last stadium, but was never greater than half the maximum response of the adult stage. The adult adipokinetic response doubled from the first to the fourth day then gradually declined through day 16. The increased AKH response was time-correlated to fat storage in the larvae and to lipid diposits in the oocytes in the adult. Synthetic Grb-AKH activated glycogen phosphorylase in the fat body of *Acheta*. The amount of Grb-AKH present in the CC changed very little throughout the last larval stadium and through the first 9 days of the adult stage, averaging about 15 pmol/gland pair. A second peptide (a hexadecapeptide) was isolated from the CC of *Acheta* and sequenced. Its structure is identical to a putative diuretic hormone previously described in *Acheta*.

Introduction

The corpora cardiaca (CC) of insects are neurohaemal organs that store and release neurosecretory products synthesized in the brain. In addition, the CC also contain intrinsic glandular cells producing a variety of peptide hormones affecting physiologic, metabolic and developmental processes [1]. Those that regulate sugar and lipid metabolism comprise a group of related octa-, nona- or decapeptides, a number of which have been sequenced in the last 5 year [2]. In cockroaches, stick insects and some beetles (*Tenebrio*) the CC peptides stimulate the release of trehalose from the fat body when injected back into the same insect [2]. These are termed hypertrehalosaemic hormones (HrTH). In locusts, crickets, and some Lepidoptera the intrinsic CC peptides stimulate the release of diacylglycerides from the fat body when injected back into the same insect [2]. These are termed adipokinetic hormones (AKH) or hyperlipaemic hormones. However, CC extracts or the peptides isolated from the CC from most insect species have a hypertrehalosaemic effect when injected into *Periplaneta* and a hyperlipaemic affect when injected into *Locusta* [2], indicating that the receptor system is as important as the peptide sequence. *Acheta* fits this pattern, because CC extracts cause a hypertrehalosaemic response in *Periplaneta* and a hyperlipaemic response in *Locusta* [3]. When the CC extracts from *Acheta* were injected back into *Acheta* there was a hyperlipaemic response but no glycaemic response [3].

The adipokinetic response of *Locusta* was low in young adults then increased to a plateau and then gradually declined again to low levels [4]. The re-

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sponses of adult *Acheta* over 8 days old was half that of younger adults when injected with synthetic locust AKH [3]. In general, it was known that the hyperlipaemic response of larval insects to CC extracts was less than that of the adult forms [4, 5, 6]. It has been suggested that the reduced responsiveness may involve a less efficient lipophorin in the larvae [4] or reduced levels of JH [7]. Reversed-phase high pressure liquid chromatography (RP-HPLC) chromatograms have been used to quantitate the AKH content of the CC in *Locusta,* where it was shown that the AKH content increased almost 3-fold during the adult stage [8].

The first goal of this research was to separate and sequence the AKH (or AKHs) that occur in the CC of *Acheta*. With regards the function of the peptide, we were especially interested in the time and age responses. We also wished to examine the change in AKH content of the CC in relation to age, using HPLC chromatograms for quantification. We planned to compare the AKH content of the CC with the response of the fat body to a constant dose of synthetic *Acheta* specific AKH during development. The sequence information would also be of phylogenetic interest since the sequence is known for the AKH's in several other Orthoptera, including another cricket species [2].

Methods

Rearing and handling

Crickets were cultured on Purina cricket chow at 30 °C on a 14:10 LD cycle as previously described [9]. Newly molted last instar females were collected daily, separated from males and were designated 0-day-old when the adult emerged. Crickets were anaesthetized by a 5 sec exposure to CO_2, which was shown to have no effect on lipid titers [10]. Blood was collected from the base of the foreleg as outlined previously [3].

Separation

Corpora cardiaca from adult *Acheta* were collected in cold (4 °C) water, and extracts made as described previously [11]. After vacuum drying, the pellet was suspended in a mixture of 25% solution B: 75% solution A (corresponding to 15% acetonitrile) and injected onto a Nucleosil C18 column for reversed-phase high performance liquid chromatography (RP-HPLC) in batches of 50 to 100 glands using equipment described earlier [12]. A gradient from 43% solution B (57% solution A) to a 53% solution B over a period of 20 min at a flow rate of 1 ml/min was used. Solution A was distilled water + 0.11% TFA and solution B was 60% acetonitrile + 0.1% TFA. The active (adipokinetic) fraction was collected manually, and the combined material from a total of 215 corpora cardiaca was used for the determination of the primary structure.

Enzymatic deblocking and pulsed-liquid phase sequencing

The dried active material after RP-HPLC was enzymatically deblocked using L-pyroglutamate aminopeptidase as described previously [13]. After an incubation of 50 min the solution was chromatographed on RP-HPLC using the same solvents as above. The gradient was from 25% B to 60% B over 30 min at a flow rate of 1 ml/min. Several fractions were manually collected, dried by vacuum centrifugation and used for pulsed-liquid sequencing.

Automated Edman degradation was performed with a pulsed-liquid phase sequencer (Model 477A, Applied Biosystems, Foster City, U.S.A.) connected to an on-line phenylthiohydantoin (PTH) amino acid analyzer (Model 120A, Applied Biosystems). Sequencing reagents and solvents were from Applied Biosystems. Sequencing and PTH analysis was carried out with standard programmes.

AKH Contents of CC's

Each sample consisted of the corpora cardiaca from 10 crickets collected in 500 µl of 4 °C water, homogenized and lyophilized. For each age group three samples were collected and processed. The lyophilized homogenates were redissolved in 80% methanol and centrifuged. The supernatant was injected onto a C18 reverse phase column and an isocratic mobile phase of 30% methanol (70% water containing 0.1% TFA) was employed. Peak heights of the CC extracts and synthetic Grb-AKH standards were compared to estimate the CC content during larval and adult development.

Phosphorylase activity

The fat body extraction method and the assay for phosphorylase activity were essentially as previously described [14]. 15 min after injection of the synthetic Grb-AKH, 2–3 day old females were briefly immersed in liquid nitrogen to halt all enzyme activity, then the fat body was scraped from the abdomen as it thawed. The fat body was homogenized in triethanolamine acetate buffer (pH 7, 50 mM) containing 5 mM EDTA and 20 mM NaF and centrifuged for 5 min at 10,000 × *g*. The layer below the upper fat layer was used for the phosphorylase assay in the direction of glycogen breakdown.

Results

Separation

A crude methanolic extract of 90 glands from *A. domesticus* was fractionated on an analytical RP-HPLC column in a gradient mode using trifluoracetic acid as the ion-pairing reagent (Fig. 1). This method proved to be successful in isolating a fraction with a retention time of *ca.* 9.5 min, which elevated lipids in crickets and corresponded to a distinct absorbance peak (Fig. 1, top). The retention time of the active fraction was different from that of several other known peptides (Fig. 1, bottom), but was identical to that of the adipokinetic hormone of the cricket *Gryllus bimaculatus* (Grb-AKH) as previously reported [3]. Another batch of 150 corpora cardiaca of *A. domesticus,* showed a double peak at 9 to 10 min (Fig. 2). It was not possible to separate these peaks further. We decided, therefore, to keep this material separated. In this fashion, we ended up with 2 separate samples: one designated A, collected from 90 corpora cardiaca, showing only one peak (example Fig. 1, top) and a second, designated B, collected from about 150 corpora cardiaca, showing a double peak (as in Fig. 2). Both samples were subjected for sequence analysis.

Sequence data

Both samples were incubated with pyroglutamate aminopeptidase and chromatographed on RP-HPLC (see Fig. 3 and 4). Sample A showed a new absorbance peak already 5 min (Fig. 3, top) after incubation with the enzyme, and almost complete digestion was achieved after 30 min (Fig. 3,

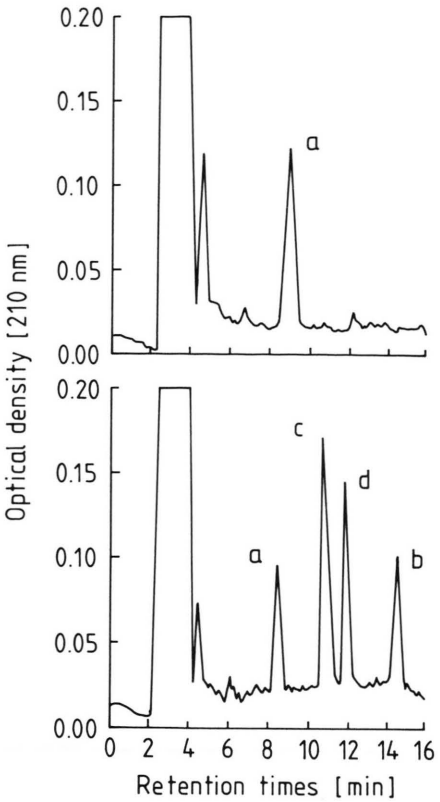

Fig. 1. Top: Separation of *Acheta* adipokinetic hormone (sample A) from a water/methanolic extract of 90 CC's with a gradient from 43% solution B (57% solution A) to a 53% solution B over a period of 20 min at a flow rate of 1 ml/min on a reverse phace C18 column. Solution A was dist. water + 0.11% TFA and solution B was 60% acetonitrile + 0.1% TFA. The peak (a) is homogenous. Bottom: Retention times of Pea-CAH-I (a), Pea-CAH-II (b) (300 pmol each), Lom-AKH-I (c), and Lom-AKH-II (d) (100 pmol each) run with the same gradient.

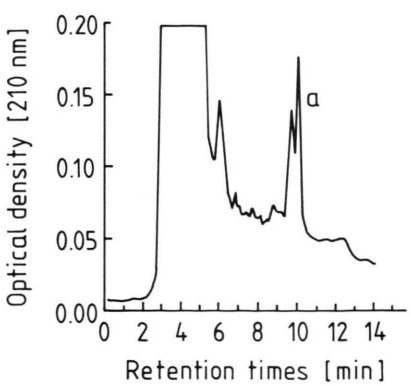

Fig. 2. Separation of *Acheta* adipokinetic hormone from a water/methanolic extract of 150 CC's (sample B) with the same gradient as described in Fig. 1. Note that peak (a) is not homogenous, but contains a double peak.

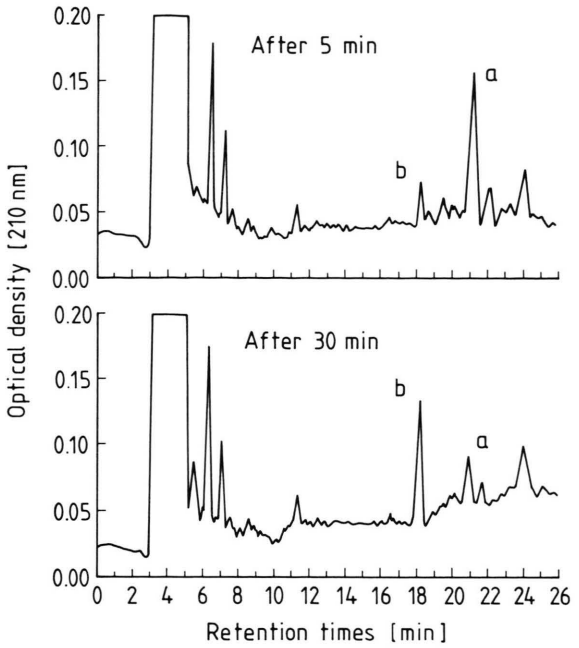

Fig. 3. The enzymatic deblocking of *Acheta* adipokinetic hormone (sample A, from Fig. 1) by means of incubation with pyroglutamate aminopeptidase and the separation of the product. The gradient was from 25% B to 60% B over 30 min at flow rate of 1 ml/min. After 5 min of digestion the chromatogram (top) shows mostly the presence of the substrate Grb-AKH (a), but after 30 min the chromatogram of the same digestion mixture shows the predominance of the product Grb-AKH minus the pGlu (b).

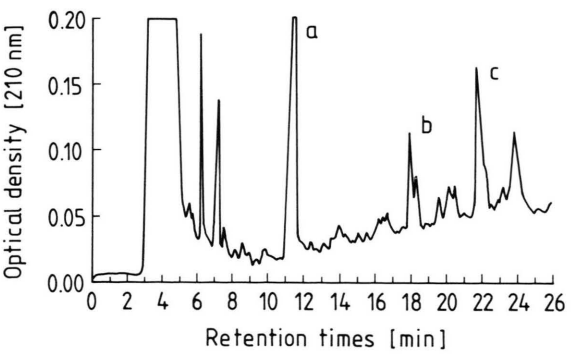

Fig. 4. The enzymatic deblocking of sample B (from Fig. 2) by means of incubation with pyroglutamate aminopeptidase and the RP-HPLC separation of the digestion products after 30 min. The gradient was from 25% B to 60% B over 50 min at a flow rate of 1 ml/min. Peak a: unknown, not a peptide. Peak b: same as b in Fig. 3 (Grb-AKH). Peak c: a hexadecapeptide (probably diuretic hormone, see Results).

Table I. Amino acid sequence of adipokinetic hormone of *Acheta domesticus* by automated Edman degradation.

Amino acid	Sample A recovery [pmol]	Sample B, peak b recovery [pmol]
VAL[1]	24	34
ASN[2]	40	36
PHE[3]	31	34
SER[4]	16	10
THR[5]	19	15
GLY[6]	15	15
(TRP[7])*	11	6
_[8]	–	–
_[9]	–	–

* Peak barely above background (see Results).

bottom). The new absorbance peak had a retention time about 3 min less than the intact molecule. This peak yielded the sequence given in Table I. The last amino acid was detected in cycle 7 (tryptophan), and the next two gave no degradation product. The tryptophan peak was barely above background, and its identity based on the degradation procedure is not certain. Sample B was chromatographed with a shallower gradient and a digestion of 30 min resulted in 3 major peaks labelled a, b and c. These were manually collected and sequenced. Peak a yielded no data, apparently not being a peptide; peak b had the same sequence as the peptide from sample A (Table I) and peak c was found to be a longer peptide and did not belong to the AKH/RPCH-peptide family (Table II).

Table II. Amino acid sequence of peak c from sample B.

Amino acid	Recovery [pmol]
ARG[1]	116
ASP[2]	136
ILE[3]	241
PHE[4]	211
HIS[5]	46
ALA[6]	139
GLU[7]	90
THR[8]	72
ASP[9]	99
ILE[10]	64
PHE[11]	48
GLU[12]	35
VAL[13]	26
PRO[14]	21
LYS[15]	3
_[16]	–
_[17]	–

Together with the N-terminal pyroglutamate residue, which was split off by enzymatic digestion, the sequence of the adipokinetic hormone from *A. domesticus* turns out to be an octapeptide, and the other substance a hexadecapeptide. The structure of the octapeptide is identical to the peptides separated from the corpora cardiaca of *G. bimaculatus* (Grb-AKH; [15]) and the grasshopper *Romalea microptera* (formally called Ro II, [13]); the other peptide has previously been sequenced from *A. domesticus* CC and was claimed as a putative diuretic hormone [16].

Time- and dose-responses

5–6 consecutive samples were collected from each cricket for the time-response study to minimize error. 3–5 day old adult virgins were used for the time- and dose-response curves because these animals produced a consistently high response (Fig. 5). The base line of the lipid titre in 5 day old female crickets was 30 ± 2 mg/ml and the maximal increase in response to the hormone was 20 ± 2 mg/ml, or about 66%. The ED_{50} of Grb-AKH was about 0.8 pmol and for Lom-AKH-I *(Locusta)* it was about 1.2 pmol (Fig. 3). The maximum hyperlipaemic response was achieved with 2 pmol for Grb-AKH and with about 5 pmol for Lom-AKH-I. The peak response to both peptides occurred about 2 h after injection (Fig. 6); the hyperlipaemic response of Grb-AKH was about 25% higher.

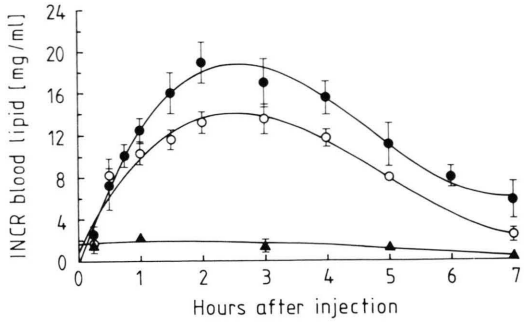

Fig. 6. Time-response curves for 3–5 day old virgin females. For Grb-AKH (closed circles) 5 pmol was injected and each point represents $n = 5$. For Lom-AKH-I (open circles) 50 pmol was injected and each point represents $n = 8$. The closed triangles represent water controls ($n = 5$).

The variation of fat body response with age

The response of the fat body during days 6 and 7 of the last larval instar to Grb-AKH was as high as in the older adults (Fig. 7). There was a generally increasing trend in response during the last larval instar, declining on the last day. Likewise there was an increasing AKH response during the first 3–4 days of the adult stage, after which the response gradually declined over the next 10 days.

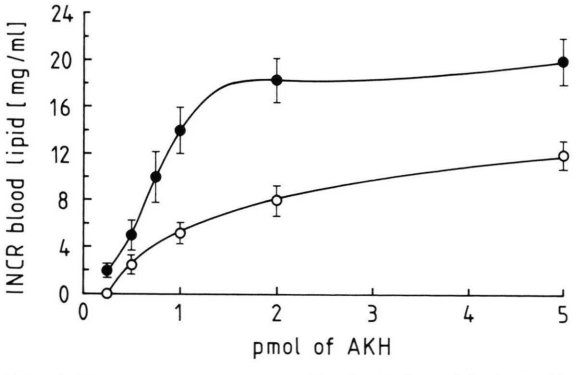

Fig. 5. Dose-response curves for 3–5 day old virgin females. The increase in blood lipids was measured 60 min after injection of the hormone. For each Grb-AKH point (closed circles) $n = 5$ and for each Lom-AKH-I point (open circles) $n = 8$.

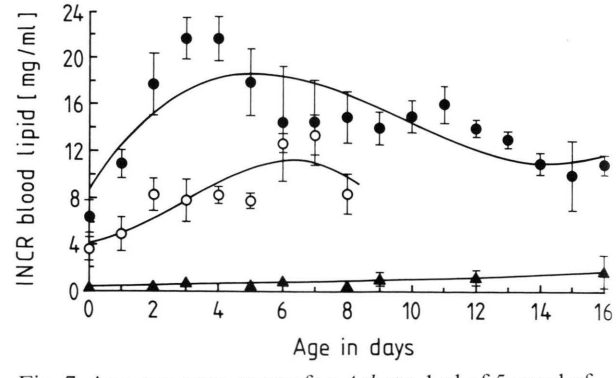

Fig. 7. Age-response curves for *Acheta*. 1 µl of 5 pmol of synthetic Grb-AKH was injected and the increase in blood lipids measured after 60 min. Each point for adults (filled circles) $n = 5$, for larvae (open circles) $n = 6$. The closed triangles represent water-injected adult controls ($n = 5$).

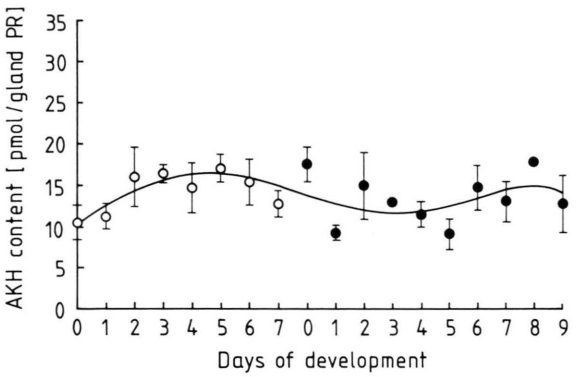

Fig. 8. Grb-AKH content of corpora cardiaca. For each sample the CC's from 10 crickets were homogenized in cold water and lyophilized. Open circles are larvae and closed circles are adults. For each age three samples were collected, $n = 3$. Methanolic extracts were injected onto a reverse phase C18 column and an isocratic mobile phase of 30% acetonitrile (0.1% TFA) run. The AKH concentrations were derived by comparison of peak heights of CC extracts compared to that of synthetic Grb-AKH standards.

AKH content of corpus cardiacum

The content of the CC in *Acheta* did not vary significantly from an average of about 15 pmol throughout the last larval instar and through the first 9 days of life of the virgin adult female (Fig. 8). The AKH content of 16 day old virgin females was also little different, 11.6 ± 0.78 ($n = 4$ pooled samples), indicating no significant change through day 16.

Stimulation of the phosphorylase a system

Acheta adipokinetic hormone (Grb-AKH) activates the fat body glycogen phosphorylase a system as evidenced by the increased percent of phosphorylase a (Table III). Handling crickets also appeared to stimulate the system, as indicated by the relatively high percent phosphorylase a in the controls (40%).

Discussion

Primary sequence

RP-HPLC of the methanolic extract of *A. domesticus* CC showed in some samples one absorbance peak associated with adipokinetic activity in *A. domesticus,* in other samples two absorbance peaks which ran close to each other. Brief enzymatic digestion of these fractions with pyroglutamic aminopeptidase yielded deblocked peptides which were accessible for sequence analyses by Edman-degradation. Sequential degradation revealed an octapeptide as the adipokinetic hormone of *A. domesticus* and a hexadecapeptide for the coeluting peptide. Although not proven in this study it is assumed that the octapeptide is blocked at its C-terminus, as all other members of the AKH/RPCH-family have a primary amide at the C-terminus. It was only indirectly shown here, because the natural peptide from *A. domesticus* CC extract had the same retention time as the synthetic peptide Grb-AKH on RP-HPLC. Thus, we have shown that *A. domesticus* contains the same peptide as the other cricket analyzed so far, *G. bimaculatus* [15] and, furthermore, an identical peptide was also identified as one of the two members of the AKH/RPCH-family in the CC of the grasshopper, *Romalea microptera* [13].

There are several instances of the same peptide occurring in two or even three different species (in two different genera) [2], but this is the first instance of the same AKH peptide occurring in three different genera. It has been suggested [17] that the nomenclature of a peptide should be based on the first report, which for this hormone was for *Gryllus bimaculatus* and thus the name of the peptide in *Acheta* should be Grb-AKH. As more peptides from the CC's of more insects are sequenced their relationship will undoubtedly contribute to understanding phylogenetic concepts in the genera or families involved. In cockroaches it was found that

Table III. The effect of synthetic Grb-AKH on the phosphorylase a system on the fat body of anaesthetized *Acheta.*

Conditions	% Phosphorylase *a*	*n*
Injected 3 µl dist. water	42.2 ± 9.5%	10
Injected with 4 pmol Grb-AKH	70.9 ± 4.9%	5

Animals were sacrificed exactly 15 min after injection.

there was a good agreement of the phylogenetic tree constructed by morphological and physiological data and the existance of specific hypertrehalosaemic hormones [18]. The occurrence of an identical AKH molecule in two cricket genera, *Acheta* and *Gryllus,* can be rationalized by their close relationship. The occurrence of the same peptide in *Romalea* could indicate common ancestry, but more work needs to be done and many more peptides identified.

AKH characteristics

The dose response curves for *Acheta* indicate that the receptor sites have a higher affinity, that is, a better receptor fit, for its own AKH than for that of the locust. In studies of naturally occurring members of the AKH/RPCH-family and assessing their hyperlipaemic activity in *Locusta,* it was shown that the locust's own AKH (Lom-AKH-I) had the greater affinity (lowest ED_{50}) and the higher ED_{max} [2, 19, 20]. Even comparing the two AKH's in *Locusta,* Lom-AKH-I had the greater affinity (lowest ED_{50}) and the higher ED_{max} [19, 20]. The time to maximum response and time to recovery after injection of Grb-AKH and Lom-AKH-I was typical of that observed in *Carausius* [21], *Tenebrio* [22], *Manduca* [23] and adult *Locusta* [24]; a maximum response in about 90–120 min with recovery to baseline levels in about 6 h.

Effect of age on fat body response

The response of adult *Acheta* to synthetic cricket AKH was from 2 to 3 times greater than that of the last larval stadium (this study). In the last instar the response doubles by day 6. In the adult stage of *Acheta* the response triples from day 0 to day 4 then gradually declines over the next 12 days. A previous study had indicated that the response of adult *Acheta* to synthetic locust AKH (Lom-AKH-I) decreased after about 6–8 days [3]. Similar changes in response to AKH with age have been reported for other insects. The hyperlipaemic response of *Locusta* to injections of CC extracts of adult *Tenebrio* increased 3-fold over the age period from day 0 to day 15 of the donor beetle [6]. Likewise the response of *Locusta* to CC extracts of *Carausius* doubled with older donors [5]. During the last instar the response of *Locusta* to extracts of its own CC was much less than during the adult stage, but there was a peak of responsiveness in the middle of the larval instar [4]. During the first few days of the adult stage of *Locusta* there was essentially no response to CC extracts then the response increased dramatically (5-times resting titres) to reach a plateau by day 8 and then declined back to a very low (almost control titres) after day 35 [4]. This response of adult *Locusta* to its own AKH (CC extract) is similar to that of *Acheta,* which also shows an initial increase in responsiveness followed by a gradual decline (Fig. 5).

The reason for the incomplete AKH response in young adult *Locusta* was not due to a shortage of available lipids, since ample dietary and stored lipids were present [25]. Nor was the lack of response due to a shortage of the hormone, since AKH elicits in both larvae and adults an elevation of cAMP concentration in the fat body [26]. It was suggested [4] that the reason for the lower AKH response in young locusts might involve the reduced ability to form lipoprotein A + in the blood, which is essential for the binding of the increased amounts of diacylglycerols released after AKH injection. However, injections of adult lipophorin ($A^{=}$ and C_2 proteins) into larvae did not result in adult levels of response to AKH by larval fat body, therefore larval lipophorin composition is not the cause of reduced response to AKH [27]. However, adult fat body incubated in larval blood showed a reduced adipokinetic response, indicating that other larval blood factors might restrict AKH action [27].

Allatectomy has essentially no effect on AKH-induced hyperlipaemia in *Locusta* [7, 28], indicating no role of JH in response to AKH. However, in fifth-instar adultiform larval *Locusta,* induced by precocene treatment, the hyperlipaemic response to synthetic AKH was equal to that of the adult stage [7]. This prompted the suggestion that JH deficiency in adults, may be responsible for high imaginal competence for the response to AKH [7]. This seems unlikely in adult *Acheta* because the JH titre rises shortly after the final moult [29] which is at the same time the response to synthetic AKH increases.

If neither JH nor the composition of the lipophorin explain age dependent responsiveness of the fat body to AKH, then another unidentified factor must be involved. Larval lipophorin functions in the transport of lipids to fat body storage

depots and growing tissues [27]. The increasing AKH response in larval *Acheta* is related to the rapid accumulation (storage) of lipids in the fat body which peaks on days 5–6 [30]. The increased response in the adult stage is related to the rapid transport of lipids from the fat body into the oocytes [30]. However, in *Locusta* the AKH responsiveness increased in both males and females, indicating that ovarial growth was not a critical factor [4]. The conclusion is that more work needs to be done to explain the fluctuations in AKH response in insects.

Effect of age on AKH content in CC

There was no significant change in AKH content found throughout the last larval instar, between the last instar larvae and adult stage or through the first 9 days of the adult female life in *Acheta*. In *Locusta* there was little change between early and late last instar female larvae (99 to 104 pmol/CC), and little change from larvae to young adult [8]. However, in the adult females there was a doubling from day 0 to day 10 (127 to 271 pmol/CC) and an almost 6-fold increase from day 1 to day 42 (752 pmol/CC) [8]. It was suggested that the gradual increase in AKH pool in the locust CC might be related to the time required to develop full flight potential [8]. In this regard it is interesting that adult *Acheta* lose flight capacity within 3–4 days following the last molt [30].

The content of AKH in *Locusta* was almost an order of magnitude greater than what we report here for *Acheta*. Two factors that might contribute to a larger AKH pool in *Locusta* CC would be their larger size and the capacity of the animal for long distance flight. It should also be remembered that the size of the AKH pool in the CC does not measure the production or the release rates of AKH. As far as *Acheta* is concerned, this means that more AKH could be produced and released at certain times during the life cycle even though the content of the CC remained constant. The regulation of the adipokinetic response would likely be based on both AKH release rate and the responsiveness of the target cells.

Activation of fat body phosphorylase a

The hyperlipaemic action of Grb-AKH is associated with an activation of the fat body glycogen phosphorylase a system. In the present study about 30% of the fat body glycogen phosphorylase of control, anaesthetized adult *Acheta* is in the active form, which is within the range (20%) for control *Acheta* previously reported [31]. This is about twice the values reported for control adult *Locusta* [14, 32]. *Acheta* are much more active and excitable than *Locusta* and the high percentage phosphorylase a in control *Acheta* may represent a response to agitation. Further handling did elevate the percent phosphorylase a to 40%. *Locusta* phosphorylase a could also be elevated to 30% by rotating or shaking the insects in a bottle [14]. A basic unanswered question is what does the breakdown of glycogen to glucose-1-phosphate, which is the function of the phosphorylase a system, have to do with the primary function of AKH which is to release diacylglycerols into the blood. One could speculate that the glycolytic pathway would be used to make glycerol-3-P *via* glyceraldehyde-3-P from the glucose-6-P formed from increased phosphorylase a activity. In the fat body of insects that utilize lipids for energy, fat body triacylglycerol is degraded first to 2-monoacylglycerol (plus two fatty acids), followed by reacylation to 1,2 diacylglycerol [33]. The glycerol-3-P from AKH activation of phosphorylase a activation would be used for synthesize additional diacylglycerols from the resultant extra fatty acids.

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