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Nutrient content of diet affects the signaling activity of the insulin/target of rapamycin/p70 S6 kinase pathway in the African malaria mosquito *Anopheles gambiae*

Dany Arsic *, Patrick M. Guerin

Laboratory of Animal Physiology, Institute of Biology, University of Neuchâtel, Emile-Argand 11, CP 158, 2009 Neuchâtel, Switzerland

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ABSTRACT

Regulation of female mosquito feeding and reproduction plays a central role in their disease-vector competence. In this study we show that *Anopheles gambiae* mosquitoes engorged on albumin, amino acid and saline meals the same way as on blood, whereas sucrose evoked a typical plant nectar feeding response. Among the artificial diets, only the albumin-containing ones allowed follicular development. The target of rapamycin (TOR)/p70 S6 kinase (S6K) pathway has been identified as an essential nutrient-sensing tool controlling egg development in mosquitoes under the control of regulating inputs from the insulin pathway. We assayed the early response of TOR, S6K, tuberous sclerosis (TSC2), insulin receptor (INR) and two insulin-like peptides (ILPs) by quantitative real-time PCR assessment of mRNA levels and immunoblotting of phosphorylated active TOR and S6K in *An. gambiae* ovary and brain 3 h after engorgement. We show that transcript levels of *s6k* and members of the insulin pathway are readily affected by nutrients (especially one ILP in the head) and that the TOR/S6K phosphorylation is able to react quickly to a meal to an extent which depends on the true nutritive value.

1. Introduction

Anopheles gambiae Giles is the main vector of human malaria in sub-Saharan Africa. In *An. gambiae* and other anautogenous mosquitoes oogenesis is arrested at the pre-vitellogenic phase until a blood meal is obtained, which is required for completing the maturation of the proximate follicles and deposition of an egg batch within two days. Sugar feeding is fundamental for both sexes of *An. gambiae* and repeated sugar meals are taken by individuals of both sexes to support their survival (Gary and Foster, 2001, 2006). In females this allows ovaries to develop from Christophers' stage I to the pre-gravid resting stage (Gillies, 1954, 1955) corresponding to Christophers' stage II (Fernandes and Briegel, 2005) and to develop eggs after a single blood meal. The presence of free amino acids (AAs) in the midgut lumen was highlighted as an important signal used by female mosquitoes to regulate the retention of the meal (Caroci and Noriega, 2003). Moreover, infusion of a balanced solution of AAs permitted initiation of egg development under certain conditions in several mosquito species including *An. stephensi* (Uchida et al., 2001, 2003). All these meal components are involved in the nutritional control of vitellogenesis in anautogenous mosquitoes (reviewed by Attardo et al., 2005).

Target of rapamycin (TOR or mTOR for mammalian TOR) kinase is a member of the phosphatidylinositol 3-kinase (PI3K) superfamily, which regulates gene expression, protein biosynthesis, and cell differentiation via transcriptional and translational regulatory pathways (Rohde et al., 2001). Serine 2448 phosphorylation of mTOR is directly related to AA and nutrient status (Nave et al., 1999; Reynolds et al., 2002) and is suggested to act as a controlling switch for mTOR activity (Cheng et al., 2004). One of the described TOR negative regulators is tuberous sclerosis complex 2 (TSC2). The TOR pathway is a nutrient-sensing signal transduction pathway stimulated by hormones and AAs, and it is conserved from yeast to mammals. Findings in *Drosophila melanogaster* showed that the fat body functions as an AA sensor and that nutritional signals are transduced by the TOR signaling pathway (Colombani et al., 2003). p70 S6 kinase (S6K) belongs to the AGC family of protein kinases and is activated by TOR-mediated phosphorylation at threonine 389 in mammals or homologous threonine 398 in *D. melanogaster*. Activated S6K associates with a protein complex involving TOR, which in turn results in the selective stimulation of translation initiation (see Arsham and Neufeld, 2006; Wullschleger et al., 2006 for reviews).

* Corresponding author. Tel.: +41 32 718 3082; fax: +41 32 718 3001.
E-mail address: Dany.Arsic@unine.ch (D. Arsic).

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In the mosquito *Aedes aegypti* the TOR signaling pathway activated by AAs regulates *Vitellogenin* expression in the fat body and the activation of egg development after a blood meal (Hansen et al., 2004). More recently, Hansen et al. (2005) have determined that phosphorylation of S6K is the key downstream event of the AA/TOR nutritional signaling in *Ae. aegypti*. The authors showed that TOR activity increased in ovaries after a blood meal and that S6K phosphorylation increased in fat bodies after AA stimulation *in vitro*. Hansen et al. (2005) concluded that TOR-dependent S6K activation is the central step in nutritional information transduction for egg development in mosquitoes. The importance of the ovaries themselves in the regulation of vitellogenesis arises from their ecdysone-releasing function in response to the blood meal. The retention of the blood meal was described to be prolonged by the effect of ecdysone produced by the ovaries (Cole and Gillett, 1979). Ecdysone also activates yolk protein precursor synthesis in the fat body, which previously received nutritional signals from the midgut (see Attardo et al., 2005 for a review). A causal link was established between nutrient availability and insulin-dependent growth in *D. melanogaster*, as the expression of two insulin-like peptide (ILP) coding genes in the medial neurosecretory cells proved to depend on nutrient availability and this expression was needed for normal growth and female fertility (Ikeya et al., 2002). In insects, ecdysone production by the ovary is stimulated by ILPs (Gilbert et al., 2002) and begins with the phosphorylation of the insulin receptor (INR), a receptor tyrosine kinase, as shown in *Ae. aegypti* (Riehle and Brown, 2002).

A behavioral aspect of TOR/S6K nutritional signaling was demonstrated in *D. melanogaster* where S6K in ILP-releasing neurons was shown to mediate hunger regulation of approaching food and consumptive behaviors, controlling both the quality and quantity of nutrient ingested (Wu et al., 2005). Recently the role of the TOR/S6K pathway in regulating food intake was also highlighted in the mammalian hypothalamus (Cota et al., 2006). In both studies, increased TOR- and p70 S6 kinase activity caused decreased food intake.

In the genome of *An. gambiae*, accessions with predicted homology to mammalian, *D. melanogaster* and *Ae. aegypti* TOR, p70 S6 kinase, TSC2 and INR are annotated in public databases such as Ensembl and Anobase. Seven ILP coding genes were identified in *An. gambiae* genome (Riehle et al., 2002) and their expression in different body parts at different life stages were described by Krieger et al. (2004). However, to our knowledge, there is as yet no published research on the mobilization and functioning of the insulin and TOR/S6K pathways in *An. gambiae* organs in response to diverse nutritional stimuli. As egg development from the previtellogenic state of arrest is completed within 48 h of a blood meal in this species, and knowing the key role of the ovary and of brain neurosecretory cells in the nutritional regulation of vitellogenesis, early insulin and TOR/S6K signaling in *An. gambiae* ovary and brain is clearly of interest. This study aimed at assessing the effects of diets of different chemical nature and nutritive value on female *An. gambiae* feeding behaviors and ovarian development, as well as examining the early response of *tor*, *s6k*, *tsc2*, *inR*, *ilp3* and *ilp4* in *An. gambiae* ovary and brain a few hours after the different meals. The effects of blood, albumin with AAs, sucrose and saline on the expression of these genes and on the activity of TOR and S6K in ovary and brain were respectively determined by quantitative real-time PCR and by immunoblotting of phosphorylated TOR and S6K. Indeed, the level of S6K proteins that are phosphorylated on Drosophila threonine 398 (or the homologous threonine 389 in mammals) has been used as a measure of TOR activity for several years (Dennis et al., 2001). We show that transcript levels of *s6k* and members of the insulin pathway are readily affected by nutrients (especially ILP3 in the head) and that the TOR/S6K pathway is able to react rapidly to any meal by increasing TOR and S6K phosphorylation. Further, we show that the extent of this signaling activity depends on the true nutritive value of the meal.

2. Materials and methods

2.1. Anopheles gambiae rearing

An. gambiae Giles *sensu stricto* (strain 16CSS, derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa) were reared at 28 °C, >99% relative humidity (by holding the cages in closed plastic bags with wet cotton towels) under 14:10 h (day:night) light conditions in a walk-in climate chamber. Larvae were raised in trays (30 cm × 25 cm × 6 cm, 400 larvae per tray) with 400 ml distilled water and fed pulverized Tetramin® daily (Timmermann and Briegel, 1993). In this manner homogeneous populations were maintained where all adult mosquitoes had the same size. Adults had access to 10% sucrose *ad libitum* during normal rearing but were deprived of it overnight before experimental feeding. For egg production, females were given blood meals on restrained guinea pigs.

2.2. Feeding behavior assays

The feeding behavior of *An. gambiae* females was characterized as they fed on one of the three different feeding solutions: (1) saline (8.75 g/l NaCl + 0.75 g/l $NaHCO_3$, pH adjusted to 7.4, out of several saline solutions tested this one stimulated the highest proportion of individuals to engorge (Galun et al., 1985)); (2) 100 g/l sucrose, pH 8; (3) albumin in saline (120 g/l bovine serum albumin fraction 5, saline as above, pH adjusted to 7.4).

Three-day-old female mosquitoes were individually enclosed in plastic vials closed with stretched Parafilm® and put on cotton wool soaked with one of the feeding solutions in a glass Petri dish base. Dishes containing saline and albumin solutions were held at 37 °C during experiments. To induce mosquitoes to feed on sucrose different conditions were tested (see Section 3): vials were upturned on wet filter paper without Parafilm® so that the mosquitoes were directly exposed to the unheated solution.

The mosquitoes were weighed before feeding, let to feed *ad libitum* for 10 min, their degree of engorgement was assessed (visual estimate of abdominal distention from 0 to 1, maximal value based on maximal abdominal distention normally caused by blood feeding), mosquitoes were weighed again, and finally their engorgement state was re-assessed after 3 h. Engorgement loss is the difference between the assessment just after the meal and 3 h post feeding. The weight increase following feeding was calculated. Finally this weight increase was divided by the weight before feeding and is termed "meal to body weight ratio" in Section 3.

2.3. Feeding and dissection

Five-day-old female mosquitoes were exposed to different nutrients in order to monitor eventual differential activation of the insulin/TOR/S6K pathway. The feeding systems were the same as described above. Feeding solutions were: (1) saline (as above), (2) sucrose (as above), (3) blood (mosquitoes allowed to feed on a human arm until repletion), (4) albumin plus free AAs (120 g/l bovine serum albumin fraction 5 plus 10 g/l free AAs from a dilution of 50× RPMI 1640 amino acid solution, SIGMA cat. nr. R 7131, pH adjusted to 7.4, termed "albumin + AA"). This protein level is compatible with that used by Caroci and Noriega (2003) and 10 g/l of free AAs matches the 9.7 g/l free AA concentration in *Ae. aegypti* midgut 2 h after a Kogan meal (125 g/l protein in saline, unpublished finding mentioned in Caroci and Noriega (2003)). All

mosquitoes were allowed to feed to repletion and removed from Petri dishes immediately after. Unfed females (maintained on sucrose until the day before experiments like all other mosquitoes) were included as controls. Three hours following engorgement mosquitoes were either ultra-freezed at −80 °C for RNA extraction or dissected after short chilling on ice in cold Tris-buffered saline (TBS) to obtain the ovaries and the brain and to record the anatomical destination of the meal. The composition of TBS followed Cheng et al. (2004): 50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH adjusted to 7.6. Tissue samples were prepared as described below in the Western blotting procedures.

2.4. Effect of diet on post-prandial behavior

Five-day-old female mosquitoes were fed with the different solutions described above (saline, sucrose, alanine, blood, albumin, albumin + AA) or with the 10 g/l free AAs mixture described above (termed "AAs"). Their willingness to bite was assessed immediately after engorgement by putting them on human skin. They were then attributed to one of the two categories: "did bite" or "did not bite". Other female mosquitoes from the same generation and the same rearing cage were handled the same way (enclosed in vials and exposed to the plate heater), but were not offered any diet. These unfed mosquitoes were also tested for biting behavior on human skin as controls.

2.5. Enema and biting assays

In order to investigate the effect of abdominal distention on the willingness to bite, 3–5-day-old *An. gambiae* females were exposed to human skin in the rearing cage and only those attracted were collected for further use in this assay to ensure that all females were appetent before manipulation. After short chilling on ice, mosquitoes were individually restrained in a piece of plasticine tightened around their thorax and a saline enema (Briegel and Lea, 1975) was given to "test" females through a glass electrode connected to a micrometer syringe system until standard engorgement volume was reached (ca. 1 μl). "Control" females had the tip of the electrode inserted in the hindgut through the anus but the saline solution was not injected. All the manipulated mosquitoes were kept individually in 30 ml collection tubes with wet filter paper for 2 h at room temperature and then placed in the dark for at least 20 min before being exposed to human skin again.

2.6. Effect of diet on follicle development

Five-day-old female mosquitoes were fed with the different solutions described above (saline, sucrose, alanine, blood, AAs, albumin, albumin + AA) and were held at 28 °C and > 99% relative humidity for 24 h. They were then dissected to record the developmental stage of their ovarian follicles. As controls, unfed mosquitoes were processed the same way. Tissues were fixed, mounted on glass slides and observed through an Olympus BX50 optical microscope using a digital CCD camera connected to a computer. In each mosquito the diameter of the five largest follicles of one of the ovaries was measured using analySIS software (Soft Imaging System) and the arithmetic average of these five values was calculated.

2.7. Gene expression assays

The effect of diet on the gene expression of several members of the insulin and TOR/S6K pathways was assessed by relative mRNA level quantification using quantitative real-time PCR (qRT-PCR). The identification of the genes of interest and the sequences of the specifically designed primers are given in Table 1. Primer pairs were designed using Primer3 freeware (http://primer3.sourceforge.net) so that at least one oligonucleotide span an exon–exon boundary to prevent genomic DNA amplification, and that the amplicon be no longer than 200 base pairs, where possible, to promote amplification efficiency. Transcript sequences and exon boundaries information were retrieved from Ensembl (www.ensembl.org/Anopheles_gambiae). Primer specificity was first verified *in silico* by BLAST comparisons of the oligonucleotide sequences against all *An. gambiae* genomic DNA and cDNA sequences hosted by Ensembl. Optimal annealing and amplifying conditions were then tested by gradient PCR for each set of primers.

Samples of 15–30 heads or abdomens of individuals fed on one of the different diets (as described above) were used separately for total RNA extraction (Qiagen RNeasy Plant Mini Kit, according to manufacturer instructions: 'Plant and Fungi' protocol). Resulting RNA was DNAse-treated (RQ1 DNAse, Promega) to remove genomic DNA contamination and 1 μg of treated RNA per sample served as template for reverse transcription (M-MLV H^- Point Mutant reverse transcriptase with RNAsin ribonuclease inhibitor, Promega, using oligo-dT 15-mers for priming). Resulting cDNA was

Table 1
Identification of the genes whose transcripts and/or proteins were investigated in the present study

Local gene name	Stable name	Ensembl and VectorBase gene ID[a]	Ensembl gene description	Ensembl ortholog prediction[b]	Primer pair used for quantitative real-time PCR
tor	None	AGAP007873	None	Human *mTOR* (*FRAP1*) and Drosophila *tor*	For: 5′-GGCGATTGCTTCGAGGTT-3′; Rev: 5′-ACACGAACGCTTCCAGTACC-3′
s6k	None	AGAP007333	None	Human *p70S6K* and Drosophila *s6k*	For: 5′-ATTAGAGGCGGTTAGGCATC-3′; Rev: 5′-AAAATATTCCCTCGCGCTCT-3′
inR	INR	AGAP012424	Insulin receptor	None	For: 5′-CCAACCTTACCAGGGACTGA-3′; Rev: 5′-GCATCGGGTAACAACATACG-3'
ilp3	Q6VVG9_ANOGA	AGAP010602	Insulin-like peptide 3 precursor	None	For[c]: 5′-GGTAAAGGTACTGTCCTTCCTG-3′; Rev: 5′-AGTATCTGCTGCGTGTTGTC-3'
ilp4	Q6VVG8_ANOGA	AGAP010601	Insulin-like peptide 4 precursor	None	For: 5′- TCTCCGAAAGAACACAGTTGA-3′; Rev: 5′-GGTTTCTGCCTGAACCACAT-3'
tsc2	None	AGAP011123	None	Human, Drosophila and *Aedes aegypti TSC2*	For: 5′-GACACGAACACGCAGAAGAA-3′; Rev: 5′-CACGAGTATGAGCGTGGAGA-3′
S17	RS17_ANOGA	AGAP004887	40S ribosomal protein S17	Human, Drosophila and *Ae. aegypti 40S ribosomal protein S17*	For: 5′-TTAGCACAGAATGGGTCGTG-3′; Rev: 5′-TGTTACGCAGTGGTTTCGTC-3′

Primers were specifically designed for this work or are otherwise specified. *tor*, *s6k*, *inR*, *ilp3* and *ilp4* were already identified in the genome of *Anopheles gambiae* by Riehle et al. (2002) and are now described as such in the genome databases.

[a] http://www.ensembl.org/Anopheles_gambiae; http://www.vectorbase.org.

[b] Genes detected as unique best match of the mentioned *An. gambiae* gene by automated BLAST comparisons against all genomic sequences hosted by Ensembl.

[c] Primer pair published by Krieger et al. (2004).

used as template for qRT-PCR reactions (400 ng cDNA template per reaction) using ABsolute™ QPCR SYBR® Green Mix (ABgene) according to manufacturer instructions in a final reaction volume of 25 μl. qRT-PCR reactions were run in triplicates in a Bio-Rad iCycler thermal cycler executing the following program: a 15 min enzyme activation period at 95 °C followed by 45 cycles where temperature was set to 95 °C (denaturing), 55 °C (annealing) and 72 °C (extension) for 30 s each. Amplicon amount-specific fluorescence data were collected at the end of the extension step. The amplification cycles were followed by melting curve analysis of the PCR products. That is, after 1 min at 50 °C temperature was increased by 0.5 °C every 10 s and fluorescence was recorded at each of the 90 steps. Maximal rate of fluorescence decrease occurred at a sequence-dependent temperature allowing verification of amplification specificity.

RS17_ANOGA (*S17*) was amplified in each treatment as an internal control for general expression level in the sampled tissues. Detected *S17* mRNA level (Ct_{ref} values), as automatically computed by iCycler iQ software (Bio-Rad) from fluorescence curves after amplification, was subtracted from the mRNA level (Ct_{target} value) of the gene of interest in each replicate of each treatment, in order to provide a relative quantification value ($\Delta Ct = Ct_{target} - Ct_{ref}$). Note that Ct values are logarithmic measures of and inversely proportional to mRNA levels, as they point to the cycle number at which amplicon amount-dependent fluorescence passes an automatically defined threshold. All experiments were repeated at least twice on separate cohorts of mosquitoes and results were pooled within each treatment for statistical analyses.

2.8. Western blotting procedures

Phospho-specific antibodies have been developed and are commercially available for human TOR phosphorylated on serine 2448 (pTOR) and for Drosophila S6K phosphorylated on threonine 398 (pS6K), as these phosphorylations are known to be essential for the activity of these enzymes. Protein sequence alignments implemented in Ensembl showed that human TOR serine 2448 aligns with *An. gambiae* TOR serine 2376 and Drosophila S6K threonine 398 aligns with *An. gambiae* S6K threonine 388. Moreover, threonine 398/388 resides in the S6K kinase extension domain which is highly conserved among species. Original Drosophila S6K and human TOR phosphorylated residue numbering is kept throughout this text for clarity.

Total protein extracts were prepared from 30 ovary pairs or 20 brains of blood-, albumin + AA-, saline-fed and unfed mosquitoes coming from two separate cohorts. Each sample was then divided in two aliquots. Tissues dissected 3 h post feeding were boiled at 95 °C for 5 min in a denaturing buffer (2% SDS, 5 mM DTT and 0.1% protease inhibitor cocktail in 50 mM Tris solution pH 7.5) and cooled on ice. Samples were submitted to a methanol/chloroform purification protocol followed by another 5 min boiling and 1 min centrifugation at 13400 rpm prior to separation by SDS-PAGE. Migration in 8% polyacrylamide gel lasted 90 min at 15 mA and transfer on nitrocellulose membrane lasted 100 min at 300 mA, followed by membrane staining using Ponceau S to visualize transfer result. Destained membrane was incubated for 2 h at room temperature in blocking buffer (1% bovine serum albumin [BSA] and 0.1% Tween-20 in TBS) and then at 4 °C overnight in primary antibodies diluted in blocking buffer: either 1:1000 of phospho-Drosophila p70 S6 Kinase (Thr^{398}) rabbit polyclonal antibody (Cell Signaling Technology #9209), or 1:500 of phospho-mTOR (Ser^{2448}) rabbit monoclonal antibody (Cell Signaling Technology #2976). Hybridized primary antibody was detected using a goat anti-rabbit IgG (H + L)-HRP conjugate (Bio-Rad #170-6515) as secondary antibody at a 1:3000 dilution in 5% non-fat dry milk and 0.1% Tween-20 in TBS. Hybridized secondary antibody was detected by using peroxide and ECL reagents and exposing the membrane to X-ray film.

3. Results

3.1. Feeding behaviors and diet destination

Female mosquitoes exhibited different behaviors when exposed to sucrose in comparison to the other diets. They also had different requirements for diet presentation in order to induce feeding. Mosquitoes did not feed on albumin or saline solution unless it was heated (optimum at 36 °C), pH-buffered (optimal pH 7.4) and presented under a membrane to feed through. Provided that these conditions were fulfilled, females rapidly engorged on the solution and always filled their midgut, never the crop. On the contrary, females readily fed on sucrose only when it was at room temperature, contained no buffering saline, and when they had direct access to the filter paper soaked with the solution. Other assays showed that mosquitoes fed neither on heated sucrose, on sucrose mixed with saline, nor on unheated albumin, AA, or saline solutions. Ingested sucrose solution always filled the crop, never the midgut.

3.1.1. Meal size depending on diet

The variable "meal to body weight ratio" provides a measure of the amount ingested relative to the size of the mosquito and differed significantly among the diets (ANOVA, $F_{2,144} = 20.31$, $P < 0.0001$). The values (mean ± standard deviation) were significantly higher for albumin-fed mosquitoes (1.188 ± 0.376, representing meal weights of 1–2 mg taken by mosquitoes weighing ca. 1 mg) than for saline-fed (0.889 ± 0.358) and sucrose-fed ones (0.757 ± 0.310) (Tukey post-hoc pairwise comparisons using 95% confidence intervals [C.I.]).

3.1.2. Meal retention depending on diet

During the 3 h delay between the first estimation of engorgement state (just after feeding) and the last estimation of engorgement state some female mosquitoes excreted part of their meal. The proportion of meal lost (engorgement score just after feeding − engorgement score after 3 h) differed significantly among the three diets (albumin: 0.06 ± 0.11 [mean ± standard deviation]; saline: 0.29 ± 0.24; sucrose: 0.31 ± 0.16; Kruskal–Wallis $\chi^2_2 = 52.14$, $P < 0.0001$). However, the distribution of the data did not permit pairwise comparisons. This was mainly due to the fact that 39 out of 56 albumin-fed mosquitoes did not show any sign of excretion. Saline-fed mosquitoes manifested the most dispersed meal retention data whereas albumin-fed mosquitoes the least.

3.1.3. Effect of diet on post-prandial behaviors

The proportion of mosquitoes which tried to bite when exposed to human skin was zero or very low after gorging on blood (0%, $n = 20$), albumin + AA (3%, $n = 31$) or saline (10%, $n = 20$). This proportion was intermediate for AA-fed mosquitoes (32%, $n = 19$), and very high for sucrose-fed (100%, $n = 16$) and unfed (89%, $n = 32$) mosquitoes (Fig. 1). A binomial generalized linear model was fitted to the data using R statistical software (R-Development-Core-Team, 2007) to test the effect of the diet on the proportion of mosquitoes still willing to bite after a meal. The principal effect of the diet was highly significant ($P < 0.001$). Post-hoc pairwise comparisons using Tukey contrasts indicated two groups formed by sucrose-fed and unfed mosquitoes on one hand, and by AA-, saline-, albumin + AA- and blood-fed mosquitoes on the other supported by highly significant differences ($P < 0.001$). However, the proportion of mosquitoes still willing to bite after an AA meal

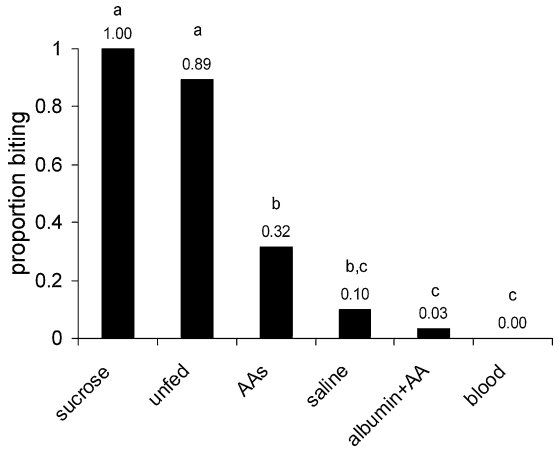

Fig. 1. Proportion of *An. gambiae* females that bit when exposed to human skin just after engorgement on either saline, sucrose, a mixture of amino acids (AAs), albumin plus amino acids (albumin + AA), or blood. Unfed mosquitoes were included as controls. Sucrose-fed mosquitoes were not deterred from biting, whereas previous engorgement on all other diets resulted in a low or extremely low proportion of mosquitoes biting when subsequently exposed to human skin. Lower case letters (a, b, c) indicate significant differences (binomial generalized linear model followed by pairwise comparisons using Tukey contrasts).

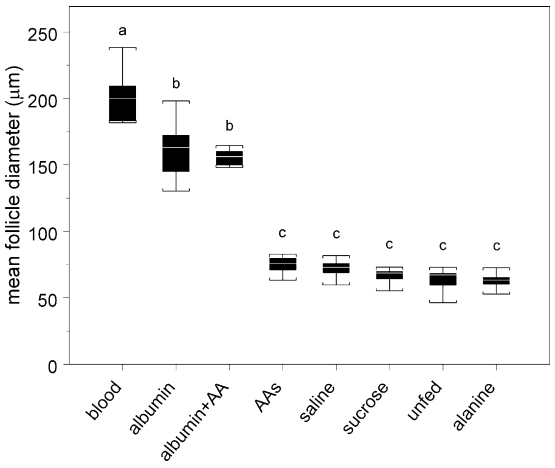

Fig. 2. Mean follicle diameter of *An. gambiae* ovaries 24 h after feeding on either saline ($n = 12$), sucrose ($n = 12$), alanine ($n = 13$), albumin ($n = 12$), a mixture of amino acids (AAs, $n = 15$), albumin plus amino acids (albumin + AA, $n = 10$), or blood ($n = 14$). Unfed mosquitoes were included as controls. In addition to blood, only the diets containing albumin were able to induce follicular development, although to a lesser extent. Box-plots represent the median (white bar) and the 25–75% inter-quartile interval (box), and whiskers extend to the last data point included in 1.5 times the inter-quartile interval above and below the box. Lower case letters (a, b, c) indicate significant differences (ANOVA followed by Tukey pairwise comparisons using 95% confidence intervals).

was significantly higher ($P = 0.02$) than after an albumin + AA or a blood meal.

3.2. Effect of abdominal distention by enema on biting behavior

The effect of abdominal distention using saline enema was clear. All females were willing to bite before manipulation, but 76% of "test" females ($n = 38$) lost any willingness to bite when offered human skin after enema. Eighty-eight percent of the "control" females ($n = 25$) were still induced by human skin to bite. The association between treatment and biting behavior was highly significant (Pearson's chi-square test with Yates' continuity correction: $\chi_1 = 22.45$, $P < 0.0001$).

3.3. Effect of diet on follicle development

In addition to blood, only the two diets containing 120 g/l albumin evoked follicular development. Twenty-four hours after feeding, the follicles of mosquitoes fed with either blood (203 ± 20 μm, mean ± standard deviation), albumin + AAs (156 ± 6 μm) or albumin alone (168 ± 25 μm) were significantly larger and further developed (oocyte enlarged to 75–90% of the follicle, full of yolk spheres, corresponding to Christophers' stage IIIb or IVa as described by Clements and Boocock (1984)) than those of mosquitoes who were fed saline, sucrose, AAs or an alanine solution or were unfed (ANOVA, $F_{7,90} = 292.59$, $P < 0.0001$, Tukey comparisons using 95% C.I., Fig. 2). Blood-fed mosquitoes had significantly larger follicles than albumin- and albumin + AA-fed ones. Follicles of AA-fed (75 ± 6 μm), alanine-fed (62 ± 7 μm), saline-fed (72 ± 6 μm) and sucrose-fed (67 ± 6 μm) mosquitoes did not differ significantly from those of unfed (64 ± 8 μm) mosquitoes (corresponding to pre-vitellogenic Christophers' stage IIa or IIb). Brown-yellow dried droplets of feces were present the day after feeding in the vials containing albumin- or albumin + AA-fed mosquitoes, whereas blood-fed mosquitoes produced darker feces. None of the female mosquitoes held for 48 h layed eggs except the blood-fed mosquitoes. Although they had most probably mated during the five days before being fed, female mosquitoes were not provided access to males after feeding. No effect of any diet treatment was observed on ovaries dissected 3 h after feeding.

3.4. Effect of diet on the mRNA level of members of the insulin and TOR signaling pathways

The mRNA levels of *tor*, *s6k*, *inR*, *ilp4* and *ilp3* were assessed by qRT-PCR from total RNA extracts of heads of female mosquitoes fed on either albumin + AA, saline or sucrose and frozen after 3 h (Fig. 3). As prospective assays showed that *ilp4* was not detected in abdomen samples, the mRNA level of another gene (*tsc2*) was assessed in addition to *tor*, *s6k* and *inR* in total RNA extracts of abdomens (Fig. 4).

In head samples the gene expression of *tor* (as inferred from mRNA levels) did not differ among treatments (ANOVA, $F_{2,23} = 0.94$, $P = 0.41$, Fig. 3). Sucrose-fed mosquitoes bore higher *s6k* mRNA levels than albumin + AA-fed ones whose levels were even lower than those of saline-fed mosquitoes (ANOVA, $F_{2,12} = 11.36$, $P = 0.002$, Tukey comparisons using 95% C.I.), whereas a difference between sucrose and saline-fed mosquitoes was only detected by using 85% C.I. *inR* mRNA levels were marginally significantly higher in sucrose-fed mosquitoes than in saline-fed ones (ANOVA, $F_{2,20} = 3.03$, $P = 0.07$, Tukey comparisons using 90% C.I.). *ilp3* mRNA levels were significantly different among treatments: highest in albumin + AA-fed mosquitoes, intermediate in sucrose-fed ones and lowest in saline-fed mosquitoes (ANOVA, $F_{2,15} = 44.03$, $P < 0.0001$, Tukey comparisons using 95% C.I.). Only trends were recorded for *ilp4* where pairwise contrasts go in the same direction as those found for *ilp3* (ANOVA, $F_{2,15} = 2.37$, $P = 0.13$, results of Tukey comparisons using 85% C.I. are shown on Fig. 3).

In abdomen samples *tor* mRNA levels were marginally significantly lower in albumin + AA- and sucrose-fed mosquitoes than in saline-fed ones (ANOVA, $F_{2,20} = 3.64$, $P = 0.04$, Tukey comparisons using 90% C.I., Fig. 4). Saline- and sucrose-fed mosquitoes showed higher *s6k* mRNA levels than albumin + AA-fed ones (ANOVA, $F_{2,19} = 5.53$, $P = 0.01$, Tukey comparisons using 95% C.I.). No difference in *inR* mRNA levels was detected among treatments (ANOVA, $F_{2,19} = 0.29$, $P = 0.75$). Albumin + AA-fed mosquitoes had lower *tsc2* mRNA levels than saline-fed ones (ANOVA, $F_{2,22} = 4.98$, $P = 0.02$, Tukey comparisons using 95% C.I.).

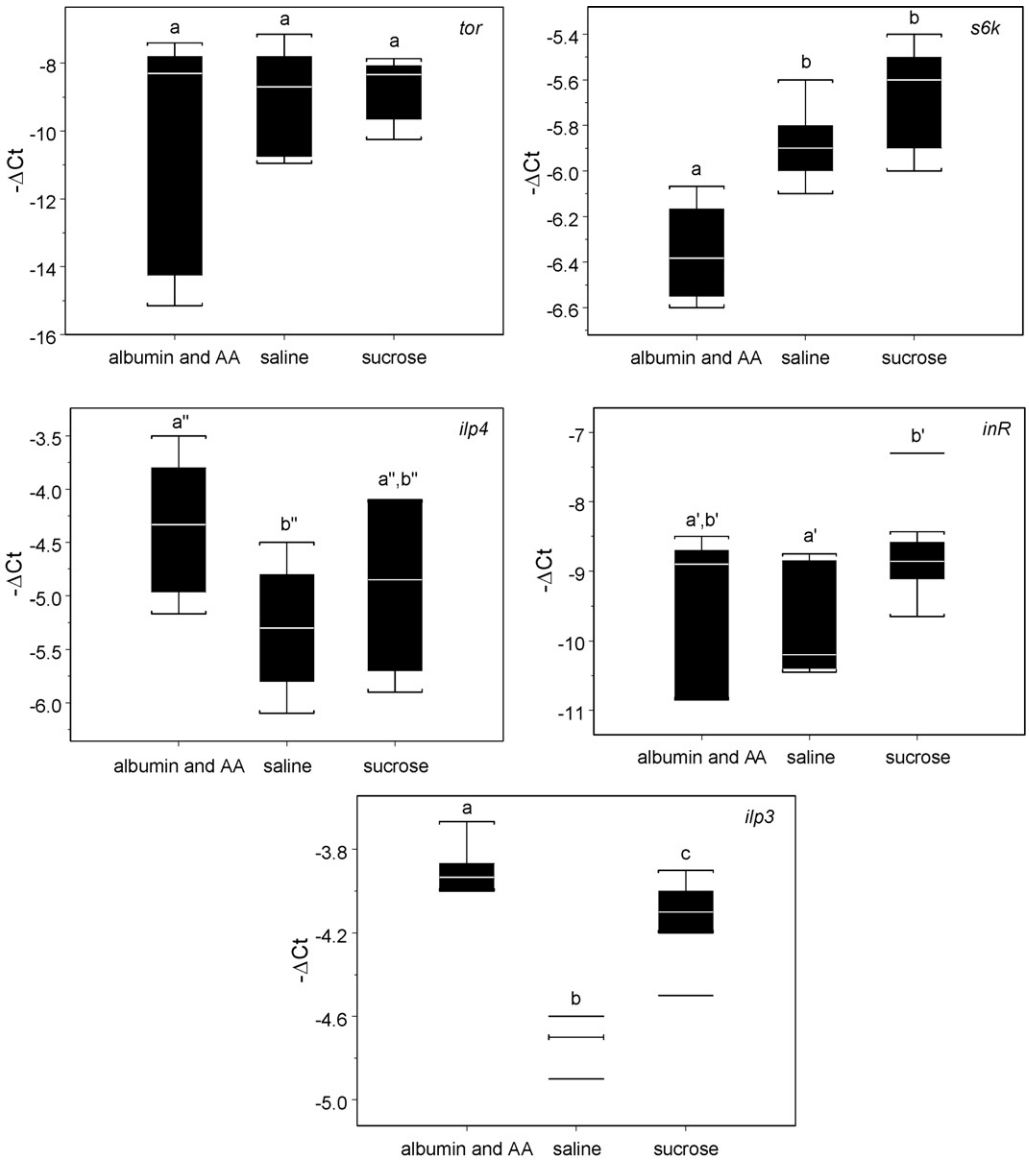

Fig. 3. Effect of diet on the gene expression of *tor*, *s6k*, *inR*, *ilp4* and *ilp3*, members of the insulin and TOR signaling pathways in the head of female *An. gambiae* 3 h after different meals. Assessment of mRNA levels in total RNA extracts from heads of mosquitoes fed on either albumin + AA, saline, or sucrose was carried out by quantitative real-time PCR using specifically designed primer pairs, SYBR® Green fluorescent labeling system and *RS17_ANOGA* as a control mRNA. Relative quantification (ΔCt values) was computed by subtracting the mRNA level (Ct value) of the control gene from the mRNA level (Ct value) of the gene of interest in each replicate in each treatment (see Section 2 for details). The sign of the ΔCt values was inverted so that a higher value corresponds to a higher mRNA level. Lower case letters (a, b, c) indicate significant differences (ANOVA followed by Tukey pairwise comparisons using 95% confidence intervals), addition of single quote (a', b') indicates marginally significant differences and double quotes (a'', b'') indicate trends detected by Tukey pairwise comparisons using 85% confidence intervals. Box-plot explanation as in Fig. 2 with additional bars representing outliers.

All significant differences described here amount to ca. 1 ΔCt unit between the means, that is, a theoretically maximal mRNA level ratio of 2.

3.5. Effect of diet on the activity of the TOR/S6K pathway

The activity of the TOR/S6K pathway was assessed by measuring the levels of the phosphorylated active forms of these enzymes (i.e. phospho(Ser^{2448})-TOR and phospho(Thr^{398})-S6K) by Western blot analysis of total protein extracts from ovaries and brains of unfed mosquitoes as well as of mosquitoes fed on either blood, albumin + AA or saline 3 h prior to dissection. Detectable levels of phosphorylated TOR proteins were only found in ovary extracts from fed mosquitoes (Fig. 5) and were higher in blood-fed mosquitoes than in albumin + AA- or saline-fed ones. Phosphorylated TOR was not detected in ovaries of unfed mosquitoes. Phosphorylated S6K was detected in the ovary extracts from all treatments at levels which increased along with the nutritive value of the meal (Fig. 5). That is, pS6K level was very high in the ovaries of blood-fed mosquitoes, slightly lower in albumin + AA-fed ones, much lower in saline-fed mosquitoes and very low (at the limit of detectability) in unfed ones. Phosphorylated S6K was also detected in all brain extracts, although at lower levels than in ovaries (relatively to the staining intensity of total protein extracts transferred on the blotting membrane), and levels varied depending on treatment (Fig. 5). Blood-fed mosquitoes hosted the highest level of cerebral pS6K, which was lower in albumin + AA-fed ones and much lower in saline-fed and unfed mosquitoes.

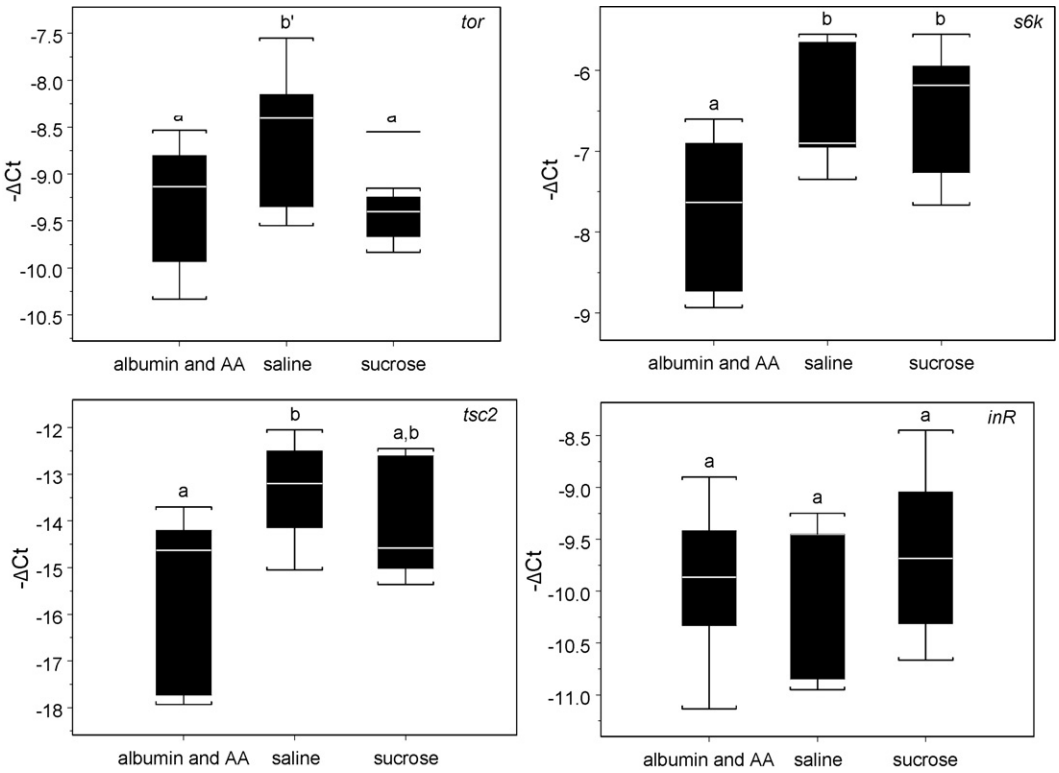

Fig. 4. Effect of diet on the gene expression of *tor*, *s6k*, *inR*, and *tsc2*, members of the insulin and TOR signaling pathways in the abdomen of female *An. gambiae* 3 h after different meals. Assessment of mRNA levels in total RNA extracts from abdomens of mosquitoes fed on either albumin + AA, saline, or sucrose was carried out and represented as explained in Fig. 3.

4. Discussion

4.1. Feeding behaviors and anatomical destination of the meals

Female mosquito feeding behaviors clearly suggest that albumin, AAs, and saline solutions (needing to be heated and directed to the midgut) were treated as blood (the critical source of protein for egg production in anautogenous mosquitoes), whereas the sucrose solution (needing to be at room temperature and directed to the crop) was treated as a different, complementary energy source, like plant nectar. Friend (1978) argued that the elevated diet temperature and the presence of a membrane induced the blood-feeding mode and that in this mode *Culiseta inornata* mosquitoes seldom feed if they detect sugar instead of blood. The present study provides evidence for the same phenomenon in *An. gambiae*. Furthermore, our data show that the blood-feeding mode can also result in full engorgement when the solution offered is only saline. This suggests that blood-like feeding is determined by the presence and concentration of a few saline components, by pH, temperature, and finally the presence of a membrane to feed through. Interestingly, it does not require the presence of free AAs nor proteins. Our data provide some of the first criteria for determining between blood- or nectar-feeding modes in an *Anopheles* species.

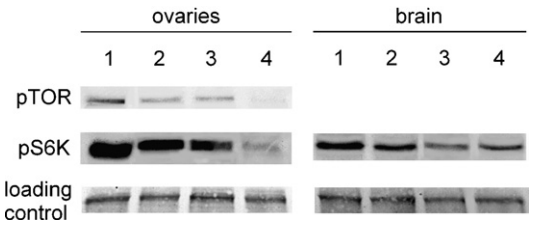

Fig. 5. Activation of the TOR/S6K pathway following ingestion of different diets by *An. gambiae* females. Ovaries and brains were dissected 3 h after the mosquitoes engorged themselves on blood (1), albumin + AA (2) or saline (3). Unfed mosquitoes (4) were included as controls. Total protein extracts were prepared from 30 ovary pairs or 20 brains per treatment and were divided in two aliquots each. Phospho(Ser^{2448})-TOR (pTOR) and phospho(Thr^{398})-S6K (pS6K) were detected by immunoblotting using specific antibodies which bind on TOR and S6K proteins only when phosphorylated on the mentioned residues respectively. Portions of the nitrocellulose membranes stained with Ponceau S after transfer are shown as controls for loading homogeneity.

The data on post-prandial behaviors show that the diets evoking the blood-feeding mode also largely prevented the mosquitoes to bite when exposed to human skin after engorgement. The strong effect of a saline meal is particularly noticeable and mirrors the outcome of the enema assays. The latter assays also proved that manipulating the perception of nutritional status by female mosquitoes is feasible and permits behavioral testing of its consequences. Moreover, this is the first report of an immediate host-seeking termination due to abdominal distention by itself in an anopheline species. It was already reported in *Ae. aegypti* by Klowden and Lea (1979). In the case of *Ae. aegypti*, this effect is consistent with the strict, non overlapping gonotrophic cycles exhibited by females of this species. But in *An. gambiae*, females can take multiple blood meals before laying eggs and their gonotrophic cycles overlap. The strong inhibition of host-seeking behaviors induced by artificial abdominal distention could be attributed to the fact that the volume injected was sufficient to activate enough stretch receptors in the abdominal and/or midgut wall to induce the behavioral switch. A similar explanation was proposed by Chambers and Klowden (1996) as they could abolish the effect of abdominal distention by transecting the ventral nerve cord. Interestingly, the tight link between the blood-fed-like behavior (refuge seeking rather than host seeking) and the artificially engorged status was illustrated by an unexpected phenomenon in

the present study. Some artificially engorged females who at first showed the expected blood-fed-like behavior of ignoring the host following the enema subsequently excreted all the saline solution during the 2 h before the biting assay. These mosquitoes were attracted to human skin again and tried to bite just like "control" individuals.

The intermediate effect of the AA mixture on post-meal biting evokes the possibility that tasting free AAs could signal an upcoming advantageous meal. Thus, the inhibition caused by abdominal distention could be reduced by the motivation to feed on what seems to be blood. This hypothesis could be verified by testing which blood components are tasted first when a female *An. gambiae* probes its host.

We conclude that the physico-chemical stimuli inducing the blood-feeding mode followed by nervous perception of subsequent midgut filling in mosquitoes are certainly the key factors that lead to the rapid post-prandial cessation of a willingness to bite, even when the meal is free of nutrients.

4.2. Ovarian follicle development depending on diet

Except for blood, the only diets causing follicular development were the solutions containing 120 g/l albumin ("albumin" and "albumin + AAs" treatments, Fig. 2). This means that the protein in the meal was assimilated and used for follicle development, as the female mosquito does with blood. This physiological response is consistent with the feeding behavior recorded for albumin, i.e. largest meals and highest retention among the artificial diets. These outcomes validate the signaling and nutritive value of the albumin-containing solutions used in these series of experiments.

The need for proteins in the meal is shown by the insufficiency of 10 g/l of a mixture of AAs to trigger follicular development (Fig. 2). The insufficiency of an AA meal for ovarian development in anopheline anautogenous mosquitoes was also demonstrated by Uchida et al. (2001, 2003) who simulated the natural increase of AAs in the hemolymph after a blood meal by infusion of AAs into the hemocoel of unfed female anopheline and culicine mosquitoes. Uchida et al. (2003) concluded that although an increase of hemolymph AA concentration and a mated status are essential factors for oogenesis in *An. stephensi*, some additional factors may be needed for full development comparable to that induced by blood meals. Among these factors, essential fatty acids, steroids and vitamines may be cited. Their absence from the albumin + AA solution could explain the failure of complete egg development in females who were fed this diet in our study. Uchida et al. (2003) ascribed the particular needs of anophelines compared to most culicine species to the physiological differences described by Briegel (1990). Namely, anophelines have lower larval caloric reserves such that blood proteins may be diverted to the synthesis of maternal lipid and protein deposits resulting in low efficiency of blood meal utilization for oogenesis. A modest supply of AAs may not provide enough substrate for both maternal reserves and vitellogenesis in anophelines.

4.3. Effect of diet on the mRNA level of members of the insulin and TOR signaling pathways

The main consequences recorded in the head of *An. gambiae* females 3 h after an albumin + AA meal were the decreased *s6k* and increased *ilp* mRNA levels (including trends for *ilp4*), whereas a sucrose meal increased *ilp3* mRNA levels and marginally those of *inR* and *s6k* compared to saline-fed mosquitoes.

In the abdomen an albumin + AA meal decreased *tor* (marginally), *s6k* and *tsc2* mRNA levels, whereas a sucrose meal had no effect but a marginally decreased *tor* mRNA level compared to saline-fed mosquitoes.

However, differences never exceeded a transcript level ratio between treatments of 2 (one unit ΔCt increment corresponding maximally to a doubling in amplified product). The small amplitude of mRNA level differences could be attributed to relatively small sample sizes (which increased variance) and to the short time allowed after feeding until freezing.

An. gambiae ilp3 and *ilp4* transcripts were also detected predominantly in head RNA samples by Krieger et al. (2004) using reverse transcription PCR. They localized ILP proteins by immunostaining in medial and lateral neurosecretory cells using a monoclonal antibody to synthetic bombyxin II, a silkworm ILP. Nutrient-dependent expression of certain insulin-like peptides in neuroendorcine cells in the brain of Drosophila was causally linked to insulin-dependent growth by Ikeya et al. (2002) who showed that expression of *dilp3* and *dilp5* was repressed by food withdrawal. Nutritionally triggered expression of *ilp3* (also found in our results) was suggested by Krieger et al. (2004) to explain the rarity of *ilp3* transcripts in their samples. Krieger et al. (2004) argued for a neuro-hormonal role of *ilp3* and *ilp4* due to their near-exclusive transcript localization in the female head.

Our results indicate that an albumin + AA meal was enough to rapidly trigger *ilp* transcription in the brain of fasting mosquitoes, even more than a sucrose meal. This contrasts with the prominent role of carbohydrates in insulin transcriptional regulation well known in mammals, as blood glucose level regulates the *insulin* gene expression in the pancreas via an autocrine loop (Xu and Rothenberg, 1998). However, *An. gambiae* cerebral insulin pathway may be more strongly influenced by sucrose than albumin + AA, as *inR* and *s6k* mRNA levels were marginally increased after a sucrose but not after an albumin + AA meal compared to saline-fed mosquitoes. Indeed, INR and S6K are primary mediators of downstream regulatory consequences of the insulin signaling pathway. The mediator role of neural *s6k* expression and kinase activity in feeding behavior regulation by the cerebral insulin-like system was demonstrated in Drosophila by Wu et al. (2005). Insulin receptor transcription in *Ae. aegypti* ovaries was shown to vary during the gonotrophic cycle with peaks just after blood meal and after oviposition (Riehle and Brown, 2002). Such transcriptional changes were not detected in the present study for abdominal *inR*, which is likely to result from the fact that pre-vitellogenic ovaries represent only a very small proportion of the abdominal tissues. Still, our data suggest that *inR* (and *s6k*) transcription is able to rise in the head in response to a sucrose meal. Together with the absence of post-prandial biting repression, this suggest a mechanism of sensing and regulating storage of nutrients providing only energy (essentially carbohydrates) which would be independent of mechanisms responding to nutrients that provide building blocks for oogenesis. Such an independence is supported by the different feeding modes (especially diet destination and the existence of the crop) exhibited by mosquitoes offered sucrose compared to other solutions (as described above).

Neither albumin + AA nor sucrose meals significantly increased *tor* mRNA levels in any body part compared to saline-fed mosquitoes. This is coherent with the results of Hansen et al. (2005) who highlighted only a small increase in *tor* mRNA level in *Ae. aegypti* ovaries 4 h after a blood meal and no measurable change in the other abdominal organs. Large differences were only detected after 24 h and only in the ovaries (Hansen et al., 2005). Albumin + AA feeding even decreased *s6k* mRNA levels in both head and abdomen as well as abdominal *tsc2* mRNA levels compared to saline-fed mosquitoes. Knowing the inhibitory function of TSC2 on TOR-mediated nutritional signaling, a *tsc2* transcription decrease after an albumin + AA meal is not illogical.

One possible explanation of the decreased *s6k* mRNA levels would be that increasing signaling effort triggered by the high AA concentration in the meal begin by consuming *s6k* transcripts for translation faster than they are produced. Hansen et al. (2005) did not find any change in abdominal *Ae. aegypti s6k* mRNA level 4 h after a blood meal except for malpighian tubules, but at low levels. A more comprehensive explanation should take into account the numerous functions and the complexity of the whole TOR/S6K pathway and its feedback loops (Arsham and Neufeld, 2006; Inoki et al., 2006; Wullschleger et al., 2006; Manning and Cantley, 2007). Future studies should also specifically address the ovarian expression of the genes addressed here.

4.4. Effect of diet on the activity of the TOR/S6K pathway

TOR and S6K activity was measured by phospho(Ser^{2448})-TOR and phospho(Thr^{398})-S6K levels for the first time to our knowledge in *An. gambiae* brain and ovary extracts and showed important variation depending on the nutrients present in the meal.

Our results point to a rapid start of TOR phosphorylation in the ovaries in response to feeding. We infer that TOR-mediated nutritional signaling was activated and responded by increasing TOR phosphorylation as soon as the mosquito engorged on a solution, even a nutrient-free one (saline). The higher pTOR level found in ovary extracts after a blood meal compared to all other treatments could reflect the fact that a high concentration of AA is not the most potent signaling elicitor and that a blood meal contains other components that are rapidly sensed and recognized as most important for supporting vitellogenesis. The supplementary effect of blood is indeed consistent with its exclusivity in supporting full egg development and oviposition. Abdominal distention and the consequent activation of stretch receptors in the abdominal walls and in the midgut following any meal can be suggested as one of the first triggers of the basal, nutrient-independent pTOR response, even though AA and other components are also readily sensed by the midgut and fat body. Together with mRNA levels analyses, our results suggest that early nutrient sensing primarily affects TOR activity rather than gene expression.

Two technical issues contributed to the fact that detected pTOR levels were low overall. First, TOR is a heavy protein of 282 kDa and sensible amounts (but similar among treatments) of proteins heavier than 250 kDa remained in the polyacrylamide gel despite long transfer times. Secondly, the phospho-specific antibody to pTOR has been optimized by the manufacturer for immunohistochemical applications and had never been tested for Western blotting. So, additional method optimization might have helped achieve a better labeling.

The stronger labeling of pS6K found in ovary extracts of albumin + AA-fed mosquitoes compared to saline-fed ones and of saline-fed compared to unfed mosquitoes is interesting. This shows that an albumin + AA meal was capable of augmenting S6K activity in the ovaries within only 3 h from the fasting state where S6K activity was very low and illustrates the signaling response ability of the ovaries to a meal capable of triggering follicle development (see above). pS6K labeling was even stronger in ovaries of blood-fed females, indicating that the supplementary value of a blood meal for oogenesis is reflected as early in this organ. Hansen et al. (2005) found that phosphorylation of *Ae. aegypti* S6K rose markedly after a blood meal in the fat body and even more significantly in the ovaries. This increase, beginning from a very low level, was clearly detectable after 4 h in the fat body but just after 2 h in the ovaries even though the level of total S6K did not show any change. We show that saline solution was handled by female mosquitoes as if it had been blood as they engorged on it and filled their midgut. Although saline meals did not allow follicular development, but repressed post-prandial willingness to bite, ovaries responded by increasing S6K activity compared to the unfed status.

Cerebral S6K activity was also correlated to the true nutritive value of the meal. Compared to unfed mosquitoes pS6K levels were only increased by protein-rich diets (blood and albumin + AA). This contrasts with the positive effect of a saline meal on ovarian pS6K level. It may be that midgut distension, when not reinforced by AA sensing, has only local signaling consequences. Increased cerebral pS6K levels following meals that repress biting willingness comply with attenuated hunger responses recorded in Drosophila larvae following S6K activity up-regulation in ILP-releasing neurons (Wu et al., 2005). The low levels of cerebral pS6K in saline-fed *An. gambiae* despite biting repression could echo the fact that biting willingness was assayed immediately after feeding, whereas tissue dissection followed 3 h post feeding, during which saline-fed mosquitoes showed highest values of meal loss. Thus, we suggest that a critical factor for S6K activity, in addition to AA and protein sensing, is the "current" midgut distension state. These two factors are not independent as sensing of meal nutritive value has an influence on its retention.

Altogether, our results strongly suggest that TOR/S6K signaling is very sensitive and responsive and that its output is not "all or none", but increases continuously with an increasing nutritional value of the meal as measured by the mosquito in terms of meal size, post-prandial biting repression and oogenesis triggering ability. Consequent to wide phylogenetic conservation of the TOR/S6K pathway, the regulatory mechanisms unveiled in this study are probably shared by many animals. Further studies should assay the effect of sucrose on TOR and S6K phosphorylation and check the temporal coordination of TOR and S6K activity with egg development to detect whether the reset to basal values awaits oviposition, knowing that gonotrophic cycles overlap in *An. gambiae*.

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