Molecular functional characterization of appetence maturation and its nutrient-dependent control in the African malaria mosquito *Anopheles gambiae*

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par

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Molecular functional characterization of appetence maturation and its nutrient-dependent control in the African malaria mosquito *Anopheles gambiae*

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Résumé

Le moustique *Anopheles gambiae* (anophèle) est le principal vecteur du paludisme (malaria) en Afrique sub-saharienne. Les repas sanguins fréquents pris par les femelles adultes sur des êtres humains multiplient les occasions de transmission des parasites sanguins du genre *Plasmodium* responsables du paludisme. Une meilleure compréhension de la régulation des comportements de recherche d'hôte entrepris par les moustiques femelles, ainsi que de leurs comportements alimentaires et de leur reproduction est une condition indispensable à l'avancement de la recherche visant à réduire la transmission du paludisme.

Dans cette étude, nous avons premièrement cherché à mieux comprendre la maturation de l'appétence des femelles anophèles pour un hôte. Nous avons enregistré leur attirance nocturne vers un corps chaud au cours des premiers jours de leur vie adulte. Puis, nous avons suivi une approche de génomique fonctionnelle visant à détecter d'éventuelles corrélations entre l'attirance et l'expression de plusieurs gènes impliqués dans la régulation des comportements de recherche d'hôte. L'appétence des femelles anophèles a montré une augmentation exponentielle avec l'âge des moustiques avec un saut particulier entre les jours 4 et 5. Cela correspond à la période à laquelle les réserves de glycogène ont sensiblement baissé de leur valeur maximale des jours 1-2 et où les réserves de lipides sont au maximum, ce qui pourrait refléter un état de maturité « optimale » du moustique en vue de la production d'œufs. Nos résultats ont révélé que les niveaux de transcrits des gènes *period* (*per*), *insulin-like peptide 4* (*ilp4*) et *p70 s6 kinase* (*s6k*) étaient positivement corrélés à l'âge et, donc, à l'appétence, mais n'étaient pas plus élevés chez les moustiques appétants âgés d'un jour comparés aux non-appétants du même jour. Cela suggère que l'expression de ces gènes n'est pas seulement liée au comportement, mais nécessite une maturation et peut-être aussi une insémination. Nous spéculons que les niveaux d'ARNm de *per* augmentent dans le cerveau en anticipation des tâches de recherche de nourriture. Le catabolisme du glycogène qui a lieu après le jour 2 pourrait occasionner une augmentation du glucose disponible, stimulant la transcription d'*ilp4*.

Cette étude a aussi eu pour but d'identifier les facteurs décisifs déclenchant l'oogenèse et l'inhibition concomitante du comportement de recherche d'hôte. Pour ce faire, nous avons mesuré l'effet d'aliments de différentes natures chimiques et valeurs nutritionnelles sur les comportements de nourrissage, la propension à piquer après un repas, et sur le développement

des follicules ovariens 24 heures après le repas de femelles anophèles. Dans l'optique de manipuler la sensation qu'a le moustique de son propre état nutritionnel, nous avons aussi testé l'effet de la distension abdominale sur le comportement de recherche d'hôte. Cette étude montre que le mode d'ingestion normalement évoqué par le sang peut être induit par une combinaison de facteurs physico-chimiques et ne requiert pas la présence d'acides aminés ou de protéines dans l'aliment. Nous démontrons que l'induction de ce mode d'ingestion résulte en l'inhibition du comportement de piquer après le repas. La distension de l'estomac (intestin moyen) pourrait être le médiateur-clé de ce phénomène, puisque le remplissage artificiel de l'estomac par injection anale (enema) de solution saline provoque aussi l'inhibition du comportement de piquer. De plus, nous établissons qu'une certaine concentration de protéine dans le repas est nécessaire et suffisante pour commencer l'oogenèse, mais seul le sang a permis le développement complet des œufs dans nos expériences, ce qui prouve que le moustique femelle doit trouver d'autres composés essentiels à partir d'un hôte.

Comment le moustique sait s'il a faim ou s'il est repu est une autre question posée dans ce travail. Nous avons mesuré l'effet d'aliments de différentes natures chimiques et valeurs nutritionnelles sur la réponse précoce des gènes *target of rapamycin* (*tor*), *s6k*, *tuberous sclerosis complex 2*, *insulin receptor*, *ilp3* et *ilp4* dans des femelles anophèles quelques heures après le repas. Les effets sur l'expression de ces gènes de repas composés soit de sucre, de solution saline ou d'albumine additionnée d'acides aminés ont été déterminés par PCR quantitative en temps réel dans des extraits de têtes et d'abdomens. L'activité des enzymes TOR et S6K dans l'ovaire et le cerveau à la suite de ces repas ou d'un repas sanguin a été déterminée par la détection des formes phosphorylées de ces enzymes par la technique du *western blot* en utilisant des anticorps spécifiques. De plus, l'effet de ces différents nutriments sur la présence et l'activité de ces deux enzymes a été déterminé dans l'ovaire et le cerveau par immunohistochimie. En fait, le niveau d'enzymes S6K qui sont phosphorylées sur le résidu thréonine 398 (chez la drosophile) ou 389 (site homologue chez les mammifères) est une mesure de l'activité de l'enzyme TOR. Nous montrons que les niveaux de transcrits de *s6k* et de membres de la voie de l'insuline sont rapidement affectés par les nutriments ingérés par l'anophèle (surtout *ilp3* dans la tête), et que la voie TOR / S6K est capable de réagir rapidement à tout repas par une augmentation de la phosphorylation de TOR et S6K. Qui plus est, nous montrons que l'étendue de cette activité de signalement dépend de la valeur nutritive réelle du repas. L'immunolocalisation de ces enzymes fournit des informations sur la question très débattue de leur localisation sub-cellulaire et documente ce que nous interprétons comme des signes de communication inter- et intra-cellulaire dans des cellules folliculaires.

Abstract

Anopheles gambiae mosquitoes are the main vector of human malaria in sub-Saharan Africa. The frequent blood meals taken by female *An. gambiae* increase the transmission of *Plasmodium* blood parasites. A better understanding of the regulation of host-seeking and feeding behaviors in this mosquito is a prerequisite for further efforts to cut malaria transmission.

Our first aim was to portray better the maturation of appetence for a host in female *An. gambiae*. We addressed host-seeking behaviors by recording the nocturnal attractedness to a warm body on the first days of adult life. Then, we implemented a functional genomics approach which assayed potential correlation between appetence and the expression of candidate genes implicated in the regulation of host-seeking behaviors. The appetence of *An. gambiae* females was shown to increase exponentially with age, with a marked increase between days 4 and 5, at least in the tested conditions. This corresponds to the period at which glycogen stocks have well decreased from peak values at days 1-2 and lipids stocks are at highest levels, which could reflect an "optimal" maturity of the mosquito for oogenesis. Our results revealed that the transcript levels of *period* (*per*), *insulin-like peptide 4* (*ilp4*) and *p70 s6 kinase* (*s6k*) were positively correlated to age and thus, to increasing appetence, but were not higher in attracted than in non-attracted mosquitoes on day 1. This suggests that the expression level of these genes is not only linked with behavior, but requires maturation and maybe also mating. We speculate that the levels of brain *per* mRNA increase in anticipation of the challenges of foraging. Glycogen catabolism taking place after day 2 could result in an increase of available glucose, which could trigger *ilp4* transcription.

This study aimed also at identifying the critical factors triggering oogenesis and the concomitant inhibition of host-seeking behavior. Therefore we assessed the effects of diets of different chemical nature and nutritive value on female *An. gambiae* feeding behaviors, post-prandial willingness to bite, as well as on the development of ovarian follicles 24 h after the meal. We also tested the effect of abdominal distention on host-seeking behavior in an attempt to manipulate the sensation that the mosquito has of its own nutritional state. This study reports that blood-like feeding can be induced by a combination of physico-chemical factors and do not require the presence of amino acids or proteins in the diet. We show that once this feeding mode

has been induced, post-meal biting is inhibited. Midgut distention may be a key mediator of this phenomenon, as saline enemas were able to inhibit biting as well. Moreover we demonstrate that a certain concentration of protein in the meal is needed and sufficient to trigger the start of oogenesis, but only blood allowed full egg development in our assays, which proves the need for the female mosquito to find other essential compounds from a host.

Another question addressed in this work is how the mosquito knows it is hungry or satiated. We assessed the effects of diets of different chemical nature and nutritive value on the early response of *target of rapamycin* (*tor*), *s6k*, *tuberous sclerosis complex 2*, *insulin receptor*, *ilp3* and *ilp4* in *An. gambiae* females a few hours after different meals. The effects of albumin plus amino acids, sucrose and saline on the expression of these genes were determined by quantitative real-time PCR in head and abdomen extracts, and the activity of TOR and S6K in the ovary and brain following these meals or a blood-meal was determined by immunoblotting of phosphorylated TOR and S6K. In addition, the effect of these nutrient types on the presence and activity of both enzymes in ovarian and brain cells was determined by immunohistochemistry. Indeed, the level of S6K proteins that are phosphorylated on Drosophila threonine 398 (and the homologous threonine 389 in mammals) is a measure of TOR activity. We show that transcript levels of *s6k* and members of the insulin pathway are readily affected by nutrients in *An. gambiae* (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. Immunolocalization of these enzymes provides data on the debated question of their sub-cellular localization and documents what was interpreted as signs of inter- and intra-cellular communication in follicular cells.

Keywords (Mots-clés)

Anopheles gambiae; host-seeking; feeding mode; blood feeding; sugar feeding; diet destination; oogenesis; ovary; brain; midgut; nutritional signaling; *period*; *takeout*; *target of rapamycin*; *p70 S6 kinase*; *insulin-like peptide*; *insulin receptor*; gene expression; quantitative real-time PCR; western blotting; immunohistochemistry; malaria; hematophagy

(*Anopheles gambiae*; recherche d'hôte; mode de nourrissage; repas sanguin; repas de nectar; destination anatomique du repas; oogenèse; ovaire; cerveau; intestin moyen; signalement nutritionnel; *period*; *takeout*; *target of rapamycin*; *p70 S6 kinase*; *insulin-like peptide*; *insulin receptor*; expression des gènes; PCR quantitative en temps réel; western blotting; immunohistochimie; paludisme; hématophagie)

General introduction

When and why mosquitoes want to bite us, and why to ask the question

And appetite, a universal wolf,
So doubly seconded with will and power,
Must make perforce a universal prey,
And last eat up himself.

Shakespeare, *Troilus and Cressida*

The parasite-host relationships have been one of the most potent selective force and evolutionary drive in the history of life on Earth. Parasites and hosts have always competed to evolve resistance against each other's arms and defenses. Nevertheless, this does not mean that any attempt to relieve the burden of infectious diseases is vain. Six out of the 14 currently listed neglected tropical diseases (NTDs) are transmitted by insect vectors: onchocerciasis is carried by black flies, leishmaniasis by sandflies, Chagas disease by "kissing bugs", lymphatic filariasis and dengue by mosquitoes, and sleeping sickness by tsetse flies. Leishmaniasis and lymphatic filariasis deform and disfigure to such an extent that those affected can be ostracized. Through administration of new, inexpensive medicines, millions are now protected from these diseases and therefore from the risk of social stigma, enabling them to live fulfilling social lives (WHO, http://www.who.int/features/factfiles/neglected_tropical_diseases/ntd_facts/en/index1.html). In 2006 the WHO Department of Control of Neglected Tropical Diseases issued a publication where it summarizes the main achievements in reducing the prevalence of several diseases and bringing some of them on the road to eradication (*Neglected Tropical Diseases, Hidden Successes, Emerging Opportunities*. downloadable from http://whqlibdoc.who.int/hq/2006/ WHO_CDS_NTD_2006.2_eng.pdf).

"Appetite for food and appetite for sex are two of the most important, if not the most important, driving forces underlying the design for living" stated V. G. Dethier (1976) in his renowned book *The Hungry Fly*. A central concept in this work is "appetence". It has something to do

with the sensation of appetite that is familiar to us, but in hematophagous insects it refers to the behavior of searching for a host and feeding on it, and to the willingness or readiness to engage in such behaviors. The conduct related to the search for food was named appetitive behavior.

Hematophagous insects are found in six of the 28 orders of Insecta. Within the order Diptera, there are 12 families that include blood-sucking species, many of which are important transmitters of diseases that affect humans and livestock. Hematophagy in insects is believed to have evolved through at least two routes (Waage 1979, cited by Klowden 1997). In some insects, it may have been the result of a long-term intimate association with a host, in which feeding habits progressed from organic matter, dung, and sloughed skin, and finally to skin and blood. The other route to hematophagy, by which mosquitoes are believed to have traveled, may have been the result of predaceous or plant-feeding insects that already had mouth parts and digestive tracts that were preadapted for piercing tissues, shifting their feeding preferences to the richer resource of blood (Waage 1979, cited by Klowden 1997). Blood feeding has evolved independently in many groups of the Insecta, in some cases even within the same family, like in the Culicidae (mosquitoes). This regimen is associated with a diversity of life styles. As summarized by Davis and Friend (1995) there are (a) obligate parasites (e.g. tsetse flies) that must feed on blood for both reproductive and energy requirements, (b) insects that must feed on blood for reproductive purposes, like some mosquitoes (e.g. *Anopheles gambiae*), but that can and do feed on plant resources to meet their energy needs, and (c) facultative parasites that can feed on either blood or plants for both reproductive and energy requirements; some insects may not express hematophagy until their second gonotrophic cycle, like some mosquito species producing their first batch of eggs using their larval reserves. The second category of insects (b, above) includes anautogenous mosquitoes that will feed at least once on a host before laying eggs. Usually, anautogenous mosquitoes are frequent blood feeders, which renders them perfect vectors of a cohort of infectious diseases.

Infectious diseases are the first cause of mortality in most of the developing parts of the world. In particular, mosquitoes are vectors of malaria, dengue, yellow fever, lymphatic filariasis, chikungunya and West Nile virus. Mosquitoes of the genus *Anopheles* are the unique vectors of human malaria (Fig. 1). Today approximately 40% of the world's population, mostly those living in the world's poorest countries, is at risk of malaria (Fig. 2). This disease causes more than 300 million acute illnesses and at least one million deaths annually – a situation which has given rise to a strong international effort to support research in this field (e.g. the Roll Back Malaria Partnership, http://www.rollbackmalaria.org).

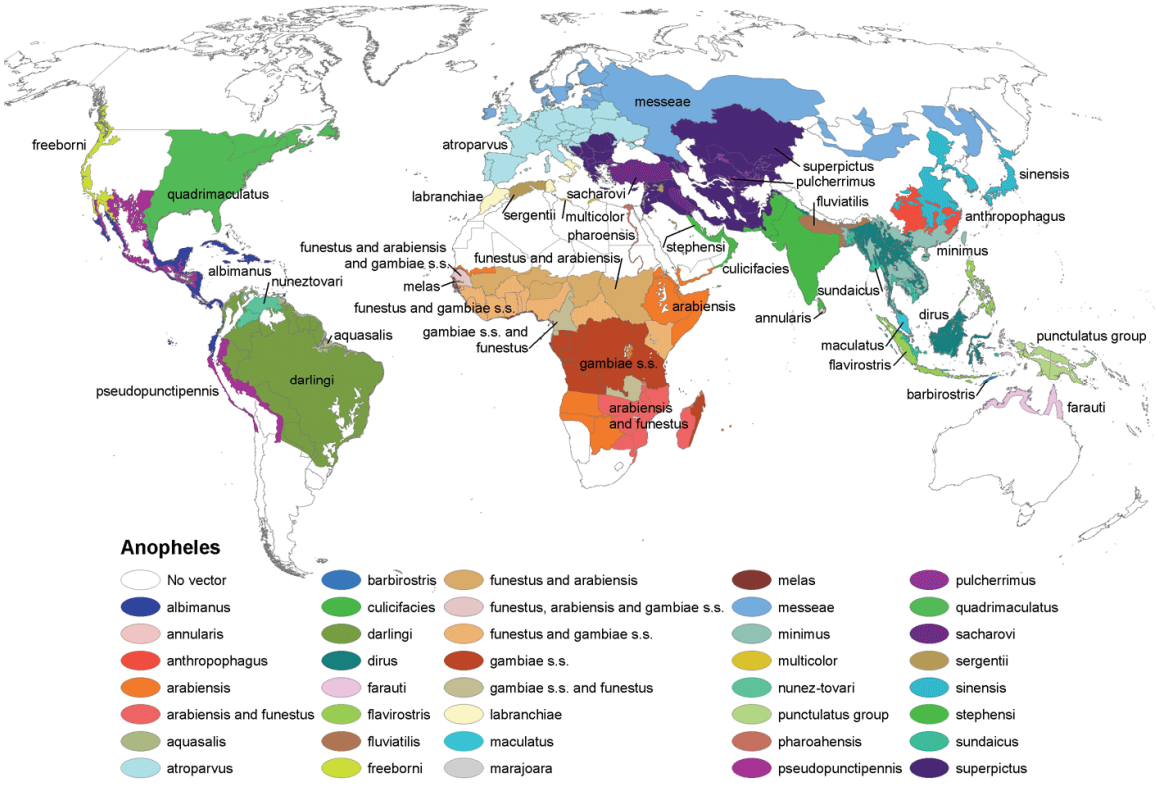

Figure 1 Global distribution of dominant or potentially important malaria vectors (Kiszewski et al. 2004).

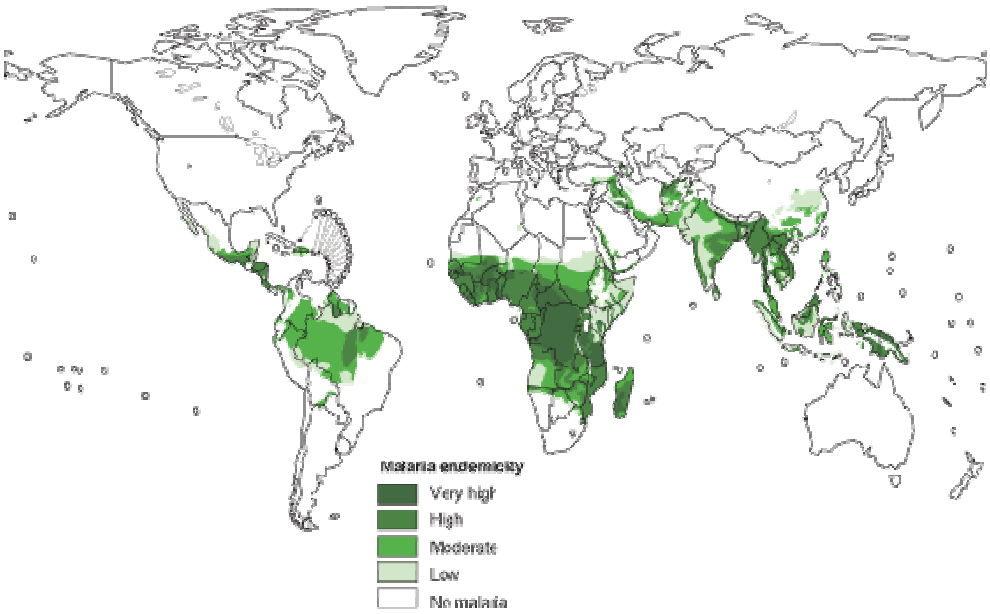

Figure 2 Global distribution of malaria transmission risk in 2003 (World malaria report 2005, http://rbm.who.int/wmr2005/).

Anopheles gambiae sensu lato refers to a complex of morphologically indistinguishable species. This species complex consists of *An. arabiensis, An. bwambae, An. merus, An. melas, An. quadriannulatus* and *An. gambiae sensu stricto* (*Anopheles gambiae* Giles). *An. gambiae s. str.*, which is the main vector of human malaria in sub-Saharan Africa, has been discovered to be currently diverging into two different sub-groups — the Mopti (M) and Savannah (S) molecular forms — though the two sub-groups are still considered to be a single species.

Like all mosquitoes, anophelines go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature. The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10-14 days in tropical conditions. Adult females lay 50-200 eggs per oviposition. Eggs are laid singly directly on water and are unique in having floats on either side. Eggs are not resistant to drying and hatch within 2-3 days, although hatching may take up to 2-3 weeks in colder climates (life-cycle information was retrieved from the U.S. Centers for Disease Control and Prevention website, http://www.cdc.gov/malaria/biology/mosquito/).

Mosquitoes are also successful as disease vectors due to their reproductive capacity, which results in large numbers of these vectors being generated in a short period of time. Therefore, a better understanding of the regulation of female mosquito feeding and reproduction is of central importance and may reveal new ways to disrupt the process of disease transmission.

In this study we focused on *An. gambiae* females' host-seeking and feeding, together with the internal signaling of food and its consequences. Specifically, we asked the following questions:

- When in her lifetime is one female *An. gambiae* seeking for a host?
- Is there a development of this behavior with age?
- How is it explained by her physiology?
- What factors induce this female to gorge?
- How does she know she is hungry or replete?
- How are the different nutrients ingested sensed by the mosquito?
- What consequences do these nutrients induce on mosquito behavior and egg production?

To answer these questions we tested the behavior of mosquitoes toward a heated surface as a function of their age and we examined their feeding habits when offered different diets (e.g. blood, protein, amino acids, sugar or saline) under different conditions. The physiological grounds of these processes were addressed by molecular investigations. We selected candidate genes potentially involved in feeding behavior regulation and in the response to nutrients (e.g. members of the insulin pathway), and assayed their body-part gene expression levels as a function of mosquito age as well as after ingestion of different diets. Furthermore, we focused on an important and widely conserved signaling pathway involved in nutrient-sensing and regulation of growth, the Target of rapamycin (TOR) / p70 S6 kinase (S6K) pathway. The levels and activity of these two key signaling enzymes were followed in the female mosquito brain and ovary to track early signs of nutritional signaling in response to different diets.

Molecular tools are totally adequate in the study of physiology (as it consists, for example, of molecular metabolic or signaling pathways) and especially in the case of disease vectors, as the pharmacological responses humanity is recruiting are molecules engineered against other molecules, and are intended to assume a predefined function in the ocean of interacting molecules that is a living organism.

We profited from the fact that *An. gambiae* was the first mosquito whose genome was fully sequenced. Ever since, no other mosquito genome has been so extensively studied and characterized. Unfortunately, this has led to substantial changes in the prediction of which sequence corresponds to which gene and which transcript. The total genome assembly was re-built several times and several sequences initially assigned to specific genes were retired. Between 2002 and 2004, an international effort followed the publication of *An. gambiae* genome sequence. Biotechnological engineering was enthusiastically favored as the new approach in the control of disease-vector mosquitoes. Novel technologies were developed such as the *Plasmodium/Anopheles* GeneChip® (Affymetrix) where all know *Plasmodium falciparum* and *An. gambiae* transcripts are spotted onto a single microarray chip. Subsequently, a batch of studies using this tool was rapidly issued. Large browsable datasets of transcripts (most of which only characterized by an accession number) more or less expressed after a blood meal or after infection were published to the web.
However, many researchers soon realized the lack of repeatability the microarray technology suffers from and the limits of what can be interpreted and applied from large sets of uncharacterized transcripts. In addition, mosquitoes are still not *Arabidopsis* or *Drosophila*: they represent substantial difficulty to genetic engineering, even if this is going to improve in the

next years. Furthermore, mosquito population replacement by transgenic strains in nature has nearly been abandoned as a serious strategy to foresee.

Still, when we started this project, in 2004, we were convinced, like many other researchers, that functional genomics would provide useful data. For example, the reconstitution of regulatory pathways (or interaction maps) from gene expression data was among our starting aims. Indeed, we constantly kept in mind that the more we understand, the more we can act. For instance, a better knowledge of the involvement of key genes in the control of foraging or in nutritional signaling paves the way to find one of them suitable for transgenesis or as a pharmacological target. Moreover, due to the conservation among organisms of the metabolic and signaling pathways addressed in this work, some results could be useful for further research on human diseases associated with insulin resistance or p70 S6 kinase malfunctioning.

This study was also motivated by the will to achieve challenging synergies between several areas of competence: classical insect physiology and behavioral ecology on one hand, and high-end molecular methods on the other hand. Collaborations between several research groups of the University of Neuchâtel had to be initiated, and these molecular methods had to be set up to answer our questions regarding insect physiology. All these challenges were very interesting to surmount.

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Chapter 1

The maturation of appetence in *Anopheles gambiae* females: behavioral monitoring and functional genomics

1. Introduction

Life-history traits of anopheline mosquitoes and their contrasts with those of culicines were extensively studied by Hans Briegel and his co-workers, especially in relation to the physiological bases of mosquito development, reproduction and ecology.

After eclosion, insects enter a maturation period, the teneral phase, which lasts about one day in mosquitoes (see Briegel 2003 for a review). This is a very important period for anatomical differentiation and maturation of behavior, for the hormonal and digestive systems, vitellogenesis, and also the flight muscles. The time required before the whole set of blood-sucking habits become manifested is the central focus of this study up to the gene expression level.

One of the most influential differences between the two mosquito subfamilies is that anophelines are described as "skinny" mosquitoes with very limited teneral reserves at adult eclosion, by contrast to culicines, which are entitled "fat" mosquitoes (Briegel 1990a, b).

The development of responsiveness to host cues and the factors affecting it were mostly studied in *Aedes* species (see Clements 1999, chapter 31), which revealed endogenous regulation of host-seeking by the *Aedes aegypti* Head Peptide I (Brown et al. 1994). By contrast, much fewer data is available for *Anopheles* species, which are known to bear extensive differences in physiology and ecology compared to aedines (Briegel 1990a, b; Klowden and Briegel 1994; Briegel 2003).

In nature, *An. gambiae* is crepuscular and nocturnal. Swarming is crepuscular and starts, under an open sky, about 10 min after sunset and lasts for 20 min, during which time the light intensity falls from ca. 55 to 1 lux (Marchand 1984). Sugar-fed virgin *An. gambiae* females and

males (kept separately) exposed to 12:12 h (light : dark) conditions with 75 min artificial dawn and dusk became increasingly active during the first 4 days after eclosion (Jones and Gubbins 1978). The peak of flight activity they showed at dusk increased over the days, but the populations sustained only a low level of flight activity throughout the scotophase, while remaining totally inactive during photophase (Jones and Gubbins 1978). Inseminated females were active from the start of the scotophase and exhibited higher activity throughout the scotophase than virgins of the same age, but a lower peak at dusk (Jones and Gubbins 1978).

The development of appetence in *An. gambiae* females was investigated by Fernandes and Briegel (2005) who offered a human arm every 4 h to inexperienced females. The first successful blood meals occurred within half a day post-eclosion, but at least one more day was needed for most females to fed successfully, depending on sugar availability. However, this apparent switch on by day one of blood-feeding behavior should not hide the importance of maturation. Reserve acquisition is prioritized over vitellogenesis in teneral *An. gambiae* females and oogenesis is not allowed as early as first blood meals (Briegel and Hörler 1993).

Further characterization of *An. gambiae* feeding habits came as multiple blood meals taken by females were highlighted by Briegel and Hörler (1993). The authors showed that females kept on sucrose from eclosion and fed on a human host after 12 h avidly refed when offered the same host within 12 or 24 h. Multiple blood meals can be taken at short intervals in this species, even within a single night, occasionally leading to a daily oviposition rhythm (Hörler and Briegel 1995). The term of gonotrophic discordance was coined by Swellengrebel (1929, cited by Clements 1992) to describe the situation where females require more than one blood meal to complete a single gonotrophic cycle. In *Anopheles*, multiple feeding is facilitated by an efficient mechanism of blood protein concentration during feeding (Briegel and Rezzonico 1985).
All these behavioral adaptations of anophelines appear to compensate the low lipid contents per female (Briegel and Hörler 1993). Low energy reserves at eclosion (Briegel 1990a) form the metabolic basis for this multiple blood-meal requirement. Female *Anopheles* appear to rely on a massive protein input from the blood meal for reproduction and for the acquisition of additional lipid and protein reserves (Briegel and Hörler 1993). The length of the mosquito gonotrophic cycle is among the most important of the bionomic parameters, because it is thought to be an estimate of the frequency at which the insects contact their hosts and, thus, an estimate of the opportunities for acquisition and transmission of parasites (Klowden and Briegel 1994).

Our aim was to portray better the maturation of appetence in female *An. gambiae*. We first addressed host-seeking behaviors by recording the evolution of nocturnal attractedness to a warm body on the first days of adult life. Then, we sought to deepen the characterization into the sources of appetence maturation through a functional genomics approach which assayed potential correlation between appetence and the expression of candidate genes implicated in the regulation of host-seeking behaviors.

The identification of genes endorsing functions linked to feeding behaviors has been the focus of many recent studies in a wide range of organisms (e.g. the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, the honey bee *Apis mellifera*, the model rodents *Mus musculus* and *Rattus norvegicus*, as well as humans).

The first assembly of the *An. gambiae* genome was published in 2002 (Holt et al. 2002) as a premiere for mosquitoes. Since, specialized on-line genome browsers host this data (Ensembl: www.ensembl.org, Anobase: www.anobase.org, GenBank at NCBI: www.ncbi.nlm.nih.gov) and implement an ever-growing wealth of sequence characteristics and bioinformatic tools. This has made possible the identification of genes putatively assuming the same functions in the regulation of feeding behaviors in *An. gambiae* than in the organisms previously studied. We retrieved much relevant and useful information from the *Drosophila* genome with the help of the specialized genome browser Flybase (http://flybase.bio.indiana.edu) and its smart hierarchical system of gene ontology terms referring to biological processes. Namely, the insulin signaling pathway, known in mammals to respond to sugar nutrients, was shown in the central nervous system of *D. melanogaster* to participate, in relation to the neuropeptide Y-like system and the p70 ribosomal S6 kinase, to the regulation of food acquisition through mechanisms sensitive to and reacting to nutritional status (Shen and Cai 2001; Ikeya et al. 2002; Wu et al. 2003; Wu et al. 2005a; Wu et al. 2005b; Lingo et al. 2007). Another important mechanism identified is amino acid signaling mediated by the Target of rapamycin (TOR) pathway, whose activity was shown to respond to nutrient availability in the rat brain and correlates with subsequent regulation of food intake (Cota et al. 2006). In mosquitoes, members of these pathways were identified in *Ae. aegypti* and *An. gambiae* and their roles in nutritional signaling and regulation of reproduction physiology were recognized, as described in more detail in Chapter 3. The first mosquito insulin receptor was found in *Ae. aegypti* (Graf et al. 1997). Genes coding for insulin-like peptides (*ilp1-7*), an insulin receptor (*inR*), an insulin receptor substrate (*IRS*) and for the enzymes TOR (*tor*) and S6 kinase (*s6k*) were already identified in *An. gambiae* genome by Riehle et al. (2002). *An. gambiae* insulin-like peptides were further characterized and their expression localized by Krieger et al. (2004).

The control of food intake by the circadian rhythms is clearly apparent in the case of nocturnal mosquitoes, but the molecular basis of its functioning is less obvious and only recent studies on *Drosophila* have linked circadian clock genes to feeding behaviors. The circadian clock, as described by Sarov-Blat et al. (2000), consists of a feedback loop in which clock genes are rhythmically expressed, giving rise to cycling levels of mRNA and protein. In *Drosophila*, *period* (*per*) and *timeless* (*tim*) are among the characterized genes believed to participate in central pacemaker control. In addition to circadian clock entrainment, their annotation points to biological processes such as rhythmic behaviors, circadian sleep/wake cycle, locomotor rhythm and response to thermal stimuli (Flybase gene ontology annotation). Furthermore, *takeout* (*to*) was identified as a circadian clock-regulated output gene in *Drosophila* by Sarov-Blat et al. (2000), who proposed a pathway in which *to* conveys temporal and food status information to feeding activities, including locomotor activity.

As feeding frequency is used for modeling transmission of mosquito-borne diseases, this study finds all its significance. Indeed, anopheline feeding is often independent of the ovarian cycle, and thus relying strictly on the length of the gonotrophic cycle to assess feeding frequency will result in significant underestimates of transmission rates, as concluded Klowden and Briegel (1994). Therefore, a better understanding of the factors affecting *An. gambiae* feeding habits could seed up-to-date population control strategies. Another benefit of such investigation is that the genes recognized as regulating feeding behaviors could serve as targets of transgene assays aiming at modifying feeding rhythms in such a way that pathogen transmission would be prevented without reducing mosquito fitness.
To our knowledge, this study is the first investigation on the functional genomics underlying feeding behaviors in *An. gambiae*, and the first to address the important genes *s6k*, *period* and *takeout* in this species.

2. Materials and Methods

2.1 Mosquito rearing

Anopheles 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 x 25 x 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 Behavioral assays

2.2.1 Monitoring the development of appetence behavior

Female mosquitoes use warm convective air currents among other cues to localize their host at short range. Attraction and probing (trying to bite) behaviors were observed in female *Ae. aegypti* and *Culex quinquefasciatus* exposed to warm air coming from a heat source (Howlett 1910). Similar responses including flight re-direction were also observed toward a warm moist convection current coming from a finger tip (Daykin et al. 1965). Thus, in this study a heated area served as a simple and reproducible host cue.

To monitor the development of appetence behaviors through the first days of imaginal life of *An. gambiae* mosquitoes, 1000 pupae coming from our synchronized rearing were left to eclose for one night in a 35 cm-wide, 35 cm-deep, 55 cm-high Plexiglas cage under normal rearing condition except for a ca. 90% relative humidity. Adults (two thirds were males) had a constant access to 10% sucrose *ad libitum*. On each of the first five days after the night of eclosion, 1 h after the beginning of the scotophase two black discs of 6 cm diameter were placed on one side of the cage, 10 cm away from each other and 15 cm from the top of the cage. One of the discs was connected to a heating device so that the temperature of the portion of wall where the disc was placed rapidly reached 37°C and stayed at this temperature, as controlled inside the cage. Simultaneously, mosquitoes were activated by blowing inside the cage so that all mosquitoes had the same chance to perceive the warm area at the beginning of the experiment. The use of human breath was the simplest and most efficient way of activating the mosquitoes in the cage, although the application of an air pulse with a controlled CO_2 concentration, as from a gas bottle, would have constituted a more standardized protocol. Attraction of mosquitoes toward the discs was followed by video-recording the scene during 12-15 min using a webcam (ToUcam PCVC 740K, Philips) on to a PC under constant near-infrared (950 nm) illumination coming from a lamp (WFN-II, Model LED 30, Videor Technical E. Hartig GmbH, Germany) located 1 m away opposite the discs.

To assess appetence, the number of mosquitoes attracted on each disc was counted each 10 s from the beginning to the end of the recording period.

2.2.2 Appetence- and age-dependent mosquito sampling for gene expression assays

To investigate the gene expression of several candidate genes potentially implicated in feeding behavior regulation in maturing *An. gambiae*, mosquito populations were established the same way as for appetence development assays described above, except that smaller cages (18.5 cm-wide and -high, 25 cm-deep) and fewer pupae (250) were used. On days 1, 2 and 5 after the night of eclosion, a 10 W incandescent bulb was switched on under the table on which the cage was placed and left until the time of experiment (1 h after artificial sunset) to provide the minimum illumination needed to work. Appetence assays used the same equipment as described above, except that the control disc (not heated) was placed on the opposite side of the cage. Each day, 10 to 15 females which were unambiguously attracted to and trying to bite the warm disc were collected and immediately frozen at -80°C. On day 1 the same number of females flying in the cage but clearly not interested in the warm disc were sampled as “not attracted”. On days 2 and 5 nearly all females were attracted to the warm disc with a rapid turnover so that virtually no females could be unambiguously recognized as “not attracted”.

2.3 Bioinformatics

Putative homologs of candidate genes identified in other organisms and their transcript sequences were retrieved in *An. gambiae* genome by transcript or peptidic sequence comparisons using BLAST family tools implemented on NCBI and Ensembl websites (see Results for more information on each gene). Query sequences were taken from GenBank, Flybase, or Ensembl. Primer pairs were designed using Primer3 freeware (http://primer3.sourceforge.net) so that the amplicon be no longer than 200 base pairs, when possible, to promote amplification efficiency. 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. The identification of the genes of interest and the sequences of the specifically designed primers are given in Table 1.1.

2.4 Gene expression assays

Pools of 10 to 15 attracted or non-attracted female mosquitoes (as described above) sampled on days 1, 2 or 5 after eclosion were homogenized in liquid nitrogen in a microcentrifuge tube using a pestle and 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, 25 min at 37°C, STOP solution added, then 10 min at 65°C) 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) which lasted 1 h at 42°C followed by 5 min at 94°C. Resulting cDNA was used as template for qRT-PCR reactions (400 ng cDNA template per reaction) using ABsolute™ QPCR SYBR® Green Mix (ABgene) according to manufacturer instructions and specifically designed primers (0.2 µM final concentration of each primer) 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, 54°C for *per*, *to1* and *to4*) 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 (corresponding to PCR product denaturation) 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. This ribosomal protein-coding gene was already used as a control in gene expression assays in *An. gambiae* by Nirmala et al. (2005). Detected *S17* mRNA level (Ct_{ref} values), as automatically computed by iCycler iQ software (Bio-Rad) from fluorescence curves after amplification, was averaged among replicates of each treatment and then subtracted to 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. Thus, the true mRNA level ratio between two samples differing by one ΔCt unit depends on amplification efficiency and ranges from 1 to 2. Amplification efficiency was different among genes but similar among treatments, as observed from amplification curves. As

a consequence, only within-gene comparisons are meaningful. All experiments were repeated at least twice (and usually three or four times) and results were pooled within each treatment for statistical analyses.

3. Results

3.1 Behavioral appetence assays: attraction to a warm body as a function of mosquito age

The development of appetence in *An. gambiae* females after eclosion was monitored through assaying their attraction to a warm area created on their cage 1 h into the scotophase of the five first days of their imaginal life. On the first night the infrared video-recording system was not set up yet and numbers of attracted females were directly observed at six time-points using the same heating device, but near the end of artificial dusk instead of during scotophase. Each night, females were rapidly attracted to the warm area and tried to bite it as if it were a host. Peak census was reached within 60 to 100 s after installation of the discs, followed by gradual decrease (Fig. 1.1). The slope of the decrease was higher on successive nights. Virtually no males were attracted to the warm area, whereas they were present on the control disc. Each night a few mosquitoes stayed on the control disc as anywhere else on the cage walls. These mosquitoes showed very low turnover rates and their number did not show any trend with the passing days. By contrast, turnover of females on the warm area was very rapid. The peak instantaneous number of attracted females on the warm area increased with age and even doubled each night from day 2, but occurred at nearly the same time every night. Correspondingly, the time until the number of attracted females decreased to a plateau phase also increased with age. It should be noted here that maximal instantaneous numbers of mosquitoes on the discs are limited by the effect of crowding.

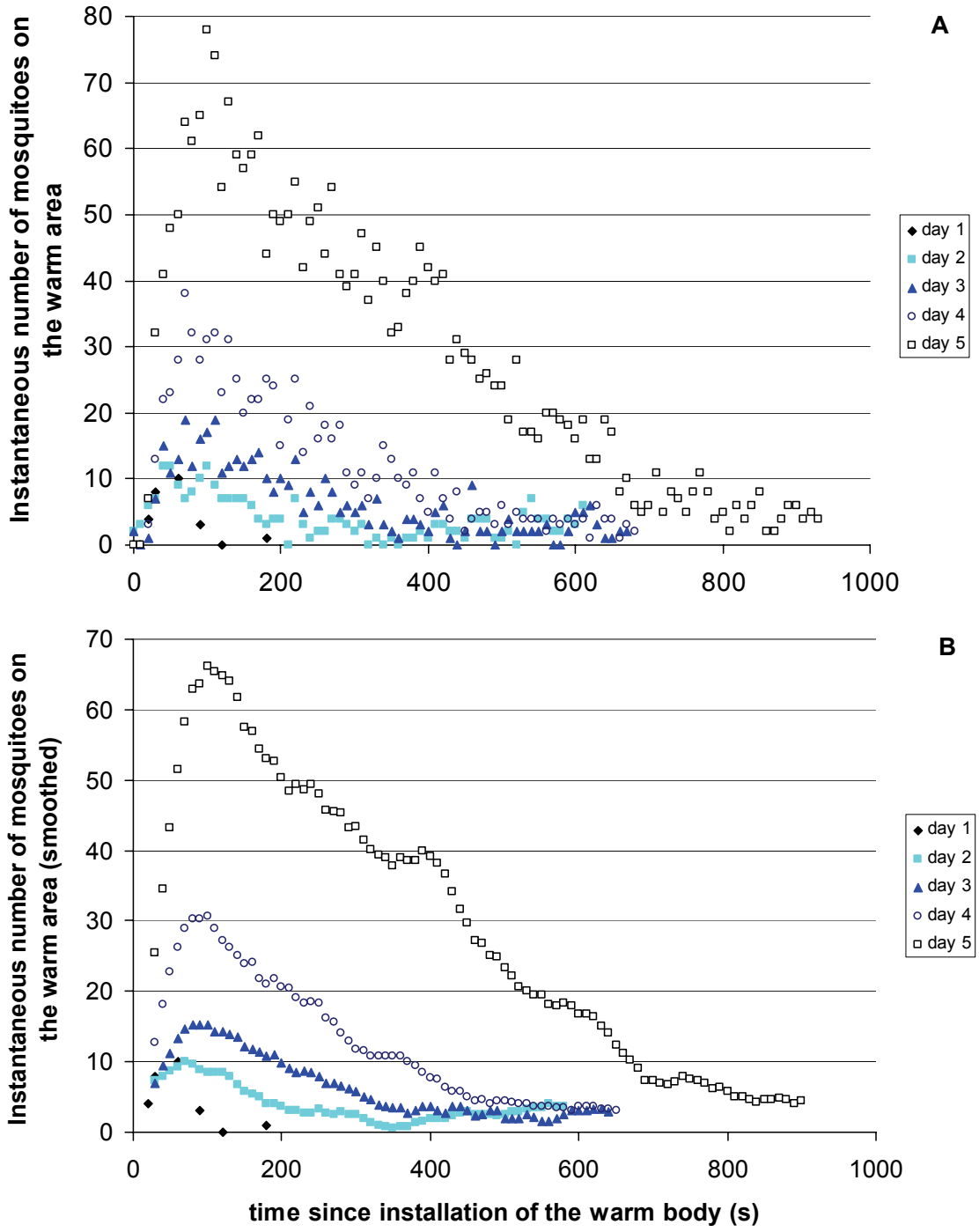


Figure 1.1 Behavioral monitoring of the development of appetence in *An. gambiae*. Time-course of the number of *An. gambiae* mosquitoes attracted to a warm black disc (37°C) installed on a wall of their cage on the five first nights after eclosion, 1 h into the scotophase. A similar but unheated control black disc was simultaneously put 10 cm away from the 6 cm heated disc and the scene was video-recorded in the dark by using infrared lighting and an infrared-sensitive webcam. Attracted mosquitoes were counted every 10 s on each disc and raw (A) and smoothed (60-s running average) (B) data for the warm disc are

plotted here. Note that day 1 values (same on A and B) correspond to direct observation at 6 time-points as the infrared recording system was not set up yet. Females were rapidly attracted to the warm area and tried to bite it, whereas a very low number of mosquitoes (including males) stayed on the control disc just as anywhere else in the cage. Peak instantaneous number of attracted females doubled every day from day 2.

3.2 Candidate genes identified with putative roles in host-seeking behaviors

s6k, *inR*, and *ilp4* were already identified in the genome of *An. gambiae* by Riehle et al. (2002) before being included and described as such in the genome databases (Table 1.1). *An. gambiae* genes corresponding to *Drosophila period*, *takeout* and *tuberous sclerosis complex 2* (*tsc2*, an inhibitor of TOR) were found by running BLAST protein sequence comparisons using *Drosophila* sequences as queries against all *An. gambiae* predicted peptides hosted by Ensembl. Probable homology was further confirmed by the automated ortholog prediction implemented in Ensembl gene reports and functional equivalence was ascertained by comparison of the gene ontology terms and predicted protein domains.

These investigations started when the *An. gambiae* genome was very poorly annotated and we could hardly ever rely on gene description. Ortholog prediction has also much progressed since then. Whole genome assembly has changed (from MOZ 2 to AgamP3) and countless new gene builds (sets of predicted coding sequences) have been released since we began to search *An. gambiae* sequences. This has led to an appreciable number of surprises and difficulties. Indeed, several predicted coding sequences and peptides on which preliminary assays had started were subsequently retired from the database. Some were only affected by changes in exon prediction, but other sequences were totally removed, leaving open questions as to the identity of the amplified products.

Other genes were tested (see description and results in Annex A1) but not all could be successfully amplified, and results were very difficult to interpret because these assays were run in the starting "trial-and-error" period when the experimental design was not definitive. Moreover, several gene sequences assayed were affected by database updates, two gene sequences were even totally retired from the genome assembly and replaced by different sequences attributed to the same gene.

Table 1.1 Identification of the genes whose transcript levels were investigated in the present study. Primers were specifically designed for this work. *s6k*, *inR*, and *ilp4* were already identified in the genome of *Anopheles gambiae* by Riehle et al. (2002) and have since been included and described as such in the genome databases.

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
s6k	none	AGAP007333	none	human *p70S6K* and *Drosophila s6k*	For: 5'-ATTAGAGGCGGTTAGGCATC-3' Rev: 5'-AAAATATTCCCTCGCGCTCT-3'
per	Q6VFD3_ANOGA	AGAP001856	period (fragment)	*Drosophila* and *Ae. aegypti period*	For: 5'-AACCAATTCTCCGACGACAC-3' Rev: 5'-GATATCTCGCCCAGCATCAC-3'
ilp4	Q6VVG8_ANOGA	AGAP010601	insulin-like peptide 4 precursor	none	For: 5'-TCTCCGAAAGAACACAGTTGA-3' Rev: 5'-GGTTTCTGCCTGAACCACAT-3'
inR	INR	AGAP012424	insulin receptor	none	For: 5'-CCAACCTTACCAGGGACTGA-3' Rev: 5'-GCATCGGGTAACAACATACG-3'
tsc2	none	AGAP011123	none	human, *Drosophila* and *Aedes aegypti TSC2*	For: 5'-GACACGAACACGCAGAAGAA-3' Rev: 5'-CACGAGTATGAGCGTGGAGA-3'
to1	Q86PT4_ANOGA	AGAP004263	putative antennal carrier protein TOL-2	*Drosophila takeout*	For: 5'-TGGTGAACTGCGATTTTCTG-3' Rev: 5'-CGGAAGATTTTCGTGAAAGC-3'
to4	none	AGAP004262	none	none (paralog of *to1*)	For: 5'-AGTGGACCGGTAAGCTCGT-3' Rev: 5'-GCTGATATCGCAGGCTTCA-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'

[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

3.3 Gene expression depending on age and appetence

The mRNA level (as measured by ΔCt values computed following qRT-PCR) of two of the tested genes did not show any significant variation nor trend depending on age and attractedness: *inR* (ANOVA, $F_{3,36}$= 1.526, P = 0.58, fig. 1.2) and *tsc2* (ANOVA, $F_{3,34}$= 0.662, P = 0.23).

Three genes showed a mRNA level increase with age. Namely, *ilp4* mRNA levels followed an increasing trend over the passing days and was significantly higher at day 5 than at days 1 and 2 (ANOVA, $F_{3,46}$= 7.508, P < 0.001, followed by Tukey post-hoc pairwise comparisons using 95% confidence intervals [CI]). Attracted mosquitoes showed higher *per* and *s6k* mRNA levels at day 2 and 5 than at day 1 (ANOVA, *per*: $F_{3,29}$= 19.496, P < 0.0001, *s6k*: $F_{3,55}$= 6.723, P < 0.001, both: Tukey comparisons using 95% CI).

Two genes presented a decreasing pattern of mRNA levels with age in attracted mosquitoes. *to1* levels were lower at day 2 and 5 than at day 1 (ANOVA, $F_{3,32}$= 13.260, P < 0.0001, Tukey comparisons using 95% CI) and marginally lower at day 5 than at day 2 (Tukey comparisons using 90% CI) and *to4* levels were lower at day 5 than at day 1 and 2 (ANOVA, $F_{3,19}$= 9.810, P < 0.001, Tukey comparisons using 95% CI).

Differences between attracted and non-attracted mosquitoes (sampled at day 1) were only present as a significantly lower *per* mRNA level in attracted mosquitoes.

All significant differences described here amount to ca. 1 ΔCt unit between the means, that is, a theoretically maximal mRNA level ratio of 2. Exceptions are only found in *per* transcripts, whose levels increased by 2.2 ΔCt units between attracted mosquitoes at day 1 and day 5, corresponding to a theoretically maximal mRNA level ratio of 4.6.

A problem coming from the reagents used for DNAse treatment of total RNA samples was discovered only after the experiments. That is, the DNAse enzyme was not of "amplification grade", i.e. developed to allow further use of samples for PCR, and the reagent used to stop its activity ("STOP solution" in Materials and Methods) was suspected to exert some PCR-inhibitory activity as it contains EGTA, a chelating agent known to be able of such inhibition. As these reagents were carried in cDNA template samples, they probably interfered with PCR amplification and added random variance on Ct values.

As oligo-dT primers were used for cDNA synthesis, mRNA sequences were retro-transcribed from the poly-A tail. Consequently, target sequences located far from the poly-A tail may have experienced lower reverse-transcription efficiency, resulting in a lower relative abundance of these sequences in the final cDNA sample. Thus, qRT-PCR quantification of amplicons located far from the poly-A tail of their corresponding mRNA (due to primer design) may have been

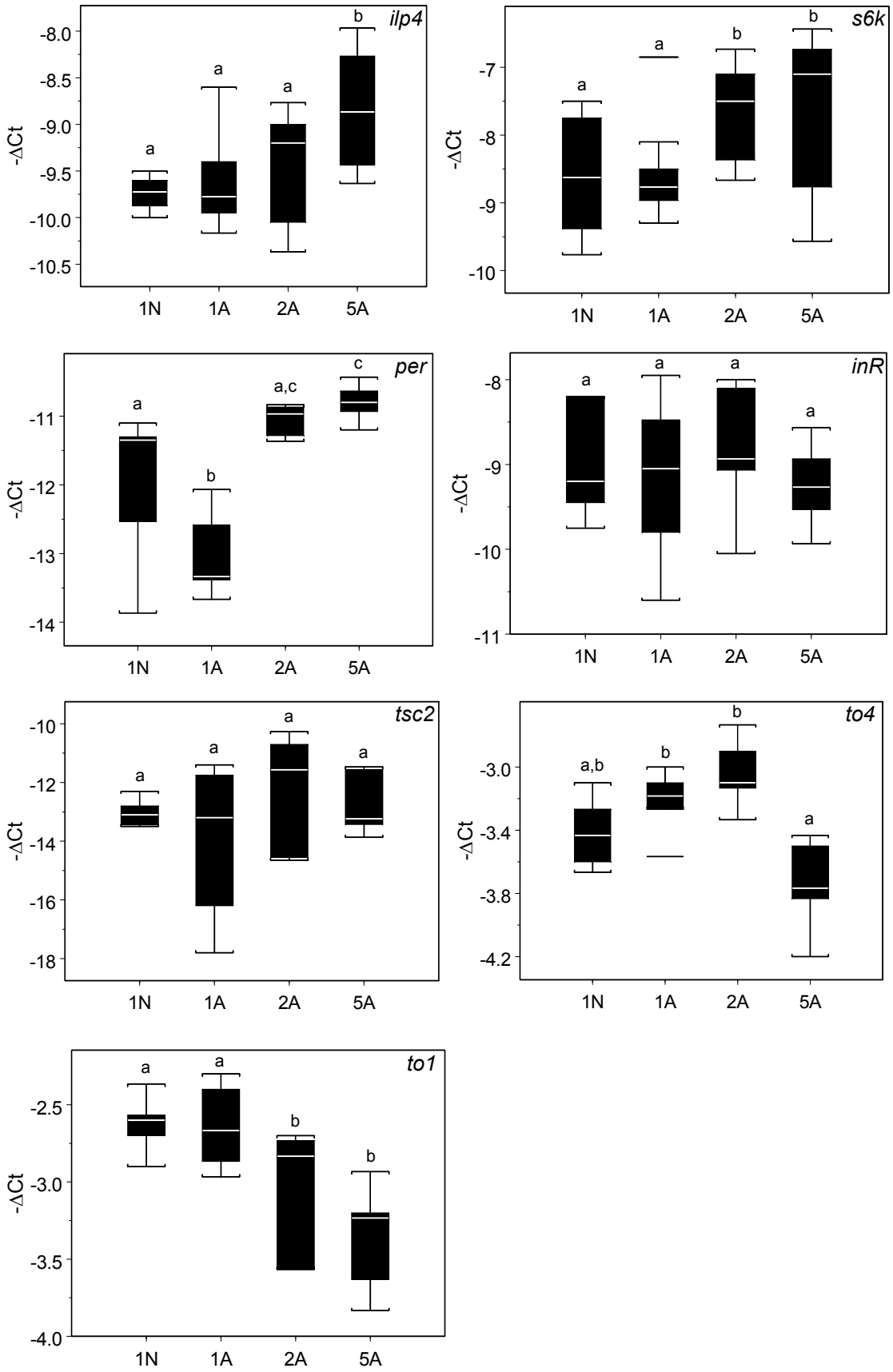
biased downward compared to amplicons located closer to the poly-A tail. We had no reliable means to measure this potential effect, but it is likely to have affected all treatments in a similar way.

Figure 1.2 (next page) Gene expression of *ilp4*, *s6k*, *per*, *inR*, *tsc2*, *to4* and *to1*, in female *An. gambiae* depending on age and attractedness to a warm body. Mosquitoes were sampled 1 h after artificial sunset as attracted ("A") or non-attracted ("N") to a warm body (37°C) on days 1, 2 and 5 after eclosion. Assessment of mRNA levels in total RNA extracts 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 to the mRNA level (Ct value) of the gene of interest in each replicate in each treatment (see Materials and Methods for details). The sign of the ΔCt values was inverted so that a higher value corresponds to a higher mRNA level. 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).

4. Discussion

4.1 Monitoring the maturation of appetence

The appetence of *An. gambiae* females (as measured by patterns of attractedness to a warm body) was shown to increase exponentially with age, with a marked increase between days 4 and 5 (Fig. 1.1), at least in the tested conditions. As the experimental population was synchronized, the increasing numbers of attracted females are more likely to result from increasing individual appetence rather than from an increasing proportion of females having overcome an all-or-none maturation threshold. A comparable but less clear-cut threshold may exist in the need to build up maternal reserves (by using all nutrients available, including blood) before being able to develop eggs from a blood meal. Increase of individual appetence is supported by evidence coming from repellent assays in *Ae. aegypti*, where 9-day-old females were absolutely not deterred from biting by repellent doses that were sufficient to keep them away when 3-day-old (Patrick Guerin, personal communication).


ilp4
-8.0
-8.5
-9.0
-9.5
-10.0
-10.5
-ΔCt
a
a
a
b
1N
1A
2A
5A
s6k
-7
-8
-9
-10
a
a
b
b
per
-11
-12
-13
-14
a
b
a,c
c
inR
-8
-9
-10
-11
a
a
a
a
tsc2
-10
-12
-14
-16
-18
a
a
a
a
to4
-3.0
-3.4
-3.8
-4.2
a,b
b
b
a
to1
-2.5
-3.0
-3.5
-4.0
a
a
b
b

The development of appetence in *An. gambiae* females was already investigated by Fernandes and Briegel (2005) who offered a human arm every 4 h to inexperienced females which had permanent access to water or 10% sucrose after eclosion. A few females tried to bite at 4 h, but none successfully fed until 8 h post-eclosion. Ninety-five percent of water-kept females had fed within 16-20 h, whereas in females offered access to sugar the 95% threshold was delayed to 36-40 h (Fernandes and Briegel 2005). The latter time frame corresponds to the age of mosquitoes sampled on day 2 in the present study. Fernandes and Briegel's (2005) results support our observation that only a portion of the female population was attracted to the warm body on day 1, whereas no more females could be unambiguously declared as not attracted from day 2. The assays run by Fernandes and Briegel (2005) draw a black and white picture of appetence development as the authors measured the successful outcome of appetence-related behaviors. However, a finer view is needed to account for true maturation. Briegel and Hörler (1993) reported that although biting, feeding, and digestion were possible at 12 h, female metabolism was not yet competent to allow oogenesis. Synthesis of yolk components developed only after 24 h of imaginal life, indicating the priority of reserve acquisition over vitellogenesis in teneral females, according to the authors.

The critical importance of the low teneral reserve levels as grounds for multiple blood meals and subsequent gonotrophic discordance in several anopheline species was emphasized by Briegel and Hörler (1993). This effect may as well influence the timing of appetence development and its differences between *Ae. aegypti* and *An. gambiae* (Briegel 2003) as these species have different teneral reserve levels and different lipogenesis efficiency from sugar meals.

Briegel (1990a) showed that *ad libitum* access to sucrose for several days allows *An. gambiae* females to build glycogen and lipid stocks (Fig. 1.3). The author found that the maximal glycogen content was reached between day 1 and day 2 after eclosion, followed by a gradual decrease. The sharp increase in attractedness pictured in our results between days 4 and 5 corresponds to the period at which glycogen stocks have well decreased from peak values at days 1-2 and lipids stocks are at highest levels (Briegel 1990a, Fig. 1.3). These metabolic parameters could reflect an "optimal" maturity of the mosquito, where fat bodies and ovaries are completely developed and ready for oogenesis. Repeated observation during routine care of the mosquito colony suggest that appetence is even higher in older mosquitoes than those formally assayed here, but it may well be that these old mosquitoes would use a blood meal for their own survival and lipid reserves rather than producing the largest possible number of eggs and dying afterward. Such a "selfish" behavior was observed in old *Ae. aegypti* females (Briegel et al. 2002).

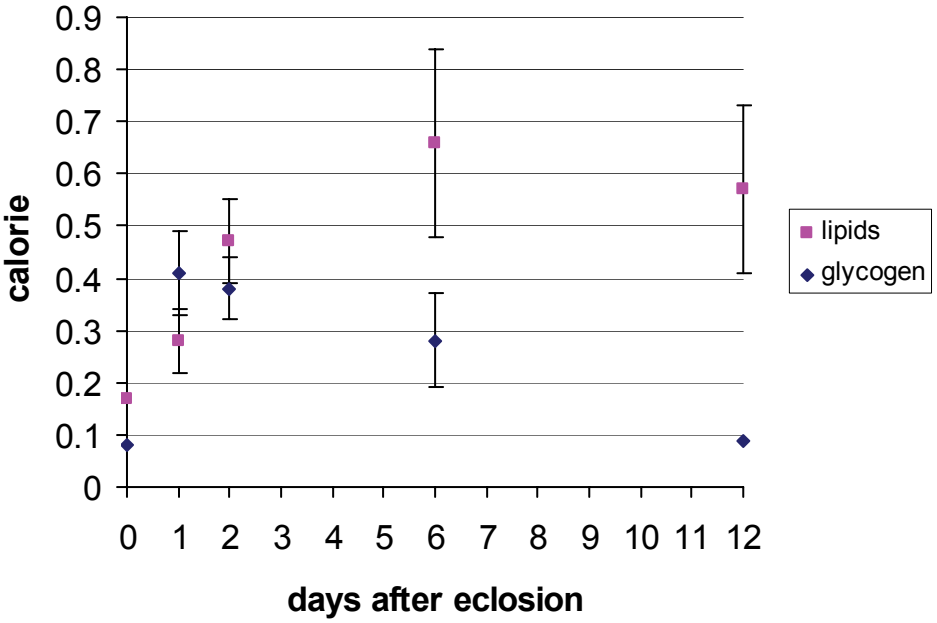


Figure 1.3 Effect of feeding female *Anopheles gambiae* 20% sucrose *ad libitum* during several days on the synthesis of glycogen and lipid reserves. Caloric values per female are given with the associated standard error when available. Day 0 corresponds to teneral reserves of lipids and total carbohydrates, not only glycogen. Data from Briegel (1990a).

A possible confounding effect which could be raised is that on day 1 more non-attracted mosquitoes would have recently fed on sucrose than attracted ones. However, this is not highly probable because differences in *ilp4* or *s6k* transcript levels would have been observed in response to a recent sucrose meal (see Chapter 3), and also because a recent sucrose meal does not decrease attractedness to human skin nor biting willingness, as demonstrated in Chapter 2.

Several tracks have been proposed to explain *An. gambiae* nocturnal habits. One states that biting at night when hosts are less active and less sensitive was advantageous (and thus positively selected) to avoid host defense during extended feeding times as required by simultaneous blood meal protein concentration (Briegel and Rezzonico 1985).

Knowing the role of insemination (Jones and Gubbins 1978), it would have been interesting to monitor populations exclusively composed of females to disentangle the extent of variation respectively due to maturation and to mating. Still, Gillies (1954) stated that the first blood meal could be taken independent of mating, because reaching the "pre-gravid" maturity state was considered more important. Furthermore, *An. gambiae* females were observed taking blood

meals before mating in the field (Charlwood et al. 2003), which is consistent with our record of appetent females on day 1.

In nature, another important factor affecting host-seeking behaviors is mosquito infection by malaria parasites. Anderson et al. (2000) showed that infection by the sporozoites of *Plasmodium falciparum* significantly reduced survival of blood-feeding *An. gambiae* by increasing the probability of dying during their nocturnal feeding period. Koella et al. (2002) reported that different stages of the malaria parasite *P. gallinaceum* can differentially affect the host-seeking behavior of its mosquito vector *Ae. aegypti*. Females carrying transmissible stages of the parasites had an increased threshold volume of ingested blood before being inhibited to seek a second host than females infected with non-transmissible stages (Koella et al. 2002). Rivero and Ferguson (2003) documented an increase in sugar feeding in *An. stephensi* mosquitoes infected by *P. chabaudi* and suggested that it could be a particular mechanism associated with parasite virulence. All these studies require us to remain modest regarding the appropriate conclusions to draw from the developmental mechanisms described in the present work. Our findings depict a basal pattern that is submitted to strong influences in nature ranging from larval food availability (and its far-reaching consequences on adult teneral reserves) to adult infection by malaria parasites.

4.2 Maturation of appetence as tracked by functional genomics

The mRNA levels of candidate genes potentially linked to feeding behaviors were assessed as a function of age and appetence of female *An. gambiae* mosquitoes. Our results revealed that the transcript levels of several genes (*per*, *ilp4* and *s6k*) were positively correlated to age and thus, to increasing appetence, but were not higher in attracted than in non-attracted mosquitoes on day 1. This suggests that the expression level of these genes is not only linked with behavior, but requires maturation and maybe also mating, as females were certainly unmated before their first night of imaginal life (between day 1 and day 2).

The very clear increase in *per* gene expression with age (and correlated appetence) is remarkable, in view of its previously described roles in honey bees and mammals. Toma et al. (2000) found that the level of *per* mRNA in the brain of bees was positively affected by age and the "forager" status of a bee. They speculated that the levels of brain *per* mRNA increase in anticipation of the challenges of foraging. This could also be the case in *An. gambiae* females, where increased *per* mRNA levels were present from day 2, the typical age from which *An.*

gambiae females begin to have their first blood meals, provided that they find a host. The role of *Period* genes in circadian anticipatory behavior in mice was investigated by Feillet et al. (2006) who found that *Per2* mutants did not show food-anticipatory activity because of their incapacity to anticipate 24 h cycles of food availability. The lower *per* mRNA level found in attracted *An. gambiae* females compared to non-attracted ones contrasts with the general positive effect of age and suggests that *per* transcription does not control attractedness, but rather reflects the maturity of mosquitoes that are not only willing to bite but also fully disposed to develop eggs after one or several blood meals.

The mRNA levels of *ilp4* were especially increased at day 5 compared to younger mosquitoes. The reason for this clear step could reside in the mosquito metabolism. Glycogen catabolism taking place after day 2 (Briegel 1990a, Fig. 1.3) could result in an increase of available glucose, which could trigger *ilp4* transcription. Nutrient-dependent expression of insulin-like peptides was demonstrated in *D. melanogaster* (Ikeya et al. 2002), has been suggested in *An. gambiae* (Krieger et al. 2004), and is addressed and discussed in Chapter 3. A similar explanation could be evoked for increased *s6k* transcript levels at days 2 and 5 compared to day 1, as this enzyme is activated in *D. melanogaster* ILP-releasing neurons in response to nutritional inputs in order to regulate hunger-driven behaviors (Wu et al. 2005a). Transcriptional response of *s6k* to nutrients is addressed in Chapter 3.

Our results highlighted two genes (*to1* and *to4*) whose expression level was negatively correlated with age, especially when comparing day 5 to younger mosquitoes. The only study showing a variation with age of a takeout-like gene highlighted an increase in the abdomen and a decrease in the head of *A. mellifera* of a protein-coding gene that is not ortholog to *D. melanogaster takeout* (Hagai et al. 2007). The expression of several *takeout/juvenile hormone binding protein* family genes were shown to vary upon nutritional status in *B. mori* larvae (Saito et al. 2006). Moreover, *takeout* expression was shown to be induced by starvation in *D. melanogaster* and refeeding reversed the starvation effect (Sarov-Blat et al. 2000). The authors presumed that clock regulation of *takeout* expression contributes to metabolic and even behavioral fluctuations that are relevant to food and feeding, namely either anticipating food availability or responding to the absence of food. The regulation of feeding behavior and locomotor activity by *takeout* was further addressed in *D. melanogaster* by Meunier et al. (2007) who showed by mutation that *takeout* regulates food intake according to food availability notably by increasing sensitivity of gustatory neurons to sugars after starvation. Meunier et al. (2007) also showed that *takeout* is needed to increase locomotor activity during starvation. The decreasing *to1* and *to4* mRNA levels observed in the present study with age could reflect either the building-up of energy stocks from the multiple sugar meals (lipogenesis peaked only 6 days

after eclosion (Briegel 1990a)), or, conversely, symptoms of starvation due to the lack of a blood meal. The latter explanation is however less probable because *to1* transcript level was already lower on day 2, although no starvation signs are yet expected on the second day of imaginal life when sugar is provided. Nonetheless, the possibility remains that *to1* and *to4* transcription be differentially regulated, as these paralogs may not share all of their functions. Indeed, both bear odorant-binding and hormone-binding protein domains, but only To1 protein has an ATP-binding domain (InterPro domains listed on Ensembl gene reports).

We found that *inR* and *tsc2* mRNA levels did not show any age- or attractedness-dependent variation. Both genes are implicated in nutritional signaling (the insulin-signaling pathway and amino acid signaling, respectively). Thus, a similar nutritional status, as far as external food sources are concerned, among the mosquitoes sampled could explain this fact. Indeed, the transcription of both genes was shown to react to a protein-rich meal (see Chapter 3), and *Ae. aegypti* insulin receptor expression was shown to vary through a gonotrophic cycle (Riehle and Brown 2002).

A notable point in our results is that appetence state as measured by attractedness at day 1 is poorly reflected by differences in gene expression level. This raises the possibility that motivated foraging-related behaviors be mainly regulated by protein interactions rather than transcriptional changes, at least on the first day of imaginal life. Furthermore, as whole mosquitoes were used for total RNA extraction, significant gene expression changes could have occurred in relatively small body parts or organs (i.e. ovary) without being detected.

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Annex A1: First series of gene expression assays

A1.1 Candidate genes

Other candidate genes and pathways were identified in different organisms to play a role in regulation of feeding behaviors. The genes addressed in this first series of experiments are listed and described in Table A1.1.

The *foraging* (*for*) gene encoding a cGMP-dependent protein kinase (PKG) has been linked with foraging behaviors in *D. melanogaster* (Osborne et al. 1997), as well as its orthologs *Amfor* in *Ap. mellifera* (Ben-Shahar et al. 2002) and *elg-4* in *C. elegans* (Fujiwara et al. 2002). Differences in *for* expression have been clearly linked with differences in food-dependent locomotion in all the cited organisms. In each case, two opposed phenotypes are associated with the presence of particular alleles at this locus. For example, in *Drosophila* the natural variant having one for^R allele is called "rover" because it moves a greater distance while feeding than does the other natural variant called "sitter" which is homozygous for the recessive allele for^s (Osborne et al. 1997). This pair of phenotypes, due to different allelic equipment at the *for* locus, are also known to exist in nematodes ("roamer" and "dweller") and in bees (forager and nurse) (Fitzpatrick and Sokolowski 2004; Fitzpatrick et al. 2005). Two genes are known to encode PKG in *An. gambiae*: ebi6403 and agCG54791 (Fitzpatrick and Sokolowski 2004), and the latter corresponds to the gene *cGKII* addressed in this study (Table A1.1). This name was chosen because sequence comparisons revealed a better match with vertebrate type II cGMP-dependent protein kinase which is named cGKII.

Moreover, a neuropeptide F was isolated from adult *Ae. aegypti* (Stanek et al. 2002). It is a member of the neuropeptides F/Y superfamily (Stanek et al. 2002) that are notably involved in regulation of feeding-related behaviors and circadian rhythms in vertebrates. The *Drosophila* NPF (*DmNPF* gene) is suggested to play a role in feeding behavior and digestion, and even in the response to food stimuli in larvae (Shen and Cai 2001). Wu et al. (2005a) identified in *Drosophila* larvae a neural pathway involving neurons expressing ILP, NPF, or its receptor NPFR1 that differentially regulates food preference but not ingestion rate. Involvement of NPF and its receptor in the regulation of motivated foraging by fasting *Drosophila* larvae was further characterized by Wu et al. (2005b) and Lingo et al. (2007). A mutation in a putative NPF receptor was also found to affect feeding behavior in *C. elegans* (de Bono and Bargmann 1998). Expression of the neuropeptide F was confirmed in both the brain and midgut of female *Ae. aegypti* and its highest hemolymph titer was found before and 24 hours after a blood meal, a pattern that was consistent with an inhibitory effect on reproduction (Stanek et al. 2002). Such

neuropeptide F genes were identified in *An. gambiae* genome, e.g. *AgamNPFF* (Riehle et al. 2002), and NPF and NPFR transcripts were found in all life stages in *An. gambiae* (Garczynski et al. 2005), but their precise role are not documented as yet.

An important intracellular mediator of insulin signaling in a very wide range of organisms is the Insulin-receptor substrate encoded by the gene *chico* (Flybase name, generally known as *insulin receptor substrate, IRS*). A putative homolog was already identified in the genome of *An. gambiae* by Riehle et al. (2002) before being included and described as such in the genome databases.

In teneral adult female mosquitoes an increase in juvenile hormone (JH) signals that ecdysis of the adult has finished and that reproductive processes should begin (Hagedorn 1994; Klowden 1997). JH allows the fat body to become competent for hormonal response to ecdysone (Zhu et al. 2003). The balance between ecdysone and JH is known to regulate egg production in insects and was shown to be crucial in controlling the genetic response of the ovarian cells to nutritional signals (Terashima and Bownes 2004). Namely, ecdysone titers are increased under nutritional shortage and, above a certain threshold, promote apoptosis over oogenesis. Terashima and Bownes (2004) characterized the controlling role of the expression of an early ecdysone response gene (*Broad-Complex*, *BRC*) in the choice between vitellogenesis and apoptosis in relation to the nutritional environment during oogenesis. A putative homolog to *Drosophila BRC* was newly identified in *An. gambiae* genome in this work by BLASTP comparisons of peptidic sequences against all *An. gambiae* predicted peptides in Ensembl using the *Drosophila* peptide sequence retrieved from Flybase as a query. The resulting *An. gambiae* best match scored a 90% amino acid identity with the query.

In *Drosophila*, *dgcα1* encodes a soluble guanylyl cyclase alpha subunit. Guanylyl cyclases catalyze the formation of cyclic guanosine monophosphate (cGMP) which activates PKG encoded by the *foraging* gene. Riedl et al. (2005) reported that *Drosophila* larvae with inactivating mutations in *dgcα1* showed both increased PKG activity and foraging locomotion. The putative ortholog of *dgcα1* in *An. gambiae* genome was found by BLAST sequence comparisons in Ensembl and confirmed as such by the automated ortholog prediction implemented in this genome browser as well as by comparing the protein domains description. It was termed *Gyc-α* for this study. The proteins respectively encoded have 66% of identical amino acids between *Drosophila* and *An. gambiae*.

This first series of experiments addressed the mRNA levels of the candidates genes listed in Table A1.1 as a function of mosquito age and appetence. The aim was to find genes whose expression pattern could be consistent with a regulatory role in female *An. gambiae* appetence.

A1.2 Results and interpretation

Mosquitoes were sampled nearly the same way as described in the Materials and Methods section in the main text of this chapter, except that the last sample occurred on day 3 and not day 5. In addition, mosquitoes which never were given access to sucrose were also assayed. In both populations (with or without access to sucrose) attracted females were also sampled during the afternoon of day 2 (second half of photophase) and were used to assay the expression of a few genes only.

The amplification of several genes failed despite multiple trials with different primers and amplification conditions. That was the case for *NPFR*, *NPYR* (another NPY receptor-like gene resembling *NPFR*), and *Gyc-α*. Results from the successfully amplified genes suffered from a general low repeatability, but differences between some samples could still be repeated and interpreted. That is, *NPF* showed a ca. 1-ΔCt-unit decrease in day-1 attracted females compared to non-attracted ones sampled the same day, as well as a further ca. 1-ΔCt-unit decrease in attracted mosquitoes between days 2 and 3. These differences were observed in both populations with or without access to sucrose. In addition, *cGKII* expression levels in both the sucrose and sucrose-free populations were stable or slightly decreased with age and appetence state. By contrast, *BRC* and *tim* mRNA levels were ca. 1 ΔCt unit higher in attracted females than in non-attracted ones on day 1 and, interestingly, showed a decrease in the afternoon of day 2 before increasing again a few hours later in scotophase-sampled mosquitoes to reach day-1 levels. The photophase decrease was deeper in sucrose-deprived mosquitoes. This could be the first report of a circadian cycling of *BRC* and *tim* gene expression in *An. gambiae* and, what is more, the first report of an effect of sucrose availability on the amplitude of such cycling so early in imaginal life. Both *BRC* and *tim* mRNA levels were ca. 2 ΔCt units higher in day-3 sucrose-deprived mosquitoes, which showed clear signs of starvation but fierce appetence, than on day 2. Day-1 attracted females showed a ca. 1-ΔCt-unit increased *chico* and similarly decreased *ilp3* mRNA levels compared to non-attracted ones.

Unfortunately, *An. gambiae tim* and *chico* sequences used in this work to design primers were retired from Ensembl and the primers we used do not match with the new sequences currently assigned to these genes. Therefore, we cannot be sure of the identity of the amplified products.

The results of this first series of gene expression assays lead to the conclusion that *NPF* and *cGKII* expression do not seem to be correlated to appetence, but evidence exists for such a correlation in the case of *chico*, *BRC* and *tim* (as far as their identity can be assumed). Thus, the most promising pathways to look at for appetence control would be intracellular insulin signaling and early ecdysone response genes.

Table A1.1 Identification of the genes whose transcript levels were investigated in the present study. Primers were specifically designed for this work unless otherwise specified. *NPF*, *ilp3* and *chico* (*insulin receptor substrate*) were already identified in the genome of *Anopheles gambiae* by Riehle et al. (2002) and have since been included and described as such in the genome databases. However, sequences attributed to *tim* and *chico* accessions have changed recently and the primers provided here do not match with these new sequences.

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
NPF	NPF	AGAP004642	Neuropeptide F	*Ae. aegypti NPF*	For[c]: 5'-GACGATGGCGTCAGGCAC-3' Rev: 5'-CATGTCTAGATATTTGTTGGTAGCTG-3'
cGKII	none	AGAP008585	none	*Ae. aegypti cGMP-dependent protein kinase*	For: 5'-CGCTCATTAGGGAGGACAAG-3' Rev: 5'-ACATCCATTAGCTCCGGTTG-3'
BRC	none	AGAP002165	none	*Drosophila* and *Ae. aegypti broad-complex core-protein*	For: 5'-CGAGCTACTGAAGAGCACACC-3' Rev: 5'-CTTAAAATTTCGGCCGTCTTC-3'
tim	none	AGAP008288	none	*Drosophila* and *Ae. aegypti timeless*	For: 5'-GTCCTGCAACGGGAAGTTAG-3' Rev: 5'-GCTTGCTGCTCTCCTTCATC-3'
ilp3	Q6VVG9_ANOGA	AGAP010602	insulin-like peptide 3 precursor	none	For[d]: 5'-GGTAAAGGTACTGTCCTTCCTG-3' Rev: 5'-AGTATCTGCTGCGTGTTGTC-3'
chico	none	AGAP001877	none	*Drosophila chico* (*insulin receptor substrate 1*)	For: 5'-CGAAGATGTGGGCATTCAC-3' Rev: 5'-CGATGATGGAATCCTGCAC-3'
NPFR	none	AGAP004122	none	*Drosophila NPFR1*	For[c]: 5'-GCACAACCGTCATCCACCATGG-3' Rev: 5'-CGGGACATCAGGACATCAGCTC-3'
NPYR	GPRNPY2	AGAP004123	putative neuropeptide Y receptor 2	*Drosophila NPFR1*	For: 5'-ATGGTCCTTTGCGATAATGC-3' Rev: 5'-CGATGTGACGATGGAGAATG-3'
Gyc-α	none	AGAP000280	none	*Drosophila head-specific guanylate cyclase* and *Ae. aegypti guanylate cyclase α 1*	For: 5'-TGTACAAGCACTTTGACGAG-3' Rev: 5'-GCATCTTGATGTGCTGACC-3'

[c] these primer pairs were published by Garczynski et al. (2005)

[d] this primer pair was published by Krieger et al. (2004)

Chapter 2

Determinants of feeding habits, post-prandial host-seeking and oogenesis in *Anopheles gambiae* females: what nutrients are really needed for

1. Introduction

Mosquitoes have polytrophic meroistic ovaries containing between ca. 20 and 500 ovarioles depending on the species, as reviewed by Clements (1992, section 22.1), and between 100 and 200 per ovary in *Anopheles gambiae* (Fiil 1976). Ovarioles consist of a germarium and two follicles each. The latter are composed of one oocyte and seven nurse cells and are enclosed in a follicular epithelium. 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. During oogenesis, the follicular epithelial cells allow passage of yolk protein precursors which are internalized by receptor mediated endocytosis and deposited in the oocyte. Nurse cells synthesize large amounts of ribosomal RNA (rRNA) that are transported to the oocyte cytoplasm via intercellular canals; the nurse cells and the oocyte thus form a syncytium. Oocyte maturation is completed whithin two days, epithelial cells have secreted a chorion and the nurse cells degenerate.

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; Uchida et al. 2003). All these meal

components are involved in the nutritional control of vitellogenesis in anautogenous mosquitoes (reviewed by Attardo et al. 2005).

In the mosquito *Aedes aegypti* the target of rapamycin (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; Hansen et al. 2005), notably by promoting the transcription of rRNA and ribosomal proteins. 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). The nutritional signaling mediated by the TOR pathway is specifically addressed in Chapter 3.

The effect of larval nutrition and subsequent adult reserves on hormonally regulated oocyte maturation has been studied in *Ae. aegypti* (Caroci et al. 2004; Noriega 2004; Telang et al. 2006; Shiao et al. 2008). Telang et al. (2006) proposed a model in which high levels of glycogen and protein reserves (maybe stored or processed in the fat body) surpass a threshold set in the nervous system that activates ovarian ecdysteroid production and inhibits juvenile hormone (JH) biosynthesis by the corpora allata (CA), which together allow vitellogenesis and egg maturation. When glycogen and protein levels are below this threshold, the CA secretes high JH levels, ovarian ecdysteroid production is low, and egg maturation is delayed or arrested. Larval nutrition determines the level of adult teneral reserves and, *ipso facto*, how much is still to be acquired by the meals to overcome the threshold and develop eggs. Shiao et al. (2008) produced evidence for nutrient reserves-dependent JH, as a mediator of fat body competence, to permit the TOR-signaling response to AAs derived from the blood meal.

The phagostimulatory action exerted on mosquitoes by different compounds has been studied for a long time and has revealed extensive differences between species. Galun (1987) classified the blood feeders into three categories on the basis of their gorging response to blood components: (1) those that recognize blood through properties of the plasma and thus feed equally well on whole blood and on plasma; (2) those that feed on plasma, but to a much lesser extent than on whole blood; and (3) those that require information limited to the cellular fraction and are reluctant to gorge on plasma unless some cell components are added to it. Mosquitoes of all anopheline species studied by Galun et al. (1985) were reported to feed on platelet-poor plasma as avidly as on whole blood and the plasma effect could be fully reproduced by 0.15 M

NaCl plus 10^{-3} M $NaHCO_3$ in *An. freeborni*, *An. stephensi* and *An. gambiae*. By contrast, three culicine mosquitoes, *Culex pipiens* (Hosoi 1959), *Ae. aegypti* (Galun et al. 1963), and *Culiseta inornata* (Friend 1978) are among the insects which require the cellular fraction and are stimulated by adenine nucleotides (e.g. ATP) dissolved in isotonic saline (reviewed in Galun 1987). *Ae. aegypti* showed a log-linear dose response increase in engorgement (proportion of females gorging) with ATP concentration, whereas anophelines fed on ATP solutions showed no correlation between the ATP concentration and engorgement (Galun et al. 1985).

Previous studies addressed the consequences of abdominal distention by itself and documented a termination of host-seeking behavior in *Ae. aegypti* (Klowden and Lea 1979) as well as the induction of egg development in an autogenous strain of *Ae. albopictus* (Chambers and Klowden 1996). These works made use of the anal injection technique described as "enema" by Briegel and Lea (1975).

This study aimed at identifying the critical factors triggering oogenesis and the concomitant inhibition of host-seeking behavior. Therefore we assessed the effects of diets of different chemical nature and nutritive value on female *An. gambiae* feeding behaviors, post-prandial willingness to bite, as well as on the development of ovarian follicles 24 h after the meal. We also tested the effect of abdominal distention on host-seeking behavior in an attempt to manipulate the sensation that the mosquito has of its own nutritional state. This study reports that blood-like feeding can be induced by a combination of physico-chemical factors and does not require the presence of AAs or proteins in the diet. We show that once this feeding mode has been induced, post-meal biting is inhibited. Midgut distention may be a key mediator of this phenomenon, as saline enemas were able to inhibit biting as well. Moreover we demonstrate that a certain concentration of protein in the meal is needed and sufficient to trigger the start of oogenesis, but only blood allowed full egg development in our assays, which proves the need for the female mosquito to find other essential compounds from a host.

2. Materials and Methods

Anopheles gambiae populations were reared as described in Chapter 1.

2.1 Feeding assays

The feeding behavior of *An. gambiae* females was characterized as they fed on one of 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 V, 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 results): 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 results.

2.2 Post-prandial biting assays

Five-day-old female mosquitoes were exposed to different nutrients in order to monitor an eventual differential influence on post-prandial motivation for a blood meal. 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) 10 g/l free AAs from a dilution of 50x RPMI 1640 amino acid solution containing a blend of all natural AAs minus alanine and glutamine (SIGMA cat. nr. R 7131, pH adjusted to 7.4, termed “AAs”), (5) albumin plus free AAs (120 g/l bovine serum albumin fraction V plus 10 g/l free AAs in saline as above, 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. Mosquito

willingness to bite was assessed immediately after engorgement by putting them on human skin. They were then attributed to one of 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 heated plate), but were not offered any diet. These unfed mosquitoes were also tested for biting behavior on human skin as controls.

2.3 Follicle development assays

Five-day-old female mosquitoes were fed with the different solutions described above (saline, sucrose, blood, AAs, albumin, albumin+AA) or with a single-AA solution (10 g/l L-alanine in saline, pH adjusted to 7.4) 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 in 4% paraformaldehyde, 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.

2.4 Enema and biting assays

In order to investigate the effect of abdominal distention on the willingness to bite, 3- to 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, Fig. 2.1). “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. Female mosquitoes that showed a clear behavior (either not attracted at all or trying to bite) were immediately frozen for RNA extraction (procedures were as described in Chapter 1).

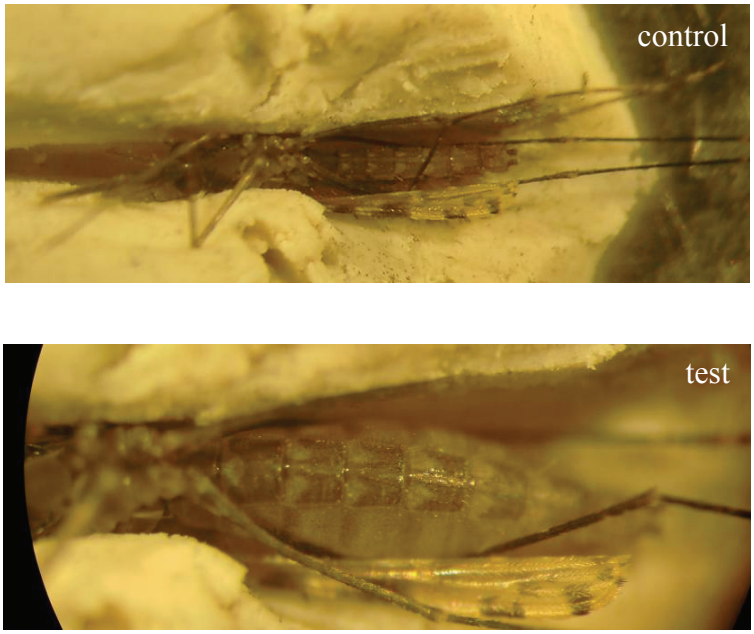

Figure 2.1 Pictures of "control" (upper plate) and "test" (lower plate) *An. gambiae* females after sham manipulation or enema. They were restrained in plasticine just the time needed to carry out the manipulation. The plasticine was tightened around the thorax of the mosquitoes so that their abdomen was free to enlarge.

3. Results

3.1 Effect of diet composition on feeding habit (including diet destination), meal size and retention, post-prandial biting, and follicular development

3.1.1 Feeding mode and diet destination

Female mosquitoes exhibited different behaviors when exposed to sucrose in comparison to the other diets (Table 2.1). 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, pH-buffered 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.

Table 2.1 Behavioral differences exhibited by female *Anopheles gambiae* exposed to sucrose in contrast to other diets.

diet	10% sucrose	all other diets
diet condition needed for feeding[a]	room temperature pH = 8 (no NaCl/$NaHCO_3$ buffering) direct contact with soaked filter paper	heated (optimum ca. 36°C) pH buffered to 7.4 membrane to feed through
diet destination	crop	midgut
feeding behavior	repeated meals	single meal, full engorgement

[a] Mosquitoes did not feed unless these conditions were adhered to.

3.1.2 Meal size

The variable "meal to body weight ratio" provides a measure of the amount ingested relatively 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- (0.889 ± 0.358) and sucrose-fed ones (0.757 ± 0.310) (Tukey post-hoc pairwise comparisons using 95% confidence intervals, Fig. 2.2).

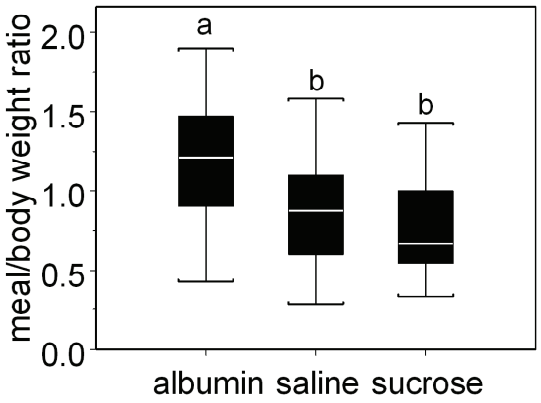

Figure 2.2 Meal weight to initial body weight ratio for female *An. gambiae* fed on albumin, saline, or sucrose. This ratio was higher in mosquitoes fed with albumin than in mosquitoes fed with other diets. Box-plots represent the median (white bar) and the 25%-75% inter-quartile interval (box), and 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) indicate significant differences (ANOVA followed by Tukey pairwise comparisons using 95% confidence intervals).

3.1.3 Meal retention

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 (Kruskal-Wallis $\chi^2_2 = 52.14$, $P < 0.0001$, Fig. 2.3). 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.

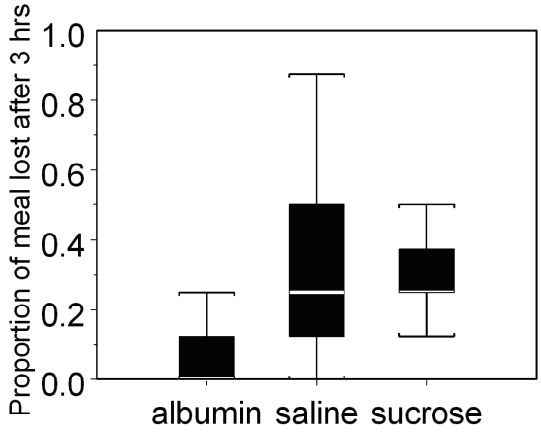

Figure 2.3 Proportion of albumin, saline and sucrose meals lost 3 h after feeding by *An. gambiae* females. This proportion was significantly different between treatments. Lowest losses were mainly in albumin-fed mosquitoes, most of whom (70%) did not show any visible excretion. Saline-fed mosquitoes manifested the most dispersed data and highest values for meal loss. Box-plot explanation as in Fig. 2.2.

3.1.4 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. 2.4). 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 indicate 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 was significantly higher ($P = 0.02$) than after an albumin+AA or a blood meal.

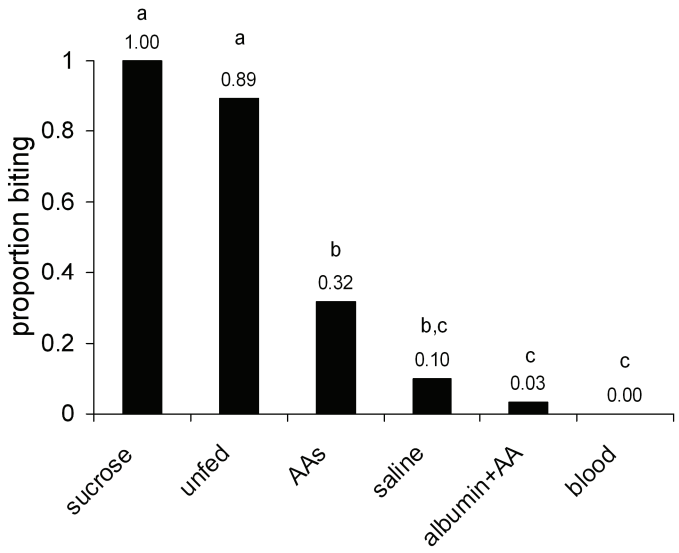

Figure 2.4 Proportion of *An. gambiae* females that bit when exposed to human skin just after engorgement on either saline, sucrose, a mixture of amino acids (AAs), albumin plus amino acids (albumin+AA), or blood. Unfed mosquitoes were included as controls. Sucrose-fed mosquitoes were not deterred from 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).

3.1.5 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 µm ± 20 µm, mean ± standard deviation), albumin+AAs (156 µm ± 6 µm) or albumin alone (168 µm ± 25 µm) were significantly larger and further developed (oocyte enlarged to 75-

90% of the follicle, full of yolk spheres, corresponding to Christophers' stages IIIb or IVa as described by Clements and Boocock (1984)) than those of mosquitoes fed saline, sucrose, AAs or an alanine solution or unfed (ANOVA, $F_{7,90} = 292.59$, $P < 0.0001$, followed by Tukey post-hoc pairwise comparisons using 95% confidence intervals, Fig. 2.5). Blood-fed mosquitoes had significantly larger follicles than albumin- and albumin+AA-fed ones. Follicles of AA- (75 µm ± 6 µm), alanine- (62 µm ± 7 µm), saline- (72 µm ± 6 µm) and sucrose-fed (67 µm ± 6 µm) mosquitoes did not differ significantly from those of unfed (64 µm ± 8 µm) mosquitoes (corresponding to previtellogenic Christophers' stages 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.

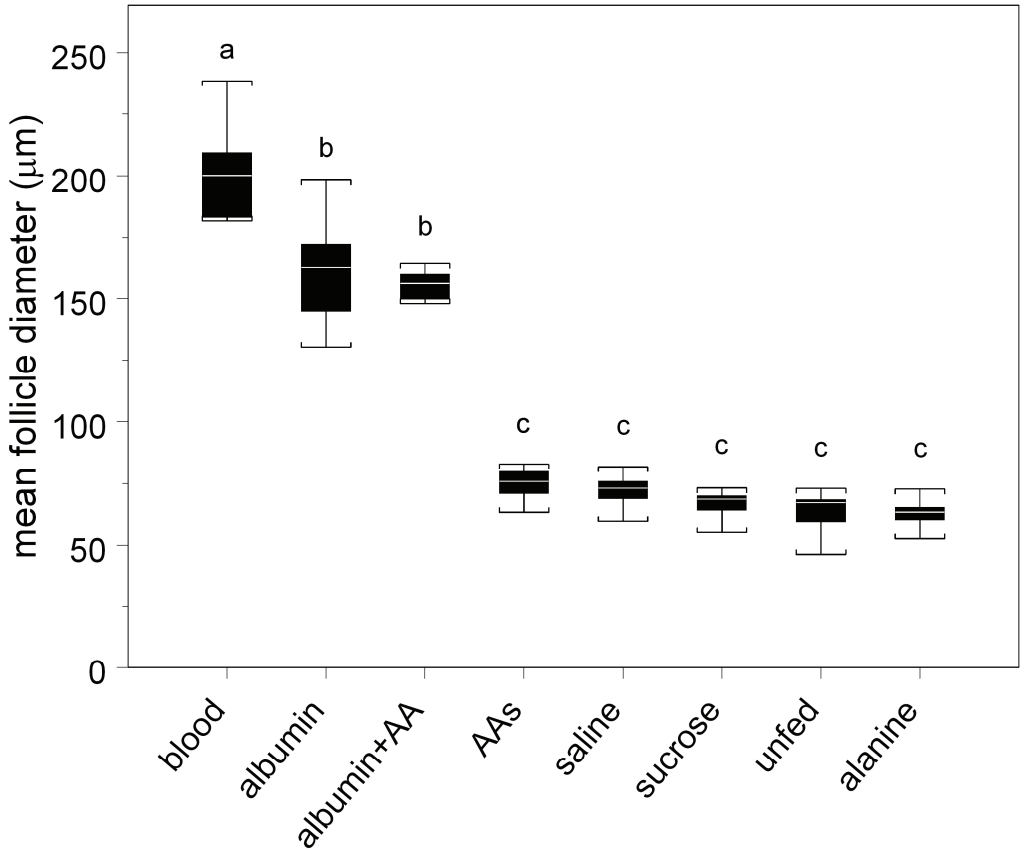

Figure 2.5 Mean follicle diameter of *An. gambiae* ovaries 24 h after feeding on either saline (n = 12), sucrose (n = 12), alanine (n = 13), albumin (n = 12), a mixture of amino acids (AAs, n = 15), albumin plus amino acids (albumin+AA, n = 10), or blood (n = 14). Unfed mosquitoes were included as controls.

In addition to blood, only the diets containing albumin were able to induce follicular development, although to a lesser extent. Lower case letters (a, b, c) indicate significant differences (ANOVA followed by Tukey pairwise comparisons using 95% confidence intervals). Box-plot explanation as in Fig. 2.2.

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 experiment, but 76% of "test" females (n = 38) lost any willingness to bite when offered human skin after manipulation. Eighty-eight percent of the "control" females (n = 25) were still induced by human skin to bite (Table 2.2). The association between treatment and biting behavior was significant (Pearson's chi-square test with Yates' continuity correction: $\chi_1 = 22.45$, $P < 0.0001$).

Table 2.2 Host-seeking inhibition by artificial abdominal distention. Treatments: "test" females were given a ca. 1 µl saline enema, whereas "control" females had the tip of the glass electrode inserted in the anus but no solution was injected. When the mosquitoes were exposed to human skin 2 h after manipulation the behavioral outcome was registered to be "not attracted" or "tried to bite" only when the situation was clear-cut. Numbers of mosquitoes provided in the table are followed by the percentage (within each treatment).

	Behavioral outcome	
Treatment	not attracted	tried to bite
test	29 (76.3%)	9 (23.7%)
control	3 (12%)	22 (88%)

The mRNA samples extracted from "test" and "control" mosquitoes obtained from two repetitions of this experiment were unfortunately found to be too degraded for use in gene expression assays. Figure 2.6 shows the outcome of the agarose gel electrophoresis verification of RNA integrity.

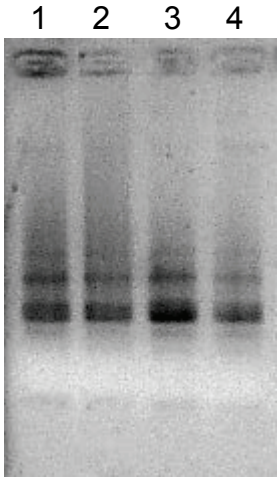

Figure 2.6. 1% agarose gel separation of 1 µg total RNA extracted from "test" (saline enema mosquitoes, lanes 1 and 3) and "controls" (lanes 2 and 4). All samples formed a smear and the gel shows 28S molecules broken down into different fragments. 16S molecules and derived fragments were apparently small enough to exit the gel during migration.

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. Both nectar- and blood-specific feeding modes have been described and clearly distinguished for several decades (e.g. Trembley 1952; Day 1954). Nectar meals are first directed to the crop, whereas blood meals are directly pumped into the midgut. The phagostimulatory nature of the food has been identified as the predominant factor controlling the feeding mode (Trembley 1952; Friend and Smith 1977), and sucrose solutions were shown to generally induce the nectar-feeding mode. Friend et al. (1988; 1989) and Schmidt and Friend (1991) showed that sucrose solutions of a concentration of 0.2 M or higher induced crop-only filling in nearly all tested *Culiseta inornata* mosquitoes. The sucrose concentration used in the present study was 0.29 M. The control of diet destination was shown in *C. inornata* to be mediated by the differential stimulation of chemosensory receptors on the

mouthparts (Friend et al. 1988; 1989). The fact that the mosquitoes tested in the present study did not feed on heated sucrose is consistent with the results of Friend (1978) who found that female *C. inornata* seldom fed on sugar solutions that were as warm as blood or contained behind a membrane. Friend (1978) argued that the elevated diet temperature and the presence of a membrane induced the blood-feeding mode and that in this mode the 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.

The fact that the artificial diets proposed were treated as either blood or plant nectar supports their validity as meaningful stimuli for the signaling pathway under study. Our 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 which 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 only 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.

Some effects of abdominal distention or, more precisely, gut distention, on early events in digestion physiology further support the signaling ability of this anatomical process. Lemos et al. (1996) found that trypsin mRNA accumulates in *An. gambiae* female gut within the first day of imaginal life and that gut epithelium distention induces the secretion of the enzyme into the lumen independently of the nutrient content of the meal. Abdominal distention caused by a nutrient-free meal was able to evoke an increase in gut aminopeptidase activity as a blood meal did, but the effect lasted only for the six first hours post feeding, whereas blood-meal induced aminopeptidase activity increased for more than 20 h (Lemos et al. 1996). The authors suggested that the "early" release of trypsin enzyme could serve to sense the presence of a protein substrate in the meal and subsequently produce a signal (small peptides or AAs) ultimately resulting in activation of late trypsin genes.

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.

By contrast to albumin-, AA- or saline-fed mosquitoes, sucrose-replete ones were never deterred from biting. During our assays sucrose solution was directed exclusively to the crop, whereas all other diets filled exclusively the midgut. Although all meals distended the abdomen, sucrose meals that arrived only as far as the crop may not have excited the midgut stretch receptors, nor the midgut endocrine cells. The existence of crop-associated stretch-sensitive neurons has still to be investigated in mosquitoes. Indeed, the consequence of a sucrose meal for female mosquitoes should theoretically elicit a different signaling mechanism than a blood meal, and especially in the ovaries, because sucrose alone cannot support egg development.

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.

Future studies would interestingly address the characterization of post-prandial refuge-seeking behaviors in female mosquitoes engorged on blood as well as on other diets of lower nutritive value.

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. 2.5). 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 alanine and even of a mixture of AAs to trigger follicular development: follicular development was observed 24 h after the meal neither in AA-, alanine- nor in saline-fed mosquitoes (Fig. 2.5). The absence of follicular development in alanine-fed mosquitoes is consistent with the findings of Attardo et al. (2006) that alanine is not among the AAs whose presence is essential for vitellogenesis (i.e. transcription of *Vitellogenin*). The AA level of 10 g/l used here corresponds to the midgut luminal content reported in *Ae. aegypti* 2 h after ingestion of a Kogan meal containing a total protein concentration of 125 g/l (Caroci and Noriega 2003). The need for the takeup of a defined set of AAs in the meal (and consequently in the hemolymph) as a condition for ovarian development and vitellogenesis was demonstrated by Uchida (1992; 1998) and Uchida et al. (2001) in several *Culex* and *Aedes* species and by Attardo et al. (2006) in *Ae. aegypti*. These critical AAs are leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, valine, cysteine, arginine, and asparagine (Uchida 1998). However, the mixture of AAs containing all of these critical AAs also failed to support egg development in our experiments with *An. gambiae*. The insufficiency of an AA meal for ovarian development in anopheline anautogenous mosquitoes was further 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 mosquitoes. Infusion of a mixture of 17 AAs resulted in activation of ovarian development in most *Culex* species (Uchida et al. 2001), whereas other species including *An. stephensi* showed low responses (Uchida et al. 2003). Induction of ovarian development in a high proportion (78%) of treated female *An. stephensi* required 48 h of infusion with a 10% (w/v) AA mixture and prior mating. The AA concentration used in the present study was 1% (w/v) in the feeding solutions and the extent of the consequent hemolymph AA titer increase is difficult to estimate. 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 to complete egg development that we observed in *An. gambiae* females fed this diet. Uchida et al. (2003) ascribed the different outcome in *An. stephensi* compared to most *Culex* species to the physiological differences between anophelines and culicines described by Briegel (1990a; 1990b, see also Chapter 1). 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.

In the field, another critical factor affecting vitellogenesis in *An. gambiae* is infection by malaria parasites. The rodent malaria parasite *Plasmodium yoelii nigeriensis* causes a significant reduction in *An. gambiae* fecundity with a 41% reduction in the number of eggs produced in the gonotrophic cycle immediately following an infective blood meal (Ahmed et al. 1999). This effect has also been observed in female mosquitoes naturally infected by *P. falciparum* (Hogg and Hurd 1997). Ahmed et al. (2001) stated that this was not the result of depleted nutrient intake as mosquitoes undergoing infection-associated fecundity reduction took same sized blood meals than other mosquitoes in their study, and knowing that digestion proceeded normally in those infected mosquitoes (Hogg and Hurd 1997; Jahan et al. 1999). The mechanism of fecundity reduction could reside in impaired vitellin uptake by the ovaries and subsequent follicle resorption (Ahmed et al. 2001).

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Chapter 3

Nutritional signaling

1. Introduction

Nutritional signaling in mosquitoes starts with food chemoreception (involving gustatory receptors), abdominal distention following ingestion then excites stretch receptors sending messages to the brain (see Chapter 2) and eventually amino acids in the meal are sensed by specific signaling pathways (as developed below). These pathways modulate many processes in several tissues should the meal contain proteins. Early proteolysis assumed by gut peptidases (e.g. early trypsin or carboxypeptidase) activates subsequent peptidases to complete digestion and meal-dependent (but still uncharacterized) signals are sent to the brain. The CNS and associated neurosecretory structures then send signals of nutritional status addressed to peripheral organs (e.g. juvenile hormone, insulin-like peptides [ILPs]) and to the central control of foraging (Shen and Cai 2001; Wu et al. 2005a; Wu et al. 2005b; Lingo et al. 2007). Circulating humoral neuro-endocrine factors (ILPs, ovarian ecdysteroidogenic hormone [OEH]) command the ovaries for ecdysone production and release. Simultaneously, fat bodies are mobilized for reserve build-up or protein synthesis which consequently acts on vitellogenesis and oogenesis in addition to the control of host-seeking behaviors (see Chapter 2). The cycle is closed by the renewed hunger sensation (empty midgut and crop, low reserves, low circulating factors such as ILPs and ATP) signaling to the brain to promote foraging behaviors anew and leading to another meal.

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 amino acid (AA) availability 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* have shown that the fat body functions as an AA sensor and that nutritional signals are transduced by the TOR signaling pathway (Colombani et al. 2003). An important mechanism by which TOR affects cell function is through translational regulation (Hay and Sonenberg 2004). p70 S6 kinase (S6K) belongs to the AGC family of protein kinases and is activated by TOR-mediated phosphorylation at threonine 389 in mammals and at the 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).

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*. These 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. 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 insulin-like peptide-releasing neurons was shown to mediate hunger regulation of food approach and consumptive behaviors, controlling both the quality and quantity of nutrient ingested (Wu et al. 2005a). These authors suggested that the conserved S6K signaling pathway might be crucial for regulating hunger-driven behaviors in diverse animals. 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. This rises the interest to study such signaling in a mosquito whose feeding habits determine malaria parasite transmission. An up-to-date representation of currently known components and interactions forming the TOR signaling network has been published by Soulard and Hall (2007).

Insect vitellogenesis and oogenesis has been extensively studied for a fairly long time (see Hagedorn and Kunkel 1979 as an early review; Briegel et al. 2003; Attardo et al. 2005). Production of yolk components by the fat body and of cellular components by the nurse cells and their import into the developing oocytes are among the key early steps.
A structural description of mosquito ovaries and follicles is given in Chapter 2. To help interpret the results a primary follicle is represented (Fig. 3.1).

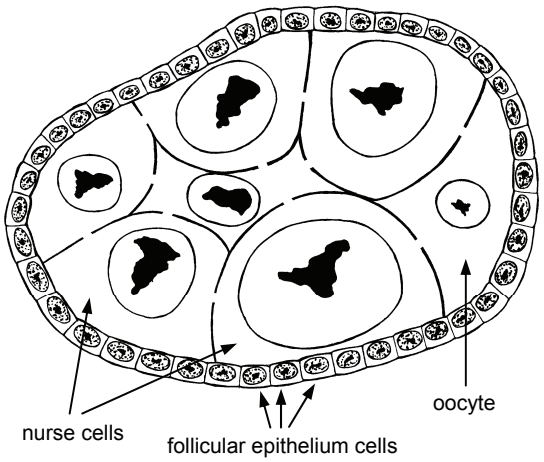


Figure 3.1 Schematic representation of a primary follicle at the pre-vitellogenic state. Seven nurse cells with large nuclei and one oocyte with a small nucleus are enclosed by hundreds of small follicular epithelial cells. The nurse cells and the oocyte are connected by inter-cellular bridges, thus forming a syncytium. Nucleoli are represented as black areas in each nucleus.

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 the *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 the early response of *tor*, *s6k*, *tsc2*, *inR*, *ilp3* and *ilp4* in *An. gambiae* females a few hours after different meals. The effects of albumin plus AAs,

sucrose and saline on the expression of these genes were determined by quantitative real-time PCR in head and abdomen extracts, and the activity of TOR and S6K in the ovary and brain following these meals or a blood-meal was determined by immunoblotting of phosphorylated TOR and S6K. In addition, the effect of these nutrient types on the presence and activity of both enzymes in ovarian and brain cells was determined by immunohistochemistry. Indeed, the level of S6K proteins that are phosphorylated on *Drosophila* threonine 398 (and 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 in *An. gambiae* (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. Immunolocalization of these enzymes provides data on the debated question of their sub-cellular localization and documents what was interpreted as indications of inter- and intra-cellular communication in follicular cells.

2. Materials and Methods

An. gambiae colonies were maintained as described in Chapter 1. The composition of the diets and the method followed to induce the female mosquitoes to feed are described in Chapter 2. *An. gambiae* gene sequences were retrieved as described in Chapter 1, and the gene expression assay methodology is also described in Chapter 1. The technical issues originating from RNA sample treatment with DNAse and from reverse transcription that were described in Chapter 1 also affected the results presented here. The genes whose products (transcripts or proteins) were addressed in this study are identified and the corresponding primer pairs are presented in Table 3.1.

2.1 Mosquito dissection

Mosquitoes were dissected 3 h following engorgement, after short chilling on ice, in cold Tris-buffered saline (TBS) to retrieve the ovaries and the brain (Fig. 3.2). Some blood-fed females were dissected only after 24 h and their ovaries were submitted to immunohistochemistry as described in Results. 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.

Table 3.1 Identification of the genes whose transcripts and/or proteins were investigated in the present study. 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 have since been described as such in the genome databases.

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[d]	AGAP004887	40S ribosomal protein S17	human, *Drosophila* and *Ae. aegypti 40S ribosomal protein S17*	For: 5'-TTAGCACAGAATGGGTCGTG-3' Rev: 5'-TGTTACGCAGTGGTTTCGTC-3'

[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)

[d] The use of a ribosomal protein-encoding gene as a reference in these gene expression assays could raise some concerns knowing that one of the consequences of the nutritionally regulated TOR signaling is to promote the transcription of ribosomal proteins. However, *S17* was already used as the reference gene in gene expression studies addressing the effect of multiple blood meals in *An. gambiae* from 3 h to 8 days after the first blood meal (Nirmala et al. 2005). In our assays *S17* mRNA levels never varied by more than 1 Ct unit among compared treatments and showed a high reproducibility within treatments. Furthermore, *S17* Ct values never showed a trend to be lower (corresponding to higher mRNA levels) in mosquitoes fed the albumin+AAs diet that should stimulate TOR signaling and ribosome synthesis the most.

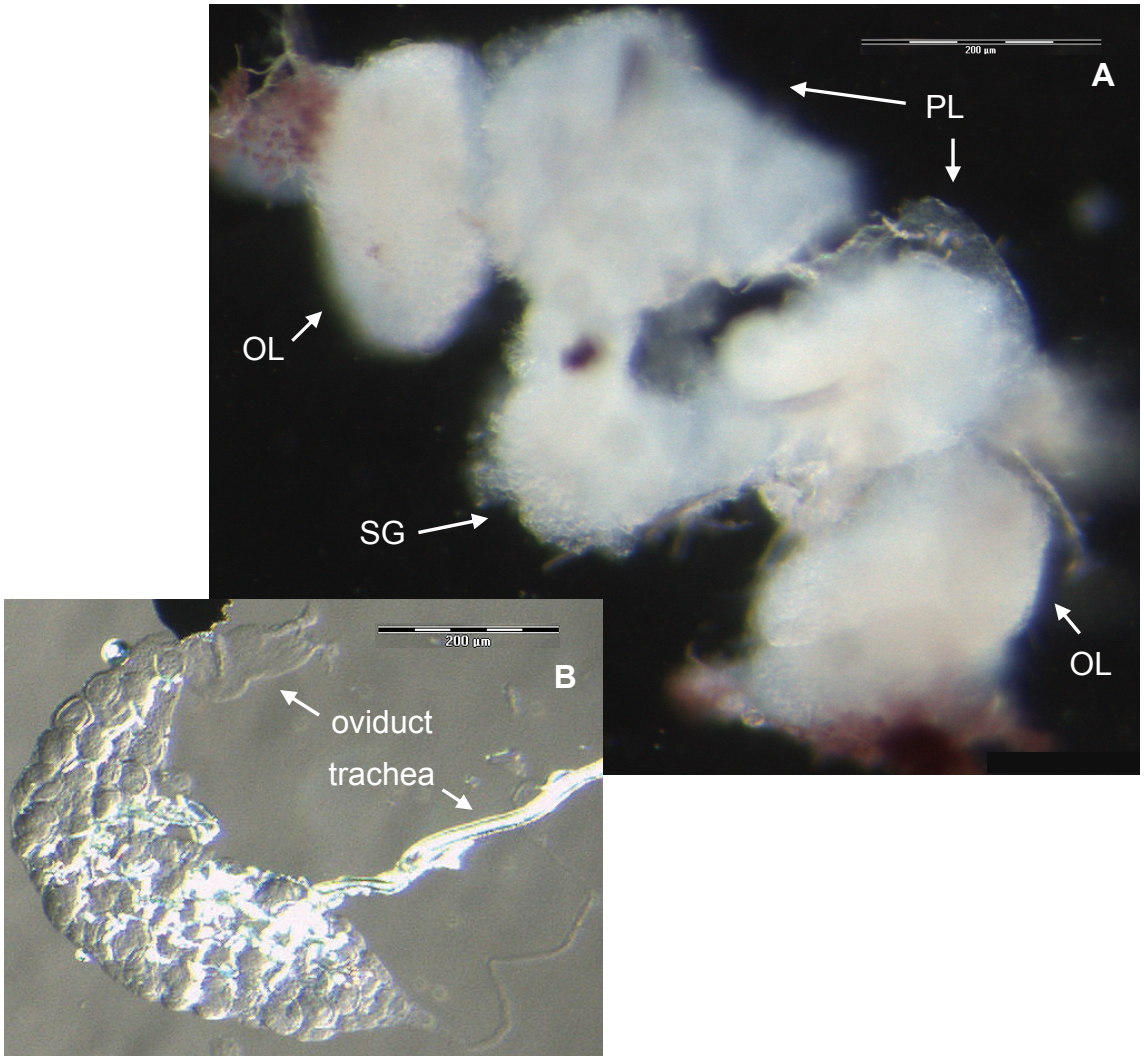


Figure 3.2 Micrographs of a brain (A) and an ovary (B) dissected from a female *An. gambiae*. Cerebral lobes are designated as follows on micrograph A: protocerebral lobes (PL), optical lobes (OL) and subesophageal ganglion (SG). All mouth and digestive tract parts were removed from the brain pictured.

2.2 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 used throughout this text for clarity.
Total protein extracts were prepared from 30 ovary pairs and 20 brains of blood-, albumin+AA-, saline-fed and unfed mosquitoes and were divided in two aliquots each. 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 13'400 rpm prior to separation by SDS-PAGE. Migration in 8% polyacrylamide gel lasted 90 min at 15 mA and transfer to the nitrocellulose membrane lasted 100 min at 300 mA, followed by membrane staining using Ponceau S to visualize any transfer. 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 (Thr398) rabbit polyclonal antibody (Cell Signaling Technology #9209, Table 3.2), or 1:500 of phospho-mTOR (Ser2448) rabbit monoclonal antibody (Cell Signaling Technology #2976, Table 3.2). 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.

2.3 Immunohistochemistry

Mosquito ovaries and brains dissected 3 h after feeding were transferred into drops of TBS on poly-L-lysine-coated glass slides and submitted to a whole-organ immunohistochemistry protocol modified from Brown and Cao (2001) and from Kaufmann and Brown (2006). During the following procedure the reagents were changed while the organs remained attached to the surface of the slide: tissues were fixed in 4% paraformaldehyde in TBS for 90 min at room temperature, permeabilized by applying 0.1% Triton X-100 in TBS for 30 min, washed twice in TBS for 10 min each, non-specific sites were then blocked using TBS-GS-T (5% goat serum SIGMA cat. nr. G 9023, 0.1% Tween-20 in TBS) for 2 h at 4°C. Next, the tissues were incubated overnight at 4°C in a 1:100 dilution of the primary antibody in TBS-GS-T. The primary antibodies used are listed and described in Table 3.2. After three 30-minute washings with TBS-GS-T at 4°C, secondary antibody was applied at 1:2000 dilution in TBS-GS-T to visualize primary labeling by confocal fluorescence microscopy. The secondary antibody was

Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG (2 mg/ml, Molecular Probes) and incubated overnight at 4°C. Negative controls consisted of tissues untreated with primary antibody. Tissues were washed 3 x 30 min with 0.1% Tween-20 in TBS at 4°C and mounted on the same slide in a 1:9 TBS:glycerol solution with a cover glass. All treatment combinations were replicated on several mosquitoes. Images were acquired with a Leica DM 6000 confocal microscope. All microscope settings remained unchanged between treatments of an experiment targetting one type of enzyme.

3. Results

3.1 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 3 h after (Fig. 3.3). 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. 3.4).

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. 3.3). 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 post-hoc pairwise comparisons using 95% confidence intervals [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. 3.3).

Table 3.2 Primary antibodies used in this study.

Antibody name	Type	Manufacturer	Target protein	Target sequence (immunogen according to manufacturer's data)	Applications for which it was optimized by the manufacturer	Suitability for use on mosquito samples[a]
Phospho-mTOR (Ser2448) (49F9) Rabbit mAb (IHC specific)	rabbit monoclonal	Cell Signaling Technology #2976	mTOR, phosphorylated on human serine 2448	residues surrounding phosphorylated human mTOR serine 2448	immunohistochemistry on frozen or Paraffin sections	yes (phospho-specific, target sequence conserved among organisms)
mTOR Antibody	rabbit polyclonal	Cell Signaling Technology #2972	mTOR	residues surrounding serine 2481 of human mTOR	western blotting, immunoprecipitation	no (target sequence different between mammals and insects)
Phospho-Drosophila p70 S6 Kinase (Thr398) Antibody	rabbit polyclonal	Cell Signaling Technology #9209	p70 S6 kinase, phosphorylated on *Drosophila* threonine 398	residues surrounding phosphorylated *Drosophila* p70 S6 kinase threonine 398	western blotting	yes (phospho-specific, target sequence conserved)
Rabbit (polyclonal) Anti-p70S6 Kinase [pThr^{389}] Phosphospecific Antibody, Unconjugated	rabbit polyclonal	BioSource #44-920G	mammalian p70 S6 kinase phosphorylated on threonine 389	region of human p70 S6 kinase that contains threonine 389	western blotting	yes (phospho-specific, target sequence conserved)
p70 S6 kinase (C-18)	rabbit polyclonal	Santa Cruz Biotechnology #sc-230	vertebrate p70 S6 kinase	residues at the C-terminus of rat p70 S6 kinase	western blotting, immunoprecipitation, immunocytochemistry	no (target sequence different between mammals and insects)

[a] according to verifications by western blotting and immunogen sequence comparison with *An. gambiae* sequences (see Results and Discussion).

In abdomen samples *tor* mRNA levels were only 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. 3.4). 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.). All significant differences described here amount to ca. 1 ΔCt unit between the means, that is, a theoretically maximal mRNA level ratio of 2.

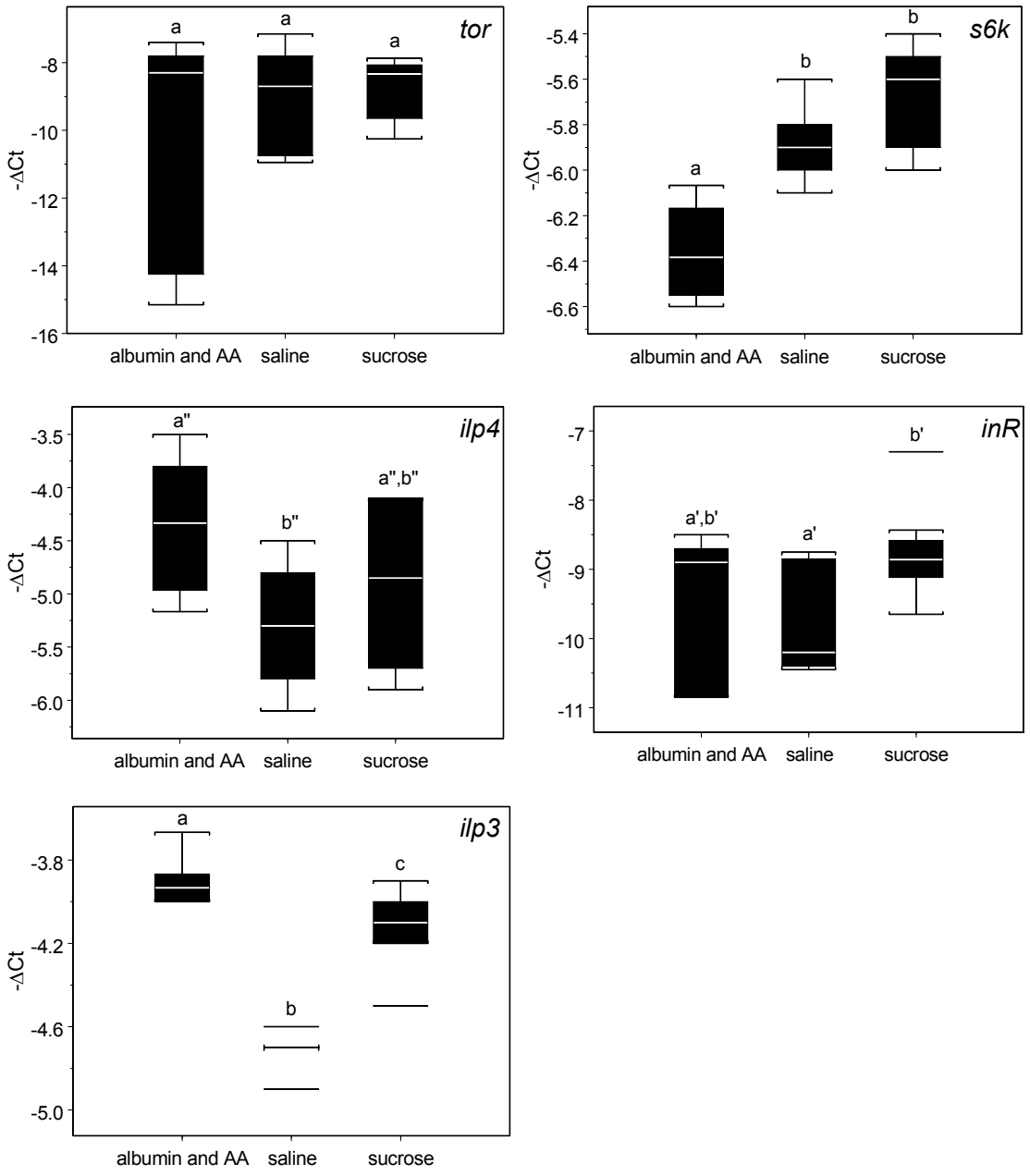


Figure 3.3 (previous page) Effect of diet on the gene expression of *tor*, *s6k*, *inR*, *ilp4* and *ilp3*, members of the insulin and TOR signaling pathways in the heads of female *An. gambiae* 3 h after different meals. Assessment of mRNA levels in total RNA extracts from heads of mosquitoes fed on 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 to the mRNA level (Ct value) of the gene of interest in each replicate in each treatment (see Materials and Methods for details). The sign of the ΔCt values was inverted so that a higher value corresponds to a higher mRNA level. 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. Additional bars represent outliers. 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.

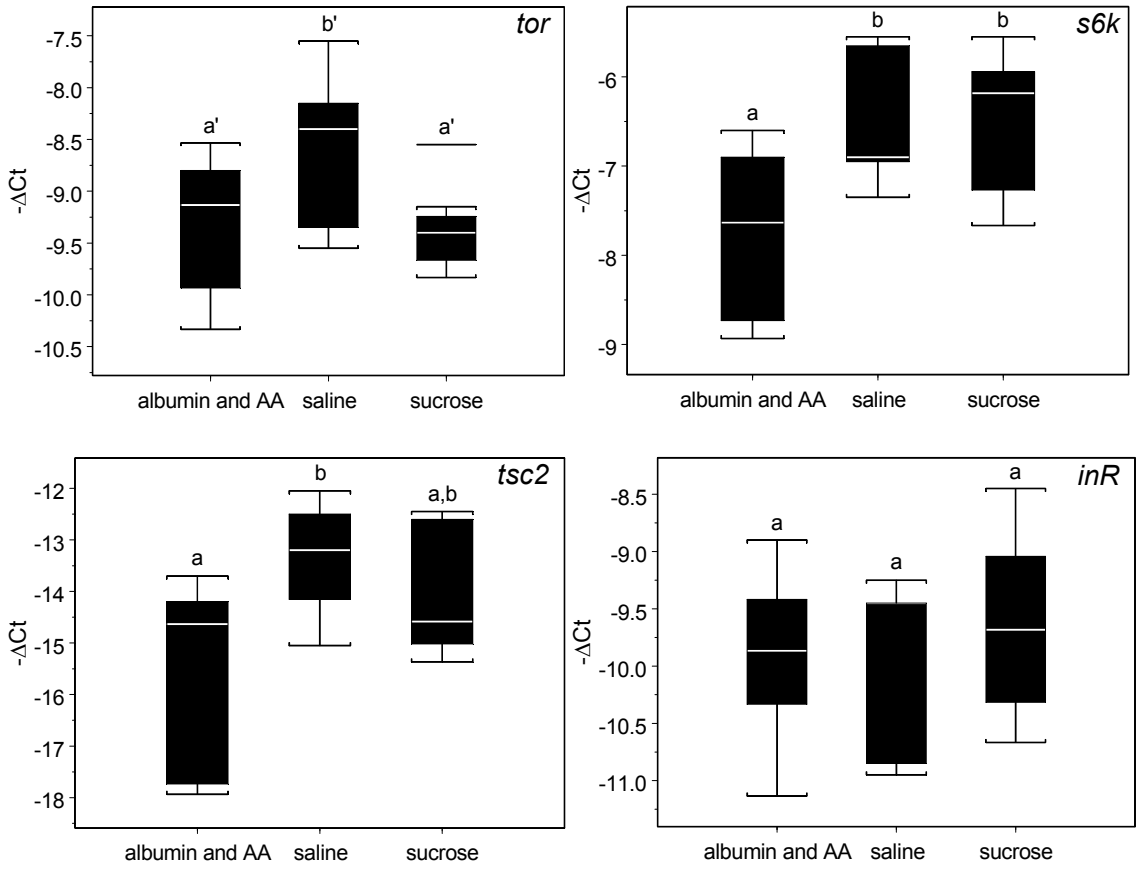


Figure 3.4 Effect of diet on the gene expression of *tor*, *s6k*, *inR*, and *tsc2*, members of the insulin and TOR signaling pathways in the abdomens of female *An. gambiae* 3 h after different meals. Assessment of mRNA levels in total RNA extracts from abdomens of mosquitoes fed on either albumin+AA, saline, or sucrose was carried out and presented as explained in the legend to Fig. 3.3.

3.2 TOR and S6K activity depending on nutritional stimulation (diet presence and composition)

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. The levels and localization of these enzymes were also investigated through whole-mount immunohistochemistry on entire ovaries and brains of mosquitoes treated the same way. Secondary antibody-only controls systematically resulted in total absence of labeling.

3.2.1 pTOR level in the ovary depending on diet composition

By means of western blotting, detectable levels of phosphorylated TOR proteins were only found in ovary extracts from fed mosquitoes (Fig. 3.5) 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 and not in any brain sample, but in the latter case it is probably due to an insufficient transfer of heavy proteins from the SDS-PAGE gel to the nitrocellulose membrane, as confirmed by post-transfer coloration of the gel. Immunohistochemical detection of pTOR was only achieved in ovaries and no difference between treatments was observed (see below under “Oocyte-nurse cells exhanges”).

3.2.2 pS6K level in ovary and brain depending on diet composition

3.2.2.1 pS6K levels in ovary and brain as detected by western blotting

Phosphorylated S6K was detected in the ovary extracts from all treatments at levels which increased along with the nutritive value of the meal (Fig. 3.5). 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. 3.5). 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.

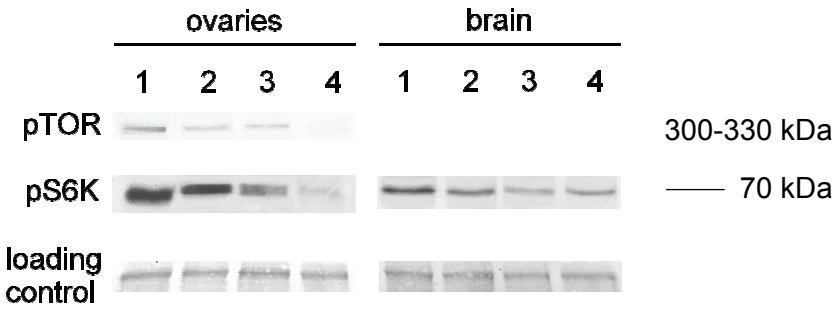


Figure 3.5 Activation of the TOR / S6K pathway following ingestion of different diets by *An. gambiae* females. Ovaries and brains were dissected 3 h after the mosquitoes engorged themselves on blood (1), albumin+AA (2) or saline (3). Unfed mosquitoes (4) were included as controls. Total protein extracts were prepared from 30 ovary pairs and 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. pS6K was detected at a molecular weight of 70 kDa, as expected, whereas the bands labeled by the pTOR-specific antibody were above the heaviest weight marker (200 kDa) and could correspond to ca. 300-330 kDa. Portions of the nitrocellulose membranes stained with Ponceau S after transfer are shown as controls for loading homogeneity.

3.2.2.2 pS6K immunohistochemistry

Immunohistochemistry using the phospho-Drosophila p70 S6 Kinase (Thr398) antibody revealed labeled areas at the surface of nurse cell nuclei in ovaries of mosquitoes fed one of the different diets or unfed and dissected 3 h after the meal (Figs. 3.6-7). The localization of these labeled areas is described below under "Nucleus-cytoplasm exchanges in nurse cells". By observing many organs from all treatments, we noted that the strongly labeled areas were present in a larger number of primary follicles in blood- and albumin+AA-fed mosquitoes than in saline-fed or unfed ones (Fig. 3.6). By contrast, strongly labeled nurse cell nuclei were found in nearly all secondary follicles in all treatments (Fig. 3.7). Unfortunately, the method failed to stain any structure in brains. Increased primary antibody concentration or incubation time could have helped, but it should be kept in mind that this antibody was developed and optimized by the manufacturer for western blotting applications. We tried to carry out immunohistochemical assays on pieces of fat body (isolated or still attached to the abdominal wall), but our protocol was not suited for these mainly lipidic organs which were dissolved and washed away by the Triton X-100 permeabilizing.

In preliminary experiments use was made of another antibody designed for recognizing mammalian phospho(Thr^{389})-p70 S6 kinase (described in Table 3.2). The same experimental design was followed as described above, but with two additional treatments: 10% sucrose and 10 g/l L-alanine in saline. pS6K was detected in the nucleus of all follicular, nurse, and oocyte cells. The higher the nutritive value of the diet the stronger the labeling of phospho-S6K in the ovaries of the mosquitoes (Fig. 3.8). The fluorescence level was maximal in blood-fed mosquitoes, slightly lower in albumin+AA-, sucrose- and alanine-fed ones, and clearly lower in saline-fed mosquitoes, whose ovaries exhibited a very uneven labeling intensity among follicles. Unfed mosquitoes exhibited only a barely detectable phospho-S6K labeling. The suitability of this antibody for mosquito samples was questioned (as discussed below) but we concluded that our immunohistochemistry results were not impaired and could be interpreted.

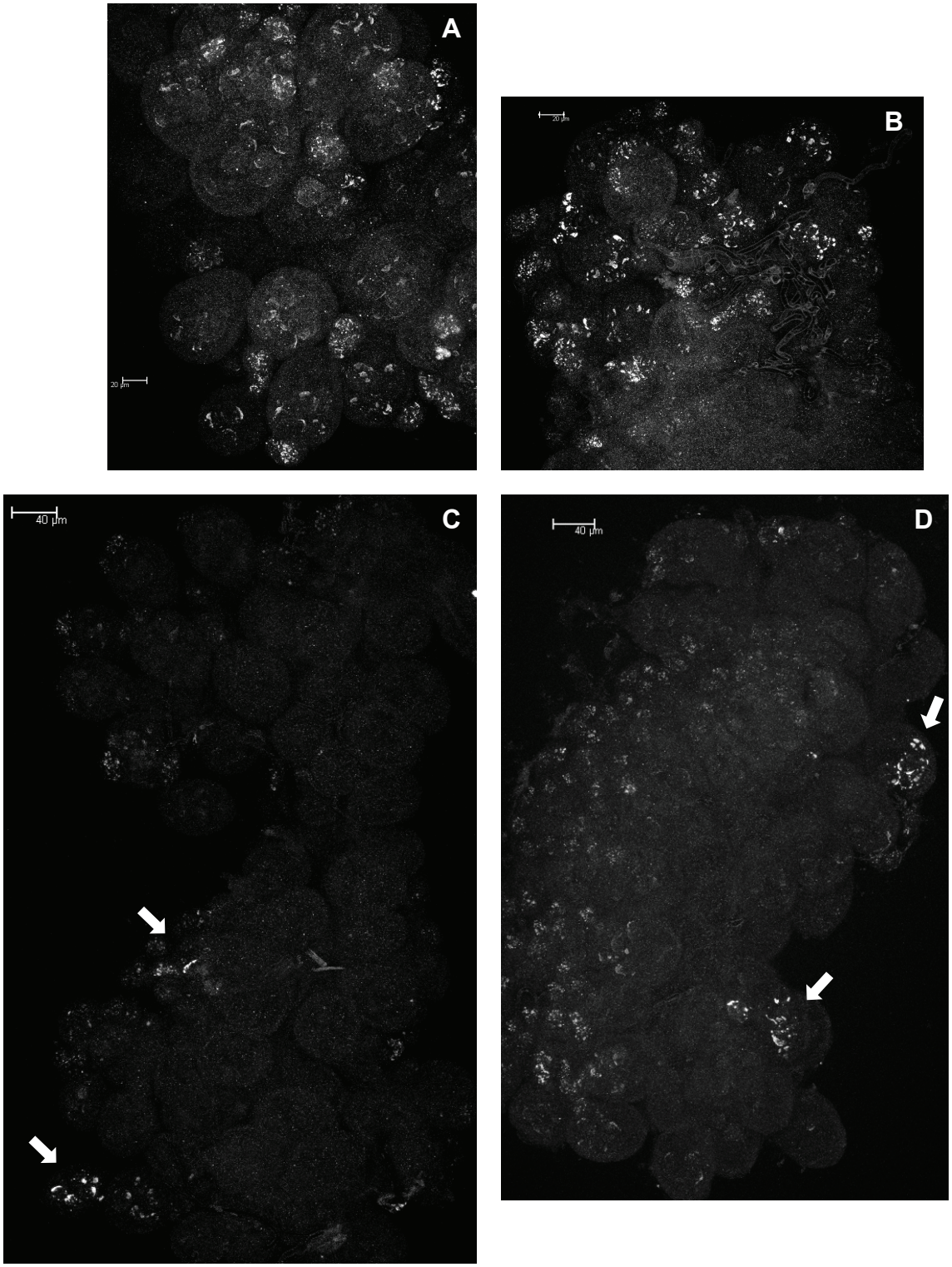


Figure 3.6 Confocal micrographs showing immunostaining of phospho(Thr^{398})-p70 S6 kinase proteins in ovarian cells 3 hours after *An. gambiae* females had fed on either blood (A), albumin+AAs (B) or saline (C), and in unfed females (D). These micrographs result from the superimposition of 10 to 20 layers. When present, labeled proteins were localized in the nucleus of nurse cells. Nearly all secondary follicles were labeled, but the number of primary follicles hosting strong pS6K labeling varied depending on the

diet and was higher in blood- and in albumin+AA-fed mosquitoes than in saline-fed and unfed ones (only at white arrows on C and D). This is based on the observation of many samples, which is difficult to illustrate comprehensively on a single figure. Scale bars represent 20 µm on micrographs A and B, and 40 µm on micrographs C and D. Micrographs A and B were taken using a 40x objective and micrographs C and D using a 20x objective.

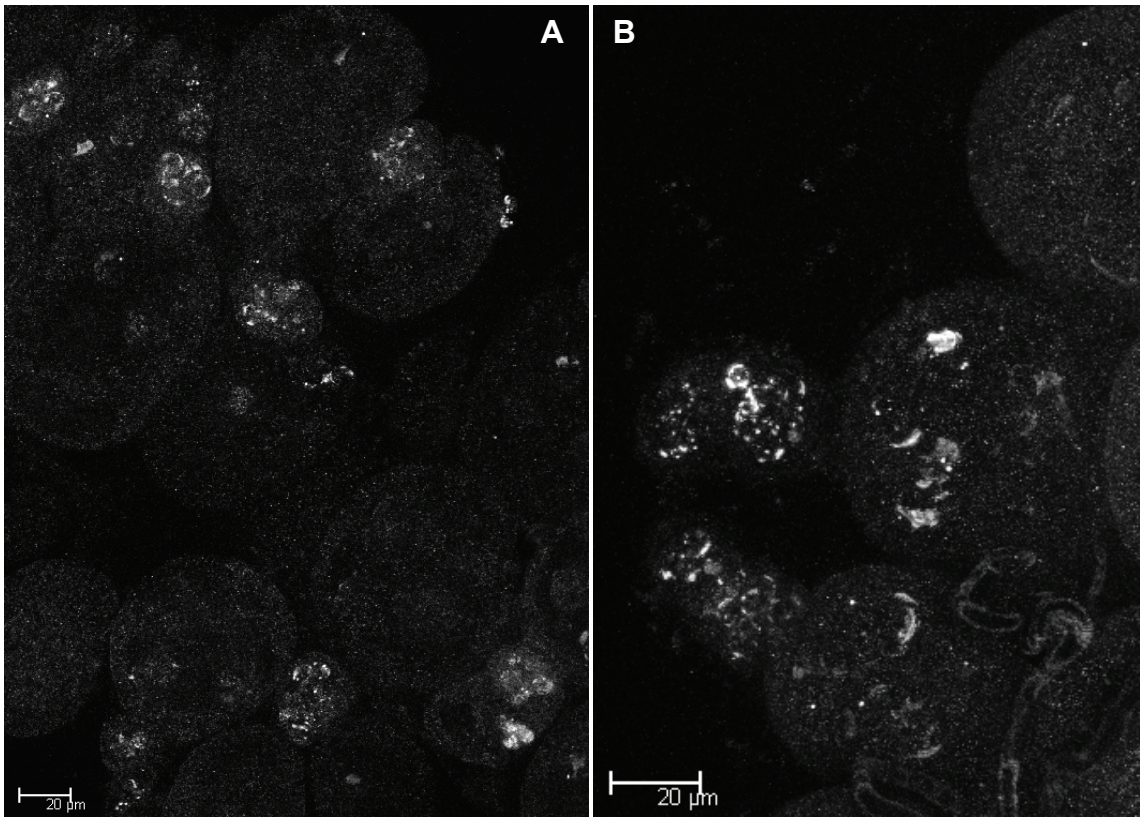


Figure 3.7 Confocal micrographs showing immunostaining of phospho(Thr^{398})-p70 S6 kinase proteins in secondary ovarian follicles of blood-fed *An. gambiae* females 3 hours after the meal. Micrographs A and B were each made of a single layer. All nurse cell nuclei bore strongly labeled areas, regardless of treatment. This could be interpreted as a sign of intense transcriptional and translational activity in these cells and be related to follicular maturation (see text).

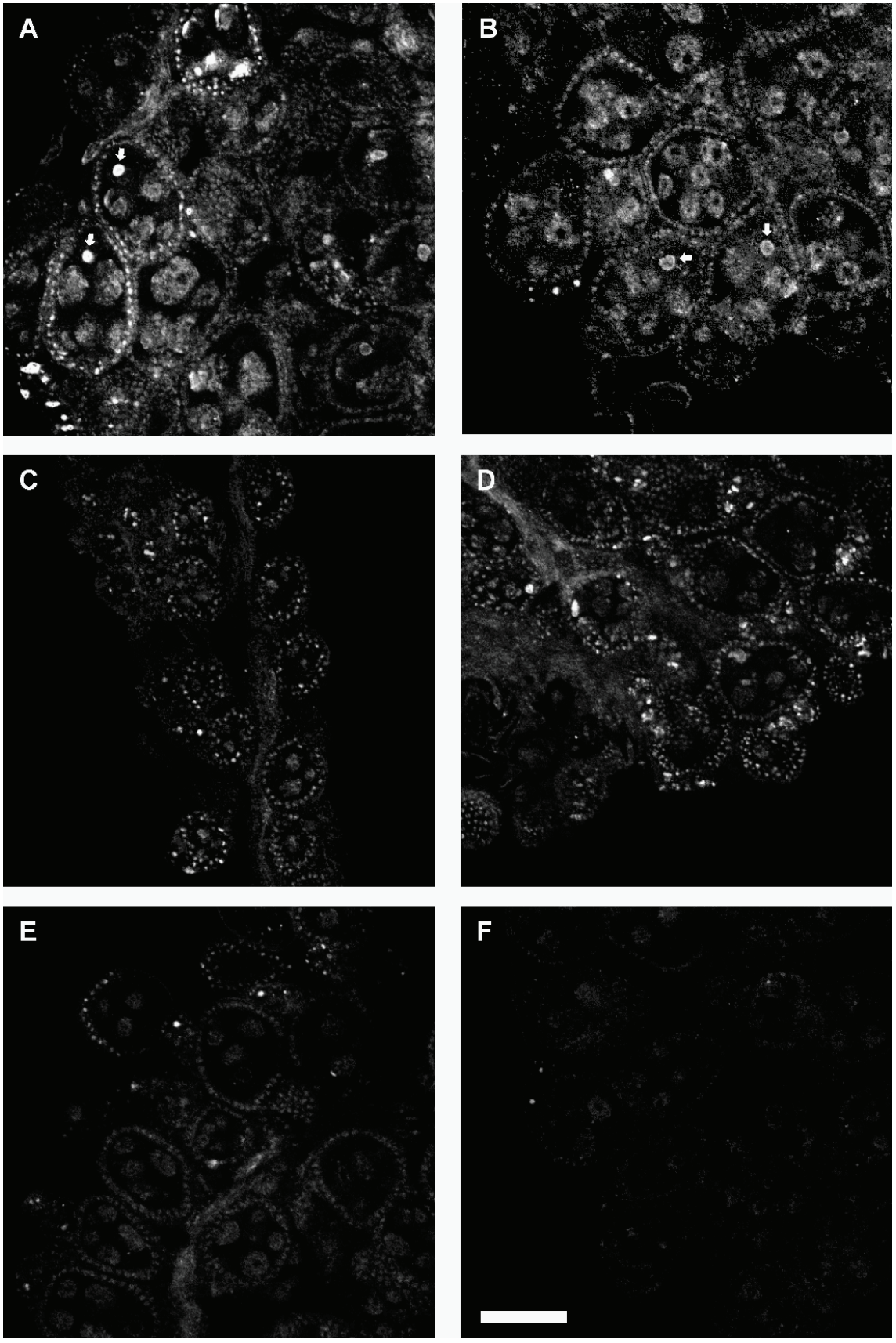

A
B
C
D
E
F

Figure 3.8 (previous page) Confocal micrographs showing immunostaining of phospho(Thr^{398})-p70 S6 kinase proteins in ovarian cells 3 hours after *An. gambiae* females had fed on either blood (A), albumin plus amino acids (B), alanine (C), sucrose (D), saline (E), or unfed (F). In this preliminary assay we used the mammalian p70 S6K phospho-specific antibody described in Table 3.2. When present, labeled proteins were localized in the nucleus of all follicular cells and in larger amounts in oocyte nuclei (white arrows on A and B). Phospho-p70 S6 kinase levels varied to a large extent depending on the diet and were much higher in blood- and in albumin plus AA-fed mosquitoes. All micrographs are single-layer and were taken with a Leica DM RBE confocal microscope. Scale bar represents 50 μm on all micrographs.

3.3 Indications of inter- and intracellular communication in follicular cells following nutritional input

3.3.1 Oocyte-nurse cells exchange: pTOR and "pseudo-TOR" immunolocalization

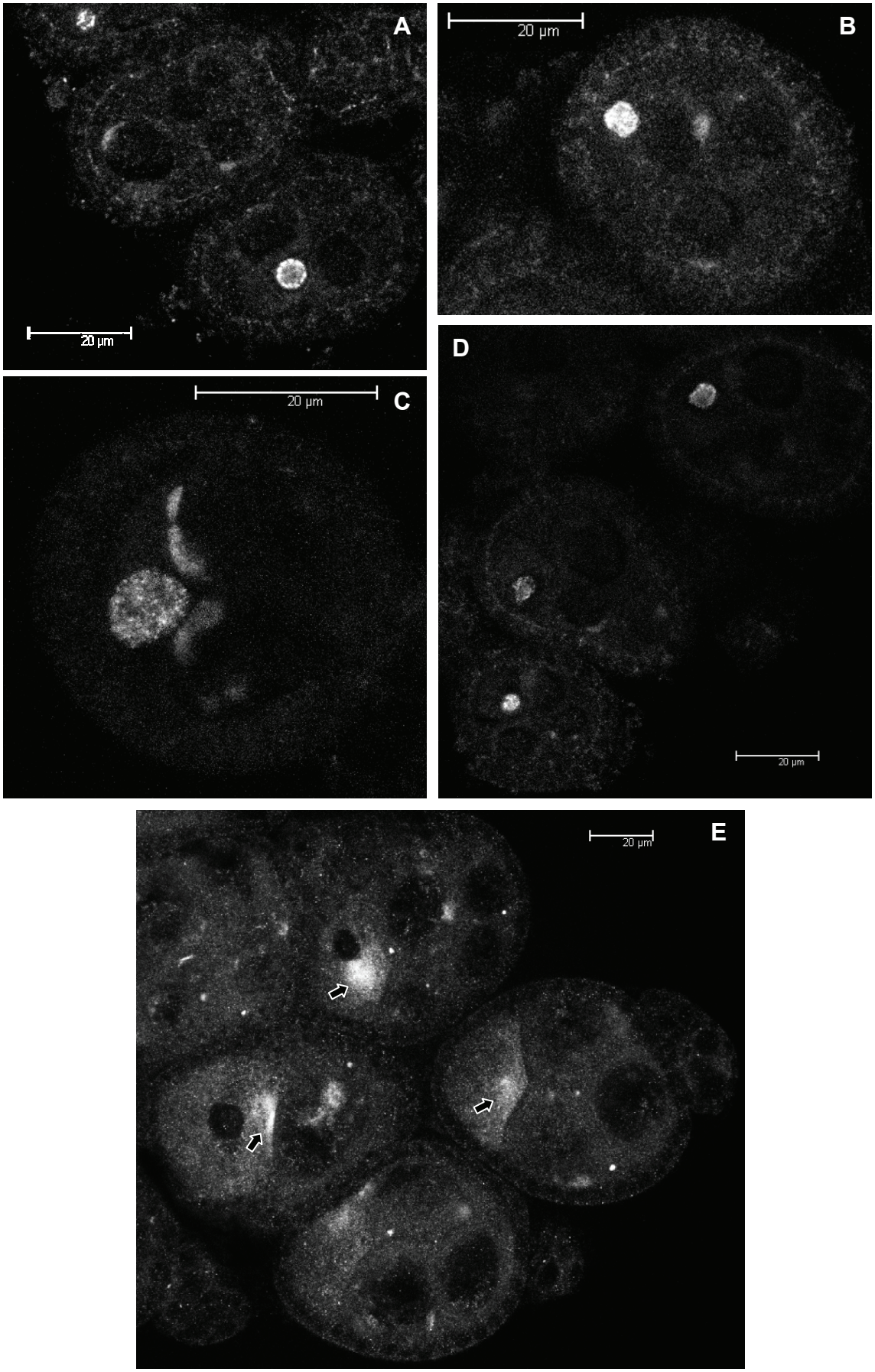
Immunohistochemistry using the phospho-mTOR (Ser^{2448}) rabbit monoclonal antibody on ovaries of *An. gambiae* females fed one of the different diets described above or unfed revealed consistant labeling of primary follicle oocyte nuclei, follicular epithelium cells cytoplasm and some areas at the surface of nurse cells nucleus (Fig. 3.9). No difference was observed between diet treatments. Interestingly, in some follicles the labeled areas at the periphery of nurse cell nuclei were orientated toward the oocyte (Fig. 3.9C). These could indicate molecular exchange between oocyte and nurse cells.

In preliminary experiments we made use of another antibody that was designed for recognizing mammalian TOR regardless of its phosphorylation state ("mTOR antibody" described in Table 3.2). Following the same experimental design described in Materials and Methods, primary follicles of blood-fed females showed strong labeling in the oocyte cytoplasm, especially in the area between oocyte nucleus and the borders with the neighboring nurse cells (Fig. 3.9E). Furthermore, ovaries of blood-fed females that were dissected 24 h after feeding showed labeling emanating from the primary follicle oocyte nuclei toward the nurse cells (Fig. 3.10) and some nurse cell nuclei bore oocyte-orientated labeling at their borders (Fig. 3.10A). Such orientated labeling patterns could also indicate communication between oocyte and nurse cells. By using this antibody we noticed a strong effect of feeding on the labeling intensity in ovarian cells (Annex A2). We also used this antibody on midgut preparations from female mosquitoes fed on different diets to detect eventual labeling of midgut-associated neurons and endocrine

cells, but no labeling was ever detected. Several verifications led us to question the suitability of this antibody for mosquito samples (as discussed below).

Figure 3.9 (next page) Confocal micrographs showing immunostaining of phospho(Ser^{2448})-TOR proteins in primary follicle cells 3 hours after *An. gambiae* females had fed on either albumin+AAs (A, B and D) or sucrose (C). Oocyte nucleus was strongly labeled in nearly all primary follicles in all treatments (including unfed mosquitoes), and labeling was also present in follicular epithelium cells cytoplasm, as well as in some areas at the surface of nurse cell nuclei. These areas were sometimes orientated toward the oocyte (C), indicating potential communication between nurse cells and the oocyte. Micrograph E shows the labeling obtained by using a mammalian TOR antibody that is indifferent to the phosphorylation state of this enzyme ("mTOR antibody" described in Table 3.2). Despite uncertainty regarding the labeled protein identity (see text), labeling was mainly present in oocyte cytoplasm (at least in blood-fed mosquitoes, see Annex A2) and was especially strong between the oocyte nucleus and the nurse cells (arrows on micrograph E), which may also indicate oocyte-nurse cell communication. All micrographs are single-layer.

Figure 3.10 (2 pages forward) Confocal micrographs of *An. gambiae* female ovaries dissected 24 hours after a blood meal and labeled with a primary antibody designed to bind mammalian TOR regardless of its phosphorylation state ("mTOR antibody" described in Table 3.2). Despite uncertainty regarding the labeled protein identity (see text), labeling was notably present as emanating from primary follicle oocyte nuclei in the direction of nurse cells (white arrows). In one follicle (A, top left, white arrows) a nurse cell nucleus and the oocyte showed labeling orientated toward each other. These orientated labeling patterns could indicate exchange between oocyte and nurse cells. Micrographs are single-layer.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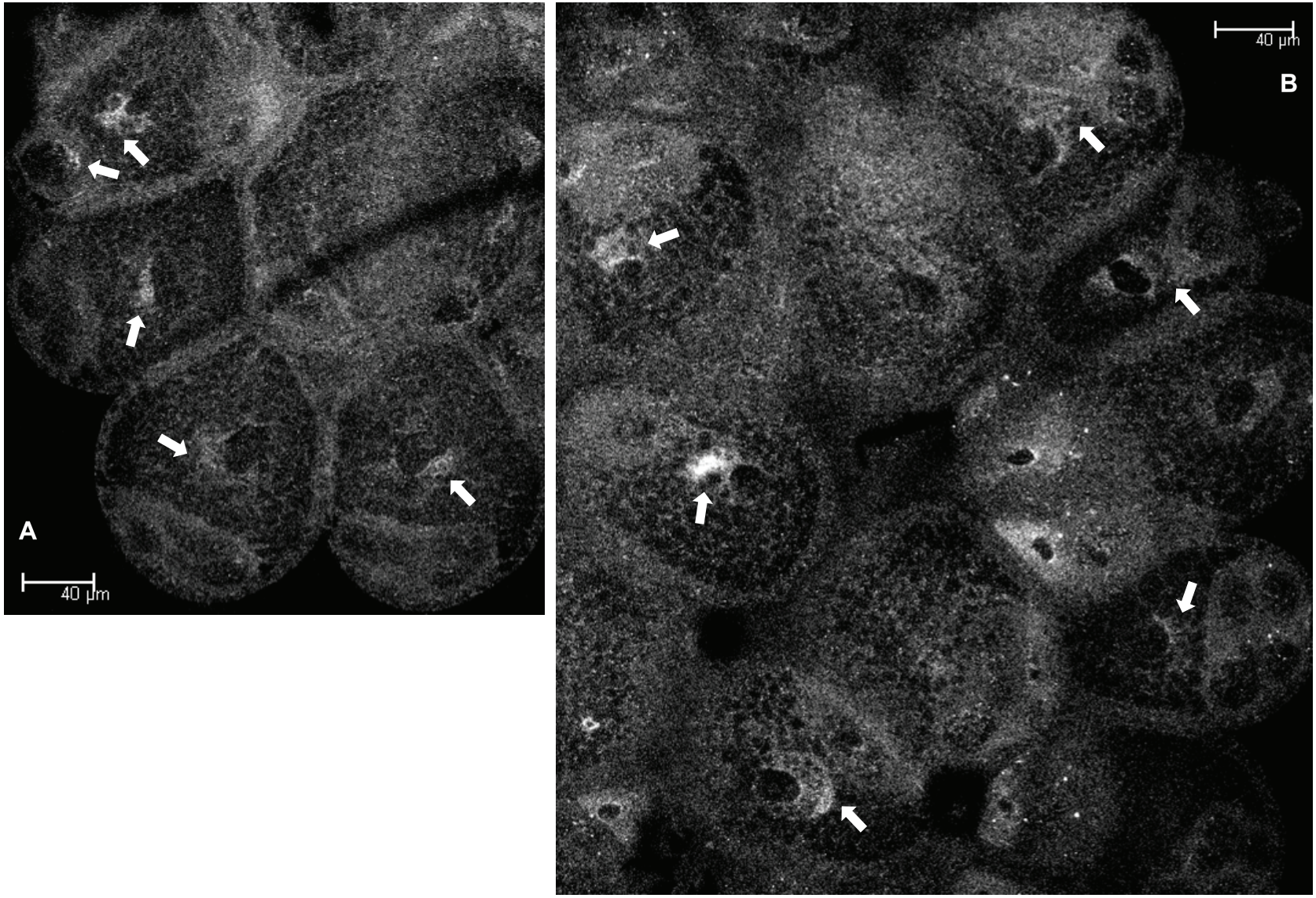
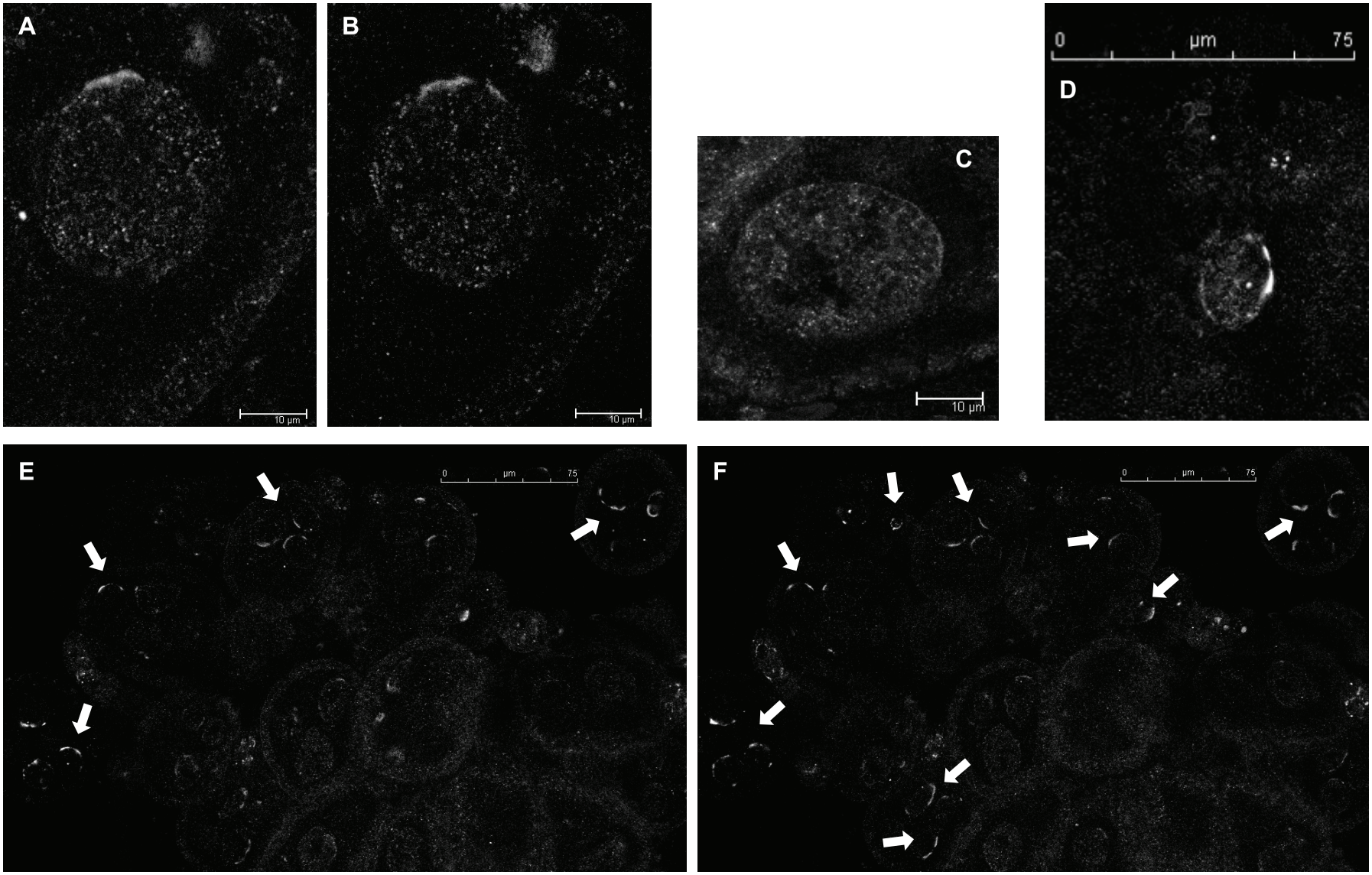

A
40 µm
B
40 µm

3.3.2 Nucleus-cytoplasm exchange in nurse cells: pS6K and "pseudo-S6K" immunolocalization

Phosphorylated S6K, as detected by immunohistochemistry using the phospho-Drosophila p70 S6 Kinase (Thr398) antibody, was located at the surface of primary follicle nurse cell nuclei as a fine network enveloping the nucleus (all nurse cells, Fig. 3.11A and B), sometimes forming ring-shaped labeling (Fig. 3.11D-F), and as strongly labeled areas emanating from some nuclei like *aurorae boreales* (not in all primary follicles, Fig. 3.12). These emanations could reveal molecular exchange between the nucleus and cytoplasm of primary follicle nurse cells.
Preliminary experiments made use of another antibody targeting the mammalian p70 S6 kinase regardless of its phosphorylation state ("p70 S6 kinase C-18" described in Table 3.2). These assays also revealed honeycomb-like networks at the surface of the nurse cells nuclei (Fig. 3.11C). However, the suitability of this antibody for mosquito samples was later questioned (as discussed below).

Figure 3.11 (next page) Confocal micrographs showing the immunolocalization of phospho(Thr^{398})-p70 S6 kinase proteins in the nuclei of primary follicle nurse cells. Two types of labeling are illustrated here: the networks enveloping the nuclei (A, B and C) and the ring-shaped labeling observed delimiting some nuclei (D, E and F). Micrograph C shows a nurse cell nucleus labeled with the total p70 S6 kinase antibody (C-18) used in preliminary experiments described in the text (Results, section 3.3.2).

Figure 3.12 (2 pages forward) Confocal micrographs showing the immunolocalization of phospho(Thr^{398})-p70 S6 kinase proteins in the nuclei of primary follicle nurse cells. Some nuclei showed labeled emanations at their surface (arrows on micrographs A and G, with detailed views on the other micrographs). This might be interpreted as a sign of nucleo-cytoplasmic exchange (see text). Micrographs A and G are superimpositions of 10 to 15 layers, whereas all others are single-layer. Micrographs A to F represent the same follicle from an unfed mosquito, whereas another follicle from a saline-fed one is shown on micrographs G to J. This type of labeling was found in all treatments. Micrographs B to F share the same scale shown on B, and micrographs H to J share the scale shown on H and I.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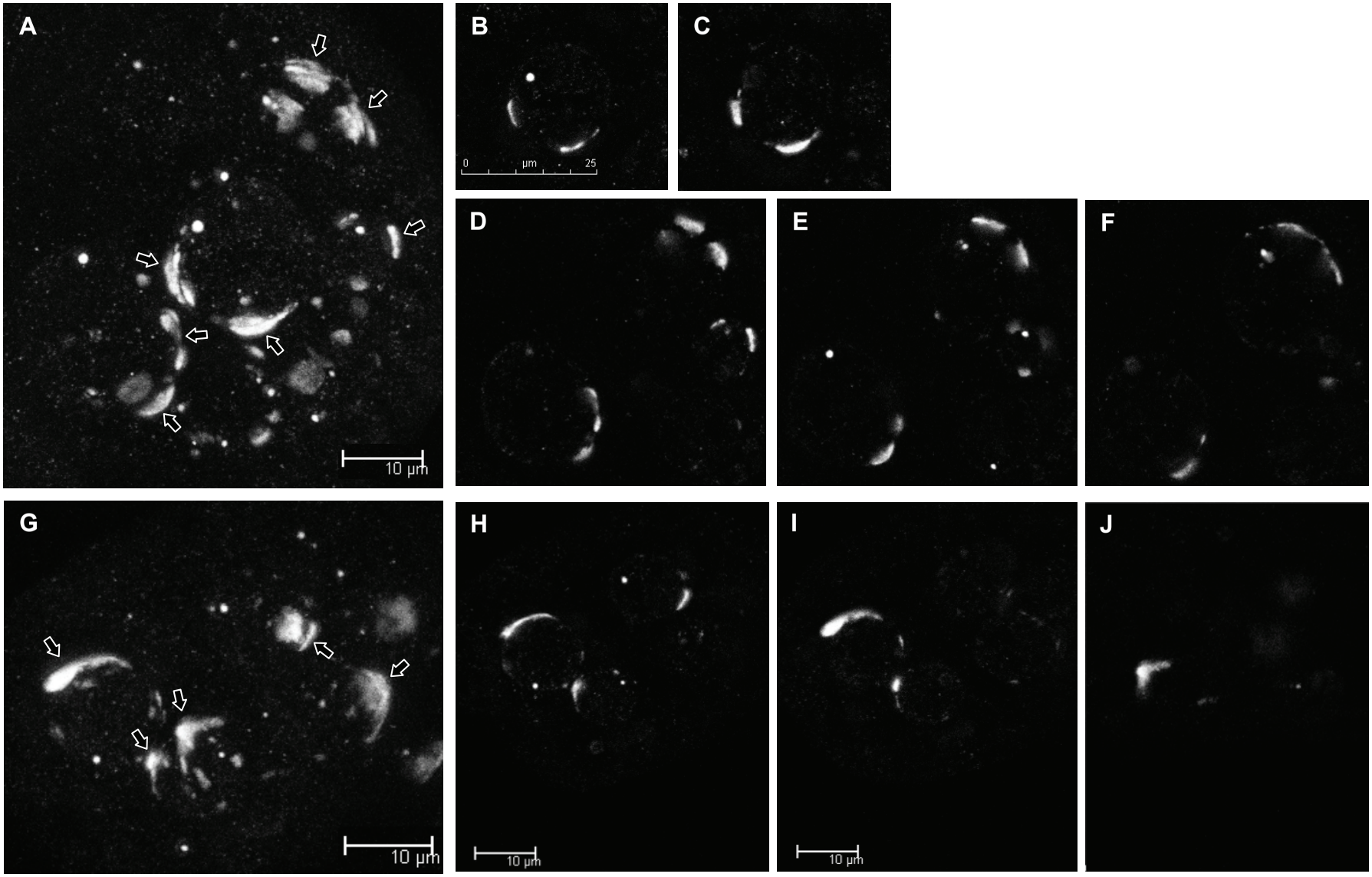
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H
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4. Discussion

4.1 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* 3 h after an albumin+AA meal were the decreased *s6k* and increased *ilp3* 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 of 2 between treatments (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 (with 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 *insulin* gene expression in the pancreas via an autocrine loop (Xu and Rothenberg 1998). However, the *An. gambiae* cerebral insulin pathway may be more strongly influenced by sucrose than by albumin+AA as the *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. (2005a). Insulin receptor transcription in *Ae. aegypti* ovaries was shown to vary during the gonotrophic cycle with peaks just after a 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 (see Chapter 2), this suggests 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 in Chapter 2).

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 then 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 begins 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 levels 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.

4.2 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 level 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 western blotting 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 level 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 (but migrates as if it weighed more than 300 kDa) and notable amounts (similar among treatments) of proteins heavier than 250 kDa remained in the polyacrylamide gel despite allowing long transfer times. Secondly, the phospho-specific antibody to pTOR was optimized by the manufacturer for immuno-histochemistry on frozen or paraffin-embedded sections and had never been tested for western blotting. This could also explain the difficulty experienced to label pTOR proteins in this study where we used whole-mount immunohistochemistry. In this context, additional method optimization might have helped achieve a better labeling. An additional factor is that this antibody was designed to recognize the human sequence surrounding the phosphorylated serine 2448, which shows some differing residues compared to mosquito sequence. This may have lowered the detected levels, but the detected protein identity is most probably trustworthy, as this antibody is monoclonal and phospho-specific.

The stronger labeling of pS6K found through western blotting of 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 the

activity of TOR and, as a consequence, that of S6K in the ovaries within just 3 h from the fasting state where TOR and S6K activity was very low, thus illustrating 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 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 (see Chapter 2). Although saline meals did not allow follicular development, but repressed post-prandial willingness to bite (see Chapter 2), ovaries responded by increasing S6K activity compared to the unfed status. These changes were mirrored in our immunohistochemical assays, especially when using the mammalian p70 S6 kinase phospho-specific antibody. By using the *Drosophila* antibody we found that ovarian pS6K labeling was more frequent in mosquitoes fed a protein-rich diet than in others, but no difference was noticed between saline-fed mosquitoes and unfed ones. The contrast between the results obtained with these two antibodies may arise from differing protocol requirements and suitability to the use on whole organs. Both were designed for western blotting applications. A phospho-specific antibody designed for mammals (Upstate #07-018) was already used by Hansen et al. (2005) to detect pS6K in *Ae. aegypti* fat bodies and ovaries by western blotting. Indeed, this phosphorylation site and the surrounding residues are well conserved among animals. However, when we used our phospho-specific mammalian S6K antibody for western blotting on *An. gambiae* ovarian samples, many bands were labeled in addition to the expected pS6K. We observed that the p70 S6K and its p85 splice variant were recognized and that their levels were very high in blood-fed mosquitoes and extremely low in unfed ones. The other bands were present at higher protein weights (between ca. 100 and 200 kDa) and everything heavier than ca. 200 kDa was labeled. This “parasitic” labeling was very strong in all treatments, even in unfed mosquitoes. We conclude that all these heavier proteins were not labeled in the immunohistochemical assays as the latter revealed no strong labeling in ovaries of unfed mosquitoes. Thus, the immunohistochemical assays are likely to have shown the labeling of pS6K only, allowing subsequent interpretation.

Cerebral S6K activity as inferred from western blotting data 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, have 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 up-regulation in ILP-releasing neurons (Wu et al. 2005a). The low levels of cerebral pS6K in saline-fed *An. gambiae* despite biting repression could reflect 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 (Fig. 2.3 in Chapter 2). 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 the nutritive value of a meal has an influence on its retention (Fig. 2.3).

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 regulation 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*. Furthermore, monitoring of cerebral S6K activity throughout the first days of imaginal life could reveal a potential correlation with the onset of appetence maturity (see Chapter 1) and even maybe with the circadian rhythm of flight activity.

4.3 Ovarian sub-cellular localization of TOR and S6K

Phosphorylated TOR proteins were mostly found in primary follicle oocyte nucleus in the present study. TOR proteins have been reported to shuttle between the cytoplasm and nucleus of mammalian cells (Kim and Chen 2000) and this shuttling is needed for TOR downstream signaling through S6K activation (Kim and Chen 2000; Bachmann et al. 2006). Li et al. (2006) found that a significant fraction of TOR-complex-1 (TORC1, described by Wullschleger et al. 2006) is localized in the nucleus, where it may have several targets involved in rDNA transcription, maintenance of nucleolar structure and nuclear import/export (Martin et al. 2006). This is consistent with the fact that signaling kinases are often found bound to genes (Pokholok et al. 2006).

In this study pS6K was uniquely located in nuclei. The *Drosophila* antibody stained only the nurse cells nucleus, whereas the antibody dedicated to mammalian pS6K labeled all nuclei (albeit to a nutrient type-dependent intensity). This difference is difficult to explain, but the explanations developed above may be evoked.

Another example of differing suitability among antibodies is the p70 S6 kinase (C-18) antibody, which was already used by Hansen et al. (2005) for detecting total S6K in *Ae. aegypti* fat bodies and ovaries by western blotting assays. However, when we used this antibody by western blotting on *An. gambiae* ovarian samples for detection of total S6K, many bands were labeled in a wide range of protein weights higher and lower than the expected value. Moreover, we verified the source sequence that the manufacturer used to produce the antibody and compared it with homologous insect sequences. By doing so, we noted that the S6K C-terminus region that was used is very different between mammals and insects. Therefore, and notwithstanding that western blotting procedures could be questioned, we cannot guarantee the true identity of the proteins labeled by this antibody in the immunohistochemical stainings.

The networks highlighted in this study on nurse cell nuclei could either be linked to nucleoplasmic actin filaments or, externally, to rough endoplasmic reticulum (RER) enveloping the nucleus, as S6K is known to be associated with such structures. There are two isoforms of ribosomal S6 kinase, termed S6K1 and S6K2, that have cytoplasmic and nuclear splicing variants. The cytoplasmic form of S6K1 (termed S6K1 II or p70 S6 kinase) is predominantly cytosolic in serum-starved NIH3T3 cells *in vitro* but translocates to the nucleus following serum or growth factor stimulation (Panasyuk et al. 2006 and references therein). Nucleocytoplasmic shuttling has been reported for the cytoplasmic form of S6K, but its regulation has not been elucidated (Edelmann et al. 1996; Kim and Chen 2000; Valovka et al. 2003). Sub-cellular localization of S6K remains a debated question. Our results on the nuclear localization of active S6K in ovarian cells of *An. gambiae* following ingestion of different nutrients could be explained by the findings of Kim and Kahn (1997), who reported that insulin rapidly phosphorylates and activates nuclear S6K. The authors showed that activation of nuclear S6K in response to insulin occurred faster than the activation of cytosolic S6K, although the extent of phosphorylation of nuclear S6K was lower than that of cytosolic S6K. The rapid activation of nuclear S6K in response to insulin may serve as a molecular switch to turn on nuclear insulin-sensitive targets, such as transcription factors, DNA binding proteins and enzymes involved in gene expression (Kim and Kahn 1997).

4.4 Indications of inter- and intra-cellular communication in primary follicles following nutritional input

Active TOR proteins labeling in the nucleus of some nurse cells showed oocyte-orientated patterns (Fig. 3.9C). Knowing that the role of the nurse cells is to synthesize high amounts of maternal components (ribosomes, mRNAs, internal membranes, etc.) to be imported into the developing oocyte and that TOR is implicated in transcriptional regulation, these labeling patterns could reflect such synthesis of gene products intended for oocyte shipment. It would constitute the first report to our knowledge of gene product synthesis localized in the nucleus of a cell in the same direction as the future destination of the gene product. Alternatively, in view of the proximity between oocyte and nurse cells nuclei in this case (Fig. 3.9C), one could speculate about a coordinating signaling between these cells meant to synchronize nurse cell synthesis activity with oocyte needs in the light of nutritional status. However, such orientated labeling was not observed more often after protein-rich diets and the case discussed here comes from a mosquito fed sugar, which permits maturation of teneral ovaries, but not oogenesis in *An. gambiae* (see Chapter 2). As a consequence, we do not know what sort of nutritional input determines the suggested orientated synthesis or coordinated signaling.

Orientated labeling revealed by using the mTOR antibody (Figs. 3.9E and 3.10) further supports some kind of communication between oocyte and nurse cells, although the identity of what was labeled cannot be assured. This antibody was already used by Hansen et al. (2005) for immunohistochemical detection of total TOR in *Ae. aegypti* fat bodies. However, when this antibody was used for detection of total TOR by western blotting on *An. gambiae* ovarian samples, several bands were labeled at lower protein weights than the expected value and no band was ever detected at the expected protein weight. Moreover, we verified the source sequence that the manufacturer used to produce the antibody and compared it with homologous insect sequences. In this manner it was established that the sequence of residues surrounding human TOR serine 2481 is very different between human and insects and even between fruit flies and mosquitoes. Therefore, this antibody is very unlikely to have ever labeled TOR proteins in mosquito tissues and the true identity of the proteins labeled in our immunohistochemical assays remains a question. Still, three hours after a meal, nurse cell-orientated labeling in oocyte cytoplasm was mainly found in blood-fed mosquitoes. Tissues dissected 24 h post-meal and represented on Fig. 3.10 also come from blood-fed mosquitoes, but no otherwise-treated mosquitoes were held for 24 h to provide comparison. Therefore, it may be that such orientated labeling in oocyte cytoplasm is influenced by nutrient type, but this deserves further investigation using techniques that provide identifiable labeling.

The emanations of active S6K observed at the surface of some nurse cell nuclei most probably bear a role in the functioning of this enzyme, knowing its reported shuttling between nucleus and cytoplasm (as discussed above). The nucleus surface or neighborhood might be the defined location of S6K phosphorylation and, in turn, of downstream S6K activity on ribosomal protein S6. This would be consistent with the immunolocalization of active TOR proteins observed near the surface of some nurse cell nuclei (Fig. 3.9) as S6K phosphorylation on threonine 398 (*Drosophila* sequence numbering) is carried out by TOR kinase activity. Moreover, much RER is located around the nucleus. TOR and S6K proteins are known to be associated with this type of cellular component and with the ribosomes embedded therein. Indeed, the fact that some steps in TOR signaling are likely to occur on the surface of intracellular vesicles is acknowledged (reviewed in Arsham and Neufeld 2006). The pS6K emanations were found in more follicles in mosquitoes fed a protein-rich diet (blood or albumin+AA) than in those fed a nutrient-free solution or unfed. This is coherent with a stronger TOR and S6K activity following meals apt to initiate oogenesis. S6K sub-cellular translocation during egg development might be observed in future studies, as Yu et al. (2006) noted that S6K was mainly labeled in the nucleus in mouse spermatocytes but in cytoplasm in sperm, and suggested that there may be a translocation of S6K during spermatogenesis.

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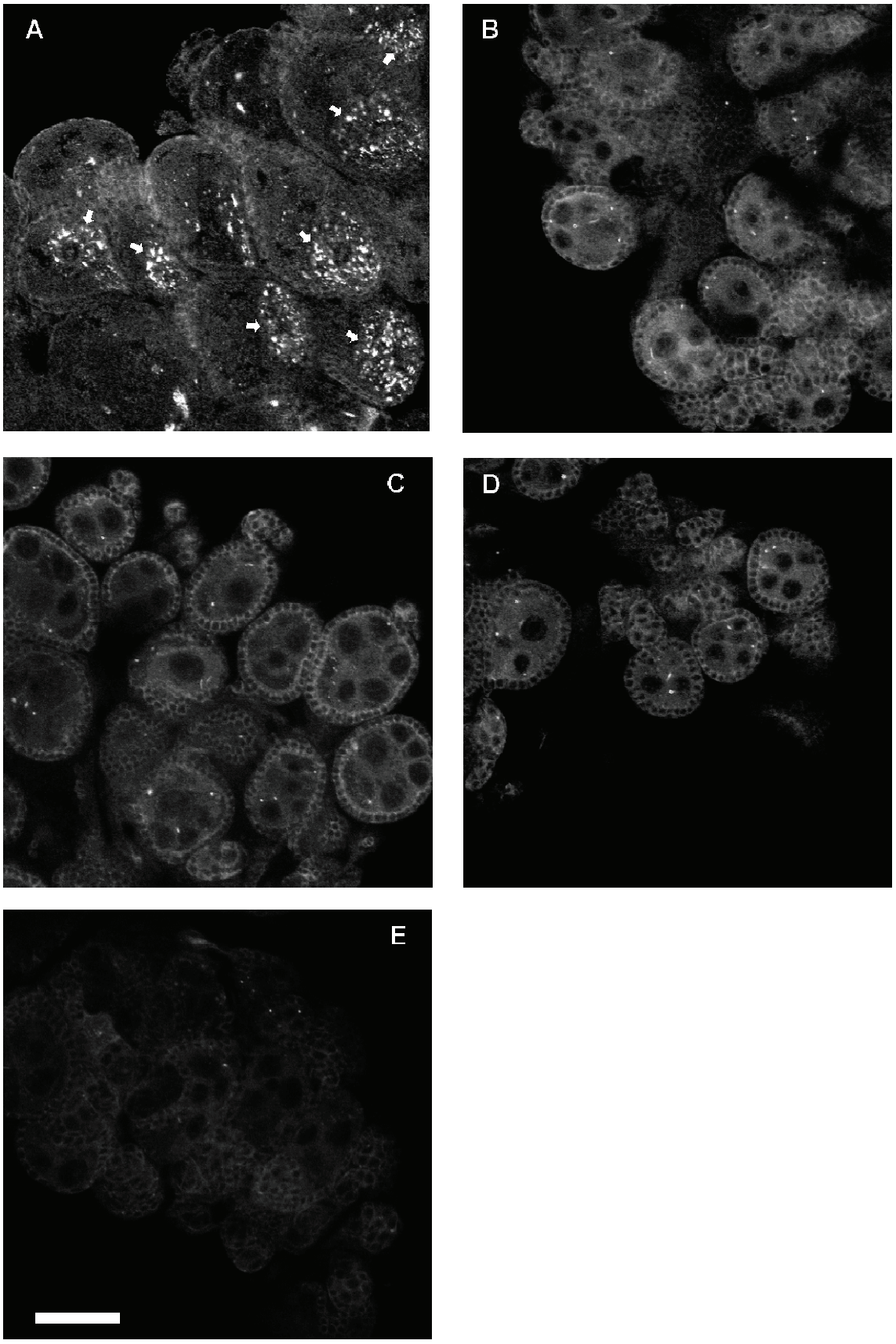
Annex A2: Preliminary immunohistochemical assays targeting total TOR in the ovaries of female *An. gambiae* following different meals

Before its incompatibility with insect samples was established (as described above), the mTOR antibody (described in Table 3.2) was used for detecting differences in TOR levels and localization in ovarian cells of female *An. gambiae* fed on different diets 3 h prior to dissection or unfed. The clearest labeling differences allowed the treatments to be regrouped into "fed" and "unfed" mosquitoes. Labeling intensity of the ovarian cells showed only limited differences among mosquitoes fed on the different diets (Fig. A2.1). However, mosquitoes fed albumin+AAs revealed a more widely distributed and more homogenous labeling compared to mosquitoes fed other diets. Furthermore, only blood-fed mosquitoes showed strongly labeled granules in oocyte cytoplasm (Fig. A2.1A). By contrast, unfed mosquitoes displayed only very weak labeling, hardly detectable from background (Fig. A2.1E). Unfortunately, due to the uncertainty regarding the identity of the proteins that were labeled by this antibody in mosquito tissues, any functional interpretation is hindered. The labeling highlighted in these assays seem to reflect the importance of the diet for supporting oogenesis and reveals a rapid reaction in the ovaries to the nutritive value of the meal. Such a rapid reaction is coherent with pTOR and pS6K levels detected by western blotting (see Results).

Figure A2.1 (next page) Confocal micrographs showing immunostaining obtained by using the mTOR antibody (described in Table 3.2) in ovarian cells 3 h after *An. gambiae* females had fed on either blood (A), albumin plus amino acids (B), sucrose (C), saline (D), or unfed (E). Labeled proteins were localized in the cytoplasm (granules in the whole oocyte cytoplasm of blood-fed mosquitoes, white arrows on A) and close to the plasma membrane in nurse cells (B, C and D). Labeling was manifest in all treatments, but very low in unfed mosquitoes (E). Micrographs were taken with a Leica DM RBE confocal microscope. Scale bar represents 50 μm on all micrographs.


A
B
C
D
E

Annex A3: Immunolabeling in nervous tissues of female *An. gambiae*: a map of putative neurosecretory structures in the brain and thoracic ganglia

Before being aware of its unsuitability for insect samples (as described above), we used the antibody targeting mammalian TOR ("mTOR antibody" described in Table 3.2) for immunohistochemistry on whole brains (including subesophageal ganglion [SG], thoracic ganglia [TG] and a piece of nerve cord in-between [CNC]) of female *An. gambiae* fed blood, albumin+AA, AAs, sucrose or saline, as well as of unfed females. Our aim was to detect an eventual change in TOR protein presence (level and localization) depending on nutritional input.

Many cells and axonal networks were labeled in all nervous tissues mentioned. Throughout numerous repetitions of such assays we noticed that labeling appeared to be independent of treatment and that there was no clear difference between tissues dissected 30 min or 3 h after feeding. Interestingly, despite uncertainty on labeled protein(s) identity, small groups of large cells were consistently labeled at several places in the brain and appeared to be connected by axonal networks running through the SG and the CNC (Figs. A3.1-3) and reaching other large cells in the TG (one cell per ganglion, Fig. A3.4). These labeling patterns are compatible with an interpretation identifying the large cells as neurosecretory cells. It this case, the whole figure could compose a map of neurosecretory structures in the central nervous system of this mosquito (Fig. A3.5).

Note: the figures follow at the end of the text.

Figure A3.1 Confocal micrographs of an *An. gambiae* female brain labeled with the general mammalian TOR antibody described in the text. Inset shows the whole brain in standard green scale representation of Alexa Fluor® 488 dye with the designation of brain lobes: optical lobes (OL), protocerebral lobes (P) and subesophageal ganglion (SG). Another color regime is used in the main panel to enhance contrasts. Several groups of large cells as well as widely distributed axonal networks were strongly labeled. The size and localization of the labeled cells is compatible with a neurosecretory function, but the labeled protein(s) identity is not certain (as explained in Chapter 3 main text). Unlabeled tracheae are visible as dark "rivers". The large dark structure between the P lobe (top) and the SG is the pharyngeal pump. Scale bars represent 100 µm in inset and 50 µm in the main panel.

Figure A3.2 Confocal micrographs of an *An. gambiae* female brain labeled with the general mammalian TOR antibody showing strongly labeled large cells and axonal networks (explanations as in the legend to Fig. A3.1).

Figure A3.3 Confocal micrographs of pairs of labeled cells found in *An. gambiae* female brains by using the general mammalian TOR antibody described in the text. In all pairs the cells appeared to be connected to each other. Most of these cells were round-shaped and of a diameter larger than 10 μm, but some showed the typical triangular shape of endocrine cells (B, bottom cell).

Figure A3.4 Confocal micrographs of *An. gambiae* female thoracic ganglia labeled with the general mammalian TOR antibody described in the text. Down left is anterior. Axons coming from the subesophageal ganglion were labeled (arrowheads on A and B), as well as one large cell per thoracic ganglion (A). Arrows on A indicate the cells labeled in the anterior ganglia. Abdominal ganglia may also host such cells, but these tissues were not dissected out. Micrograph A is a superimposition of 22 layers and micrograph B is a superimposition of 20 layers, which results in a strong background. Scale bars represent 25 μm on both micrographs.

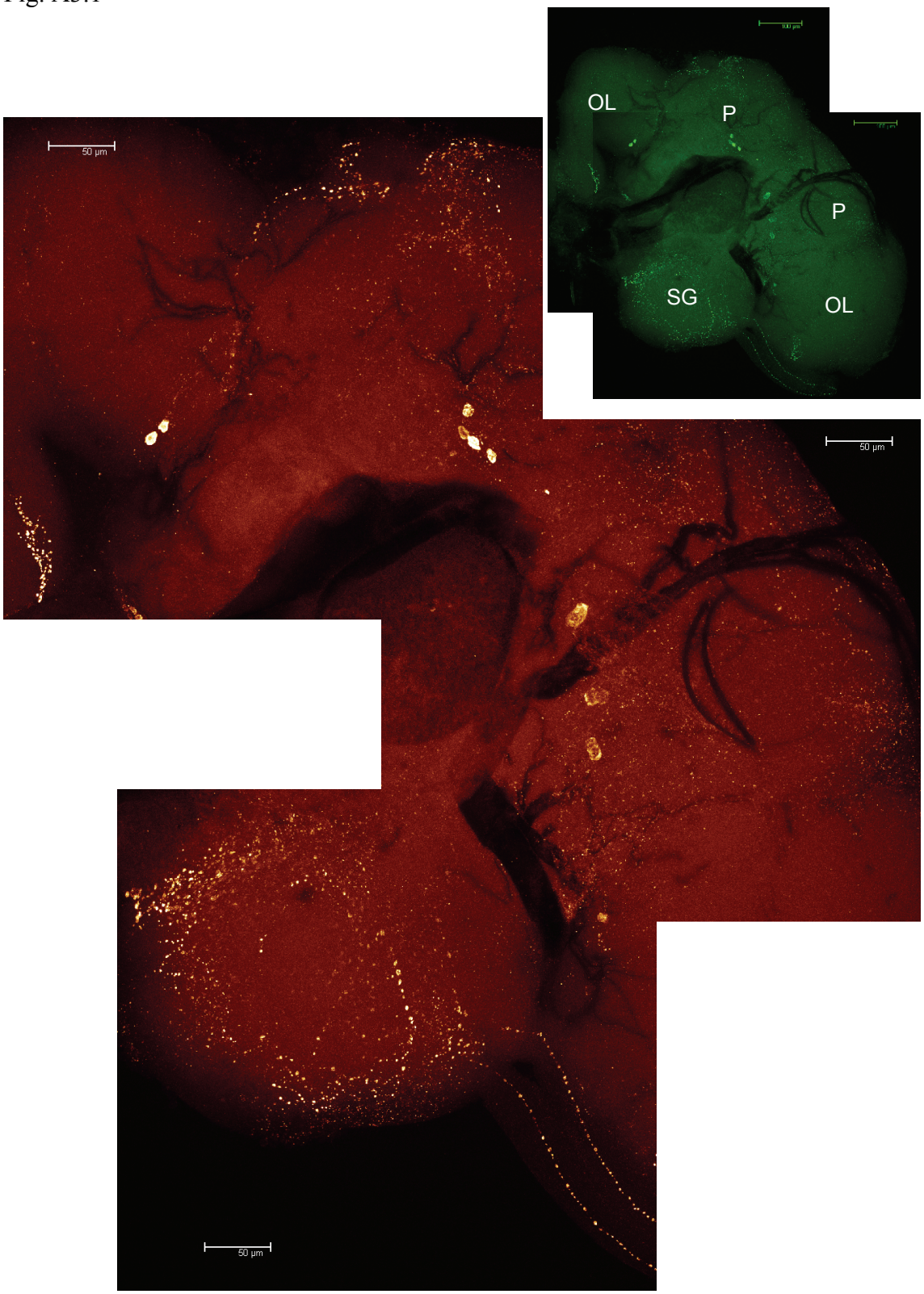
Figure A3.5 Schematic representation of an *An. gambiae* brain (A: front view, B: rear view) showing the optical lobes (left and right), the protocerebral lobes (top), the subesophageal ganglion (center) and the beginning of the "cervical nerve cord" connecting the subesophageal and the thoracic ganglia. All structures that were labeled by the general mammalian TOR antibody in *An. gambiae* female brains are summarized by the green drawing, resulting from observation of many confocal micrographs. Only structures that cannot be seen from the front view are represented on B. This could represent a map of brain neurosecretory structures. However, several items may have been missed and the actual connections existing between the cells and networks represented here are certainly far more intricate than what was found. For example, medial neurosecretory cells were never clearly identified and neither were important neurosecretory tissues such as the *corpora allata*. Therefore, and knowing that no difference was noticed between diet treatments, any unambiguous functional interpretation of this labeling is restricted.

Similarities between the labeling patterns described here and the representation of clock neurons in adult *D. melanogaster* brain described by Nässel and Homberg (2006, Fig. 9 therein), NPF-INR neurons depicted in the same organism by Wen et al. (2005), and OEH neurons in female *An. gambiae* brain (Brown and Cao 2001) were noticed. Moreover, some of the cell pairs highlighted in the present study could well correspond to the 4 pairs of sulfakinin (a satiety factor) cells found in the brain of the blow flies *Calliphora vomitoria* and *Phormia regina* by Duve et al. (1994; 1995) and Downer et al. (2007). All these similarities, together with comparisons with the anatomy of dipteran brain neurosecretory cells, comprehensively

described and illustrated by Shiga (2003), support the involvement of the structures labeled here in the neuroendocrine system. Medial neurosecretory cells (located at the top of the protocerebral lobes boundary) were apparently not labeled in our assays, but they may simply have been missed. However, the lack of specificity of our labeling and the absence of a difference between diet treatments prohibit any unambiguous functional interpretation.

Co-localization of TOR proteins with ILPs and NPF would be very interesting, but no antibody exists to label these proteins in *An. gambiae* tissues. An alternative would be to use mRNA *in situ* hybridization.

Fig. A3.1



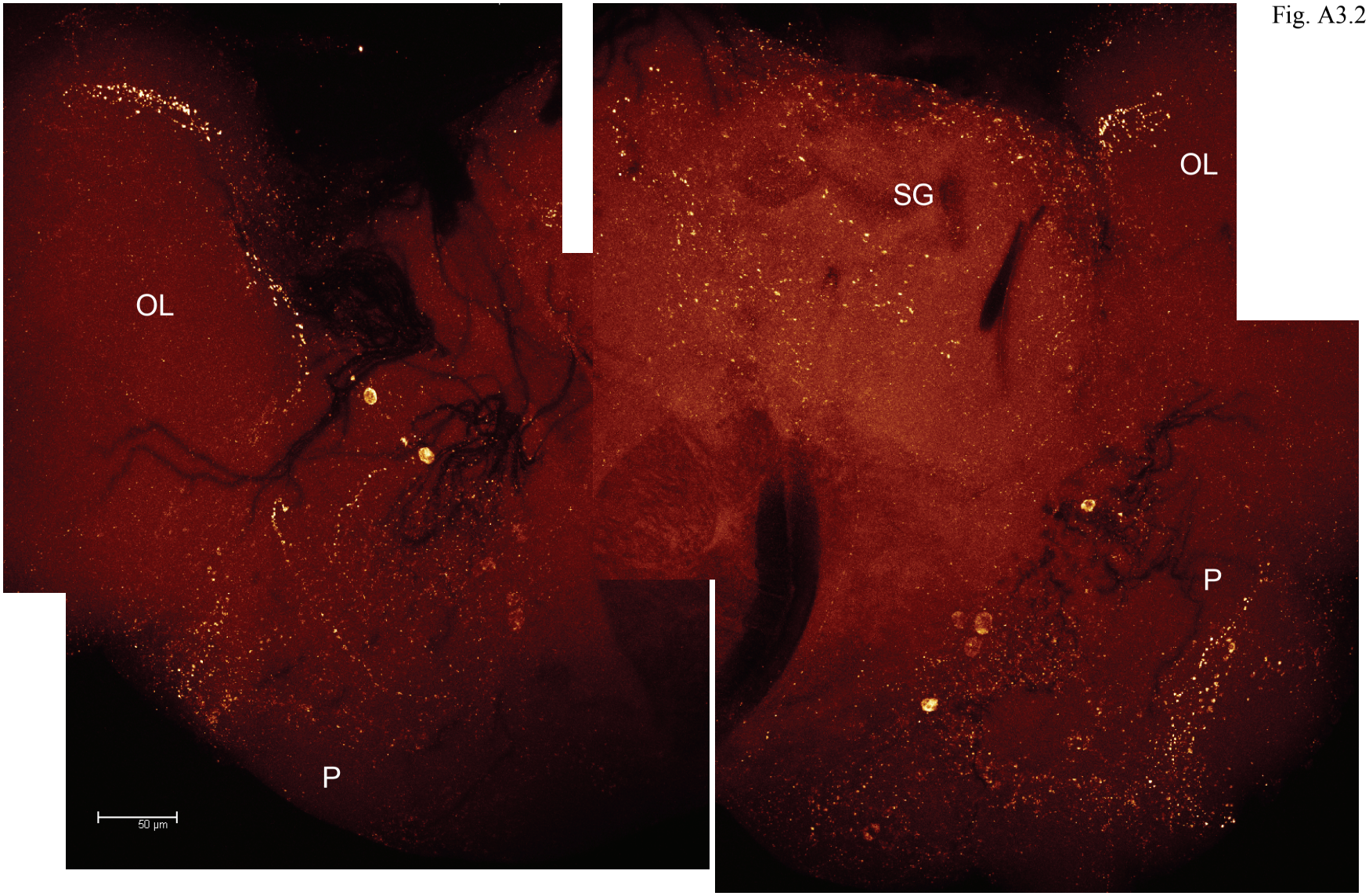


Fig. A3.2

Fig. A3.3

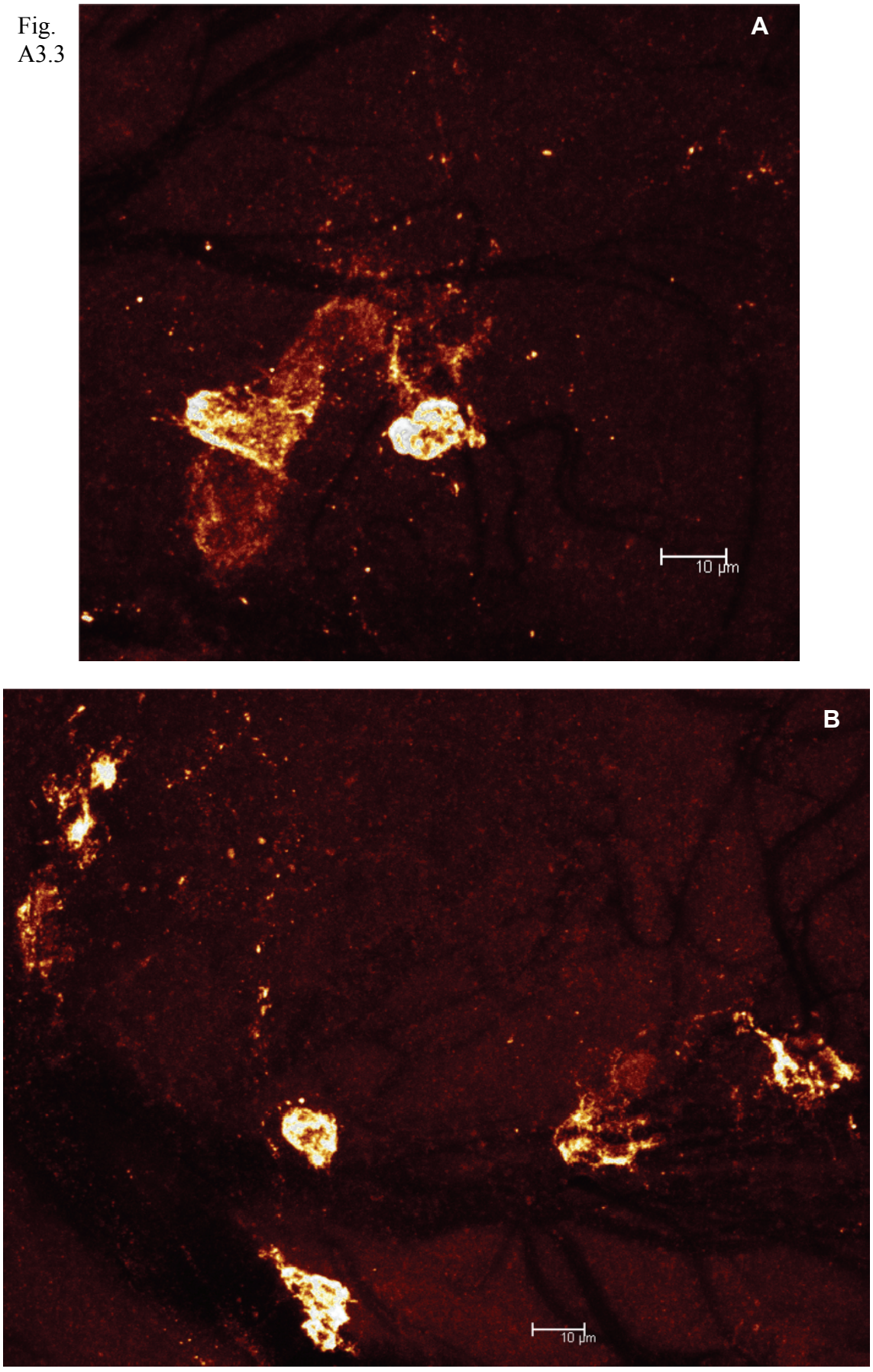
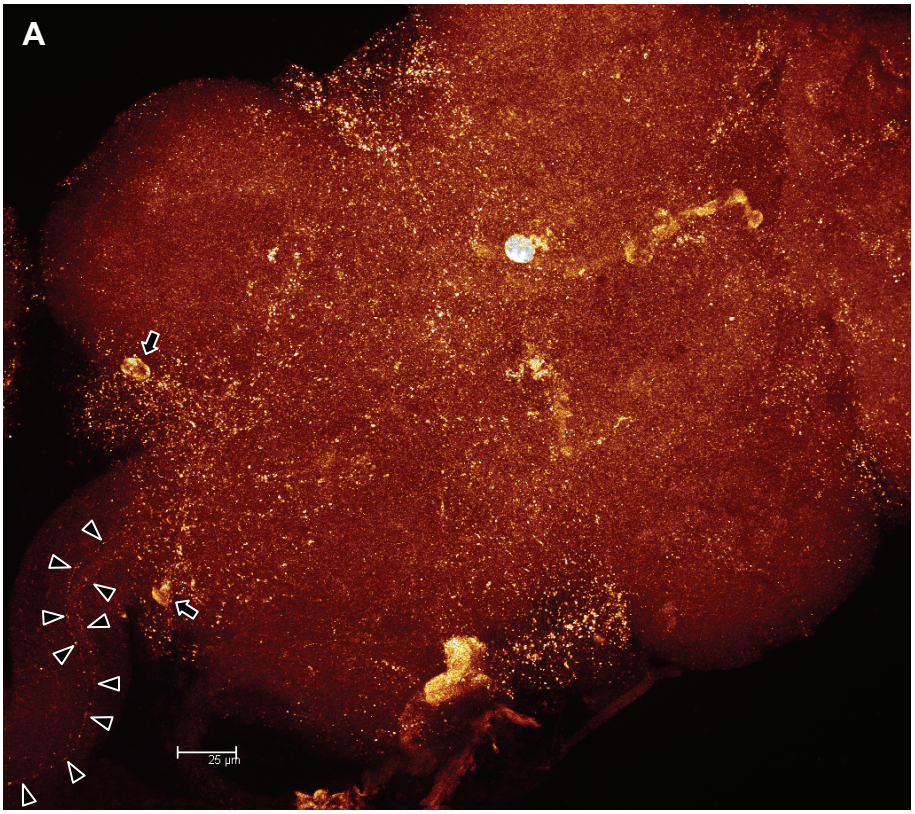


Fig. A3.4



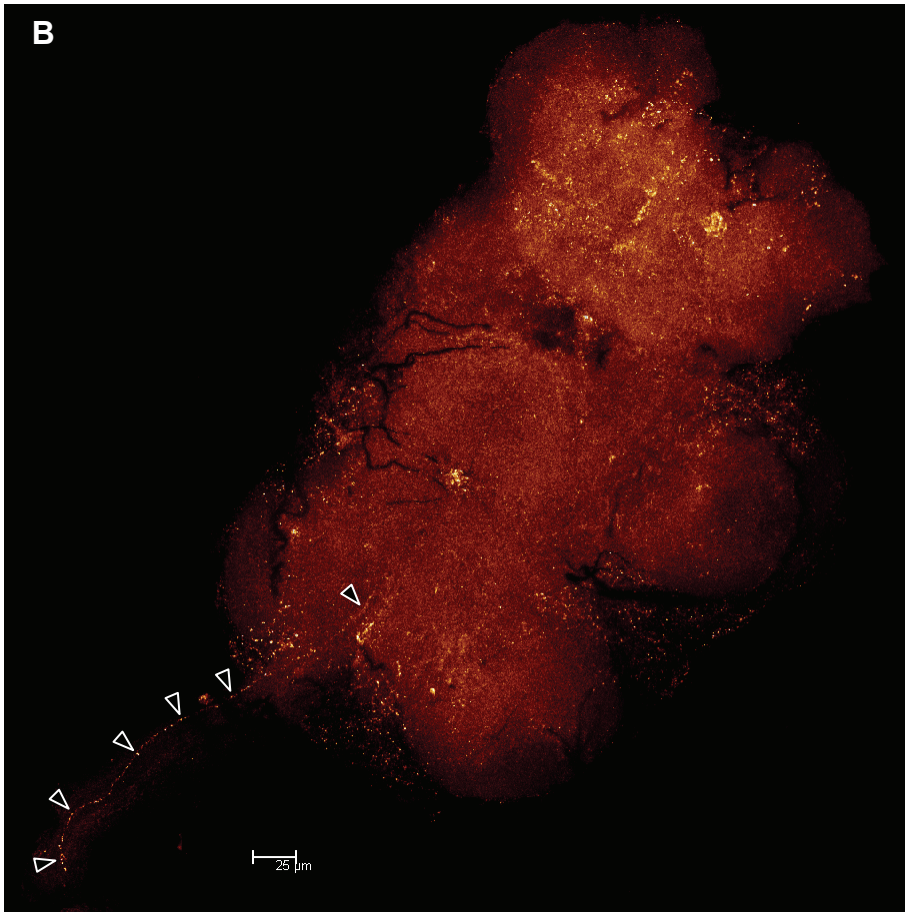
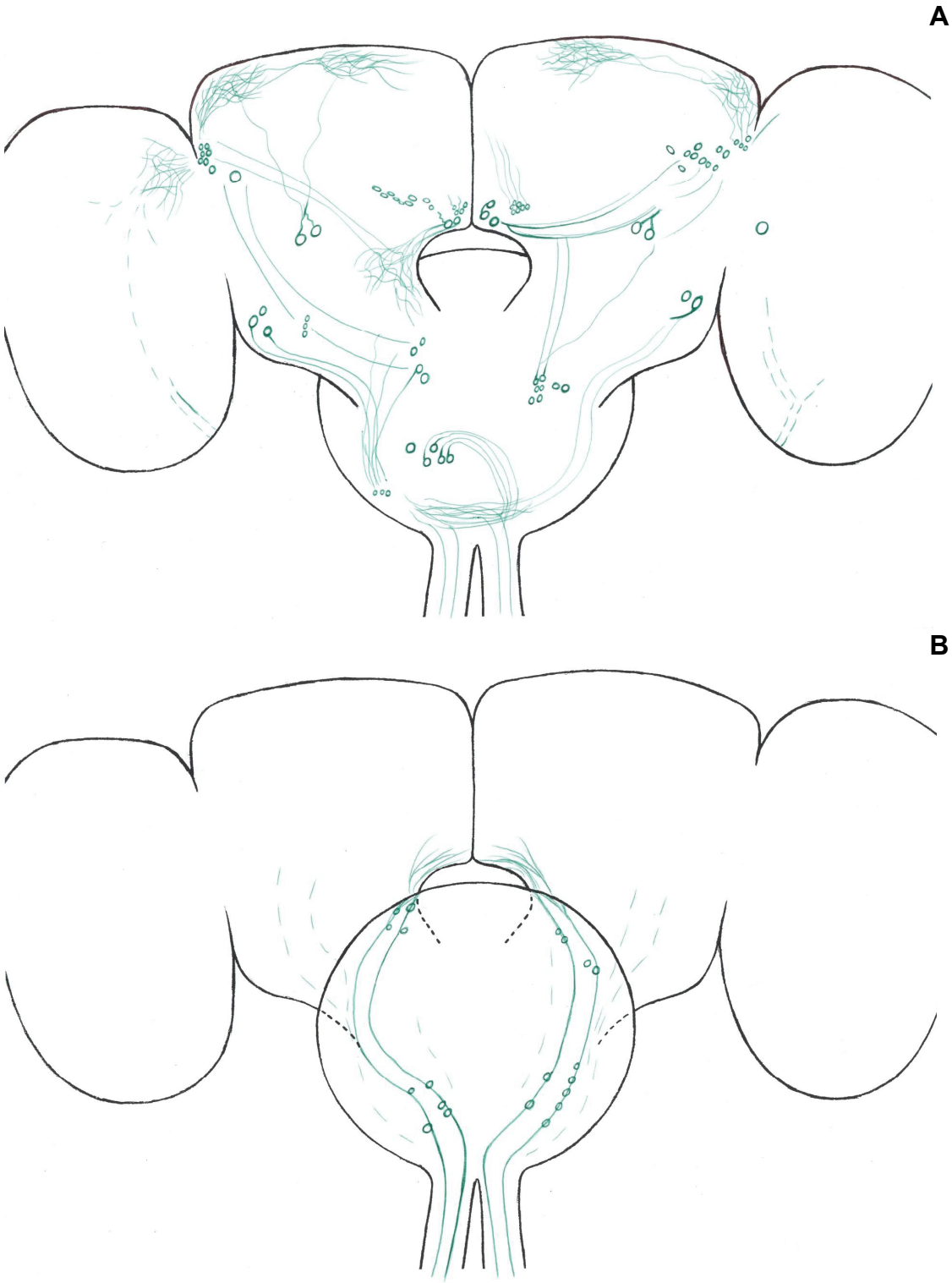


Fig. A3.5



General discussion

This study focused on the maturation of appetence, on the feeding habits and on their regulation and consequences in the African malaria mosquito *Anopheles gambiae*.

Key factors influencing the foraging behaviors of this mosquito arise from physiological aspects of life-history traits of anophelines. Anophelines mostly eclose as "skinny" "under-nourished" under-developed adults, and this is only partly dependent on larval food availability. The low teneral reserves are the principal concern of young adults. Coupled with the under-developed ovaries, this commands a delay before a female *An. gambiae* is able to successfully develop eggs for the first time.

Young females will rapidly engage in host-seeking but cannot successfully bite and gorge on host blood before 12 h of eclosion (Briegel and Hörler 1993), the time needed for mouth parts and digestive apparatus maturation. This delay forces the young mosquitoes to find a shelter during their first day, as they eclose at night and the 12 h or so will bring them to the next night, where this crepuscular/nocturnal species is active.

Then, during their first night after eclosion, *An. gambiae* mosquitoes must care about the completion of their reserves and the final maturation of their organs. From this point on, female *An. gambiae* can be described as opportunists. Host-seeking becomes more resolute night after night (see Chapter 1), but nectar meals are also taken from the first night as an adult. Mating (following mating partner finding through crepuscular swarming) can occur on the first night, but egg development is not yet possible.

Depending on their teneral reserve levels, females have to allocate a substantial amount of their first meals to the build-up of glycogen and lipid reserves and to the development of the ovarian follicles. In this species, the low level of teneral reserves is associated with a multiple blood feeding pattern and consequent gonotrophic discordance. Most females cannot develop eggs after a single blood meal, because the nutrients acquired are used for maternal reserve synthesis and development (Briegel and Hörler 1993). Nectar meals can also support maternal maturation and glycogen and lipid stocks constitution (Briegel 1990), but sugar can only bring the ovarian follicles to the previtellogenic resting stage (Fernandes and Briegel 2005) and not further (see Chapter 2). At this point, a single blood meal is sufficient for the development of an egg batch.

Our functional genomics assays revealed that the expression of several genes (*period*, *insulin-like peptide 4* and *p70 S6 kinase*) was positively correlated to age and thus, to increasing appetence (see Chapter 1). This was interpreted as reflecting organ maturation and glycogen reserve mobilization, and could be the first report of a maturation of the insulin pathway associated with the development of oogenesis ability in a hematophagous insect. Interestingly, the expression patterns of orthologs to *period* and *takeout* in *An. gambiae* and their known roles in other organisms could indicate that these genes react to the absence of a blood meal by increasing circadian food anticipation and sensitivity to host cues. The study of other candidate genes suggests that intracellular insulin signaling and early ecdysone response genes could play important roles in appetence control.

In this study we tested the conditions required to evoke the blood-feeding or the nectar-feeding mode. It was shown that even saline (without proteins or amino acids) can evoke blood-like gorging when the diet is heated, pH-buffered and presented with a membrane to feed through. In this case female mosquitoes always filled their midgut, which has itself the power to terminate host-seeking (see Chapter 2). By contrast, sugar feeding (requiring ambient temperature, direct contact with the food source and absence of pH-buffering salts) does not deter female mosquitoes to take a blood meal. The behavioral sequence ending in host-seeking termination seems to begin with the stimulation of the blood-feeding mode and is apparently not affected by the absence of nutrients in a saline meal.
In mature females, blood- and nectar meals can be taken every night and usually several times in one night, without any restriction due to oogenesis stage. Most mature females, provided all resources are available, can take blood meals and oviposit every night, although the development of an egg takes 48 h at optimal conditions. This frequent blood-feeding habit carries extensive consequences on malaria parasite transmission.

Another question addressed in this study (Chapter 3) is how the mosquito knows it is hungry or replete. What sort of signaling do the nutrients elicit shortly after a meal to inform the mosquito to stop feeding or develop eggs, for example?
Our gene expression assays revealed an effect of food on the insulin pathway in the head of female *An. gambiae*. Interestingly, insulin-like peptide expression was positively affected by a sucrose meal, which mimics the reaction of insulin to sugars in mammals and is consistent with the influence of nutrients on insulin-dependent growth in *Drosophila melanogaster*. A positive transcriptional effect of a sucrose meal is also suggested for the *insulin receptor* and *p70 S6 kinase* genes in the head of female *An. gambiae*. Furthermore, the activity of the TOR / S6K

pathway was shown to react rapidly following ingestion of any meal (even saline) by increased levels of phosphorylated TOR and S6K in the ovaries, but this was only elicited by protein-rich diets in the brain. In both organs the true nutritive value of the meal (in terms of oogenesis support) was reflected by higher phosphorylation levels following an albumin plus amino acids-meal than after a saline meal, and even higher levels after a blood meal. This early nutrient-sensing task of the TOR / S6K pathway is demonstrated here for the first time in *An. gambiae*. These responses are consistent with the early mobilization of the cerebral insulin pathway by nutrients in the meal, but the latter was more influenced by sucrose than by protein in the meal, whereas the TOR / S6K activity responded more strongly to protein-rich meals. Another contrast is the different TOR / S6K activation by a nutrient-free meal revealed in the ovaries and in the brain. Differentiated roles endorsed by TOR in the central nervous system (CNS) compared to peripheral tissues was pointed out by Arsham and Neufeld (2006). Beside being a sensor and transducer of signals within cells, TOR is critical for fat, brain and endocrine organs, which have a sentinel or communications role, dictating to the rest of the organism how to modify behavior in response to the environment (Arsham and Neufeld 2006). The authors proposed an integrated model where TOR signaling in distinct tissues responds both to local nutrient conditions and to globally acting growth signals emanating from fat tissue. In many peripheral tissues, TOR activation then leads primarily to increased cells growth, whilst in certain neurons of the CNS, TOR activation leads to down-regulation of feeding behavior. Furthermore, in endocrine tissue such as the prothoracic gland of *D. melanogaster*, insulin signaling promotes expression of the steroid hormone ecdysone, leading to decreased insulin signaling in the fat body. Arsham and Neufeld (2006) conclude that TOR measures growth-permitting inputs and regulates growth-promoting outputs on both cellular and organismal levels.

The significance of the present work also originates in the link existing between the endocrinology of insulin-like peptides and innate mosquito immunity. Studies on *An. stephensi*, a major vector of human *Plasmodium* parasites in several Asiatic countries, revealed that human insulin can induce nitric oxide (NO) synthesis in cultured cells and in the mosquito midgut via the endogen insulin signaling cascade (Lim et al. 2005). Inducible NO limits malaria parasite development in this mosquito (Luckhart et al. 1998) through the formation of inflammatory levels of toxic reactive nitrogen oxides (Luckhart et al. 2003; Peterson and Luckhart 2006; reviewed in Luckhart and Riehle 2007) that likely induce parasite apoptosis in the mosquito midgut lumen (Hurd and Carter 2004). Therefore, future studies should address the identification of parasite-derived and other inducers of mosquito nitric oxide synthase gene expression, so that this gene

and the associated regulators could turn into targets for manipulation to enhance anti-parasite resistance.

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