TALES VICARI PASCINI

CHARACTERIZATION OF SPERMATHECA-RELATED GENES IN *Aedes aegypti*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

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Abstract

PASCINI, Tales Vicari, D.Sc., Universidade Federal de Viçosa, July, 2018. **Characterization of spermatheca-related genes in *Aedes aegypti***. Adviser: Gustavo Ferreira Martins.

Aedes aegypti is an important hematophagous mosquito, with anthropophilic habits, also known as vector of important pathogens to humans, such as Yellow Fever, Dengue, Chikungunya, and, Zika viruses. *A. aegypti* females usually mate once, receiving the sperm to produce the whole offspring during their reproductive period. The spermatheca is responsible for sperm maintenance, nutrition and protection against physical and oxidative stress damage leading the long-term sperm storage process, resulting in the increase of the female fecundity. This reproductive autonomy of the females enhances their dispersion and then their vectorial capacity and the spermatheca play pivotal role providing the suitable environment, guaranteeing the sperm viability. There are three spermathecae in *A. aegypti*: two lateral and a central larger one. Considering the importance of this organ, in the present work, we reviewed different aspects of the spermatheca of insects, highlighting its importance for the reproductive success of the insects. This review also highlighted the number of spermathecae according to different taxonomic group, the role of different spermathecal parts (duct, reservoir, and gland), emphasizing their function along the processes of sperm maintenance. We also aimed to elucidate that the global gene expression in virgin and fertilized spermathecae ("spermathecomes") separately, through RNA sequencing and bioinformatics analyzes. In virgin females, there is a greater number of coding sequences related to the establishment environment to receive and to allocate the spermatozoa. In fertilized spermathecae the most representative transcripts are related to the maintenance of the spermathecal microenvironment, thus prolonging the viability of these gametes. Differentially expressed genes (DEGs) were also analyzed by comparing virgin and fertilized spermathecae. We attempted the silencing of some of these DEG by the RNAi technique, to analyze the effects of knocking-down these genes throughout the reproductive process in *A. aegypti*. The females were injected with dsRNA of eight different genes related to energy metabolism (Ae-92048), chitin-bound components (Ae-187521 and Ae-88956), transcriptional regulation (Ae-27176), hormonal regulation (AeSigP-4002), enzymatic activity (Ae-SigP-212177), antimicrobial activity (AeSigP-109183) and ion homeostasis (AeSigP-66427). The gene silencing decreased the female survival, reduced the blood-feeding intake, nutrients storage/conversion, egg production and oviposition and offspring productions. In addition, the silencing of AeSigP-66427 affecting sperm motility and impaired egg production. At last, by the *in situ* hybridization we could detect in which part or the spermatheca, the chosen target genes are expressed. The present work is pioneer to identify the collection of genes expressed the virgin and fertilized spermathecae of *A. aegypti*. Through this intriguing study, we were able to elucidate the genes and some processes related to the long-term storage of spermatozoa in *A. aegypti*. Moreover, this work provided the basis for the establishment of alternative strategies to control this vector, by reducing its reproductive capacity.

Resumo

PASCINI, Tales Vicari, D.Sc., Universidade Federal de Viçosa, julho de 2018. **Caracterização de genes relacionados à espermateca de *Aedes aegypti*.** Orientador: Gustavo Ferreira Martins.

Aedes aegypti é um mosquito hematófago, com hábitos antropofílicos, sendo considerado vetor de importantes patógenos, como os vírus da febre amarela, dengue chikungunya e zika. Fêmeas de mosquitos geralmente copulam uma única vez, recebendo os gametas para produzir toda sua progênie ao longo de seu período reprodutivo. O processo de armazenamento de espermatozoides é considerado quase que totalmente papel da espermateca, garantindo certa independência reprodutiva da fêmea, bem como a viabilidade dos espermatozoides até a fertilização dos ovos. Há três espermatecas em *A. aegypti*, uma maior central e outras duas menores laterais. O armazenamento massivo de gametas, garante o aumento da capacidade vetorial das fêmeas dos mosquitos e de sua dispersão. Diante da importância desse órgão, no presente trabalho foi realizado uma revisão bibliográfica acerca da importância da espermateca para o sucesso reprodutivo dos insetos. Essa revisão inclui aspectos morfológicos, fisiológicos e das partes que compõe esse órgão (ducto, reservatório e glândula), ressaltando sua função ao longo dos processos de encaminhamento, alocação e liberação dos gametas para a fertilização dos ovos. Posteriormente, foi proposto uma busca pelos processos moleculares existentes nas espermatecas virgens e fertilizadas de *Ae. aegypti* por meio de RNAseq e análises de bioinformática. Em fêmeas virgens pode-se observar uma maior quantidade de transcritos relacionados a diferentes processos metabólicos que estabelecem um ambiente ideal para receber e alocar os espermatozoides. Já nas espermatecas fertilizadas, a maior quantidade de transcritos encontrados se relaciona com a manutenção do microambiente espermatecal, prolongando assim, a viabilidade dos espermatozoides. Foi conduzida também uma análise comparativa dos genes diferencialmente expressos (DEG) entre as espermatecas virgens e fertilizadas. Baseado nas funções de cada gene, oito genes foram escolhidos como alvos da técnica de RNAi para se determinar o efeito do silenciamento desses genes no processo reprodutivo em fêmeas de *A. aegypti*. As fêmeas foram injetadas com dsRNA para genes alvo relacionados ao metabolismo de energia (Ae-92048), componentes ligados à quitina (Ae-187521 e Ae-88956), regulação transcricional (Ae-27176), regulação hormonal (AeSigP-4002), atividade enzimática (Ae-SigP-212177), atividade antimicrobiana (AeSigP-109183) e a homeostase de íons (AeSigP-66427). Para cada um dos genes, as fêmeas injetadas foram analisadas em relação à sua sobrevivência, alimentação sanguínea, produção e viabilidade da progênie. Por fim, por meio da hibridização *in situ*, foram identificados os locais específicos onde os genes escolhidos são expressos nas espermatecas. O presente trabalho é pioneiro a identificar os genes expressos em espermatecas virgens e fertilizadas de *A. aegypti*. Através de injeções de dsRNA, pudemos descrever os prejuízos nas fêmeas em relação a sua taxa de sobrevivência, capacidade de alimentação sanguínea, no armazenamento/conversão de nutrientes, no desenvolvimento dos ovos, bem como nos processos e oviposição e produção de progênie. Além disso, foi observado que uma redução nos transcritos de AeSigP-66427, responsável pela homeostase de íons, resulta na redução da motilidade dos espermatozoides e inviabiliza a produção de ovos. Por meio desse estudo, fomos capazes de elucidar o papel dos genes em alguns processos relacionados ao armazenamento de espermatozoides a longo prazo em *A. aegypti.* Ainda, esse trabalho nos forneceu as bases moleculares para o estabelecimento alvos específicos para o controle desse vetor, reduzindo assim, sua capacidade reprodutiva.

Apresentação

A tese apresentada é fruto dos trabalhos desenvolvidos ao longo do período de doutorado do candidato Tales Vicari Pascini no Programa de Biologia Celular e Estrutural do Departamento de Biologia Geral da Universidade Federal de Viçosa. Esse trabalho foi idealizado pelo Prof. Dr. Gustavo Ferreira Martins pertencente ao Departamento de Biologia Geral da UFV (Viçosa, Minas Gerais, Brasil) e foi realizado em colaboração com o Prof. Dr. Marcelo Ramalho-Ortigão vinculado à Uniformed Services University of the Health Sciences (Bethesda, Maryland, EUA) e o Prof. Dr. Marcelo Jacobs-Lorena vinculado ao Departamento de Microbiologia Molecular e Imunologia da Johns Hopkins University (Baltimore, Maryland, USA).

O trabalho está estruturado em dois capítulos formatados na forma de artigos científicos. O primeiro capítulo abrange a Introdução Geral e corresponde a uma revisão bibliográfica acerca da importância da espermateca dos insetos ao longo dos processos de encaminhamento, alocação e liberação dos gametas para a fertilização dos ovos. Essa revisão se encontra formatada nos padrões da revista e foi disponibilizada virtualmente em 5 de dezembro de 2016 e publicada na versão impressa n° 121 da Zoology, em janeiro de 2017 (doi: 10.1016/j.zool.2016.12.001). Ao longo do segundo capítulo, foram explorados os principais processos moleculares existentes nas espermatecas virgens e fertilizadas de *Aedes aegypti* por meio de RNAseq, análises de bioinformática e silenciamento de genes relacionados à manutenção da viabilidade dos espermatozoides no interior das espermatecas em fêmeas de *A. aegypti* ao longo de seu processo reprodutivo.

Índice

Capítulo I:

The insect spermatheca: an overview

REVIEW

The insect spermatheca: an overview

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Highlights

- The insect spermatheca receives, maintains, and releases sperm to fertilize eggs.
- Most insects have one spermatheca, although the number varies among taxa.
- The morphology of the spermatheca varies according to insect species.
- The spermatheca has the following parts: duct, reservoir and spermathecal gland.
- The sperm are nourished by secretions from both male accessory glands and spermathecal glands.

Abstract

In the female insect, the spermatheca is an ectodermal organ responsible for receiving, maintaining, and releasing sperm to fertilize eggs. The number and morphology of spermathecae vary according to species. Within the spermathecal lumen, substances in the semen and secretions from the spermathecal gland nourish the sperm. Thus, the spermatheca provides an appropriate environment that ensures the long-term viability of sperm. Maintaining sperm viability for long periods within the spermatheca is crucial for insect reproductive success; however, the details of this process remain poorly understood. This review examines several aspects of and gaps in the current understanding of spermatheca biology, including morphology, function, reservoir filling, development, and biochemistry. Despite the importance of the spermatheca in insects, there is little information on the gland secretions and their role in the maintenance and protection of male gametes. Furthermore, in this review, we highlight the current information on spermathecal gland secretions and the likely roles they play in the maintenance and protection of sperm.

Keywords: Reproduction; Reproductive system; Sperm; Insect spermatheca, Spermatozoa

1. Introduction

Insects are among the most numerous and diverse organisms on the planet. The prolific reproductive success of insects is partly due to the ability of females to store sperm in their spermatheca (Greek, *sperma* = seed; *theke* = case) or receptaculum seminis. The spermatheca is also found in other taxa, including arachnids, mollusks, annelids, nematodes and amphibians (reviewed by Hickman et al., 2014), but the spermatheca found in insects is the most well-studied to date. The insect spermatheca is ectodermal in origin, and its primary functional parts generally include the duct, reservoir, muscular pump and spermathecal gland. After copulation, sperm migrate from the proximal part (which may be the vagina, bursa copulatrix, atrium or genital chamber, depending on the insect) of the female reproductive tract, through the spermathecal duct, and into the reservoir (Tombes and Roppel, 1972; Bailey and Nuhardiyati, 2005; Oppelt and Heinze, 2007). Once the eggs have been released from the ovary, the sperm retrace this route and fertilization occurs (Lefevre and Jonsson, 1962; Tombes and Roppel, 1972; reviewed by Chapman, 2013).

The female genital tract, including the spermatheca, is involved in sperm maturation or activation (Curtin and Jones, 1961; Wandall, 1986; Klowden, 2006). Within the spermatheca, sperm are protected from mechanical damage, contact with the hemolymph, and putatively from free radicals such as reactive oxygen species (Collins et al., 2004; Al-Lawati et al., 2009; King et al., 2011). The sperm constantly move inside the spermatheca, swirling within the spermathecal lumen (see the video in the supplementary online Appendix), which organizes and stores them for release at the appropriate time during fertilization (Jones, 1973; Werner et al., 2007; Dallai et al., 2014). Insect females can thus use the sperm for fertilization according to their demand, as demonstrated by bees (Gerber and Klostermeyer, 1970; Baer et al., 2016).

Sperm storage implies a temporal separation between mating and fertilization. Therefore, sperm storage is a key mechanism underlying the reproductive independence of females from needing to mate several times and the subsequent costs associated with mating more frequently, because it ensures fertilization of eggs throughout their reproductive life. Sperm storage also suggests that sperm cells are nourished and preferentially selected, beginning when the sperm cells migrate to the spermathecal reservoir, and continuing until fertilization occurs (Ward, 2000; Franck et al., 2002; Bloch Qazi, 2003). Thus, the storage of sperm creates an opportunity for post-copulatory sperm competition and female cryptic choice of specific sperm cells for fertilization, altering the genetic background of offspring (Klowden and Chambers, 2004). These processes have been implicated in the evolution of complex sperm storage organs (SSO), including the spermatheca (Pitnick et al., 1999). A key tool to investigate sperm competition within SSO was the development of transgenic *Drosophila melanogaster* males with either green- or red-fluorescent sperm (using green or red fluorescent proteins, respectively). This allowed in vivo tracking of both the sperm's migration into a female's SSO and the fate of the sperm from different males, and performance assessment of the sperm in *D. melanogaster* (Lupold et al., 2012, 2013; Schnakenberg et al., 2012; Manier et al., 2013; Ala-Honkola et al., 2014).

The maintenance of sperm viability in the spermatheca is an intriguing and complex process. Once inside the spermatheca, ejaculatory compounds produced by glands found in the male reproductive system may promote sperm preservation, increased fecundity, and behavioral changes in the female. Therefore, the spermatheca can indirectly promote the chemical communication between males and females by means of transferring substances from the male reproductive system to the female's (Bhatnagar and Musgrave, 1971; Worthington et al., 2015). However, at least in *D. melanogaster*, compounds in the male's ejaculate have a short life span within the female reproductive system, degrading within 7 h after mating. Thereafter, it appears that the spermatheca alone sustains the sperm with its own glandular secretions (reviewed by Wolfner, 2011; Schnakenberg et al., 2012). The discovery and study of the functions of these compounds, including prostaglandins in crickets, have contributed greatly to understanding the role of the spermatheca in the reproduction of insects (Worthington et al., 2015).

Several insect species are agricultural pests, while others are vectors of disease, and therefore of medical and veterinary importance, with major negative economic and public health impacts. For this reason, there is considerable interest in blocking or reducing the currently high reproductive output of these insects, in order to control their populations (Catteruccia et al., 2005). In this context, the spermatheca could be a target organ for population control of insects because disruption of its functions would lead to decreased sperm viability, or even fecundity, and altered female sexual behavior.

Here, we review the current literature related to the spermatheca, the main SSO in female insects, as well as its main components. We consider aspects of spermathecal number, function, morphology, reservoir filling, cellular composition, development, and biochemistry. We highlight the importance of the spermatheca as an SSO, what information we have about spermathecal gland secretions, and the likely roles the secretions play in the maintenance and protection of sperm. Finally, we present a synthesis of recent findings and identify gaps in the current understanding of spermathecal biology in insects.

2. Number of spermathecae

In some lower hexapods, such as diplurans and collembolans, sperm is also stored within spermathecae. Their number varies; thus, there is one spermatheca in *Campodea* sp. (Diplura) (reviewed by Matsuda, 1976), and there are two identical spermathecae in Collembola (Dallai et al., 2008a, b). However, there is no spermatheca at all in Protura (reviewed by Matsuda, 1976). Similarly to the lower hexapods, the number of spermathecae also varies in Apterygota, from one, as reported in Zygentoma (Palit and Pal, 1999), to two spermathecae in

Archaeognatha (Bitsch, 1989). Hence, the ancestral condition for the precise number of spermathecae in Hexapoda remains an open question. However, as the number of ancestral and current species studied increases, the evolutionary scenario regarding the spermathecal number will likely be clarified.

Spermatheca has been reported in all insect orders and there is one spermatheca in most studied insect species (Table 1), and this number seems to be the ancestral condition at least for winged insects (Pterygota). A single spermatheca is present in mayflies (Ephemeroptera), reinforcing that this number is the ancestral condition in Pterygota (reviewed by Matsuda, 1976). The number of spermathecae can vary across insect families, and these variation seem to arise independently along insect irradiation. Looking at insect orders, the number of spermathecae can vary from one or two in Hemiptera to one or four in Dermaptera to none or one in both Odonata and Phthiriaptera, and most commonly from none to four spermathecae in Diptera (Table 1). In some hemipterans the true ectodermal spermathecae were replaced by mesodermal analogs, such as those found in Cimicidae (Davis, 1956) or Tingidae (Marchini et al., 2010). Curiously, *Telmatoscopus albipunctatus* (Diptera: Psychodidae) females do not have a spermatheca, requiring males to inject their immotile sperm deep into the female lateral oviducts (Burrini and Dallai, 1975).

Females in the genus *Drosophila* have three SSO: two identical spermathecae and a main storage organ, which stores up to 80% of the sperm, the seminal receptacle. Thus, when the spermathecae are stunted, as in *D. melanogaster*, sperm are stored within the seminal receptacle (Lefevre and Jonsson, 1962; Pitnick et al., 1999). In addition, in several *Drosophila* species (*D. nigrohydei*, *D. eohydei*, and *D. hydei*) the spermathecal reservoir is vestigial and does not store sperm. In this case, the expanded spermathecal duct participates in sperm allocation (Pitnick et al., 1999). Furthermore, *Anastrepha suspensa* (Diptera: Tephritidae) (Fritz and Turner, 2002) and the mosquito genera *Aedes* and *Culex* (Diptera: Culicidae) have three spermathecae (Fig. 1A) (Clements and Potter, 1967; Barreto et al., 2008; Pascini et al., 2012), and Calliphoridae (Diptera) have up to four spermathecae (Harterreiten-Souza and Pujol-Luz, 2012). The number of spermathecae in other species of insects is summarized in Table 1.

Spermathecal morphology can be sufficiently diverse to differentiate species (White and Muniss, 1972). Some genera of beetles (Borowiec and Pomorska, 2009), phlebotomine sandflies (Diptera: Psychodidae), and Muscidae flies (Couri, 2004) have spermathecae with species-specific ornamentations (Rispail and Léger, 1998; Carvalho et al., 2006). Variations in gross morphology and cuticular ornamentation can also distinguish between species of the families Machilidae (Bitsch, 1989) and Therevidae (Diptera) (Winterton et al., 1999), as well as the genus *Liodessus* (Coleoptera: Dytiscidae) (Ordish, 1985).

When the female has more than one spermatheca, they are commonly dimorphic. For example, *Aedes aegypti* has one large spermatheca (100 µm diameter) and two small spermathecae (each 75 µm diameter; Fig. 1A) (Clements and Potter, 1967; Pascini et al., 2012). Spermathecal dimorphism is also present in *Periplaneta americana* (Blatarria: Blattidae), which has two spermathecae. Here, the reservoir of spermatheca A is spherical, while that of spermatheca B is long and slender (Lawson and Thomas, 1970). The biological function of this dimorphism is unknown, but may reflect differential storage and sperm use.

Interestingly, irradiation or induced mutations in some species can artificially change the number of spermathecae. *Euborellia plebeja* and *Proreus simulans* nymphs (Dermaptera) produce what appears to be developmentally repressed supernumerary spermathecae. Typically, *E. plebeja* females have one spermatheca; however, when individuals are irradiated with gamma rays, the number of spermathecae can reach seven per female (Kamimura, 2007). The number of spermathecae is also under genetic control in *Scathophaga stercoraria* (Diptera: Scathophagidae). Here, females can have either three or four spermathecae,

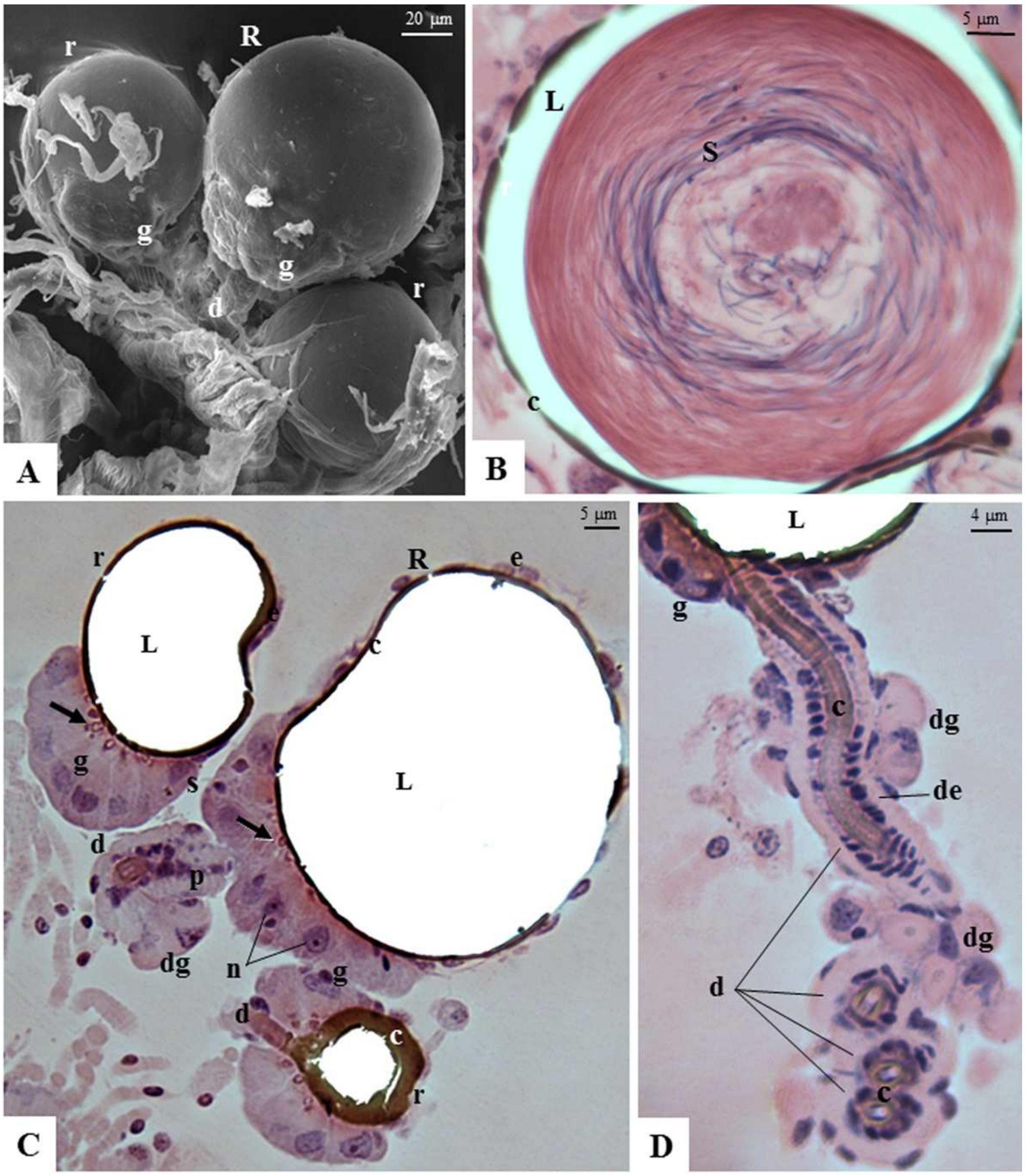

Fig. 1. Scanning electron micrograph (A) and histological sections stained with haematoxylin and eosin (B–D) of the spermathecae of *Aedes aegypti*. (A) External view of the three spherical reservoirs [one large (R) and two small (r)] with spermathecal glands (g) attached. (B) Section of sperm in a circular arrangement within the reservoir lumen. (C) Section of the reservoir (R and r) lumens that are lined by a thick cuticle (c) and a thin epithelium (e) that are continuous with their counterparts in the duct (d). Muscular tissue or spermathecal pump (p) and glandular cells (dg) are attached to the duct. The arrows point to the gland cell canaliculi where cuticle interruptions are seen. (D) Section of the spermathecal ducts (d) with lumens lined by cuticle. The columnar epithelial cells (de) are associated with duct glandular cells (dg). In (C) and (D) the reservoir contents were lost during sectioning. For

sample preparation procedures refer to Pascini et al. (2012, 2013). Abbreviations: c, cuticle; d, spermathecal duct; de, duct epithelium; g, spermathecal glands; L, reservoir lumen; p, spermathecal pump; S, sperm.

depending on the genotype (Ward, 2000). The genetic basis of this variation is unknown and the manipulation of the spermathecal number in vitro would provide more scope to study differential sperm storage and selection in a species.

In cases where the spermatheca is absent, the sperm may be stored within analogue SSOs, such as the mesodermal pseudospermathecae (Davis, 1956), or in other parts of the female reproductive system, such as the lateral oviducts (Burrini and Dallai, 1975; Marchini et al., 2010). While the biology of spermathecae has been widely studied in insects, the same cannot be said about analogue organs such as pseudospermathecae. Details including pseudospermathecae morphology, cell components, and their role in sperm competition or sperm maintenance mechanisms have been poorly explored. In the hemipteran *Cimex lectularius* (Cimicidae) two sac-like pseudospermathecae are located in the lateral oviducts. These pseudospermathecae are considered functionally similar to spermathecae, receiving and releasing sperm to fertilize eggs (Davis, 1956). In contrast, in *Stephanitis pyrioides* (Hemiptera: Tingidae) no sperm were found within pseudospermathecae, and a novel role for them as reproductive accessory glands has been proposed (Marchini et al., 2010).

3. The spermathecal reservoir

The spermathecal reservoir (also known as the chamber, receptacle, bulb, shell, sac or capsule) comprises the part of the spermatheca where sperm are stored in a parallel and/or circular array. In general, the reservoir is dilated and the most distal portion of the spermatheca is surrounded by an inner cuticle (Figs. 1A–D, 2A and 2B) (Christophers, 1960; Jones and Wheeler, 1965a, b). The reservoir can be more or less sclerotized, depending on the species, and, in hymenopterans at least, there is a positive correlation between the reservoir volume and the number of gametes it can store (Martins and Serrão, 2002; Baer, 2003; Dallai, 2014).

The reservoir cuticle is crucial for keeping the gametes isolated from the external environment and from components of the hemolymph that could cause damage to the sperm. In general, the cuticle is thick and consists of layers (more or less electron-dense) arranged according to their degree of sclerotization (Fig. 3) (Lay et al., 1999; Fritz and Turner, 2002). For instance, in *A. aegypti*, the reservoir cuticle is approximately 0.7 μm thick and has 14 levels, arranged in seven electron-dense layers that alternate with seven electron-lucent layers (Clements and Potter, 1967).

Depending on the region of the spermatheca, the composition, organization, and structure of the spermathecal cuticle differ. Consistent with the function of each component, the cuticle is generally more sclerotized in the reservoir (Fig. 1D) and more flexible in the duct (Clements and Potter, 1967; Lawson and Thomas, 1970). The cuticle of the reservoir and the spermathecal duct can be continuous (Fig. 1C) or have point of interruptions that correspond to the openings of gland cells, or even have ornamentations (Raina et al., 2007; Pascini et al., 2013).

The reservoirs have different shapes and sizes across taxa, or even within the same species, and modifications during development may cause this variation. Functionally, however, the reservoir's role is always the allocation and maintenance of sperm. The reservoir is spherical in most insects (Figs. 1A, 2A and B) (Gerber and Klostermeyer, 1970; Poole, 1970; Dallai, 1975; Dallai et al., 2012), but it can also be bilobed (e.g., ants; Fig. 2C), mushroom-shaped (e.g., fruit flies; Filosi and Perotti, 1975; Pitnick et al., 1999), kidney-shaped (e.g., orthopterans; Viscuso et al., 2015), pear-shaped (e.g., guava fruit fly; Fritz and Turner, 2002), or tubular (e.g., hemipterans; Candan et al., 2007; Borowiec and Pomorska, 2009). In *P. americana*, females have a spherical (A) and a tubular (B) reservoir. It has been suggested that the spherical reservoir preferentially provides the sperm for fertilization, while the tubular reservoir secondarily stores and provides gametes for egg fertilization. In *P. americana*, the cuticles of the two reservoirs (A and B) are distinct. In reservoir A, the cuticle is denser and highly pigmented, with a lamellar organization, similar to the exocuticle. In reservoir B, the cuticle is also lamellar, but less pigmented and less electron-dense, similar to the endocuticle (Lawson and Thomas, 1970). Furthermore, among the different species of Therevidae flies (Diptera), there are three morphologically distinct reservoirs: simple, lobed, and lobed II (Lawson and Thomas, 1970); however, their functional differences remain unknown.

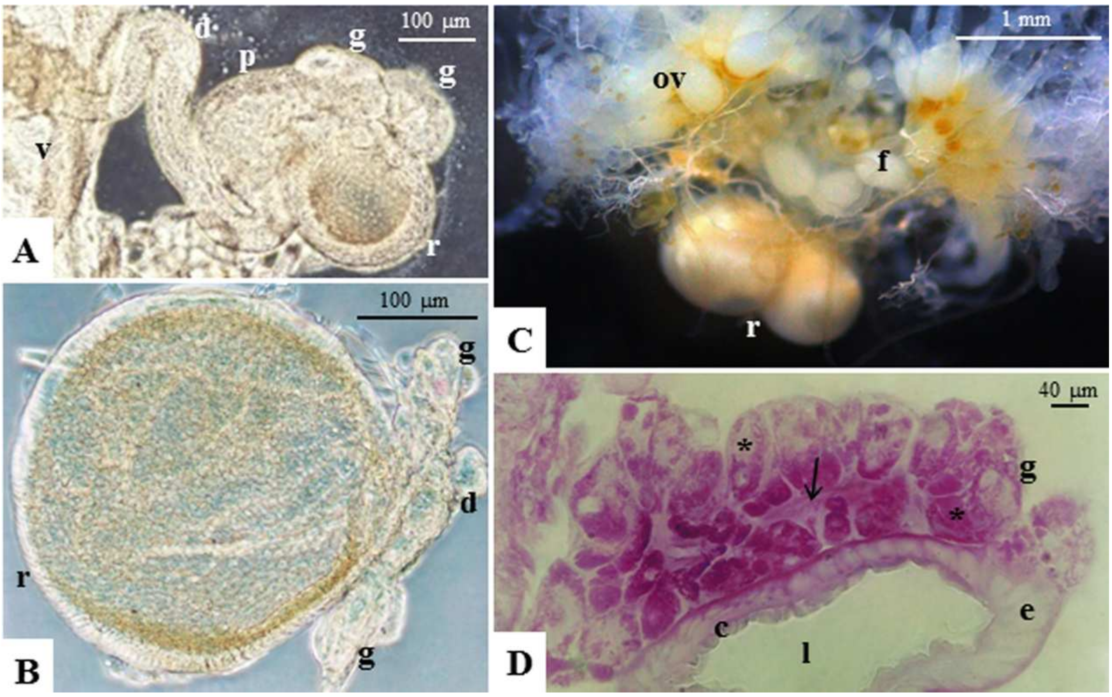

Fig. 2. Spermathecae in Hymenoptera. (A) Whole mount depicting a formaldehyde-fixed spermatheca connected to the vagina (v) of a fertilized female of the solitary bee *Thygater analis* (Apidae; Eucerini). (B) Whole mount depicting a formaldehyde-fixed spermatheca of a virgin queen of the stingless bee *Melipona quadrifasciata* (Apidae; Meliponini). The glands are attached to the reservoir. (C) Freshly dissected reproductive system (proximal portion) of a fertilized queen of the leaf-cutter ant *Acromyrmex subterraneus* (Formicidae; Attini) with a bilobate spermathecal reservoir. (D) Histological section of the spermathecal gland of a virgin queen *M. quadrifasciata* stained by PAS reaction, with gland cells (*) reacting positive for neutral polysaccharides (pink). Arrow indicates the lateral gland duct. For sample preparation procedures refer to Martins and Serrão (2002) and Pascini et al. (2012). Abbreviations: c, cuticle; d, spermathecal duct; e, columnar epithelium of reservoir; f, ovarian follicle; g, spermathecal gland; l, reservoir lumen; ov, ovary; p, spermathecal pump; r, spermathecal reservoir.

A single epithelium with a thick inner cuticle lines the spermathecal reservoir (Clements and Potter, 1967; Lay et al., 1999; Stacconi and Romani, 2011). There are two general types of epithelial organization, depending on the species: a simple epithelial layer with a single type of lining cell, or a simple epithelial layer with a glandular lining and duct-forming cells. The first type is reported in hymenopterans such as *Apis mellifera* (Hymenoptera: Apidae) (Dallai, 1975 Martins and Serrão, 2002), while the second type is present, for example, in *P. americana* (Gupta and Smith, 1969), *Zorotypus caudelli* (Zoraptera) (Dallai et al., 2012) (Fig. 3) and *Anopheles aquasalis* (Diptera: Anophelinae) (Pascini et al., 2013).

In general, the structural organization of the second type of epithelium consists of three types of cells with different functions: secretory cells (class 3 cells), duct-forming cells for transporting the secretion, and epithelial cells forming the apical cuticular layer. The cuticle of secretory cells forms a cuticular duct that connects the innermost cell with the outer cuticle of the reservoir (Fig. 3). For example, in epidermal gland cells, the duct comprises the receiving canal, located in the extracellular space of the secretory cell bordered by microvilli, as well as the conducting canal, surrounded by one or more duct-forming cells (reviewed by Quennedey, 1998). In some cases, such as in *D. melanogaster* (Filosi and Perotti, 1975) and *Locusta migratoria* (Orthoptera: Acrididae) (Lay et al., 1999), the duct-forming cells are missing. We will discuss some further aspects of the spermathecal secretory cells in Section 6.

Columnar epithelial cells are commonly seen lining the spermathecal reservoir, but in some species, the epithelial cells are flattened (Figs. 1C and 2D) and are poor in organelles such as the Golgi apparatus or rough endoplasmic reticulum (Tombes and Roppel, 1972; Sathe and Joshi, 1988; Bitsch, 1989; Gobin et al., 2006). The epithelium of the reservoir can even vary in the composition and arrangement of its cells among insects of the same family.

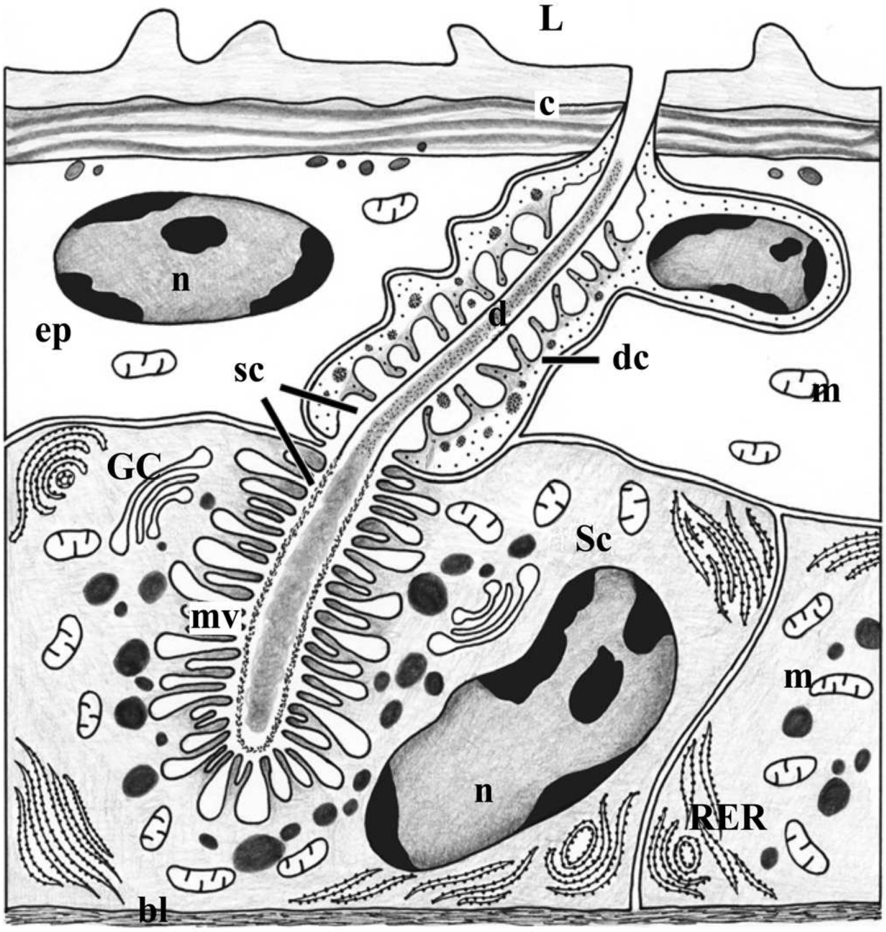

Fig. 3. Schematic drawing of the epithelium of the spermathecal reservoir of *Zorotypus caudelli* (Zoraptera) with three types of cells: epithelial cells (ep) beneath the cuticle (c), glandular or secretory (class 3) cells (G) and duct-forming cells (dc). Glandular cells have a secretory cavity (sc) bordered by microvilli (mv), which end on a duct (d) filled with electron-dense material. Modified from Dallai et al. (2012). Abbreviations: b, basal lamina; GC, Golgi complex; L, reservoir lumen; m, mitochondria; n, cell nuclei; RER, rough endoplasmic reticulum.

For instance, among Culicidae species, the reservoir is lined by flattened and interdigitated cells, as in *A. aegypti* (Fig. 1C) (Clements and Potter, 1967; Pascini et al., 2012); or as in *A. aquasalis*, the lining cells can be columnar and associated with glandular and duct-forming cells (Pascini et al., 2013).

The basal border of the reservoir columnar cells is densely compartmentalized by membrane infoldings, forming long narrow canals, associated with mitochondria, while the apical border shows irregular microvilli close to the cuticle (Dallai, 1975; Wheeler and

Krutzsch, 1994; Martins et al., 2008). It has been suggested that these characteristics result in an increased cell surface area, either facilitating the uptake of compounds in the hemolymph to be transferred to the reservoir lumen (Gobin et al., 2006; Martins et al., 2008). It appears that the role of the columnar cells is not restricted to mechanical protection or isolating sperm from the hemolymph, at least in eusocial hymenopterans. Here, these cells are supposedly active in the transport of substances between the reservoir lumen and the hemolymph, playing a role in the maintenance of the lumen milieu, together with gland cells.

Characteristics of the reservoir epithelial cells may also vary with the physiological state of the female. For example, in *A. mellifera*, the epithelium in physogastric queens is thicker than that in virgins, presumably to increase the robustness of the organ and enhance protection of the gametes. Moreover, the reservoir cells of virgin queens have projections into the lumen that are not found in fertilized queens (Poole, 1970; Dallai, 1975). In *Orchesella villosa* (Collembola), these projections are believed to provide mechanical strength to the reservoir after being filled with sperm (Dallai et al., 2008b).

Reservoirs also vary in size according to social behavior. Eusocial species have larger reservoirs than solitary species (Fig. 2A–C) (Pabalan et al., 1996; Martins and Serrão, 2002). Caste is also important; queens usually have large reservoirs, while those of workers are vestigial or even absent (Ito and Ohkawara, 1994; Schoeters and Billen, 2000). In ponerine ants, the epithelial cells in queens are columnar with a well-developed rough endoplasmic reticulum and a basal nucleus consistent with the biosynthesis and transport of substances from the hemolymph into the reservoir lumen, and they have a large number of mitochondria. However, worker ants have flattened epithelial cells that contain few organelles, suggesting the loss of transport or secretory activity in this caste (Gobin et al., 2006).

External to the spermathecal epithelium, muscle fibers surround either the entire spermathecal reservoir (Gupta and Smith, 1969; Villavaso, 1975; Bailey and Nuhardiyati, 2005) or only the duct or duct/reservoir junction where they form a sphincter or spermathecal (sperm) pump (Figs. 2A and 4A) (Dallai, 1975; Gschwentner and Tadler, 2000; Martins and Serrão, 2002). At this junction, the spermathecal cuticle is thin, facilitating contraction (Ordish, 1985; Gobin et al., 2006; Borowiec and Pomorska, 2009). In *A. suspensa* (Fritz and Turner, 2002) and *Phlebotomus papatasi* (Diptera: Psychodidae) (Ilango, 2005), the thin cuticle forms valve-like structures near the pump that are associated with circularly arranged muscle fibers that control the opening and closing of the duct, and hence the transit of sperm (Ilango, 2005).

The arrangement of the spermathecal pump muscles also varies according to the species. For example, in bees, ants and wasps, the spermathecal pump is limited to the reservoir–duct transition (Fig. 4A), forming a ring or a sphincter of multilayered concentric muscle fibers (Martins and Serrão, 2002; Gobin et al., 2006; Martins et al., 2008). This sphincter has been suggested to be involved in controlling the release of sperm and spermathecal content during fertilization (Baer et al., 2016). In *Megachile rotundata* (Hymenoptera: Megachilidae), the muscle fibers of the spermathecal pump relax at the time of fertilization, allowing the release of sperm into the oviduct where fertilization occurs. In this way, the female can control the production of drones (from unfertilized eggs) or females (from fertilized eggs) (Gerber and Klostermeyer, 1970).

In *A. aegypti*, a layer of muscle fibers runs longitudinally along the spermathecal duct (Clements and Potter, 1967; Pascini et al., 2012), while in *A. aquasalis* muscles are organized in a helical arrangement surrounding the spermathecal duct. In mosquitoes, as in other insects described above, this muscular apparatus has been implicated in the control of reservoir filling after mating, as well as the release of spermatozoa for fertilization (Giglioli, 1963), though whether these variations in the muscular organization affect sperm transport or not is unclear. In *P. americana*, the muscles cover the reservoir externally, and when they contract, a pressure is applied to the secretory cells, implicating this mechanism in not only sperm release, but also in secretion release into the reservoir lumen (Lawson and Thomas, 1970).

In *L. migratoria,* a neural loop controls spermathecal muscle contractions and thereby also controls sperm release by the reservoir during oviposition. Two synergic nerves from the ventral ovipositor nerve and from the seminal receptacle nerve to the eighth abdominal ganglion provide sensory information. This neural input alters the neural activity of the motor neurons that innervate the spermatheca and modifies spermathecal muscle contraction patterns (Clark and Lange, 2000).

In addition to sperm, the reservoir lumen also contains seminal fluid and substances produced by the spermathecal glands. Under an electron microscope, these substances appear flocculent and electron-dense, characteristics consistent with protein secretions (Clements and Potter, 1967; Fritz and Turner, 2002; Pascini et al., 2012). The spermathecal reservoir also contains its own microbiota. In *Glossina morsitans morsitans* (Diptera: Glossinidae), the endosymbiotic bacteria *Sodalis* is found throughout the female reproductive tract, and it is believed to provide benefits to its host (Balmand et al., 2013). A study identified 11 species of anaerobic bacteria and 6 aerobic species in *Coptotermes formosanus* (Isoptera: Rhinotermitidae) (Raina et al., 2007). In *Trioza alacris* (Hemiptera: Triozidae), sperm are immersed in a granular material containing gram-negative bacteria of the species *Erwinia tasmaniensis* (Marchini et al., 2014).

The knowledge about spermathecal microbiota is very limited in terms of the number of species studied and regarding the roles of the microbiota. The interplay between this specific microbiota and the spermathecal environment or sperm cells is obscure and we advise more investigations into this subject. Another gap in the literature that will need to be investigated in future is the origin of the microbiota in terms of female or male contribution. It is speculated that bacteria are released with the male ejaculate in order to provide nutrients to the sperm and, perhaps, to protect them against harmful microorganisms (Raina et al., 2007; Marchini et al., 2014).

The spermathecal reservoir also plays a crucial role in the venereal transmission of viruses from males to females. Examples include the deformed wing virus (DWV) in *A. mellifera*, and the Chikungunya virus (CHIKV) in *A. aegypti*. In these cases, the virus can be detected in the reservoir lumen after females have copulated with infected males. Later, the virus moves to the ovaries, from where it can pass to progeny (i.e., by vertical transmission) (Miranda and Fries, 2007; Mavale et al., 2010). In the case of DWV, the epidemiological significance of venereal transmission may be considerable, with the spermatheca acting as a “bridge” that amplifies virus transmission to offspring (Miranda and Fries, 2007). In the case of CHIKV, vertical transmission may maintain the virus in mosquito populations during inter-epidemic periods (Mavale et al., 2010). Besides viruses, *Wolbachia* bacteria can also be found in the female reproductive tract, including the spermatheca, after copulation, e.g. in mosquitoes such as *Culex pipiens pipiens* (Diptera: Culicidae), and *Wolbachia* infection can trigger cytoplasmic incompatibility in offspring (Beckmann and Fallon, 2013).

4. Spermathecal reservoir filling

The frequency of sperm uptake varies within insects, and females present different strategies for sperm storage. For example, species of orthopterans, beetles, lepidopterans, and termites must mate repeatedly to re-stock their sperm supplies within the spermatheca (Otronen et al., 1997; Lay et al., 1999; Drnevich et al., 2000; Seth et al., 2002; Raina et al., 2007). In contrast, in eusocial hymenopterans, females only mate for a short period early in their adulthood, but can store millions of sperm cells within their spermathecae for years (Baer, 2003; Lodesani et al., 2004). For example, in several eusocial species of bees and ants, the queens copulate with one or more males during their nuptial flight. The queens can then lay fertilized eggs for years or even decades after, generating hundreds or even millions of individual offspring (Ito and Ohkawara, 1994; Schoeters and Billen, 2000; Lodesani et al., 2004).

During copulation, the male insect deposits semen (containing sperm and seminal fluid) into the proximal portion of the female reproductive system (Fig. 4A), which may be the vagina, bursa copulatrix, or genital chamber, depending on the insect (reviewed by Chapman, 2013, and Degner and Harrington, 2016). Certain ejaculated substances, regarded as "nuptial gifts", may include immunostimulatory or antibiotic components, minerals, water and ions such as zinc. These substances can play various roles: they can nourish the sperm or be important for the sperm capacitation process, or they may reduce female receptivity or increase oviposition. In some cases, male peptides reduce the female receptiveness to males, providing the first mating male a temporal advantage over subsequent matings (see Gwyne, 2008, and papers therein).

The sperm usually migrate and/or are transported in bundles or individually (Fig. 4B) and are stored in one or more spermathecae (Werner at al., 2007; Jones and Wheeler, 1965b). In some instances, males transfer the sperm and seminal fluid directly to the spermatheca. For example, in the bug *Oncopeltus fasciatus* (Hemiptera: Lygaeidae), the flagellum of the male's aedeagus reaches all the way into the spermatheca to deposit the semen (Bonhag and Wick, 1953). Sperm can also be transferred to the reservoir within capsules smaller than spermatophores, known as spermatodoses. These spermatodoses slowly release sperm into the spermathecal reservoir (Marchini et al., 2012; Viscuso et al., 2015).

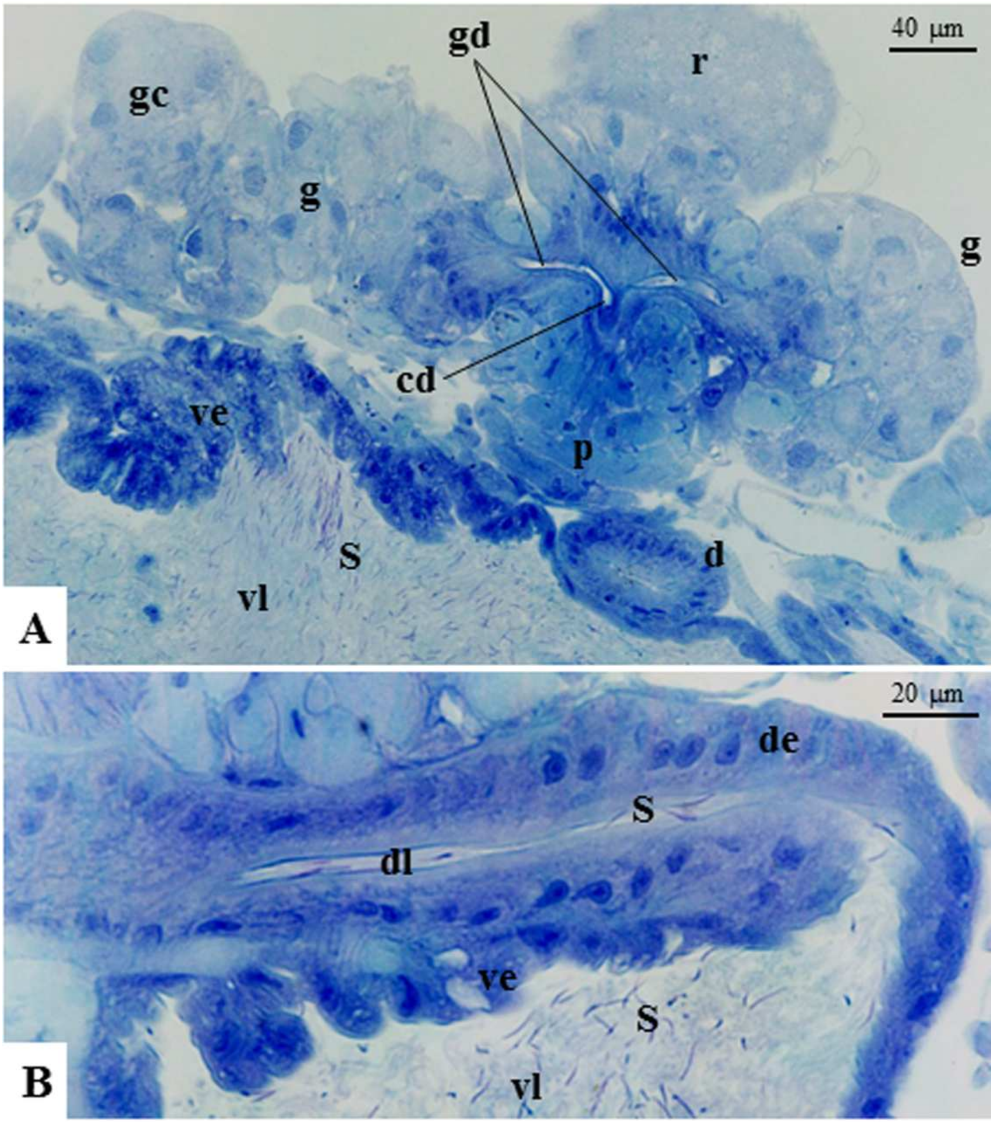

Fig. 4. Histological sections of part of the genital tract of a queen of the stingless bee *Melipona quadrifasciata* (Apidae; Meliponini) stained with toluidine blue. Sample was dissected 10 min after copulation. (A) Sperm is visible within the vaginal lumen (vl). The spermathecal glands (g) can be seen with the glandular cells (gc). The two gland branches have lateral glandular ducts (gd) that open in a common duct (cd). (B) Sperm can be seen within the vaginal lumen (vl) and within the spermathecal duct lumen (dl). For sample preparation procedures refer to Martins and Serrão (2004). Abbreviations: d, spermathecal duct (cross-sectioned); de, duct epithelium; p, spermathecal pump; r, spermathecal reservoir; S, sperm; ve, vaginal epithelium.

The speed with which the spermathecal reservoir is filled varies according to the insect. In general, the reservoir fills within a few minutes after copulation (Hartmann and Loher, 1999; Martins and Serrão, 2004; Bailey and Nuhardiyati, 2005; Werner et al., 2007). However, this process can also take several hours, as in the silkworm, *Bombyx mori* (Lepidoptera:

Bombycidae), in which the reservoir takes more than 2.5 h to fill completely (Suzuki et al., 1996). The method of sperm transport after mating also varies according to species. Sperm cells move in groups (bundles) in lepidopterans, or sperm cells can move individually, as in *Drusilla canaliculata* (Coleoptera: Staphylinidae). In the case of *D. canaliculata*, single sperm can move at an average speed of 47.5 μm s^{-1} (Werner et al., 2007; reviewed by Werner and Simmons, 2008).

The peristaltic contractions of the proximal female reproductive tract are coordinated with sperm movements, usually leading to the reservoir filling (Davey, 1958; Villavaso, 1975; Linley, 1981). However, in some cases, such as *A. aegypti*, sperm, once inside the female, find their route to the spermathecal reservoir and sperm locomotion alone seems to be sufficient to fill the spermatheca (Jones and Wheeler, 1965a, b; Degner and Harrington, 2016).

The control of sperm transfer to the SSO by semen proteins has been partially unraveled in *D. melanogaster*. Here, during the sperm migration the uterus changes from an S-shaped structure with a closed lumen to a more open and dilated conformation, opening the entrances of the SSO. The male accessory gland proteins (Acps) that are known to play a role in the post-mating uterine conformational changes appear to trigger this process (Neubaum and Wolfner, 1999; Qazi and Wolfner, 2003; Ram and Wolfner, 2007). For instance, Acp36DE seems to be a key protein for fruit fly reproduction. Acp36DE binds to the lower oviduct on the ventral side of mated females, just above the opening to the SSO. In Acp36DE-knockout females mated with wild-type males, the post-mating uterine changes are prevented, avoiding sperm migration. Additionally, sperm from Acp36DE-depleted males fail to trigger the uterine changes, and the sperm become stuck in the entrance of the SSO (Neubaum and Wolfner, 1999; Avila and Wolfner, 2009).

Soon after mating, spermatophores of male *Rhodnius prolixus* (Hemiptera; Reduviidae) release secretions that supposedly interact with the female reproductive system and trigger waves of muscle contractions, driving the sperm to the spermathecal reservoir (Davey, 1958). In the boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae), experimentally damaging the spermathecal muscles affects reservoir filling, confirming that the physical displacement of sperm depends on spermathecal muscle activity (Villavaso, 1975). Other factors can also affect reservoir filling. For instance, in *Culicoides melleus* (Diptera: Ceratopogonidae), a flexible spermatophore capsule containing spermatozoa and male accessory fluid is deposited in the female genital tract. Sperm allocation in *C. melleus* is complete within 7 min because of the release of the accessory fluid and the negative pressure generated by active fluid withdrawal from the reservoir that aspirates the sperm. Fluid withdrawal from the reservoir occurs to sustain the flow of male product toward the reservoir lumen, and to avoid spermathecal volume gain that could potentially lead to rupture (Linley, 1981).

After mating, *D. melanogaster* sperm stay immersed in seminal fluid in the uterus for 2 or 3 min, called sperm capacitation, whereby the male seminal fluid induces the sperm to swim actively toward the seminal receptacle. Once the seminal receptacle is full, the sperm are directed to the spermathecae for storage (Nonidez, 1920). Chemotaxis also plays an important role in the migration of sperm to the spermatheca. In *A. grandis*, sperm move toward the contents of the spermathecal reservoir (Grodner and Steffens, 1978). In *P. papatasi*, substances in the spermathecal lumen of virgins may attract sperm and assist in their organization (Ilango, 2005). Despite the significant role these chemotactic substances play in spermathecal filling, their chemical nature and their mechanism of action have not been elucidated to date (reviewed by Kaupp et al., 2008, and Wolfner, 2011).

The number of gametes in the ejaculate that migrate to the reservoir after copulation varies according to species (Lefevre and Jonsson, 1962; Jones and Wheeler, 1965a,b; Lizé et al., 2009). In several cricket species, the female stores 74% of the male sperm inside the spermatheca and uses 69% of this sperm for fertilization (Bretman et al., 2009). Notably, *Ceratitis capitata* (Diptera: Brachycera) fruit fly females store only ~24% of the sperm released by the male during mating (Taylor and Yuval, 1999). In *A. mellifera*, a single male is capable of ejaculating 6–12 million sperm; however, the spermatheca can store only half of this amount, which is still far more than needed for colony cycle completion. One possible reason for the differences in the number of ejaculated and stored sperm might be that males invest in sperm quantity, while females select for sperm quality (reviewed by Baer, 2003).

Species with more than one spermatheca do not fill the reservoirs simultaneously. For example, *A. aegypti* fills the largest reservoir first, followed by the two smaller ones (Jones and Wheeler, 1965a, b; Jones, 1973). Therefore, it appears that sperm are selectively stored according to reservoir capacity. Whether there is selection for spermathecal size is another unanswered question.

Once inside the reservoir, sperm are embedded within a spermathecal fluid that can promote changes in their mobility and structure (Bernasconi et al., 2002; Bretman et al., 2009; King et al., 2011). For example, in *Eyprepocnemis plorans* (Orthoptera: Acrididae), the spermathecal fluid reduces sperm motility by promoting the destabilization of the spermatozoa's tail, through the action of gp35, a 35-kDa glycoprotein that triggers the depolymerization of microtubules in the axoneme (Giuffrida et al., 1996). Reduced motility suggests a lower metabolic activity during storage, and therefore diminished oxidative stress, which would extend the lifespan of the stored sperm (Ribou and Reinhardt, 2012). In *Sciara coprophila* (Diptera: Sciaridae), sperm maturation in the spermathecae also includes changes in the configuration of the axoneme, and an elimination of the major portion of the mitochondria (Makielski, 1966); however, in this case, the spermathecal factor(s) guiding these changes are unknown.

The neural or endocrine control of reservoir filling is poorly studied. In *Sitophilus granarius* (Coleoptera: Curculionidae), peripheral nerves regulate filling by controlling the muscle fibers that surround the spermatheca (Tombes and Roppel, 1972). Furthermore, in *Blattella germanica* (Blattodea: Blattellidae) juvenile hormone (JH) triggers movements of the spermathecal duct which assist in the delivery of gametes to the reservoir (Liang and Schal, 1994). In *Gomphocerus rufus* (Orthoptera: Acrididae), JH may stimulate the production and release of proteolytic enzymes from the spermathecal duct that disrupt the spermatophore, simulating sperm release inside the female (Hartmann, 1978).

5. The spermathecal duct

There is no consensus on whether or not the spermathecal duct is a separate entity to the spermatheca, or a part of it together with the reservoir, the pump and the glandular portion. Despite this variation in the literature, in the present review we consider the spermathecal duct as part of the spermatheca, as proposed elsewhere (Snodgrass, 1935; Gupta and Smith, 1969; Dallai, 1975; Schoeters and Billen, 2000). In *D. melanogaster* a single spermathecal capsule, gland unit and duct arise from an initially undifferentiated outgrowth of the female reproductive tract in the pupa (Mayhew and Merritt, 2013), so they could be considered part of the same developmentally defined structure.

The spermathecal duct connects the spermathecal reservoir with the proximal portion of the female reproductive tract, allowing the transit of sperm in both directions. In most insects, the spermathecal duct is linear but in coleopterans of the genus *Cassida*, it is highly spiraled (Matsumura et al., 2016). A single columnar epithelium covered by a cuticle that is continuous with that of the reservoir lines the duct (Figs. 1D, 2A and 4B). In mosquitoes, the cuticle of their elongated spermathecal ducts contains resilin, which promotes elasticity, while the reservoir cuticle contains cuticulin, which provides strength and stiffness (Clements and Potter, 1967; Jones and Fischman, 1970; Pascini et al., 2012). Longitudinally or circularly organized striated muscle fibers (the spermathecal pump) surround the duct to assist the transfer of the ejaculate into the reservoir (Figs. 1C and D) (Christophers, 1960; Spielman, 1964; Clements and Potter, 1967; Pascini et al., 2012).

In bees and ants, characteristics of the duct epithelium may vary according to the reproductive status of the queen or caste (Schoeters and Billen, 2000; Gobin et al., 2006). For example, *Bombus morio* queens have a long spermathecal duct that supplies sperm with nutrients. The cytoplasm of the duct cells contains glycogen particles and numerous mitochondria that associate with the apices of the microvilli, suggesting a transport epithelium (Schoeters and Billen, 2000). In addition to these functions, in the ant *Leptothorax gredleri* (Hymenoptera: Formicidae) and in the beetle *D. canaliculata*, the ducts select the gametes to be stored in the reservoir, acting as a bottleneck. In *L. gredleri*, there is a constriction in the spermathecal duct close to the entrance of the spermathecal reservoir, through which sperm cells have to pass individually (Oppelt and Heinze, 2007). In *D. canaliculata*, the sperm can easily be stuck within the narrow spermathecal duct, blocking the spermathecal filling process (Oppelt and Heinze, 2007; Werner et al., 2007).

The duct has different roles depending on the species, e.g. acting to isolate semen from the hemolymph or secreting substances into the lumen. In *S. granarius*, material accumulates between the epithelial cells in the duct, suggesting that the cells play a role in osmoregulation by blocking the transport of substances from the duct lumen into the body cavity (Tombes and Roppel, 1972). In *G. rufus*, the duct epithelial cells secrete proteolytic enzymes that dissolve the spermatophore, enabling the release of its contents. In addition, the epithelial lining resorbs spermatophore material present in the lumen (Hartmann and Loher, 1999).

6. The spermathecal glandular portion

The glandular portion of the spermatheca generally consists of a few exocrine secretory cells that form a separate unit. This unit is either attached to the region between the duct and the reservoir (Figs. 1C, 2A and D) (Bitsch, 1989; Pabalan et al., 1996; Schoeters and Billen, 2000; Martins et al., 2005) or consists of modified epithelial cells integrated into the reservoir wall (Fig. 3) (Gupta and Smith, 1969; Lay et al., 1999; Dallai et al., 2012; Pascini et al., 2013). In the first case, the cells form a spermathecal gland unit, as in the Hymenoptera (Schoeters and Billen, 2000; Cruz-Landim and Serrão, 2002; Martins et al., 2005; Gotoh et al., 2012) and Coleoptera (Happ and Happ, 1970; Tombes and Roppel, 1972). In Hymenoptera, the secretory cells release substances into a glandular duct (Figs. 2D and 4A) that directs the secretions into the lumen of the reservoir. The precise number of glandular cells in the spermathecae is largely unknown for insects. *A. aquasalis* has approximately 95 reservoir glandular cells, and *A. aegypti* has 33–43 glandular cells in the two small spermathecae and 70–85 in the large spermatheca. In the latter case, the number of gland cells is more or less commensurate with reservoir size/demand (Pascini et al., 2012, 2013).

Glandular cells can also be attached to the spermathecal duct, but remain separate from the duct epithelial cells (Davey, 1958; Gerber and Klostermeyer, 1972; Hartmann and Loher, 1999; Raina, 2007). *A. aegypti* glandular cells are attached to the duct or the reservoir–duct transition, and each cell has its own ductule (Fig. 1C and D). The apex of each glandular cell has microvilli that are in contact with individual cell ductules, which are surrounded by a very thin epicuticle. The ductules open directly into the spermathecal lumen through the pores of the cuticle (Fig. 3) (Jones and Fischman, 1970; Pascini et al., 2012).

The spermathecal glandular portion is composed of class/type 3 glandular cells connected to the spermathecal lumen by means of a cuticular duct. The cell duct has two parts: one corresponding to the receiving canal located in the extracellular space of the secretory cell, bound by microvilli, where the secretion is provisionally stored; and another corresponding to the conducting canal, generally surrounded by an epidermal cell, from where the secretion is transported to the reservoir lumen (Fig. 3). The receiving canal is usually a simple elongated conducting canal which comprises cuticulin and an inner epicuticle (Noirot and Quennedey, 1991; reviewed by Quennedey, 1998).

The spermathecal gland cells of insects are polarized and the microvilli or infoldings of the plasma membrane may increase the surface area and facilitate transfer between the hemolymph, the cell, and the spermathecal cuticle. A permanent microvillous border can be observed at the cell apex, while the basal region is rich in endoplasmic reticulum, Golgi complexes, and mitochondria (Tombes and Roppel, 1972; reviewed by Quennedey, 1998; Fritz and Turner, 2002; Gobin et al., 2006; Dallai et al., 2012). Interestingly, the spermathecae of Collembola do not show the typical characteristics of secretory cells of insects (Dallai et al., 2008a, b).

In the family Formicidae (Hymenoptera), the spermathecal glands have two elongated branches, each with an individual duct that communicates with a common duct (Fig. 4A). In the family Vespidae, the spermathecal glands have a Y-like shape, while in Apidae the spermathecal glands consist of two elongated branches located at the junction between the duct and the spermathecal reservoir (Fig. 2B) (Martins et al., 2005; Gobin et al., 2006). In the genus *Bombus*, gland size varies according to caste; glands in queens are twice as large as the glands in the workers (Schoeters and Billen, 2000).

In *D. melanogaster*, the spermathecal glandular cells are located around each spermathecal reservoir. Each cell represents a separate secretory unit that discharges secretions directly into the spermathecal lumen (Nonidez, 1920; Filosi and Perotti, 1975). There are two subtypes of cells, dark and light, distinguished by their cytoplasm density. In both types, the nuclei are elliptic with a prominent nucleolus, and located in the basal portion of the cell. The dark cells display a well-developed rough endoplasmic reticulum and a large number of free ribosomes and mitochondria. The light cells display a poorly developed rough endoplasmic reticulum and scattered organelles throughout the cytoplasm (Filosi and Perotti, 1975; Mayhew and Merritt, 2013).

Juvenile hormones (JH) may stimulate the secretory activity of spermathecal glands. For example, in adult *Melanoplus sanguinipes* (Orthoptera: Acrididae) females, JH treatment increases leucine incorporation in the spermatheca, but does not affect the protein or carbohydrate contents (Gillott and Venkatesh, 1985). Additionally, ecdysteroid hormones appear to affect the secretory activity of spermathecal glands in *A. mellifera*, increasing or reducing the secretion of carbohydrates during the transport of sperm to the spermatheca (Wegener et al., 2013). Thus, JH and ecdysteroid hormones can influence the secretion of compounds related to the maintenance and nutrition of sperm in the spermatheca, but this remains poorly understood.

Spermathecal gland secretions probably play a role in maintaining sperm viability (reviewed by Werner and Simmons, 2008, and Wolfner, 2011). Glycogen, glycoproteins, and phospholipids are found in the gland secretions of *S. granarius*, all of which are substances that theoretically promote sperm nutrition in the spermatheca (Bhatnagar and Musgrave, 1971). Gland secretions also contain glycoproteins and mucopolysaccharides (Grodner and Steffens, 1978; Ilango, 2005; Klowden, 2006, reviewed by Wolfner, 2011). These components increase the motility of sperm in *A. grandis*, with sperm activity reduced after the experimental removal of spermathecal glands (Villavaso, 1975; Grodner and Steffens, 1978). Sugar metabolism is crucial for the reproductive output of *D. melanogaster*; glucose dehydrogenase (*Gld*) knockout females stored ~77% less sperm and delayed egg laying by 11 days compared to wild-type females (Iida and Cavener, 2004).

Little is known about the specific spermatheca-secreted gene products, including those of the gland cells required for sperm storage in *D. melanogaster*. Nevertheless, several notable functional categories are found in the lists of genes highly expressed in the spermathecae, and more broadly in response to mating. These categories include serine proteases, which may participate in signaling cascades or metabolic processes necessary for sperm maintenance or maturation, and antimicrobial peptides, which may protect sperm or females from infectious agents transferred during copulation (Ferrandon et al., 1998; Schnakenberg et al., 2011, 2012).

6. Spermatheca development

The spermatheca has been considered incompletely homologous throughout the orders of insects, arising from different developmental processes (reviewed by Matsuda et al., 1976). Depending on the type of insect developmental cycle, the spermatheca develops either during the last immature stage of development or during metamorphosis (Christophers, 1960; Raina et al., 2007). Parts of the reproductive systems of insects, including the spermatheca, originate via invaginations of the imaginal discs present on the eighth abdominal segment (Christophers, 1960). A few studies have focused on the development of spermathecae during post-embryonic development, including in-depth studies on queens of the wasp genus *Polistes*, the ant genus *Pachycondyla* (Gotoh et al., 2009), and of *D. melanogaster* (Mayhew and Merritt, 2013).

The development of the spermatheca in *Polistes* spp. begins with the invagination of the imaginal disc during the last larval stage and continues until the beginning of the pupal stage. Early in spermathecal development, the spermathecal rudiment appears as a tiny circular structure, arising during the pre-pupal and initial pupal stages. On the fourth day of the onset of pupation, the duct and the spermathecal glands are already evident. From the mid-point of the pupal stage, the reservoir begins to grow, and by the end, the reservoir wall has thickened and the muscle bundles of the spermathecal pump are established. In *Pachycondyla* ants, the gland and spermathecal duct differentiate early in the pupal stage and the reservoir wall is nearly as thick as that found in adults (Gotoh et al., 2009). In *D. melanogaster*, the spermathecal reservoir appears 24 h after the onset of pupation, forming a club-shape that persists until 36 h after pupation onset, when the reservoir lumen begins to increase in size and accumulate secretions. The most dramatic change in spermathecal morphology occurs between 42 and 48 h after pupation onset, when the spermatheca acquires a spherical shape that persists into adulthood. 36 h after pupation, a few gland cells appear and rearrange to form rosettes, thereby establishing an extracellular cavity (or end-apparatus) where glandular secretions are released. The glandular domain is complete within 72 h after the onset of pupation, which involves the death of a number of gland cells and coincides with a growing reservoir lumen (Mayhew and Merritt, 2013).

The process of spermathecal cuticle deposition during development is similar to that described for the exoskeleton, starting in the ecdysis between the last nymphal stage and the adult stage. In holometabolous insects, the process begins during metamorphosis and progresses until metamorphosis is complete, or soon after adult emergence (Christophers, 1960; Clements and Potter, 1967; Mayhew and Merritt, 2013).

Even ametabolous insects such as Machilidae (Archaeognatha) change the reproductive tract during ecdysis. In *Trigoniophthalmus alternatus* (Archaeognatha: Machilidae), two large invaginations, which allow for the establishment of the ductules, form in the middle portion of the spermathecae. After apolysis, the cells of the glandular unit undergo partial dedifferentiation. The secretory cavity of the glandular cell and its microvilli disappear, and some membrane and cytoplasmic structures are eliminated, which can be observed in the molting fluid. After ecdysis, the spermathecal cells rearrange to form buds that give rise to a new reservoir and spermathecal gland duct. Epicuticle deposition in the ductules of gland cells also occurs during this time, thereby establishing the new spermatheca that will persist throughout adulthood (Bitsch, 1989).

7. Conclusions and future directions

As we have illustrated, the current literature abounds with studies of insect spermathecal number, morphology, and filling. Despite significant morphological variation across species, the sperm storage capacity of spermathecae is broadly conserved among insects. This is not surprising, considering the importance of this organ for ensuring gamete quality and viability in insects.

The order Diptera is one of the most species-rich, ecologically innovative, and anatomically varied groups of organisms. During their evolutionary history, dipterans have transformed into an exuberant array of different shapes and sizes (Yeates and Wiegmann, 2005). This extensive anatomical variation accords with the variation observed in the morphology, function, number, and types of the female reproductive tract and SSO (Puniamoorthy et al., 2010; Krzemińska and Gorzka, 2016). However, it remains unclear whether the variations of spermathecal number, size and morphology influence the number, size or type of sperm storage in dipterans, or whether these variations differentially affect sperm attraction, competition, or selection within the reservoir. Finally, certain aspects of the spermathecal biology of dipterans, such as how the patterns in spermathecal number correlate with female longevity, life history, behavior, and/or taxonomy, remain unknown.

The spermatheca is a complex organ that creates a suitable environment for ensuring the long-term viability of male gametes within females. However, little is known about the specific substances produced by the spermatheca, or their role in the maintenance and protection of gametes (Clements and Potter, 1967; Schoeters and Billen, 2000; den Boer et al., 2009; Malta et al., 2014). The genes expressed in the spermatheca of *Drosophila simulans* (Diptera: Drosophilidae) illustrate the intricate structure and function of the spermatheca. Eleven genes have been shown to encode serine proteases, protein carriers, or antimicrobial and energy metabolism-related proteins. These genes are associated with processes that establish an environment suitable for the allocation and nutrition of sperm within the spermatheca (Prokupek et al., 2008). In addition, the increased expression of genes that code for proteases and peroxidases in the female genital tract of *Anopheles gambiae* (Diptera: Anophelinae) is presumably present in order to prepare the spermathecal environment for the reception of male gametes (Rogers et al., 2008). Although a number of recent studies characterize gene expression at the transcriptional level, it is necessary to go further to understand protein expression, particularly in the spermathecal lumen. Expression inhibition (for example, by RNA interference) of genes responsible for encoding spermathecal proteins that attract or extend the longevity of sperm is another promising avenue of research. Understanding the role of different gene products in sperm chemotaxis or preservation could provide targets for genetic strategies to prevent reproduction, for example by reducing sperm, and so controlling insect populations.

In addition to the proteins secreted by the spermatheca (den Boer et al., 2009), there is evidence in honeybees indicating that the ionic environment within the spermatheca supports sperm preservation. The high ion (Na^+/K^+) concentrations within the reservoir lumen supposedly reduce sperm motility and consequently, oxygen consumption (Verma, 1973, 1978). The importance of ions in sperm movement raises the question of whether or not spermathecae can control the ion concentration within the spermathecal milieu in order to modulate sperm activity. Additionally, spermathecal secretions function as a cryoprotectant, allowing sperm to survive cooling in honeybee queens (Harbo, 1977). Thus, it is confirmed that gamete preservation depends on the intrinsic components present within the spermathecal environment. Improved understanding of how insects maintain sperm quality within this complex environment will open new avenues of insect management by means of in vitro semen preservation and manipulation. In particular, sperm preservation will be valuable in apiculture, where large amounts of semen have to be preserved for long periods in order to artificially inseminate virgin queens.

The role of the insect spermatheca in preserving gametes is accomplished through a combination of physical protection and isolation, nourishment and chemical protection by spermathecal glandular products, and secondarily, sequestration of male seminal fluid components. Further studies are required on how these factors, together with spermathecal microbiota, preserve sperm. The experimental elimination of the microbiota would be useful to shed light on the role of microorganisms within the spermathecal milieu before or after insemination. Unravelling the endocrine control of the spermatheca and elucidating the mechanisms of chemical communication between the spermathecal lumen and the hemolymph after insemination are other important research challenges, which will provide novel insights into the role of the spermatheca in insect reproduction. Finally, it is of great interest to compare spermathecal biology across insect taxa to reveal the biological mechanisms that have, or have not, been conserved throughout the evolution of female SSO.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.zool.2016.12.001.

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Order	**Family**	**Species**	**Number of spermathecae**	**References**
Orthoptera	Acrididae	*Acrida exaltata*	1	Sathe and Joshi, 1988
		Baeacris punctulatus		Michel and Terán, 2005
		Eyprepocnemis plorans		Hartmann, 1978; Giuffrida et al., 1996
		Gomphocerus rufus		Hartmann and Loher, 1999
		Gastrimargus transversus		Sathe and Joshi, 1988
		Gomphocerus rufus		
		Gryllus domesticus		
		Hieroglyphus banian		
		Liogryllus bimaculatus		
		Teratodus monticollis		
		Locusta migratoria		Clark and Lange, 2000
		Locusta migratoria migratorioides		Lay et al., 1999
		Melanoplus sanguinipes		Gillott and Venkatesh, 1985
		Schistocerca americana		Gardner, 2004
	Gryllotalpidae	*Gryllotalpa africana*		Sathe and Joshi, 1988
	Gryllidae	*Valencia, Spain field crickets*		Bretman et al., 2009
	Tettigoniidae	*Eupholidoptera chabrieri bimucronata, Uromenus brevicollis trinacriae*		Viscuso et al., 2015
Hymenoptera	Formicidae	*Acromyrmex balzani, Acromyrmex landolti, Acromyrmex landolti balzani*		Ortiz and Camargo-Mathias, 2007
		Acromyrmex subterraneus		This study (Fig. 2C)
		Atta sexdens rubropilosa		Ortiz and Camargo-Mathias, 2007; Malta et al., 2014
		Formica pratensis, Formica rufa, Formica sanguinea, Formica truncicola		Adam, 1912
		Lasius fuliginosus, Lasius niger		
		Leptothorax tuberum		
		Myrmica laecinodis, Myrmica rubida		
		Tapinoma erraticum		
		Camponotus ligniperdus		

		Leptothorax gredleri	1	Oppelt and Heinze, 2007
		Pachycondyla (=Brachyponera) chinensis, Pachycondyla pilidorsalis		Gotoh et al., 2009
		Ponerinae (25 species)		Gobin et al., 2006
		Solenopsis saevissima richteri		Hermann and Blum, 1965
Hymenoptera	Apidae	*Andrena ovina*		Adam, 1912
		Bombus fervidus		Pabalan et al., 1996
		Halictus ligatus		
		Lasioglossum marginatum		
		Megachile relativa		
		Megachile rotundata		Gerber and Klostermeyer, 1970
		Lasius fuliginosus, L. niger		Adam, 1912
		Leiproctus spp.		Pabalan et al., 1996
		Bombus hortorum		Adam, 1912; Schoeters and Billen, 2000
		Bombus hypnorum, B. lucorum		Baer, 2003
		Bombus morio		Martins and Serrão, 2002
		Bombus pratorum, B. ruderatus		Schoeters and Billen, 2000
		Bombus terrestris		Adam, 1912; Schoeters and Billen, 2000; Baer, 2003
		Apis mellifera		Dallai, 1975
		Melipona bicolor		Cruz-Landim and Serrão, 2002; Martins and Serrão, 2002
		Melipona quadrifasciata anthidioides		Martins and Serrão, 2004 (Fig. 2E)
		Centris aenea, Centris tarsata		Martins and Serrão, 2002 (Fig. 2A)
		Epicharis flava		
		Euglossa sp., Eulaema nigrita		
		Melipona quadrifasciata		
		Plebeia sp.		
		Pseudaugochlora graminea		
		Scaptotrigona xanthotrica		
		Thygater analis, Thygater palliventris		
		Xylocopa frontalis		
	Oxaeidae	*Oxaea flavescens*		

	Vespidae	*Zethus miniatus*		Martins et al., 2005
		Mischocyttarus sp.		
		Polistes chinensis, Polistes jokahamae, Polistes snelleni		Gotoh et al., 2009
		Polistes erytrocephalus		Martins et al., 2005, 2008
		Polistes gallicus		Adam, 1912
		Polistes similimuns, P. versicolor		Martins et al., 2005
	Sphecidae	*Sceliphron sp.*		
		Microstigmus brasiliensis		
		Trypoxylon albiatarse, T. lactitarse		
	Vespidae	*Vespa crabro, Vespa germânica, Vespa norwegica, Vespa silvestris, Vespa vulgaris*		Adam, 1912
Diptera:	Culicidae	*Aedes aegypti*	3	Curtin and Jones, 1961; Jones and Wheeler, 1965; Clements and Potter, 1967; Jones and Fischman, 1970; Jones, 1973; Klowden and Chambers, 2004; Klowden, 2006; Pascini et al., 2012 (Fig. 1A)
		Aedes albopictus		Barreto et al., 2008
		Culex pipiens pipiens		Beckmann and Fallon, 2013
		Toxorhynchites theobaldi		Pascini and Martins (unpublished)
		Anopheles albimanus, Anopheles darlingi, Anopheles freeborni, Anopheles quadriannulatus	1	Klowden and Chambers, 2004
		Anopheles aquasalis		Pascini et al., 2013 (Fig. 1F)
		Anopheles gambiae		Klowden and Chambers, 2004; Klowden, 2006; Rogers et al., 2008
		Anopheles stefensi		Catteruccia et al., 2005
	Sciaridae	*Sciara coprophila*		Makielski, 1966
	Asiloidea	*Agapophytus albobasalis*		Winterton et al., 1999
	Tephritidae	*Anastrepha suspensa*	3	Fritz and Turner, 2002

	Calliphoridae	*Chrysomya albiceps*		Harterreiten-Souza and Pujol-Luz, 2012
		Chrysomya megacephala, Cochliomyia macellaria, Chrysomya. hominivorax	4	
	Ceratopogonidae	*Culicoides melleus*	2	Linley, 1981
	Glossinidae	*Glossina morsitans morsitans*		Balmand et al., 2013
	Drosophilidae	*Drosophila melanogaster*		Nonidez, 1920; Pitnick et al., 1999; Schnakenberg et al., 2011, 2012; Mayhew and Merritt, 2013
		D. wassermani, *D. nannoptera*, *D. pachea*, *D. acanthoptera*, *D. nigrospiracula*, *D. subpalustris*, *D. recens*, *D. guttifera*, *D. putrida*, *D. arizonae*		Pitnick et al., 1999
		Drosophila simulans		Prokupek et al., 2008
	Tephritidae	*Ceratitis capitata*		Taylor and Yuval, 1999
	Cecidomyidae	*Dasyneura leguminicola*	3	Metcalfe, 1933
	Scathophagidae	*Scathophaga stercoraria*		Otronen et al., 1997; Ward, 2000; Bernasconi et al., 2002
	Muscidae	*Atherigona orienalis, Muscina stabulans, Ophyra solitária, Hydroteae nicholsoni*		Couri, 2004
		Stomoxys calcitrans	2	
	Psychodidae	*Telmatoscopus albipunctatus*	0	Burrini and Dallai, 1975
		Psychoda sp.		Reviewed by Matsuda, 1976
		Phlebotomus papatasi	2	Ilango, 2005
	Asiloidea	*Ectinorhynchus variabilis*	1	Winterton et al., 1999
Coleoptera	Staphylinidae	*Aleochara bilineata*		Lizé et al., 2009; Werner et al., 1999
		Drusilla canaliculata		Werner et al., 2007
	Curculionidae	*Anthonomus grandis*		Villavaso, 1975; Grodner and Steffens, 1978
		Euscepes postfasciatus		Kumano et al., 2009
		Sitophilus granarius		Tombes and Roppel, 1972
	Dytiscidae	*Copelatus australis*		Ordish, 1985
		Homoeodytes hookeri, Hyphydrus elegans		
		Liodessus plicatus, Liodessus deflectus		
		Antiporus wakefieldi		
	Chrysomelidae	*Stolas spp.* (72 species)		Borowiec and Pomorska, 2009

		Cassida rubiginosa, Cassida vibex		Matsumura et al., 2016
	Tenebrionidae	*Tenebrio molitor*		Drnevich et al., 2000
		Tribolium castaneum		Bloch Qazi, 2003
Lepidoptera	Eriocraniidae	*Eriocrania sp.*		Reviewed by Matsuda, 1976
	Psychidae	*Solenobia walshella*		
	Sphingidae	*Sphinyx pinastri*		
	Bombycidae	*Bombyx mori*		Suzuki et al., 1996
Hemiptera:	Cicadellidae	*Balclutha incisa*		Bailey and Nuhardiyati, 2005
	Dipsocoridae	*Ceratocombus coleoptratus*		Pluot-Sigwalt and Péricart, 2003
		Cryptostemma alienum		
		Harpago maroccanum, Harpago medium		
	Pentatomidae	*Murgantia histrionica*		Stacconi and Romani, 2011
		Eurydema fieberi, E. oleraceum, E. ornatum, E. spectabilis		Candan et al., 2014
		Podisus nigrispinus		Rodrigues et al., 2008
	Lygaeidae	*Lygaeus simulans*		Gschwentner and Tadler, 2000
		Oncopeltus fasciatus		Bonhag and Wick, 1953
	Dipsocoridae	*Pachycoleus pusillimus, Pachycoleus waltli*		Pluot-Sigwalt and Péricart, 2003
	Triozidae	*Trioza alacris*		Marchini et al., 2010
		Enoplops disciger		Candan, 2008
	Coreidae	*Phyllomorpha laciniata*		García-González et al., 2004
		Leptoglossus zonatus		Souza et al., 2015
	Scutelleridae	*Odontotarsus purpureolineatus*		Candan et al., 2007
	Cimicidae	*Cimex lectularius*	0	Davis, 1956
	Reduviidae	*Rhodnius prolixus*	2	Davey, 1958
	Cicadidae	*Magicicada septendecium*	1	Reviewed by Matsuda, 1976
	Delphacidae	*Peregrinus maidis*		Tsai and Perrier, 1993
Isoptera:	Rhinotermitidae	*Coptotermes formosanus*		Raina et al., 2007
Thysanoptera:	Thripidae	*Frankmviella Occidentalis*		Dallai et al., 1996
Odonata	Coenagrionidae	*Ischnura senegalensis*		Nakahara and Tsubaki, 2007
	Petaluridae	*Petalura gigantea*		Reviewed by Matsuda, 1976
	Calopterygidae	*Mnais strigata*		
	Epiophlebiida	*Epiophlebia superstes*	0	
Entomobryomorpha	Entomobryidae	*Orchesella villosa*	1	Dallai et al., 2008b

Blattaria	Blattellidae	*Blattella germanica*	2	Liang and Schal, 1994
	Blattidae	*Periplaneta americana*		Lawson and Thomas, 1970
Archaeognatha	Machilidae	*Microcoryphia (Lepismachilis targionii, Trigoniophthalmus alternatus, Machilis sp. and Machilis rupestris)*		Bitsch, 1989
Plecoptera	Perlodidae	*Arcynopteryx compacta, Isoperla grannatica, Perlodes dispar*	1	Reviewed by Matsuda, 1976
Mecoptera	Panorpidae	*Panorpa communis*		
Siphonaptera	Pulicidae	*Spilopsyllus cuniculi*		
Trichoptera	Limnephilidae	*Anabolia nervosa*		
Psocoptera	Stenopsocidae	*Lepinotus patruelis*		
Phthiriaptera	Gyropida	*Gliricola sp.*		
	Philopteridae	*Docophorus sp.*		
	Phthiridae	*Phthirus sp.*		
	Pediculidae	*Pediculus sp.*	0	
Neuroptera	Chysopidae	*Chrysopa septempunctata*	1	
Zoraptera	Zorotypidae	*Zorotypus caudelli*		Dallai et al., 2012
		Zorotypus hubbardi		Reviewed by Matsuda, 1976
Thysanoptera	Thripidae	*Limothrips cerealium*		
		Frankmviella occidentalis		Dallai et al., 1996
Phasmatodea	Diapheromeridae	*Carausius morosus*		Reviewed by Matsuda, 1976
		Diapheromera sp.	2	
	Phasmatidae	*Leptynia sp.*		
Mantodea	Mantidae	*Stagmomantis carolina*	1	
Dermaptera	Anisolabidae	*Anisolabis littorea*		
		Euborellia plebeja		Kamimura, 2007
	Chelisochidae	*Proreus simulans*	1	
	Diplatydae	*Diplatys flavicollis*	4	Reviewed by Kamimura, 2007
Strepsiptera	Stilopidae	*Xenos vesparum*	1	Beani et al., 2005
Thysanura	Lepismatidae	*Ctenolepisma longicaudata*		Palit and Pal, 1999

Table 1. Overview of the number of spermathecae in insects.

Capítulo II

Characterization of spermatheca-related genes in *Aedes aegypti*.

Characterization of spermatheca-related genes in *Aedes aegypti*.

Abstract

Aedes aegypti is an important hematophagous mosquito, with anthropophilic habits, also known as a vector of human viruses including Yellow Fever, Dengue, Chikungunya and Zika. *A. aegypti* females usually mate once and store the sperm in three spermathecae. The spermatheca is responsible for sperm maintenance, nutrition, and protection against physical and oxidative stress damage leading the long-term sperm storage process. In this work, we unravel part of the molecular mechanism responsible for the long-term sperm storage in the spermatheca of *A. aegypti*. We generated the transcriptome of the spermathecae of virgin and fertilized females using the next-generation sequencing (RNAseq) and an enriched list of highly expressed genes of each treatment. After comparing these two physiological conditions, we performed differential expression (DEG) analyses and focused on selected expressed genes. Chosen genes were related to energy metabolism (Ae-920428), chitin components (Ae-187521 and Ae-88956), transcriptional regulation (Ae-27176), hormonal signaling (AeSigP-4002), enzymatic activity (Ae-SigP-212177), antimicrobial activity (AeSigP-109183) and ionic homeostasis (AeSigP-66427). These eight selected genes were silenced by RNAi technique, and its effect studied on virgin and fertilized females. The silencing effects vary depending on gene, showing deleterious effects on female survival, blood-feeding behavior, nutrients storage/conversion, egg development, oviposition, and fecundity. The knocking-down for AeSigP-66427 impaired the production of eggs and reduced the sperm motility. By using RNA *in situ* hybridization, we were able to show the local expression of the eight target genes. Our approach is pioneer in identifying the genes in the spermathecae along different physiological conditions of *A. aegypti* females. In addition, we highlighted some of the key mechanism in the reproductive process of the mosquito, which could provide specific targets for vector control.

Key-words: spermatheca, *A aegypti*, insect reproduction, sperm, transcriptome

1. Introduction

The prominent vectorial capacity of vector mosquitos is directly related to their high reproductive capacity, allowing them to become infected, disperse along greater distances, survive long enough to deliver more infected bites during its lifetime, spreading pathogens (Klowden 2006, Rueffler et al. 2006, reviewed by Baldini et al. 2012, Kotsakiozi et al. 2017). In this context, the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae) is a relevant model due to its high capacity of reproduction, migration and pathogen transmission. This mosquito has been one of the main targets of control strategies, being responsible to transmit severe virus such as Yellow fever, Dengue, Chikungunya and recently Zika. Furthermore, the absence of an effective vaccine and the rise of insecticide resistance has emphasized the need for new strategies of vector control (Thailayil et al. 2011, Dong et al. 2012).

In almost all insects, mating is a separate event from the egg fertilization. Once the eggs are ready for fertilization and environmental conditions are favorable, the sperm is released from the spermathecae to fertilize the eggs; initiating a new progeny (reviewed by Parker 1970, Dybas & Dybas 1981, reviewed by Sirot et al. 2009). Together with the sperm, the male can contribute/donate some semen compounds that can be responsible for sperm capacitation, nutrition and can trigger changes in the female reproductive tract, allowing sperm migration and egg development (Schnakenberg et al. 2011, reviewed by Baldini et al. 2012). Male nutrients last from hours to few days after mating, and the female spermatheca has the main responsibility to protect and nourish the sperm for long terms (reviewed by Wolfner 2011 and Pascini & Martins 2017). This organ is made of three parts main: a duct, responsible to guide the sperm migration; a reservoir or capsule, the actual sperm storage part, and the glandular portion, that produces and secrete compounds related to the sperm storage/nutrition (Clements & Potter 1967, Jones & Wheeler 1965, reviewed by Pascini & Martins 2017).

Multiple candidate factors for sperm longevity have been considered, including ions, sugars, pH, and enzymes of antioxidant and energy metabolism (Iida & Cavener 2003, Allen & Spradling 2008, Rogers et al. 2008, Cattenoz et al. 2016, Gonzalez et al. 2018, Gotoh et al. 2017). However, there is a lack of knowledge about biochemistry and physiological processes within spermathecae, being the male donation more well-known with the advance of new techniques of transgenic males (marked sperm, sterile or spermless males) (Caterruccia et al. 2005, Klowden 2006, Manier et al. 2010, reviewed by Baldini et al 2012, Gotoh et al. 2017).

The use of high-throughput sequencing is a powerful tool to decipher the global gene expression of tiny tissues. This approach coupled with silencing or gene edition (RNAi, CRISPR/Cas9 complex) is useful to characterize the functions of genes, providing an overview of all the processes that are related to specific genes (Cirimotich et al. 2009, Schnakenberg et al. 2011, Whyard et al. 2015, Kistler et al. 2015, Hammond et al. 2016, Airs & Bartholomay 2017, Gotoh et al. 2017). Because spermatheca directly influences sperm viability, the present work represents a significant advance for the comprehension of spermathecal functions in *A. aegypti* mosquitos, as the first step to unravel details of the sperm storage mechanisms in this vector. Here, we use the high-throughput RNA sequencing to screen candidate genes that contribute to sperm storage based on the differential expression provided by *in silico* analyses. In addition, the global gene expression was compared considering virgin and fertilized spermatheca by RT-PCR. The enriched spermathecal transcriptomes (named here as "spermathecomes") was generated by comparing the transcripts obtained against the transcripts of the whole bodies of male and female available at the Genbank. After this filtering, eight candidate genes were chosen from different functional groups in virgin and fertilized females for RNAi silencing to evaluate their knockdown effects on the female phenotype. Finally, by using the *in situ* hybridization labeling probes designed for the eight selected genes, we identified in which specific part of the spermathecae these genes are expressed.

2. Materials and Methods

2.1. Sample preparation and RNA-sequencing

The *A. aegypti* females (strain PP-Campos, Campos dos Goytacazes, RJ, Brazil) were collected from colonies at the Departamento de Biologia Geral at Universidade Federal de Viçosa, Minas Gerais, Brazil. The eggs were put in trays with dechlorinated tap water to hatch, in a photoperiod of 12h, 25°C ±0,5°C, the larvae were fed with turtle food (Reptolife®) and the mosquitoes with sucrose solution 10% *ad libitum.* At the same day after emergence, the adult females were separated in two different cages: cages without males with only virgin females and cages with males and females (with two-fold more males than females), to let them mate. Seven days after emergence, 600 females were dissected, totalizing 1,800 spermathecae from each group of females, in PBS (phosphate buffered solution, 0.1M, pH 7.6). All the instruments used in dissections, including needles, stereomicroscopes, forceps, and slides were cleaned before dissections with RNase AWAY® (Sigma-Aldrich, Buchs, Switzerland), in order to avoid contamination. Dissected spermathecae were transferred to centrifuge microtubes with 1mL of TRIzol® reagent [Invitrogen (Carlsbad, CA, EUA) and kept in -70 ºC until the RNA extractions. Total RNA was extracted from spermathecae pools (1,800) of each group with TRIzol® reagent according to the manufacturer`s protocol.

The RNA quality was checked in 1% agarose gel [with 1% XT MOPS [Bio-Rad Laboratories (Hercules, CA, EUA)] e 5% de formaldehyde]. The RNA integrity was analyzed by Agilent 2100 Bioanalyser® using the RNA 6000 Nano chip, both from Agilent Technologies at the K-State Integrated Genomics Facility (IGF-KSU) at the Department of Plant Pathology at the Kansas State University (Manhattan, Kansas, USA). The quantification was performed with Qubit 2.0 Fluorometer® (Thermo Fisher Scientific). The purity degree was verified by spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific) using 260, 230 and 280 nm wavelength.

For library preparation, 400 ng of high-quality total RNA was prepared using the TruSeq RNA Sample Preparation v.2 kit (Illumina®), according to the manufacturer's protocol. The libraries were normalized for 10nM and portioned in two libraries per pool. Each pool was sequenced at the MiSeq Illumina platform, using the MiSeq reagent v3 kit (Illumina®), with 600 cycles, in paired-end following the manufacturer`s protocol (http://www.illumina.com/products/truseq_rna_library_prep_kit). The read length average was of 300 bp. The library preparation and RNA sequencing proceeded at IGF-KSU.

2.2. RNA-sequencing *in silico* analysis

The transcriptome analysis was performed according to the methods described by Ribeiro et al. (2014) e Ribeiro et al. (2016). Briefly, the fastq data provided by the sequencing were trimmed, excluding <20bp reads. The read files were concatenated and assembled in single-ended by Abyss (Birol et al. 2009) e SoapdenovoTrans (Xie et al. 2014), using the k-parameter between 21-91 and a fold-change increase of 5. The fasta-generated files were compared with the coding sequences of *A. aegypti* (version 3.3) deposited in the Vector Base (Megy et al. 2012) and assembled using bast iterative and CAP3 pipeline (Karim et al. 2011). The coding sequences (CDS) were extracted based in the signal peptide signal with the open reading frame (ORF) and by similarities with other proteins from RefSeq invertebrate database do National Center for Biotechnology Information (NCBI), Diptera proteins deposited in Genbank (NCBI) and SwissProt.

To exclude the general expression of other tissues, which are not related to spermathecae in the assembled transcriptomes, were used sequences from the whole body of the virgin sugar-fed males and females deposited in Sequence Read Archives (SRA) do NCBI do bioproject PRJNA261799 (strain Liverpool) (Jiang et al. 2015). Each library was mapped over the CDS generated using blastn method, with a word size of 25, 1 gap and allowing the identity of 95% or higher. Up to five matches were allowed if and only if the scores were the same as the largest score. A chi-squared test was applied for each CDS with the aim of detect statistical differences between the paired-reads. The Bonferroni and FDR correction (Benjamini & Hockberg 1995) were applied using the P-value package version 3.3.0 from R software (Team R 2013). The normalization reads rate were compared by r1 x R2 / [R1 x (r2 + 1)] e r2 x R1/ [R2 x (r1 + 1)], in which r1 and r2 are the reads for each library (virgin and fertilized spermathecae) and R1 and R2 the number of total reads from the libraries mapped over the CDS. One unit was added avoiding division by zero. To compare transcript related expression among contigs, was stablished an “expression index” defined as a number of mapped reads mapped to a particular CDS multiplied by 100 and divided by the highest number of finding mapped reads mapped to a particular CDS (Chagas et al. 2013). The RPKM and TPM values were calculated for each mapped library (Wagner et al. 2012). To compare the genetic expression over the libraries was applied the TPM index and for absolute expression values were used RPKM values or the normalized read index as described above. Heat map graph was done with the program heatmap2 from the gplots package running within R package with default parameters and using Z scores for data normalization (Warnes et al. 2015).

The proteins were annotated in an automated manner based on a vocabulary of approximately 290 words found associated with several databases including NCBI NR light, Swissprot, Gene Ontology, CDD, KEGG, KOG, Pfam, SMART, RefSeq-invertebrates, REPBASE-RPS , rRNA and a subset of GenBank sequences containing Diptera (organism), as well as the presence or absence of peptide signal and transmembrane domains. Detailed bioinformatics analyzes can be found in Karim et al. (2011).

2.3. Transcriptome validation and qRT-PCR

For RNA-seq validation and expression values of the two different spermathecae (virgin or fertilized), eight genes from different functional groups with high expression were chosen after the transcriptome bioinformatics analysis (section 2.2, Tab. 1). The *A. aegypti* eggs (*Rockefeller* strain) were obtained in the insectary at the Immunology and Microbiology Department at Johns Hopkins University (Baltimore, Maryland, EUA). The eggs were let to hatch in distilled water; the larvae were fed with cat food (Cat Chow, Purina$^{®}$) and kept under the same insectary conditions as cited above. The newly emerged females were separated into two groups (virgin and inseminated), and dissected at day seven after emergence.

For spermathecal expression analysis, a hundred virgin and inseminated females (300 spermathecae each) were dissected on PBS and the total RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol (section 2.1). Aiming to verify if some of the expression values are part of the sperm metabolism or just triggered by their presence within the reservoir, the reservoir of a hundred inseminated reservoir were gently broken with a forceps. To the broken reservoirs were added in 1.5 mL microtubes with 100 µL of PBS and centrifuged in low speed (below 3000 rpm for 10 seconds) to avoid cell damage. The supernatant with the reservoir content was submitted to the total RNA extraction, cDNA synthesis, and expression quantification together with the virgin and inseminated spermathecae.

To verify if the selected genes are specific of spermatheca and not of other tissues, the abundance of transcripts were checked in the carcass, the midgut and ovaries before and after the egg development (after blood meal). From two pools of ten females with three days after emergence, the carcass, midgut, and ovaries were dissected and the total RNA extracted, as described above. The ovaries of females with three days after the blood meal were also dissected for total RNA extraction and compared with the other tissues.

The total RNA of samples was treated with DNAse I (Invitrogen), the solution was incubated at 37°C for 15 minutes and then inactivated at 75°C for 5 minutes. The product was precipitated with ammonium acetate in ethanol solution, resuspended in RNAse free water, followed by quantification at NanoDrop Lite Spectrophotometer (Thermo Scientific). The cDNA synthesis was conducted using 500 ng of total RNA with the Superscript III (Invitrogen) kit and random hexamers (Thermo Fisher) in 20 µL of final volume as the manufacturer`s protocol. The cDNA product was treated with RNAse H (New England Biolabs) incubated at 37°C for 10 minutes and kept in -70°C till the use.

The relative gene expression was checked by qRT-PCR, using the SYBR Green PCR Master Mix [Applied Biosystems, Thermo Fisher Scientific (Warrington, UK)], with 300 nM of each primer and 100 ng of cDNA, with a final volume of 20 µL. The gene transcripts were normalized and calculated against the ribosomal protein S7 gene (AAEL009496) as an endogenous reference; in MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL) (Applied Biosystems, Life Technologies) in the StepOne™ Real-Time PCR System (Applied Biosystems, Life Technologies). The amplification conditions were: 95°C 3min, 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

The reactions were performed using biological replicates and plate triplicates. The relative expression was determined using the comparative method of Cycle threshold (Ct), known as ΔΔCt or $2^{-\Delta\Delta Ct}$ (Pfaffl 2001) and normalized over virgin and inseminated spermathecae control groups. The statistical analysis was performed using the GraphPad Prism version 6.00 for Windows, GraphPad® Software, La Jolla California USA, www.graphpad.com”.

2.4. RNAi experiments

The knocking down assay by RNAi technique was done for eight selected genes related to related to energy metabolism (Ae-92048), chitin-bound components (Ae-187521 and Ae-88956), transcriptional regulation (Ae-27176), hormonal regulation (AeSigP- 4002), enzymatic activity (Ae-SigP-212177), antimicrobial activity (AeSigP-109183) and ion homeostasis (AeSigP-66427). The choice of the candidate genes was done considering different characteristics that perpetrate sperm storage capacity and female’s fecundity including survival, blood-feeding rate, oviposition rate, morphological characteristics of egg and rate of egg hatching (Tab. 5).

2.4.1. Double-stranded RNA synthesis (dsRNA)

The selected genes were amplified by PCR reaction using the Taq 2X Master Mix (BioLabs® Inc.) kit, with 5uM of the designed primers (Tab. 5). The PCR product was run in a 1.5% agarose gel stained with ethidium bromate. The bands in the gel were cut, purified with

ZymocleanTM Gel DNA Recovery Kit (Zymo Research) and sent for sequencing at Macrogen® (USA). The sequencing was analyzed using blastn against *A. aegypti* database (AaegL3 strain Liverpool) at Vector base (https://www.vectorbase.org/).

For double-stranded RNA synthesis, were designed primers with the same sequence for the target sequences (Tab. 5) with an addition of a T7 promoter sequence (5`GAATTAATACGACTCACTATAGGGAGA) using the MegaScript T7 transcription kit (Ambion, Austin, TX), according to the manufacturer`s protocol. The final dsRNA product was precipitated in ethanol/ammonium acetate solution, resuspended in PBS 1X (0.1M, pH 7.6) and then quantified at the NanoDrop Lite Spectrophotometer (Thermo Scientific). For dsRNA quality analysis, 6 µg of the samples were run in a 1.5% agarose stained with stained with ethidium bromate. The dsRNA was stored at -80ºC untill the nanoinjection. As a negative control, was used the EGFP sequence as a template for dsRNA synthesis (Donze & Picard, 2002). The dsEGFP microinjections were performed as a control for the target genes.

2.4.2. dsRNA nanoinjections and knockdown effects

For these experiments, mosquitos were obtained from the insectary at Immunology and Microbiology Department at Johns Hopkins University (Baltimore, Maryland, EUA), and at the insectary at Departamento de Biologia Geral at UFV using *A. aegypti* (Rockefeller strain). The mosquito rearing proceeded as described in section 2.1. Newly emerged females were separated from the males to avoid them to copulate. Virgin females were injected with dsRNA (69 nL at 46 nL/seg) using the Nanoject II Injector® (Drummond Scientific Company). The capillary needles [Drummond Microdispenser (Drummond Scientific Company, USA)] were made using the Sutter Instrument® Co. Model P-2000. A forceps was used to break the tip of the needle for injection.

For injection, the mosquitoes were anesthetized on ice, placed over a glass slide covered with filter paper and injected in the mesothorax with the dsRNA solution (69 nL at 46 nL/seg of a 3µg/µL of dsRNA in PBS solution) (Garver & Dimopolous, 2007; Drake et al. 2012). For each target gene, 500 females were injected with dsRNA for each group of females, among them three hundred were separated for survival analysis, and 200 for checking the gene expression (RT-PCR) and morphology analyzes. After the injection, females were transferred to cages and fed with 10% sucrose solution *ad libitum*.

Considering that the rate of gene silencing is highly variable and depends on a number of factors including transcript and protein turnover rates, a time course of transcripts levels along 24, 48, 72, and 96h by RT-PCR was performed (Shepard et al. 2005, Garver & Dimopolous 2007, Drake et al. 2012). We have found that silencing begins on day one post-injection; however, the inhibition reaches its peak at day three after injection (data not shown). Based on these results, for genes that showed as highly expressed in virgin spermathecae, the females were injected at day one after emergence in attempt to evaluate the effects of the gene silencing in the events that precede the pre-mating and its consequences at the mating moment. For the genes highly expressed in fertilized females, they were injected two days after emergence, to determine the effects of the silencing peak with events after the mating. At day three, all the injected females were allowed to copulate in the cage. In the next day, the males were removed from the cages and the knocking-down effects were checked. As a control group for virgin and fertilized spermathecae, 500 females were injected with dsEGFP.

2.5. Knockdown effects

To verify the effect in the dsRNA microinjections over the expression of spermathecal genes, the females were dissected in duplicate pools of ten two days after injection (for virgin) and another pool two days after mating (for fertilized). The dissection methods, as well as the total RNA extraction, RNA treatment, and RT-PCR expression, was performed as in item 2.3.

2.5.1. Survival analysis

The survival analyses of the injected females were done considering three independent replicates with 100 females each. Females were kept in plastic cages and fed with cotton soaked with 10% sugar solution *ad libitum*. To check the mating effect on the female survival, the females were allowed to mate at day 1 after dsRNA injection, for 24 hours. After this period, males and females were separated. The number of dead females was counted each day for 10 days and the dead ones were removed each day to analyze the gene knocking-down effect in the female survival.

2.5.2. Blood-feeding experiments

To check the effects of the dsRNA injection on blood-feeding behavior, a total of 100 females were separated in a cage and allowed to mate. After 24h, males were removed from the cages and 20 random females were also removed from the cage, anesthetized and their weight measured individually in a precision weighing scale (AG-200, Gehaka Ltda., São Paulo, Brazil). Weighted females were returned to the cage. In the next day, all the females in that cages were allowed to blood-feed on anesthetized mice with Ketamine hydrochloride 10% (Ketamina Agener ®) (Agener União, Embu-Guaçu, São Paulo, Brazil) and Xylazine hydrochloride 2% (Anesedan®) (Anesedan, Ceva Santé Animale, Paulinia, São Paulo, Brazil) (diluted in 1:4), under UFV Ethics Committee (Protocol 561/2016) for 30 minutes (with mouse rotation each 10 minutes). After the blood-meal, the number of the females that were blood fed was counted and another random pool of 20 females was weighted. All the females were fed with 10% sugar solution *ad libitum* and used for the oviposition rate analysis.

2.5.3. Fertility analysis

After the blood-feeding, two groups of 10 blood-fed females were individually transferred to 50 mL plastic tubes with filter paper soaked in 10 mL of distilled water and covered with fabric nets. These females were fed in cotton soaked in 10% sugar solution *ad libitum*, for 4 days. The number of live/dead females and the number of females that laid eggs were counted at the end of oviposition assay. The females that did not lay eggs were dissected and their spermathecae and ovary photographed with the light microscope Olympus BX50 with coupled camera Moticam 580 of the Departamento de Biologia Geral/UFV and compared to the control.

2.5.4. Egg morphometry

The eggs laid by the injected females from the previous experiment (2.5.3) were counted, aligned and stick to a white tape and photographed. The egg length and total area were measured in the digital images with ImagePlus software®. The measurements were done twice to avoid experimental error.

2.5.5. Fecundity analysis

To check the viability of those eggs laid by the injected females, four pools of 100 eggs were placed in a plastic cup with 100 mL of distilled water with cat food (Cat Chow, Purina®), and let to hatch. After 2 days, the number of live larvae was counted (number of hatched larvae/100 eggs). The number of hatched eggs x laid eggs was used as female fertility index.

2.5.6. Morphological analysis

To check the spermathecal phenotype after the RNAi assay, spermathecae of 10 injected females of virgin and fertilized females were dissected and fixed in Zamboni fixative solution (Stefanini et al. 1967) for two hours. Fixed samples were rinsed in PBS, dehydrated in an ascendant series of ethanol (70-100%) and embedded in Historesin (Leica Biosystems, Nussloch, Germany). Thin sections (4 µm thick) were stained with hematoxylin and eosin. The whole spermathecae from virgin and inseminated females were washed in PBS, incubated 16 h in 0.5% glycine and stained with periodic acid Schiff (PAS) reaction for detection of neutral polysaccharides, dehydrated and embedded in historesin. Sections were mounted with Eukitt® Quick-hardening mounting medium (Sigma-Aldrich, Steinheim, Germany) and photographed in the light microscope Olympus BX50 with coupled camera Moticam 580 at the Departamento de Biologia Geral/UFV. On the third day after the blood feeding, five females of each dsRNA injection group were dissected and the sperm motility checked in the freshly-dissected spermathecae under the same microscope at room temperature. The sperm motility was compared with the control group (dsEGFP) under the microscope and the ones which presented abnormal sperm motility were recorded. In case of a disruption in the sperm motility, the reservoir was gently broken by the pressure over the coverslip. The sperm motility after the reservoir breaking was also recorded. This part of the experiment was performed in duplicate for each dsRNA. The recorded videos are available at https://www.dropbox.com/sh/funva2nw3zugntw/AAACkOC9NurYVar9PeUKMbdna?dl=0

2.6. RNA *in situ* hybridization

The *in situ* hybridization was performed using the FISH Tag RNA Red Kit, with Alexa Fluor 594 dye (Life Technologies Corporation, Eugene, OR). For probes synthesis, 1µg of the purified PCR product generated in the purification step of the item 2.4.1 was used according to the manufacturer's protocol. The spermathecae were dissected, fixated for 1 hour, washed in distilled water and incubated in 500 µL of labeling solution (10% probe solution in PBS 1x) overnight. The whole tissue was washed three times with PBS, stained with 4',6-diamidino-2-phenylindole, 0.5 g/mL solution (DAPI) (1:1000) (Sigma-Aldrich, St. Louis, MD, USA) at room temperature for 1 h in the dark, mounted in 50% sucrose solution and photographed under the fluorescence microscope Olympus BX53 microscope coupled with Olympus DP 73 digital camera using a WU filter at the Laboratório de Sistemática Molecular at the Departamento de Biologia Animal/UFV.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism v.6 software (GraphPad Software) and plotted as bar graphs. The data were tested for normal distribution over Shapiro-Wilk normality test with α=0.05. Survival analysis was performed by the Kaplan-Meier method, and differences were detected by Log-rank (Mantel-Cox) test with α=0.05. Comparative analysis was made with one-way analysis of variance (ANOVA) and paired analysis with the two-tailed *t*-test with 95% confidence interval and α=0.05.

3. Results

3.1. RNA-sequencing *in silico* analysis

This study identified the plethora of genes that are transcriptionally expressed in spermathecae (namely here as "spermathecomes") of both virgin and fertilized *A. aegypti* females by RNAseq. In order to identify the transcripts related to sperm maintenance within the *A. aegypti* spermathecae, were built the spermathecae cDNA libraries from two different physiological conditions. The paired-end sequencing was performed using the Illumina Hiseq 2000 platform, resulting in a total number reads of 21,131,356 and 19,054,442 for virgin and fertilized females, respectively. To avoid possible noises, the reads were trimmed in order to eliminate small sequences (<20 bp), resulting in 20,985,437 reads for virgin females and 18,916,480 and reads for fertilized females. The trimmed reads were mapped over *A. aegypti* genome, resulting in a total of 29,248,633 coding sequences bases (CDS). From the total number of CDS bases, 22,499,473 were found in spermathecae of virgin females, with a genome coverage of 76.92% and 22.689.695 in fertilized females with a coverage of 77.57%.

The expression of housekeeping genes from the whole body was compared by the F-test (0.05, with an adjusted p-value by Bonferroni's correction for multiple comparisons) with both male and female whole bodies. Were also analyzed the transcripts annotated using the RPKM normalization method for each coding sequence mapped. A "maximum relative RPKM" was established to be used as an indicator for the "expression index", and the total number of CDS was compared by RPKM maximum relative (RPKM>1), where RPKM = 1 corresponds to the value of the constitutive expression found in the whole body of both male and female, thus proving enriched library for the two spermathecomes (virgin and fertilized). The transcripts identified in distinct clusters of the male and female differentially expressed genes common to the spermathecomes, including genes overexpressed in both virgin and fertilized spermathecomes, were grouped in a heat map graph representation (Fig. 1). The CDS were filtered and grouped according to their relative expression values among the samples (spermathecae *versus* male and whole body), with at least the double of the expression value.

In addition, the comparison of the expression values between the virgin and fertilized spermathecae was performed and were selected the differentially expressed coding sequences (DEG) presenting expression at least eight times more than the other (Tab. 1). 661 DEG were identified and annotated with at least eight times the housekeeping expression, belonging to 21 functional classes (Tab. 2). Among them, 54.41% (8,044) of those transcripts grouped in four functional groups: Unknown, conserved (2,744 - 18%), Secreted (2,216 - 14.99%), Signal transduction (1,686 - 11.41%) and Metabolism (13.98 - 9.45%). Furthermore, 111 DEG were found high expression values of least eight times in the spermathecae of virgin females when compared to the fertilized spermathecae. Based on this comparison, 77.48% of the grouped transcripts belong to four functional groups: Metabolism (43 - 38.74%), Secreted (27 - 24.32%), Signal transduction (8 - 7.21%) and Unknown, conserved (8 - 7.21%) (Tab. 3).

Different from the previous comparison (virgin *versus* fertilized spermathecae), in the inverse comparison (fertilized *versus* virgin spermathecae), only 25 DEGs were found with at least eightfold increase. The functional aggrupation was the same as cited before, comprehending 70% of the transcripts distributed among Secreted (11 - 44%), Metabolism (3 - 12%), Unknown, conserved (3 - 12%) and Signal transduction (2 - 8%) (Tab. 4).

3.2. Transcriptome validation and RT-PCR

Based on the *in silico* analysis, eight genes from five functional categories with high expression values were chosen. The chosen genes should be related with the following spermathecal functions: energy metabolism (Ae-920428-Glucose dehydrogenase), chitin components (Ae-187521- Chitin bind 4, Ae-88956- ChtBD2), transcriptional regulation (Ae-27176- Atrophin-1 protein), hormonal signaling (AeSigP-4002-DHR4 ligand), enzymatic activity (Ae-SigP-212177- N-acetylgalactosaminyl transferase), antimicrobial activity (AeSigP-109183- Kazal type serine protease inhibitor) and ionic homeostasis (AeSigP-66427- Na^+/Ca^{2+} exchanger protein). The gene identification, its related functional group, the designed primer and its relative expression compared between virgin and fertilized spermathecae is showed in Tab. 5.

The level of expression of the S7 ribosomal protein transcript (AAEL009496-RA), a constitutive gene used for expression normalization, in the *A. aegypti* spermathecae, showed the same fold-change values among virgin, fertilized and reservoir content, with no statistical difference (One-way ANOVA F=1, $R^2 = 0.25$, $P = 0.4219$) (Fig. 2A). The Glucose dehydrogenase (Ae-92048) was highly expressed in virgin spermathecae in comparison to fertilized and reservoir content (One-way ANOVA F=4.772, $R^2 = 0.9907$, $P < 0.001$). There was no difference in terms of glucose dehydrogenase expression in fertilized spermathecae and their reservoir content ($P > 0.9999$) (Fig. 2B).

Transcripts for Chitin bind 4 (Ae-187521) were detected only the virgin spermathecae being not detected in fertilized and in reservoir content (One-way ANOVA F=54.4, $R^2 = 0.9236$, $P < 0.01$) (Fig. 2C).

In Atrophin-1 protein transcripts (Ae-27176) there was a higher expression in virgin spermathecae compared with fertilized spermathecae and spermathecal content (One-way ANOVA F=17.24, $R^2 = 0.793$, $P = 0.0008$). Even though, the fertilized spermathecae seem present expression that was not statistically significant when compared with the reservoir content ($P = 0.7164$) (Fig. 2D).

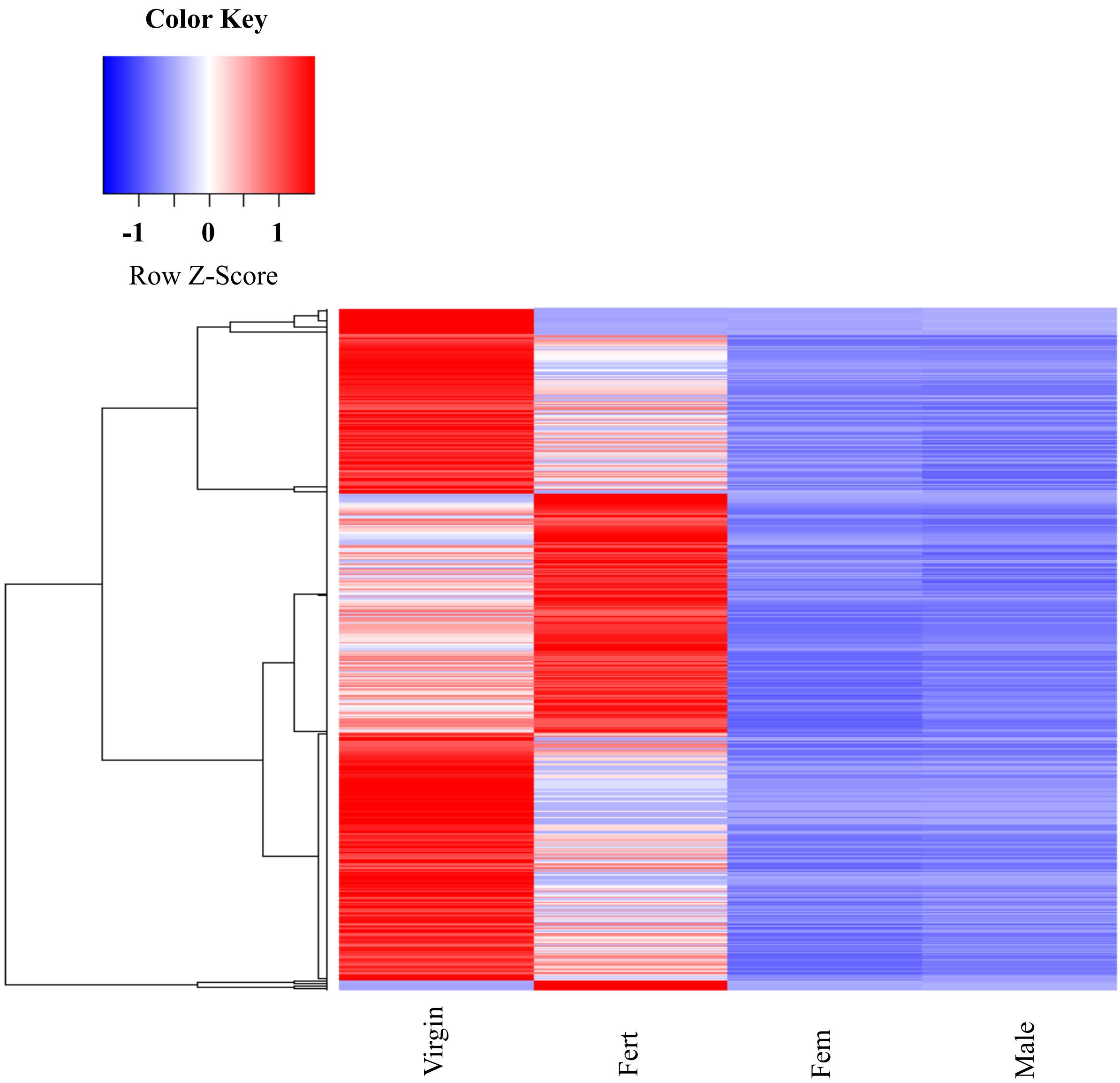


Fig. 1: Differential gene expression in *Aedes aegypti* showing the distribution of transcripts expressed in the spermathecae of *A. aegypti* females and in the male and female (Fem) whole bodies, as well as between the spermathecae of a virgin (Vir) and fertilized (Fert) females. The Z score transformed data from transcripts per million for each library is shown. The lateral clusters represent the differentially expressed transcript groups, including transcripts that appear overexpressed in virgin and fertilized (Fert) spermathecomes, better described in Tab. 2.

Condition	**CDS Number**	**Virgin 8x > Fert**	**Fert 8 x > Virgin**
All CDS	21,612	276	368
CDS with V or I library having RPKM >1	14,786	241	315
Reads of spermatheca 2x larger than whole body library *	13,555	166	58
Reads of spermatheca 8x larger than whole body library *	661	111	25
Reads of spermatheca 16x larger than whole body library *	375	93	18
Reads of spermatheca 32x larger than whole body library *	373	93	15
Reads of spermatheca 64x larger than whole body library *	175	53	10

Tab. 1: Number DEGs in the *A. aegypti* spermatheca libraries compared to the whole body of the male and between spermathecae of virgin and fertilized (Fert) females. *significant at 0.05 FDR from X^2 test with P-values adjusted with the Bonferroni correction for multiple testing.

The transcripts of DHR4 ligand (AeSigP-4002) was found only in virgin spermathecae in contrast to the other two conditions that containing gametes (fertilized spermathecae and reservoir content) (One-way ANOVA F=29.27, $R^2 = 0.8667$, P = 0.0001) (Fig. 2E).

Transcripts for N-acetylgalactosaminyl transferase (AeSigP-212177) were higher expressed in virgin spermathecae when compared with fertilized spermathecae and reservoir content (One-way ANOVA F=21.91, $R^2 = 0.8296$, P = 0.0003). Even though the fertilized spermathecae had a lower expression of this gene, it was not significant when compared with the spermathecal content (P = 0.3933) (Fig. 2F).

The ChtBD2 (Ae-88956) transcripts, were identified in all three samples (virgin, fertilized spermatheca and spermathecal content) however, only in the comparison between the spermathecae there was a higher expression in the fertilized spermatheca (One-way ANOVA F=5.724, $R^2 = 0.5599$, P = 0.0249), which was not significant compared with the spermathecal content (P = 0.0574) (Fig. 2G). For Kazal type serine protease inhibitor (AeSigP-109183) there was a higher transcripts abundance in the fertilized spermathecae (One-way ANOVA F=75.8, $R^2 = 0.944$, P < 0.0001). However, no difference was found between virgin spermathecae and spermathecal (P = 0.9808) for Kazal type serine protease inhibitor (Fig. 2H).

The expression of Na^+/Ca^{2+} exchanger protein (AeSigP-66427) was higher in fertilized spermatheca (One-way ANOVA F=74.28, $R^2 = 0.9429$, P < 0.0001). Between virgin spermathecae and spermathecal content, the expression in the last one was higher (P = 0.0009), and between fertilized and spermathecal content, there was a higher expression value (P = 0.0003) in fertilized organs (Fig. 2I).

Class	Average RPKM Virgin	SE	Average RPKM Fert	SE	Virgin/Fert	N	Percent
Cytoskeletal	169.31	26.94	168.40	29.22	1.01	437	2.96
Oxidant metabolism/detoxification	28.66	4.65	31.48	4.43	0.91	248	1.68
Extracellular matrix/cell adhesion	79.75	10.46	54.35	12.08	1.47	366	2.48
Immunity	51.65	15.26	122.43	44.96	0.42	126	0.85
Metabolism	40.96	2.61	55.03	4.23	0.74	1398	9.45
Nuclear export	12.76	2.02	14.49	2.94	0.88	54	0.37
Nuclear regulation	21.75	3.94	22.41	4.29	0.97	483	3.27
Protein export machinery	43.31	4.81	44.62	4.49	0.97	488	3.30
Protein modification machinery	59.84	8.80	65.44	9.62	0.91	398	2.69
Proteasome machinery	30.31	3.03	34.09	3.63	0.89	306	2.07
Protein synthesis machinery	258.16	22.27	507.82	45.62	0.51	425	2.87
Secreted	121.17	21.18	123.83	16.30	0.98	2216	14.99
Signal transduction	32.62	2.72	33.62	3.59	0.97	1687	11.41
Storage	102.68	36.11	193.28	71.89	0.53	21	0.14
Transposable element	13.02	1.76	13.03	1.76	1.00	711	4.81
Transcription factor	17.98	2.49	29.27	9.90	0.61	357	2.41
Transcription machinery	47.60	14.26	39.59	7.68	1.20	911	6.16
Transporters/storage	39.94	4.82	53.27	6.70	0.75	463	3.13
Unknown, conserved	221.24	49.01	144.76	29.87	1.53	2744	18.56
Unknown	339.12	92.68	210.76	50.13	1.61	934	6.32
Viral	27.87	14.58	113.85	61.82	0.24	13	0.09

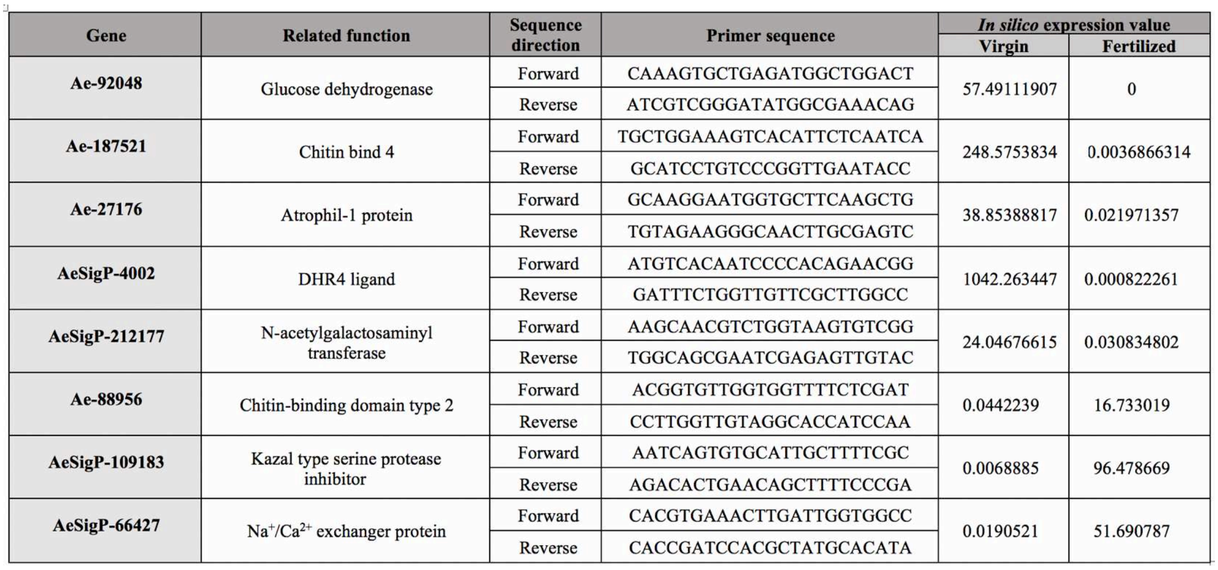
Tab. 2: Functional annotation of DEGs between virgin and fertilized of *A. aegypti* spermatheca libraries with RPKM> 1 and their ratio (proportion) among the different groups. N: number of contigs from each functional group, SE: variance of RPKM analysis.

Class	Average RPKM Virgin	SE	Average RPKM Fert	SE	Virgin/Fert	N	Percent
Cytoskeletal	386.97	0.00	15.43	0.00	25.09	1	0.90
Extracellular matrix/cell adhesion	203.64	48.76	7.78	2.26	26.17	43	38.74
Metabolism	43.54	15.98	3.64	1.90	11.96	9	8.11
Nuclear regulation	486.02	0.00	25.09	0.00	19.37	1	0.90
Protein modification machinery	20.21	15.23	0.88	0.73	22.91	5	4.50
Secreted	967.22	645.31	47.50	30.02	20.36	27	24.32
Signal transduction	35.83	11.01	2.34	0.77	15.34	8	7.21
Transcription machinery	46.21	36.70	0.73	0.55	63.33	3	2.70
Transporters/storage	260.73	0.00	11.29	0.00	23.08	1	0.90
Unknown, conserved	15.68	4.69	1.02	0.41	15.35	8	7.21
Unknown	57.21	17.26	2.78	0.90	20.57	5	4.50
Total						111	100

Tab. 3: Functional annotation of DEGs with at least eightfold expression in virgin than fertilized (Fert) spermathecae. N: number of contigs from each functional group, SE: variance of RPKM analysis.

Class	Average RPKM Virgin	SE	Average RPKM Fert	SE	Virgin/Fert	N	Percent
Oxidant metabolism/detoxification	26.81	0.00	352.90	0.00	13.12	1	4.00
Extracellular matrix/cell adhesion	0.00	0.00	2.86	0.00	28.64	1	4.00
Immunity	0.08	0.00	5.99	0.00	32.87	1	4.00
Metabolism	4.22	2.49	304.39	245.00	70.47	3	12.00
Protein modification machinery	1.42	0.00	29.55	0.00	19.41	1	4.00
Secreted	66.39	35.96	1,357.85	986.52	20.42	11	44.00
Signal transduction	46.98	38.38	445.46	293.03	9.46	2	8.00
Transposable element	7.88	7.13	104.25	94.25	13.06	2	8.00
Unknown, conserved	181.92	148.53	2,119.84	1,727.68	11.65	3	12.00
Total						25	100

Tab. 4: Functional annotation of DEGs with at least eightfold expression in fertilized (Fert) than in virgin spermathecae between the different samples. N: number of contigs from each functional group, SE: variance of RPKM analysis



Gene	Related function	Sequence direction	Primer sequence	*In silico* expression value	
				Virgin	Fertilized
Ae-92048	Glucose dehydrogenase	Forward	CAAAGTGCTGAGATGGCTGGACT	57.49111907	0
		Reverse	ATCGTCGGGATATGGCGAAACAG		
Ae-187521	Chitin bind 4	Forward	TGCTGGAAAGTCACATTCTCAATCA	248.5753834	0.0036866314
		Reverse	GCATCCTGTCCCGGTTGAATACC		
Ae-27176	Atrophil-1 protein	Forward	GCAAGGAATGGTGCTTCAAGCTG	38.85388817	0.021971357
		Reverse	TGTAGAAGGGCAACTTGCGAGTC		
AeSigP-4002	DHR4 ligand	Forward	ATGTCACAATCCCCACAGAACGG	1042.263447	0.000822261
		Reverse	GATTTCTGGTTGTTCGCTTGGCC		
AeSigP-212177	N-acetylgalactosaminyl transferase	Forward	AAGCAACGTCTGGTAAGTGTCGG	24.04676615	0.030834802
		Reverse	TGGCAGCGAATCGAGAGTTGTAC		
Ae-88956	Chitin-binding domain type 2	Forward	ACGGTGTTGGTGGTTTTCTCGAT	0.0442239	16.733019
		Reverse	CCTTGGTTGTAGGCACCATCCAA		
AeSigP-109183	Kazal type serine protease inhibitor	Forward	AATCAGTGTGCATTGCTTTTCGC	0.0068885	96.478669
		Reverse	AGACACTGAACAGCTTTTCCCGA		
AeSigP-66427	Na^+/Ca^{2+} exchanger protein	Forward	CACGTGAAACTTGATTGGTGGCC	0.0190521	51.690787
		Reverse	CACCGATCCACGCTATGCACATA		

Tab. 5: DEG selected from different functional groups (carbohydrates metabolism, sperm organization, signaling/receptors pathways, pathogens protection and ion homeostasis) as targets for this study.

The selected eight genes were not expressed only in the spermathecae. They were detected in the carcass, midgut and not developed and developed ovaries. However, different from what was proposed by the expression variation found in the *in silico* analysis from the spermatheca, the abundance of the transcript did not change as was shown between the virgin and inseminated spermathecae. There was no difference any even between the ovary before and after the egg development, which represents a key mechanism in the female reproduction. As was expected, the S7 ribosomal protein transcript (AAEL009496-RA) did not change among the carcasses, midgut and not developed and developed ovaries (One-way ANOVA: F=0.7268; R^2: 0.2142; P = 0.5641) (Fig. 3A).

The expression values also did not change among the carcass, midgut and not developed and developed ovaries for Glucose dehydrogenase (Ae-92048) (One-way ANOVA: F=2.429; R^2: 0.4767; P = 0.1404), Chitin bind 4 transcript (Ae-187521) (One-way ANOVA: F=1.286; R^2: 0.3253; P = 0.3437), DHR4 ligand transcripts (AeSigP-4002) (One-way ANOVA: F=3.048; R^2: 0.5334; P = 0.0922), N-acetylgalactosaminyl transferase (AeSigP-212177) (One-way ANOVA: F=0.1393; R^2: 0.04967; P = 0.9336), ChtBD2 (Ae-88956) (One-way ANOVA: F=0.8578; R^2: 0.2434; P = 0.5010), Kazal type serine protease inhibitor (AeSigP-109183) (One-way ANOVA: F=2.036; R^2: 0.4329; P = 0.1875) and for Na^+/Ca^{2+} exchanger protein (AeSigP-66427) (One-way ANOVA: F=1.773; R^2: 0.3994; P = 0.2298) (Fig. 3).

The transcripts abundance for Atrophin-1 protein (Ae-27176) (One-way ANOVA: F=5.915; R^2: 0.6893; $P = 0.0199$) were higher in developed ovaries ($P = 0.0349$) compared to the carcass, the midgut and the not developed ovary (Fig. 3D).

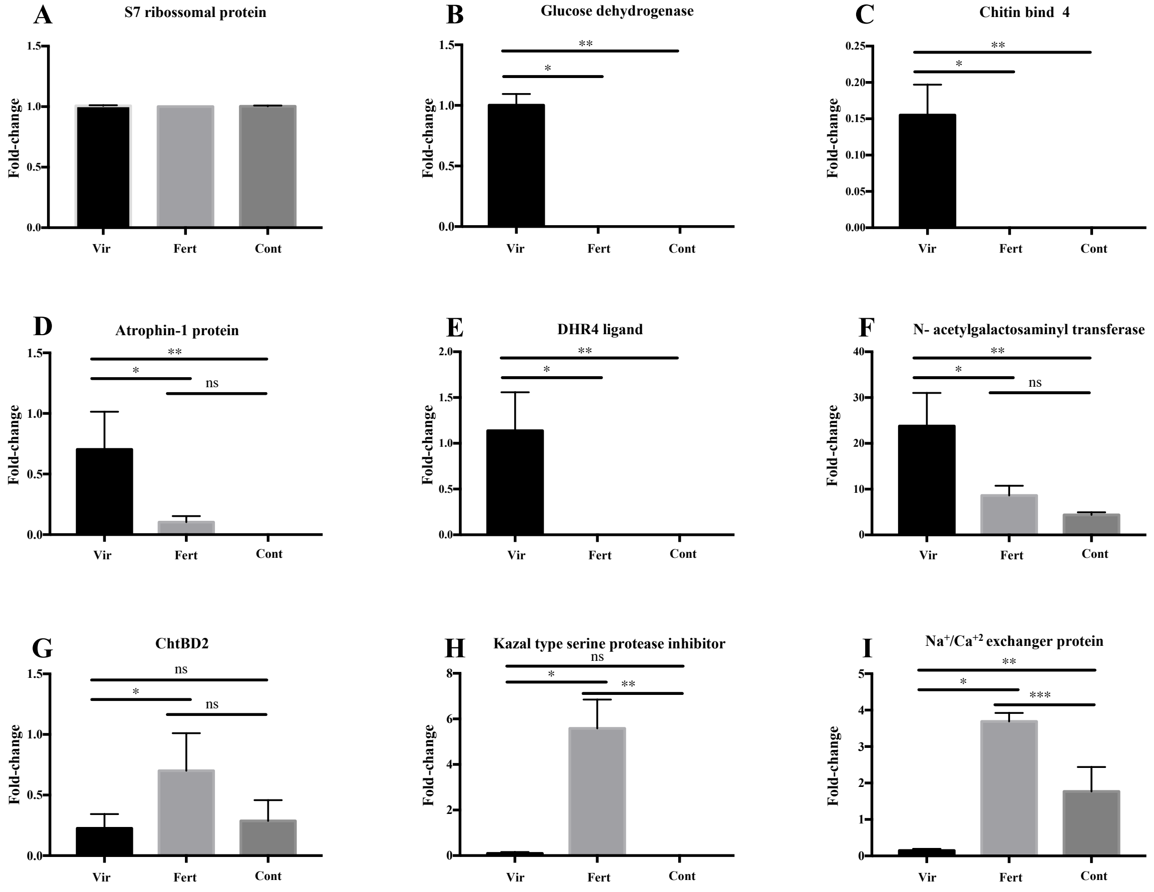


Figure 2: Relative expression of different genes in spermathecae of virgin (Vir) and fertilized (Fert) females of *A. aegypti* and of the content of the spermathecal reservoir lumen (Cont) of fertilized females by RT-PCR. For paired comparison Tukey's multiple comparisons test ($\alpha=0.005$) was used. **A:** S7 ribossomal protein transcripts (AAEL009496-RA) (One-way ANOVA: F=1; R^2: 0.25; $P = 0.4219$), **B:** Glucose dehydrogenase transcripts (Ae-92048) (One-way ANOVA: F=477.2; R^2: 0.9907; $P < 0.001$; $*P < 0.001$; $**P < 0.01$), **C:** Chitin bind 4 transcripts (Ae-187521) (One-way ANOVA: F=54.4; R^2: 0.9236; $P < 0.001$; $*P < 0.001$; $**P < 0.01$), **D:** Atrohin-1 protein transcripts (Ae-27176) (One-way ANOVA: F=17.24; R^2: 0.793; $P = 0.0008$; $*P = 0.0031$; $**P = 0.0011$), **E:** DHR4 ligand transcripts (AeSigP-4002) (One-way ANOVA: F=29.27; R^2: 0.8667; $P = 0.0001$. $*P = 0.0003$; $**P = 0.0003$), **F:** N-acetylgalactosaminyl transferase transcripts (AeSigP-212177) (One-way ANOVA: F=21.91; R^2: 0.8296; $P = 0.0003$. $*P = 0.0021$; $**P = 0.0004$), **G:** ChtBD2 transcripts (Ae-88956) (One-way ANOVA: F=5.724; R^2: 0.5599; $P = 0.0249$; $*P = 0.0303$), **H:** Kazal type serine protease inhibitor transcripts (AeSigP-109183) (One-way ANOVA: F=75.8; R^2: 0.944; $P < 0.0001$. $*P < 0.0001$; $**P < 0.0001$), **I:** Na^+/Ca^{2+} exchanger protein transcripts (AeSigP-66427) (One-way ANOVA: F=74.28; R^2: 0.9429; $P < 0.0001$. $*P < 0.0001$; $**P = 0.0009$; $***P = 0.0003$).

3.3. RNAi experiments

Knockdown effects on spermathecal expressed genes

Once the S7 ribosomal protein transcript (AAEL009496-RA) was chosen as a constitutive gene, it was expected that its expression would not change among the treatments, otherwise, it could not be used as a normalization gene. Considering this, all the expression values (abundance of transcripts) were analyzed based on the fold-change values over S7 value (Dzaki et al. 2017). The results for the silencing attempt over the eight chosen genes are shown in the Fig. 4. The dsRNA injections, including dsEGFP, did not interfere with the level of expression of S7 transcripts ($P = 03739$) (Fig. 4A).

The dsRNA injections reduced the expression of the transcripts of all the eight tested genes. In addition, there was no statistical difference among virgin and fertilized spermathecae for the target genes: Glucose dehydrogenase (Ae-92048), Chitin bind 4 (Ae-187521), Atrophin-1 protein (Ae-27176), DHR4 ligand (AeSigP-4002), N-acetylgalactosaminyl transferase (AeSigP-212177), ChtBD2 (Ae-88956), Kazal type serine protease inhibitor (AeSigP-109183), Na^+/Ca^{2+} exchanger protein (AeSigP-66427), with a $P > 0.005$ for all comparisons (Fig. 4).

Survival analysis

Once the females were injected with dsRNA, their survival rate was compared with control (dsEGFP) to check if the corresponding transcript decreases affected the female viability. For this, two different controls were used: dsEGFP injected at day 1 after emergence and dsEGFP injected at day 2 after emergence and the survival was checked for 10 days (Fig. 5).

When compared to the control (EGFP), no difference on the survival rate was found for Glucose dehydrogenase (Ae-92048) ($P = 0.6201$), DHR4 ligand (AeSigP-4002) ($P = 0.6986$), N-acetylgalactosaminyl transferase (AeSigP-212177) ($P = 0.2378$), ChtBD2 (Ae-88956) ($P = 0.3739$), Kazal type serine protease inhibitor (AeSigP-109183) ($P = 0.2996$), and Na^+/Ca^{2+} exchanger protein (AeSigP-66427) ($P = 0.3106$). However, the survival was reduced in the dsRNA treatments for Chitin bind 4 (Ae-187521) and Atrophin-1 protein (Ae-27176) in comparison to control ($P = 0.0364$ and 0.0109, respectively).

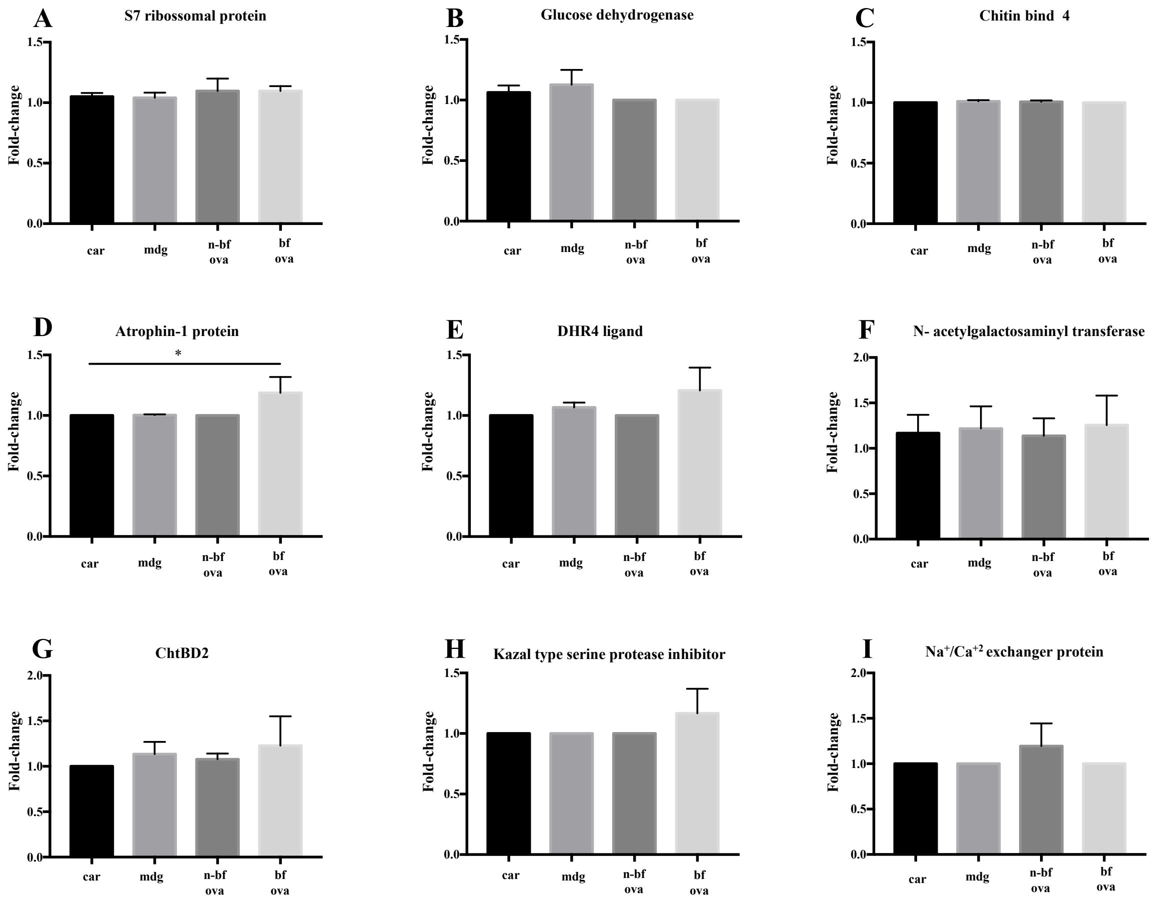


Figure 3: Relative expression of different genes in carcass (car), midgut (mdg) and ovaries of non blood-fed (n-bf ova) and of blood-fed (bf ova) females of *A. aegypti* by RT-PCR. For paired comparison Tukey's multiple comparisons test (a=0.005) was used. **A:** S7 ribossomal protein transcripts (AAEL009496-RA) (One-way Anova: F = 0.7268; R^2: 0.2142; P = 0.5641), **B:** Glucose dehydrogenase transcripts (Ae-92048) (One-way Anova: F = 2.429; R^2: 0.4767; P = 0.1404), **C:** Chitin bind 4 transcripts (Ae-187521) (One-way Anova: F = 1.286; R^2: 0.3253; P = 0.3437), **D:** Atrohin-1 protein transcripts (Ae-27176) (One-way Anova: F = 5.915; R^2: 0.6893; P = 0.0199; *P = 0.0349), **E:** DHR4 ligand transcripts (AeSigP-4002) (One-way Anova: F = 3.048; R^2: 0.5334; P = 0.0922), **F:** N-acetylgalactosaminyl transferase transcripts (AeSigP-212177) (One-way Anova: F = 0.1393; R^2: 0.04967; P = 0.9336), **G:** ChtBD2 transcripts (Ae-88956) (One-way Anova: F = 0.8578; R^2: 0.2434; P = 0.5010), **H:** Kazal type serine protease inhibitor transcripts (AeSigP-109183) (One-way Anova: F = 2.036; R^2: 0.4329; P = 0.1875), **I:** Na^+/Ca^{2+} exchanger protein transcripts (AeSigP-66427) (One-way Anova: F = 1.773; R^2: 0.3994; P = 0.2298).

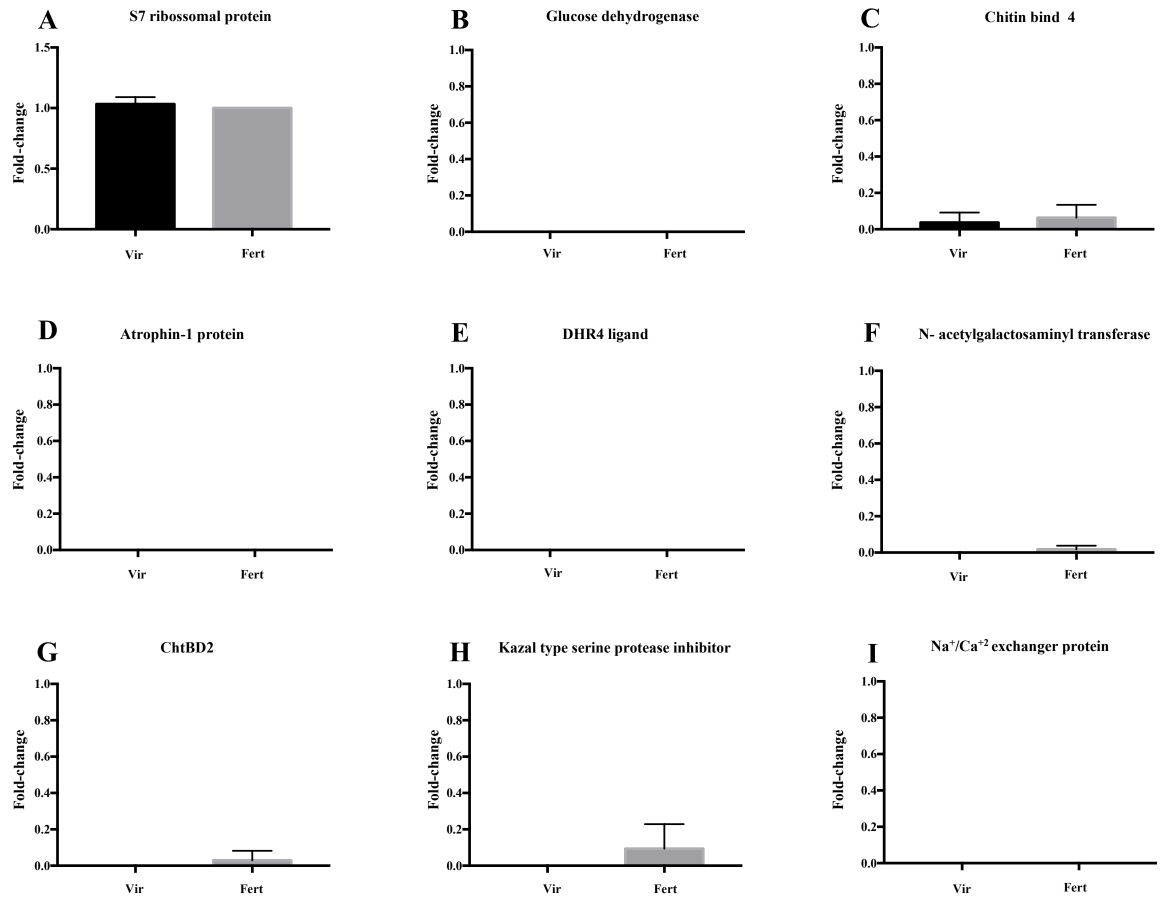


Fig. 4: Relative expression of different genes in spermathecae of virgin (Vir) and fertilized (Fert) females of *A. aegypti* after dsRNA injections by RT-PCR. For paired comparison Tukey's multiple comparisons test (α=0.005) was used. **A:** For S7 ribossomal protein transcripts (AAEL009496-RA) ($P = 0.3739$); **B:** Glucose dehydrogenase transcripts (Ae-92048) ($P > 0.005$); **C:** Chitin Bind 4 transcripts (Ae-187521) ($P = 0.6342$); **D:** Atrophin-1 protein transcripts (Ae 27176) ($P > 0.005$); **E:** DHR4 ligand transcripts (AeSigP-4002) ($P > 0.005$); **F:** N-acetylgalactosaminyl transferase transcripts (AeSigP-212177) ($P = 0.2378$); **G:** ChtBD2 gene (Ae-88956) ($P = 0.3739$); **H:** Kazal type serine protease inhibitor transcripts (AeSigP-109183) ($P = 0.2996$); **I:** Na^+/Ca^{2+} exchanger protein transcripts (AeSigP-66427) ($P > 0.005$).

Blood-feeding experiments

To verify if the dsRNA injection could interfere with the female metabolism, the blood-feeding capacity was checked. The engorged females were counted and the females were weighted before and after the blood meal (Fig. 6). However, the females injected with dsRNA for Na^+/Ca^{2+} exchanger protein (AeSigP-66427) had a lower weight after the blood meal compared to control ($P = 0.0985$) (Figs. 6C and 6D).

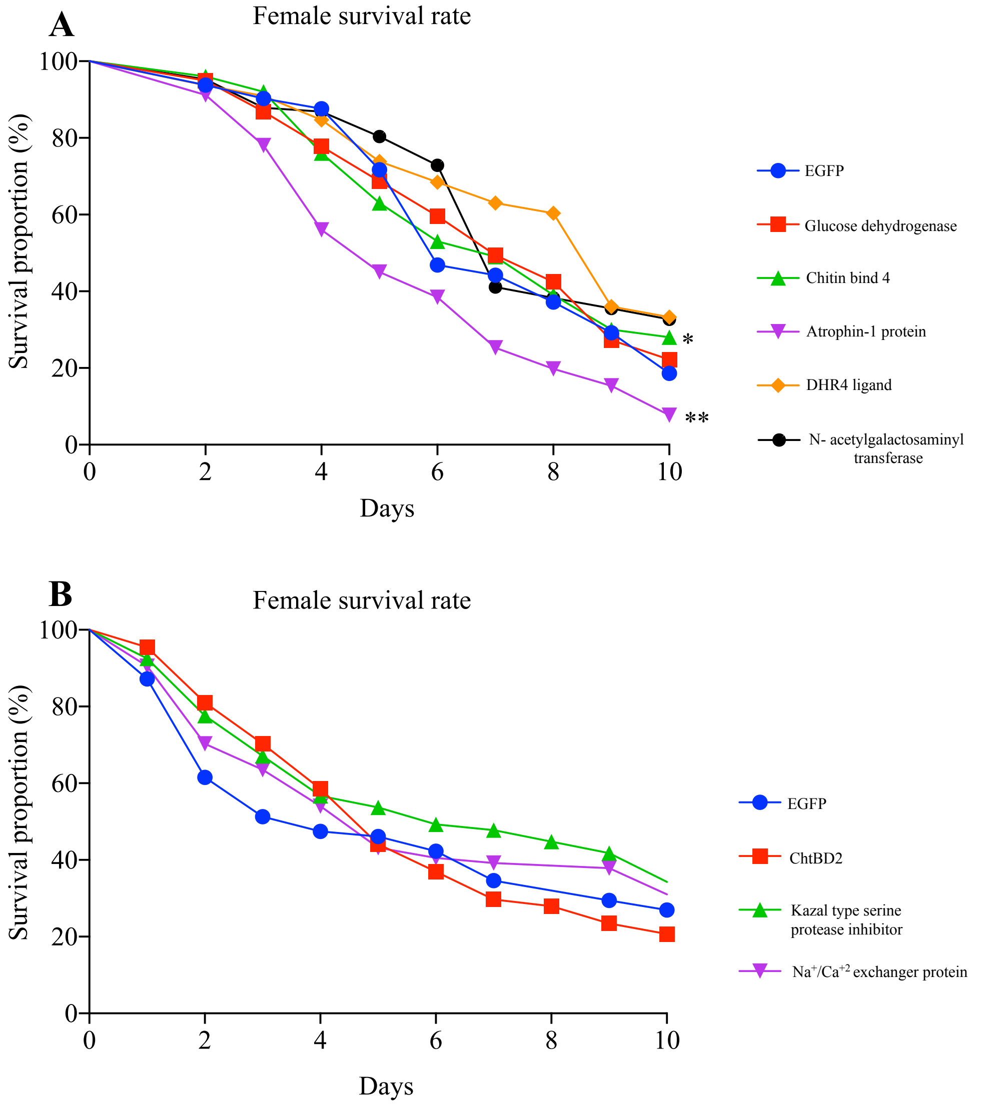


Fig. 5: Survival of *A. aegypti* females after injection of dsRNA. Each treatment was performed on 100 females, and the survival recorded over 10 days. Curves difference between each target gene and the control (EGFP) were calculated by the Log-rank test (α=0.05). **A:** Injections for genes highly expressed in virgin spermathecae (X^2 = 13.84, P = 0.0166, *P = 0.0364; **P = 0.0109); **B:** Injections for genes highly expressed in fertilized spermathecae (X^2 = 2.421, P = 0.1197).

The weight of females was higher after the injection with dsRNA for Glucose dehydrogenase (Ae-92048), DHR4 ligand (AeSigP-4002), N-acetylgalactosaminyl transferase (AeSigP-212177), Kazal type serine protease inhibitor (AeSigP-109183) and Na^+/Ca^{2+} exchanger protein (AeSigP-66427) in comparison dsEGFP controls ($P < 0.005$). Only for ChtBD2 (Ae-88956), the female weight before blood feeding was lower than the control (P = 0.0447) (Figs. 6A and 6B). After the blood meal, the weight of dsRNA-injected females for the DHR4 ligand (AeSigP-4002), N-acetylgalactosaminyl transferase (AeSigP-212177) and Kazal type serine protease inhibitor (AeSigP-109183) were also higher than control ($P < 0.0001$).

Among the treatments, only the females knocked-down for Glucose dehydrogenase (Ae-92048) and Na^+/Ca^{2+} exchanger protein (AeSigP-66427) showed a reduction in the blood meal rate in comparison to control ($P = 0.0419$ and 0.0121, respectively) (Figs. 6E and 6F).

Fertility analysis

The effect of dsRNA injections was checked considering female oviposition rate, survival rate after oviposition, fertility rate after the blood feeding (Fig. 7). The number of females that laid eggs was reduced compared to the control in Chitin bind 4 (Ae-187521) and N-acetygalactosaminyl transferase (AeSigP-212177) treatments ($P = 0.00489$ and 0.0179, respectively) (Figs. 7A and 7B). The injection of dsRNA for Na^+/Ca^{2+} exchanger protein (AeSigP-66427) inhibited the egg laying.

From those females that laid eggs even after dsRNA injections, the survival rate did not show difference among the treatments ($P > 0.005$) (Fig. 7C and 7D). From those eggs laid, the Glucose dehydrogenase (Ae-92048), Atrophin-1 protein (Ae-27176) and DHR4 ligand (AeSigP-4002) treatments did not differ from the number of laid eggs compared to the dsEGFP control ($P = 0.9024$, $P = 0.9024$, $P = 0.4343$, respectively) (Figs. 7E and 7F). In Chitin bind 4 (Ae-187521), N-acetylgalactosaminyl transferase (AeSigP-212177), ChtBD2 (Ae-88956) and Kazal type serine protease inhibitor (AeSigP-109183) treatments, the number of eggs were higher than control ($P = 0.0489$, 0.0179, 0.0235, 0.0455, respectively).

Egg morphometry

The morphometry of eggs was compared considering the length and area (Fig. 7). Females injected with dsRNA for Glucose dehydrogenase (Ae-920428), Chitin bind 4 (Ae-187521), Atrophin-1 protein (Ae-27176), DHR4 ligand (AeSigP-4002), N-acetylgalactosaminyl transferase (AeSigP-212177) and ChtBD2 (Ae-88956) laid eggs with higher length than the control ($P < 0.0001$) (Figs. 8A and 8B). There was no difference in both length and area between eggs laid by females injected with the Kazal type serine protease inhibitor (AeSigP-109183) and the control ($P = 0.9550$ and $P = 0.9991$, respectively) (Fig. 8B).

The eggs produced by females injected with dsRNA for Glucose dehydrogenase (Ae-920428) had a larger area than control ($P < 0.001$). The females of other dsRNA treatments had eggs with a smaller area in comparison to control ($P < 0.001$) (Figs. 8C and 8D).

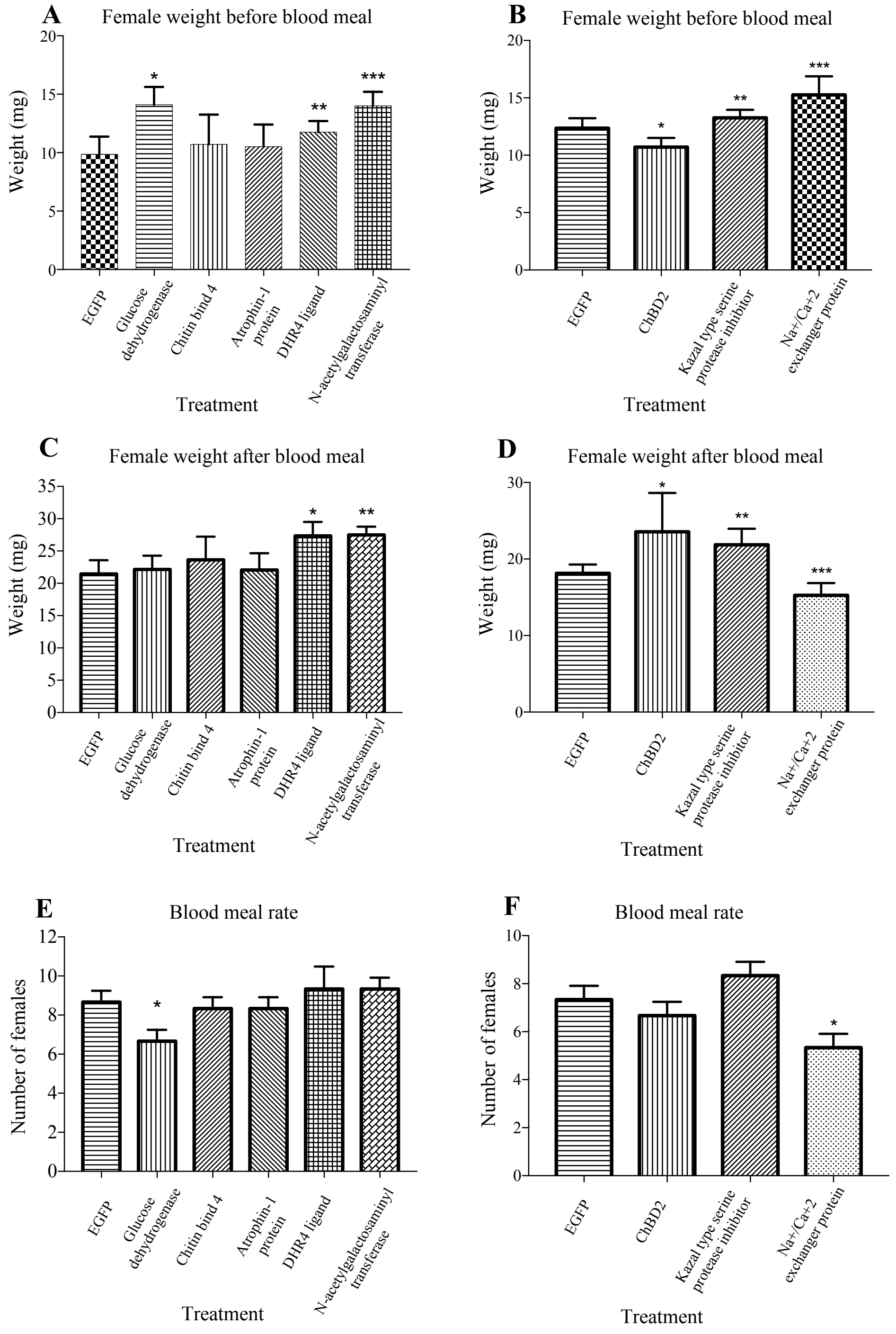


Fig. 6: Effect of dsRNA injections on the blood meal of *A. aegypti* females for target genes compared to the control (EGFP). Values for each gene were compared with the control (EGFP) by the Tukey's multiple comparisons test (α=0.005). **A:** Female weight before blood feeding of females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=23.45, R^2 = 0.5071, P < 0.0001. *P < 0.0001, **P = 0.0073, ***P < 0.0001). **B:** Female weight before blood feeding of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=63.37, R^2 = 0.7144, P < 0.0001. *P < 0.001, **P = 0.0447, ***P < 0.001). **C:** Female weight after blood feeding of females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=25.53, R^2 = 0.5282, P < 0.0001. *, **P < 0.0001). **D:** Female weight after blood feeding of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way

ANOVA: F=12.04, $R^2 = 0.3222$, $P < 0.0001$. *$P < 0.0001$, **$P = 0.0008$, ***$P = 0.0985$). **E:** Number of females injected with dsRNA for genes highly expressed in virgin spermathecae from blood-fed females (One-way ANOVA: F=5.778, $R^2 = 0.7065$, $P = 0.0061$. *$P = 0.0419$). **F:** Number of females injected with dsRNA for genes highly expressed in fertilized spermathecae from blood-fed females (One-way ANOVA: F=14.25, $R^2 = 0.8424$, $P = 0.0014$; * $P = 0.0121$).

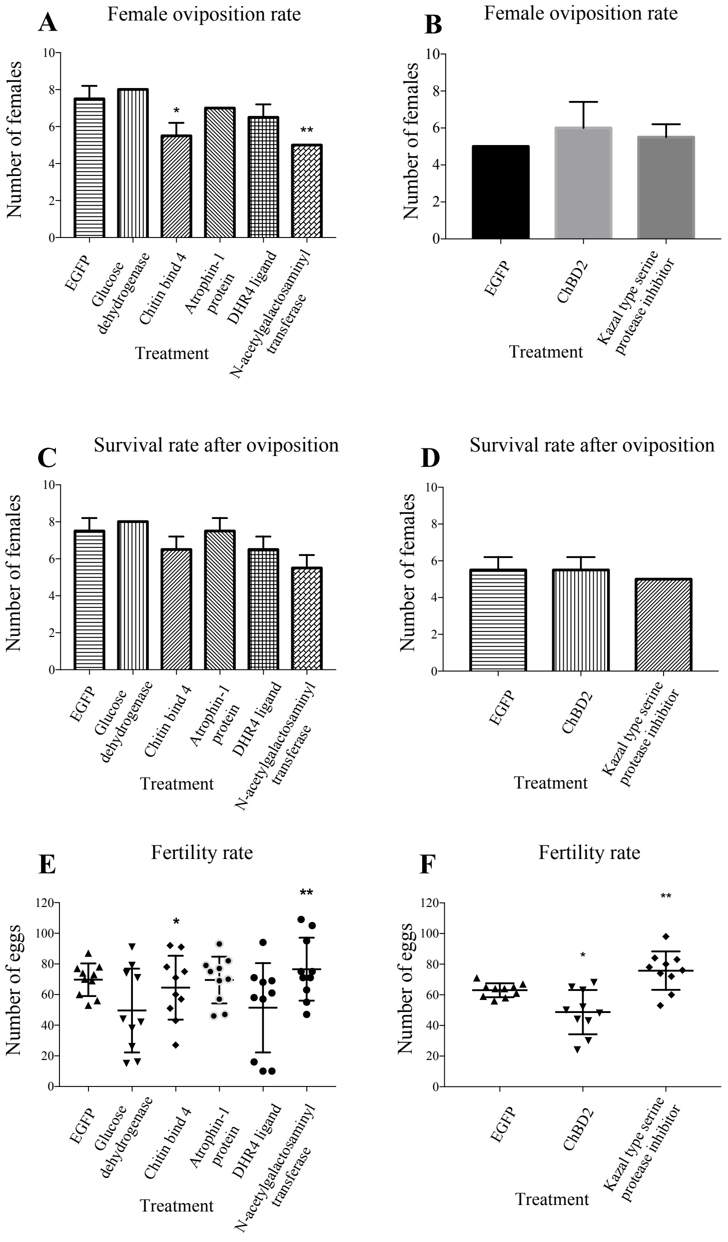


Fig. 7: Effect of dsRNA injections on the oviposition rate of *A. aegypti* females for target genes compared to the control (EGFP). Values for each gene were compared with the control (EGFP) by the Tukey's multiple comparisons test (α=0.005). **A:** Oviposition rate of females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=10.73, $R^2 = 0.8994$, $P = 0.0059$. *$P = 0.00489$, **$P = 0.0179$). **B:** Oviposition rate of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=0.6, $R^2 = 0.2857$, $P = 0.6037$). **C:** Female survival rate after oviposition of females injected with dsRNA for

genes highly expressed in virgin spermathecae (One-way ANOVA: F=4.04, $R^2 = 0.771$, P = 0.0595). **D:** Female survival rate after oviposition of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=0.3333, $R^2 = 0.2$, P = 0.8032). **E:** Fertility rate of females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=10.73, $R^2 = 0.8994$, P = 0.0059. *P = 0.0489, **P = 0.0179). **F:** Fertility rate of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=14.28, $R^2 = 0.514$, P < 0.0001. *P = 0.0235, **P = 0.0455).

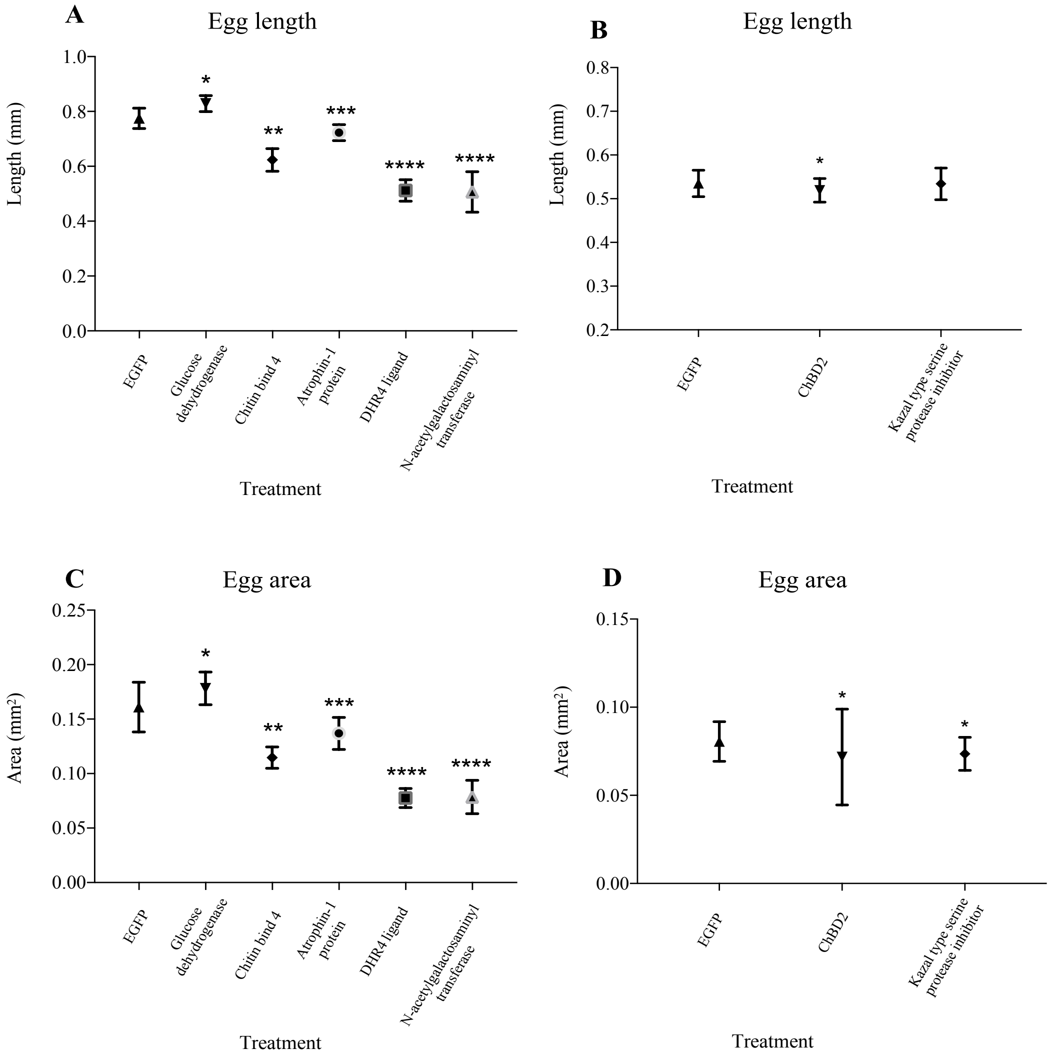


Fig. 8: Morphometry of eggs laid by *A. aegypti* females injected with dsRNA for different target genes compared to the control injected with dsEGFP. For comparison for each related gene with the control (EGFP) was used the Tukey's multiple comparisons test (α=0.005). **A:** Length of eggs produced by females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=963, R^2=0.8882, P < 0.0001. *, **, ***, ****P < 0.0001). **B**: Length of eggs produced by females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=8.213, R^2=0.05241, P-value=0.0003. *P = 0.0001). **C**: Area of eggs produced by females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=744.1, R^2=0.8409, P < 0.0001. *, **, ***, ****P < 0.0001). **D**: Area of eggs produced by females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=6.803, R^2=0.0438, P = 0.0013. *P = 0.0163).

Fecundity

The fecundity is conditioned by the number of viable eggs laid. Considering this, we have analyzed the fecundity rate of the eggs laid by the dsRNA-injected females (Fig. 9). From all the treatments, the injection with dsRNA for Glucose dehydrogenase (Ae-920428), N-acetylgalactosaminyl transferase (AeSigP-212177) and Kazal type serine protease inhibitor (AeSigP-109183) had a reduction of egg hatching compared to the control ($P = 0.0365$, 0.0002, and 0.0008, respectively).

Different from the other injections, the effects of dsRNA injection for Na^+/Ca^{2+} exchanger protein (AeSigP-66427) affected the egg development of *A. aegypti* females. Different from the ovary of control females, after the knocking-down, ovaries did not develop not even seven days after the blood meal (Fig. 10 and Video 1).

Five days after the blood feeding, no eggs were laid by treated females after the knocking-down for Na^+/Ca^{2+} exchanger protein. The filter paper or substrate for egg laying was checked for both virgin and fertilized females, and they had feces, and indication for complete blood digestion (Fig. 11).

One day after mating, the sperm motility seems to be reduced (Video 3) in treated females for Na^+/Ca^{2+} exchanger protein when compared to the control (Video 2); besides this, at day five after the blood meal, the fertilized spermathecae presented sperm with no motility (Video 4). Once the reservoir is broken, the sperm starts to swim outside of the spermathecal reservoir (Video 5).

A summary of the measurements among the control group and the dsRNA for each target gene is shown in Tab. 6.

Spermathecal morphology

The morphology of the spermathecae from all the treatments were studied in both virgin and fertilized females that were injected with dsRNA and compared with the control (Figs. 12, 13 and 14). This comparison included the duct, glandular portion, and reservoir, with no conformational alteration in the females after the injections. Surrounding the internal part of the reservoir and continuously with the spermathecal duct, a well-structured thicker cuticular layer can be seen. The glandular cells coincide with the cuticle interruptions of reservoir cuticle. In addition, within virgin spermathecae in all individuals (i.e., knocked down control), the lumen of the spermathecal reservoir is filled by a fluid.

Gene	**Related function**	**Phenotypic analysis**							
		Female weight before blood meal (mg)	**Female weight after blood meal (mg)**	**Blood meal rate (number of females)**	**Female oviposition rate (number of females)**	**Survival rate after oviposition (number of females)**	**Fertility rate (number of eggs)**	**Egg length (mm)**	**Egg area (mm^2)**
Virgin control	**dsEGFP**	9.85±1.53	21.45±2.13	8.66±0.57	7.5±0.70	7.5±0.70	69.7±10.60	0.77±0.03	0.16±0.02
Ae-92048	Glucose dehydrogenase	14.1±1.51	22.15±2.13	6.66±0.57	8±0.01	8±0.01	49.6±27.36	0.82±0.02	0.17±0.01
Ae-187521	Chitin bind 4	10.7±2.55	23.65±3.58	8.33±0.57	5.5±0.70	6.5±0.70	64.5±20.87	0.62±0.04	0.11±0.01
Ae-27176	Atrophil-1 protein	10.5±1.90	22.05±2.60	8.33±0.57	7±0.01	7.5±0.70	69.5±15.27	0.72±0.02	0.13±0.01
AeSigP-4002	DHR4 ligand	11.75±0.96	27.35±2.15	9.33±1.15	6.5±0.70	6.5±0.70	51.4±29.12	0.51±0.03	0.07±0.01
AeSigP-212177	N-acetylgalactosaminyl transferase	14±1.21	27.5±1.27	9.33±0.57	5±0.01	5.5±0.70	76.6±20.52	0.50±0.07	0.07±0.01
Mated control	**dsEGFP**	12.35±0.87	18.15±1.13	7.33±0.57	5±0.01	5.5±0.70	63±4.52	0.54±0.02	0.08±0.01
Ae-88956	Chitin-binding domain type 2	10.7±0.80	23.55±5.09	6.66±0.57	6±1.41	5.5±0.70	48.7±14.41	0.52±0.01	0.07±0.02
AeSigP-109183	Kazal type serine protease inhibitor	13.25±0.71	21.85±2.10	8.33±0.57	5.5±0.70	5±0.01	75.8±12.56	0.53±0.03	0.07±0.01
AeSigP-66427	Na^+/Ca^{2+} exchanger protein	15.25±1.61	21.4±1.46	5.33±0.57		5.5±0.70			

Tab. 6: Phenotypic analysis in *A. aegypti* females after injection of dsRNA. The values represents mean ± standard deviation.

In the fertilized spermathecae of all individuals, the sperm is organized in a circular fashion within the reservoir lumen, parallel to each other. The PAS histochemical test showed a higher color intensity in the glandular portion in the ChtBD2 (Ae-88956) and Na^+/Ca^{2+} exchanger protein (AeSigP-66427) treatments compared to the control (EGFP).

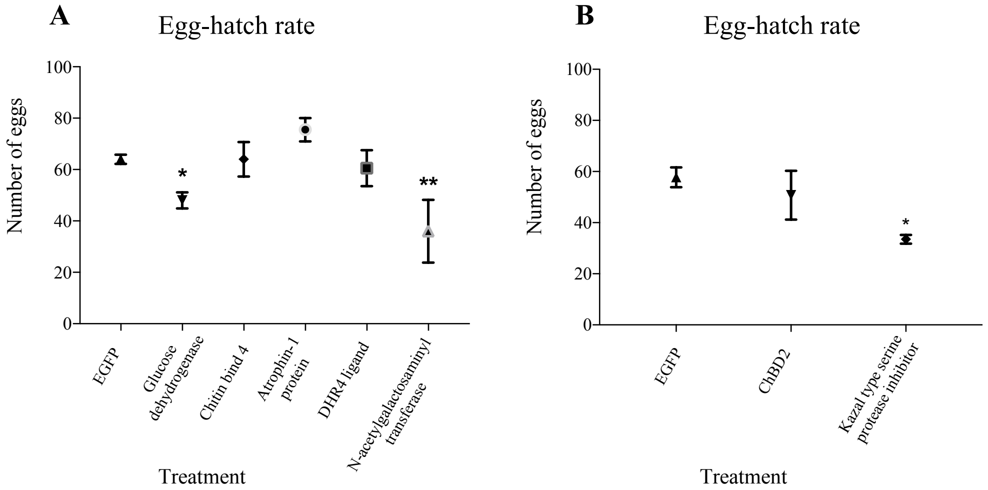


Fig. 9: Effect of dsRNA injections on the fecundity rate of *A. aegypti* females for target genes compared to the control (EGFP). Values for each gene were compared with the control (EGFP) by the Tukey's multiple comparisons test (α=0.005). **A:** Egg-hatching rate of females injected with dsRNA for genes highly expressed in virgin spermathecae (One-way ANOVA: F=16.84, R^2= 0.8239, P < 0.0001. *P-value=0.0365, **P-value=0.0002). B: Egg-hatching rate of females injected with dsRNA for genes highly expressed in fertilized spermathecae (One-way ANOVA: F=17.18, R^2= 0.7924, P = 0.0008. *P = 0.0008).

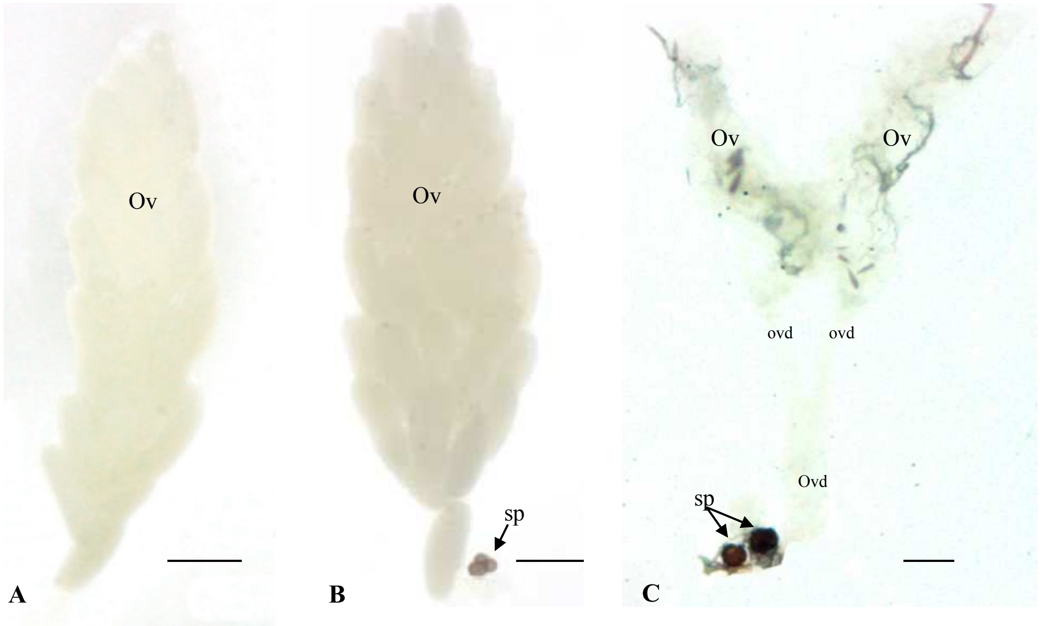


Fig. 10: Ovary morphology of *A. aegypti* females three days after blood-feeding. A: Virgin females injected with the control treatment (dsEGFP). B: Fertilized females of control injected with dsEGFP. C: Fertilized females

injected with dsRNA for target gene of Na$^+$/Ca^{2+} exchanger protein (AeSigP-66427). (Ov): Ovary, (ovd): lateral oviducts, (Ovd): common oviduct, (sp): spermathecae. Bar: 1mm.

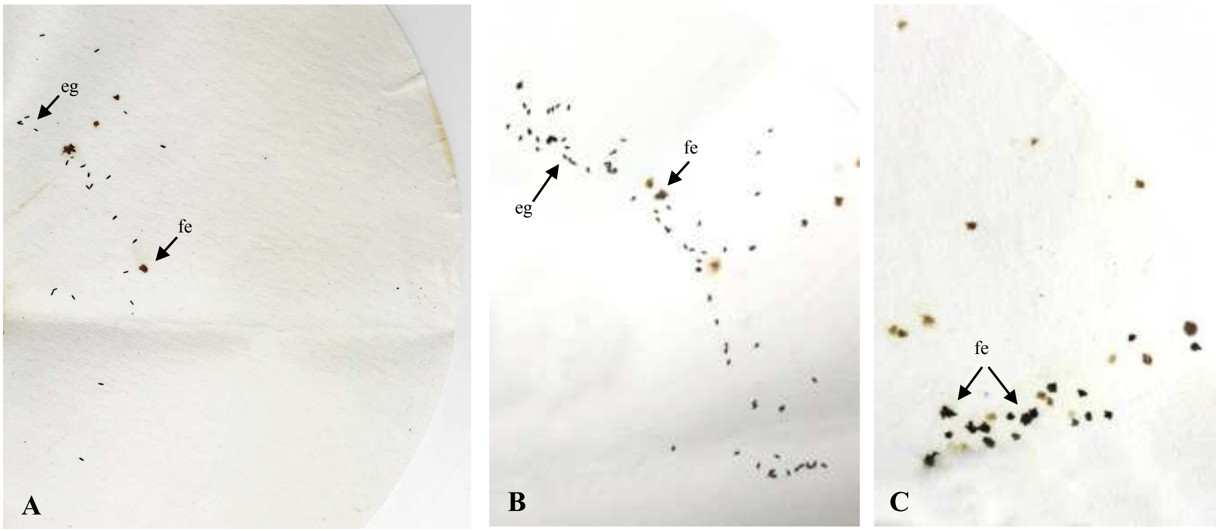


Fig. 11: Filter papers offered to females to lay eggs five days after the blood meal. **A:** Oviposition of virgin females injected with the control treatment (dsEGFP). **B:** Oviposition of fertilized females injected with the control treatment (dsEGFP). **C:** Fertilized females injected with dsRNA for target gene of Na$^+$/Ca^{2+} exchanger protein (AeSigP-66427). (eg): eggs; (fe): pigmented feces resulted from blood digestion.

A summary of the phenotypic effects provided by the knocking down effect for each target gene is shown in Tab. 7.

RNA *in situ* hybridization

In order to check in which part of spermathecal the eight target genes studied here are expressed, the *in situ hybridization* assay was performed in whole mounts of spermathecae by using labeled specific RNA sequences (probes). For the Glucose dehydrogenase (Ae-92048), the fluorescence signal was detected along the spermathecal duct, with a higher intensity in the duct of individual glandular cells. In addition, the fluorescent signal was detected in some epithelial cells of the reservoir (Fig. 15).

For Chitin bind 4 (Ae-187521) (Fig. 15) and Kazal type serine protease inhibitor (AeSigP-109183) (Fig. 17), the fluorescence signal was detected in the spermathecal duct and at the site of attachment of the glandular cells to the duct. The fluorescence intensity of the probes was higher in the attachment site of the duct of the spermathecal reservoir, where the spermathecal gland is located (Fig. 15).

	Genes highly expressed in virgin spermathecae					**Genes highly expressed in fertilized spermathecae**		
Parameters	**Ae-92048 Glucose dehydrogenase**	**Ae-187521 Chitin bind 4**	**Ae-27176 Atrophin-1 protein**	**AeSigP-4002 DHR4 ligand**	**AeSigP-212177 N-acetylgalactosaminyl transferase**	**Ae-88956 Chitin-binding domain type 2**	**AeSigP-109183 Kazal type serine protease inhibitor**	**AeSigP-66427 Na^{+}/Ca^{+2} exchanger protein**
Female survival rate		↓	↓					
Female weight before blood meal				↑	↑	↑	↑	↓
Female weight after blood meal	↓							↓
Female oviposition rate		↓			↓			**x**
Fertility rate		↑			↑	↑	↑	**x**
Egg area	↑	↓	↓	↓	↓	↓	↓	**x**
Egg length	↑	↑	↑	↑	↑	↑		**x**
Fecundity rate	↓				↓		↓	**x**

Tab. 7: Summary of the effects after the knocking down of each target gene. The arrows up (↑) and down (↓) represent an increase or decrease in the parameter, respectively. The "**x**" represents a parameter that was completed inhibited or could not be analyzed.

Transcripts for Atrophin-1 protein (Ae-27176) were detected in the gland, mainly close to the reservoir cuticle. The signal for this transcript was low in the spermathecal duct (Fig. 15). Transcripts for the DHR4 ligand (AeSigP-4002), ChtBD2 (Ae-88956) were detected only in glandular cells, in the apical portion associated with the ductule (Figs. 15 and 17). The N-acetylgalactosaminyl transferase (AeSigP-212177) transcripts were detected in the spermathecal gland and with a low intensity in the spermathecal duct. Transcripts for Na^{+}/Ca^{2+} exchanger protein (AeSigP-66427) were mostly detected in the spermathecal glandular portion close to the reservoir cuticle and in the spermathecal duct, close to the oviduct opening (Fig. 16). A summary of the fluorescence signal provided by the *in situ* hybridization for each target gene is shown in Tab. 8.

Gene	Function	Spermathecal duct epithelium	Glandular cells of spermathecal duct	Spermathecal gland	Reservoir epithelium
Ae-92048	Glucose dehydrogenase	+	-	+++	++
Ae-187521	Chitin bind 4	+	+	++	-
Ae-27176	Atrophil-1 protein	+	+	+++	-
AeSigP-4002	DHR4 ligand	-	-	++	-
Ae-88956	ChtBD 2	-	-	+++	-
AeSigP-66427	Na^{+}/Ca^{2+} exchanger protein	+++	+	+++	-
AeSigP-109183	Kazal type serine protease inhibitor	++	++	++	-
AeSigP-212177	N-acetylgalactosaminyl transferase	-	+	+++	-

Tab. 8: Intensity of fluorescent signal for different transcripts in different parts of *A. aegypti* spermathecae by *in situ* hybridization. The level of expression is represented by "+" and its absence by "-".

4. Discussion

The global gene expression of the spermathecae of the yellow fever mosquito *A. aegypti* was studied in this work. According to the enriched spermathecomes obtained by *in silico* analysis, there is a huge number of genes highly expressed in the spermatheca compared with the whole female and male bodies. Not surprisingly, 54.41% of the spermathecal transcripts represent conserved genes, with no attributed function yet, showing that the spermathecome of *A. aegypti* in an unfinished story. In spite of this, the plethora of the identified genes provided a detailed comparison between the spermathecomes of virgin and fertilized females and a physiological analysis of the spermatheca in the reproductive biology of this important vector.

By comparing virgin and fertilized spermathecae we could identify some functional groups categorized as commonly expressed genes (related with energy metabolism, cell adhesion, gene expression machinery and detoxification) (Alfonso-Parra et al. 2016). Notably, among the spermathecal transcripts in higher abundance, the higher number of them were found in the virgin spermathecome (111 DEG) when compared with the fertilized one (25 DEG).

The higher abundance expression found in the virgin spermathecome and the functional identified groups compared with the fertilized support the hypothesis that *A. aegypti* females invest a huge amount of energy to prepare the spermathecal lumen to allocate sperm. After the spermathecal filling, the expression pattern is mostly dedicated to the microenvironment maintenance to keep the sperm viability (Lensky & Alumot 1967, Verma 1973 and 1978, reviewed by Werner & Simmons 2008, Gonzalez et al. 2018).

Interference in the egg production cause massive loss of energy, damage or even no progeny development (Mathew & Rai 1975, Rezende et al. 2008, reviewed by Shukla 2015); on the other hand, any process that makes egg production more effectively enhance the offspring perpetuation. Based on that, here we analyzed the effects of the spermathecal knocking-down genes that showed highly-expressed transcripts in the *in silico* analysis in both virgin and fertilized females.

The Glucose dehydrogenase (Gld) knocking-down reduced the weight of mosquito females before the blood intake when compared to the control. This effect should be probably related to the weakness of females due to the Gld reduction. In addition, when analyzing the egg and offspring of females knocked down, the egg morphology was affected (with higher length and area), however, the larger eggs did not result in the increase of egg-hatching rate (fecundity). The same reduction was observed in *Apis mellifera* and *D. melanogaster* Gld knock-out females (Iida & Cavener 2004, Kunieda et al. 2006).

The Gld is related to the metabolism of trehalose, a non-reducing disaccharide, which is the major source of energy in insects. Trehalose is converted into glucose, which is then used to release energy (reviewed by Shukla 2015). Furthermore, some disturbance in this pathway could result in a catastrophic effect over sugar metabolism in insects (Iida & Cavener 2004, Iida et al. 2007, reviewed by Shukla 2015).

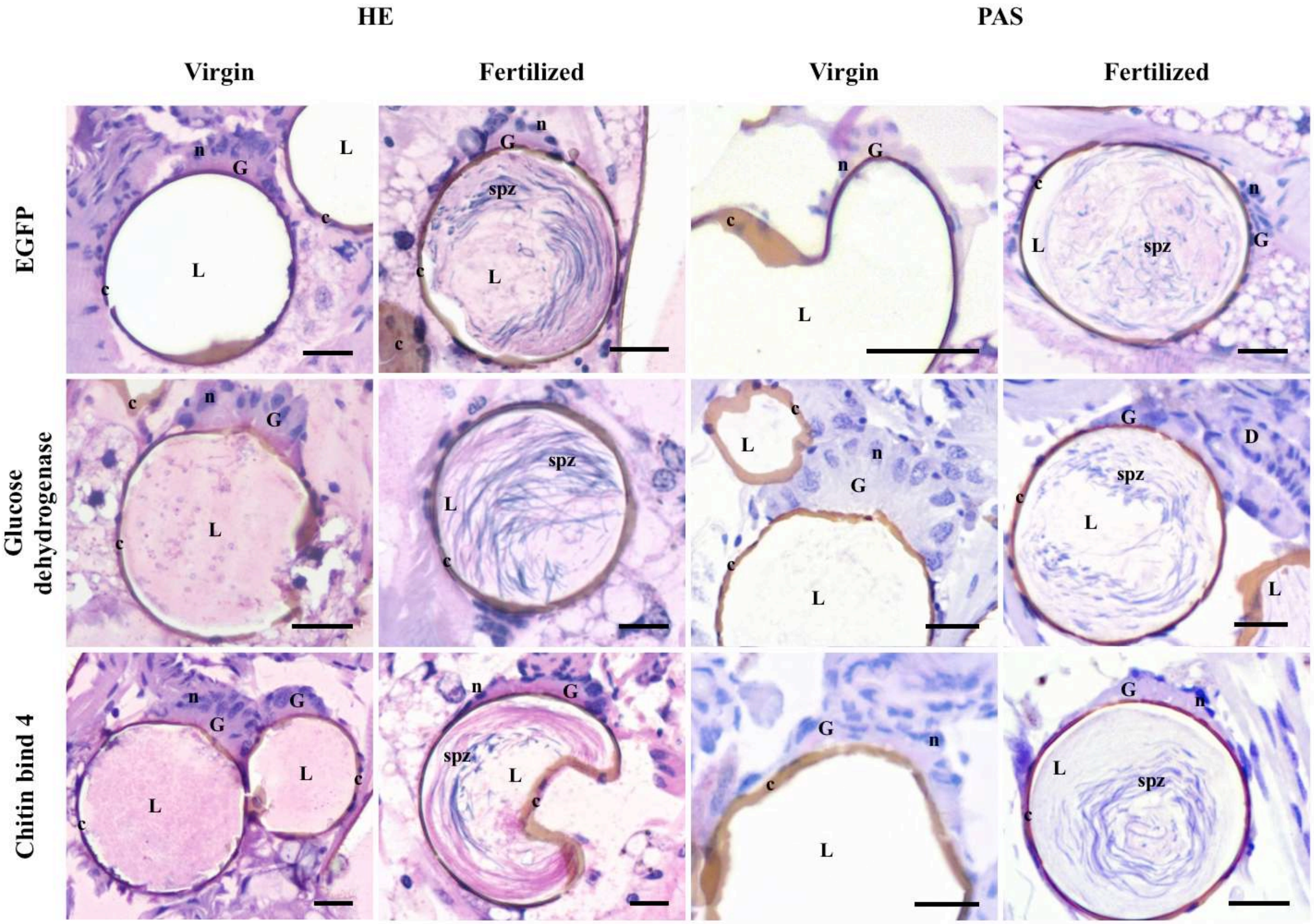


Fig. 12: Histological sections of *Aedes aegypti* spermathecae after the knockdown of virgin and fertilized spermathecae. The knocked-down treatments can be compared with EGFP (control). Sections were stained with HE (left) and PAS reaction (right). **(**L) reservoir lumen, (G) spermathecal gland**, (**n) cell nucleus**, (**spz) spermatozoa**, (**c) reservoir cuticle. Bar: 10 µm.

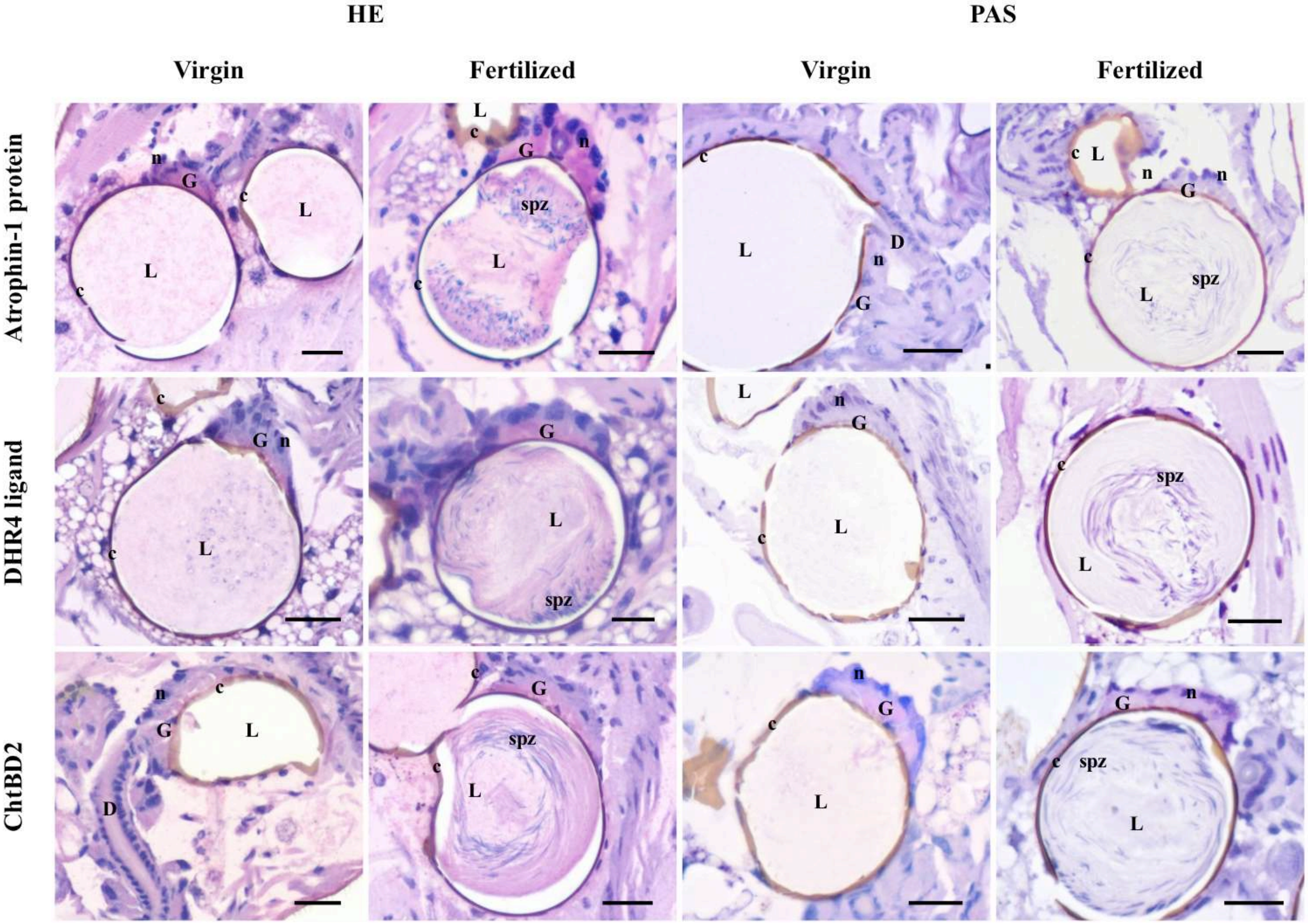


Fig. 13: Histological sections of *Aedes aegypti* spermathecae after the knockdown of virgin and fertilized spermathecae. The knocked-down treatments can be compared with EGFP (control) from Fig. 12. Sections were stained with HE (left) and PAS reaction (right). **(**L) reservoir lumen, (G) spermathecal gland**,** **(**n) cell nucleus**,** **(**spz) spermatozoa**,** **(**c) reservoir cuticle. Bar: 10 µm.

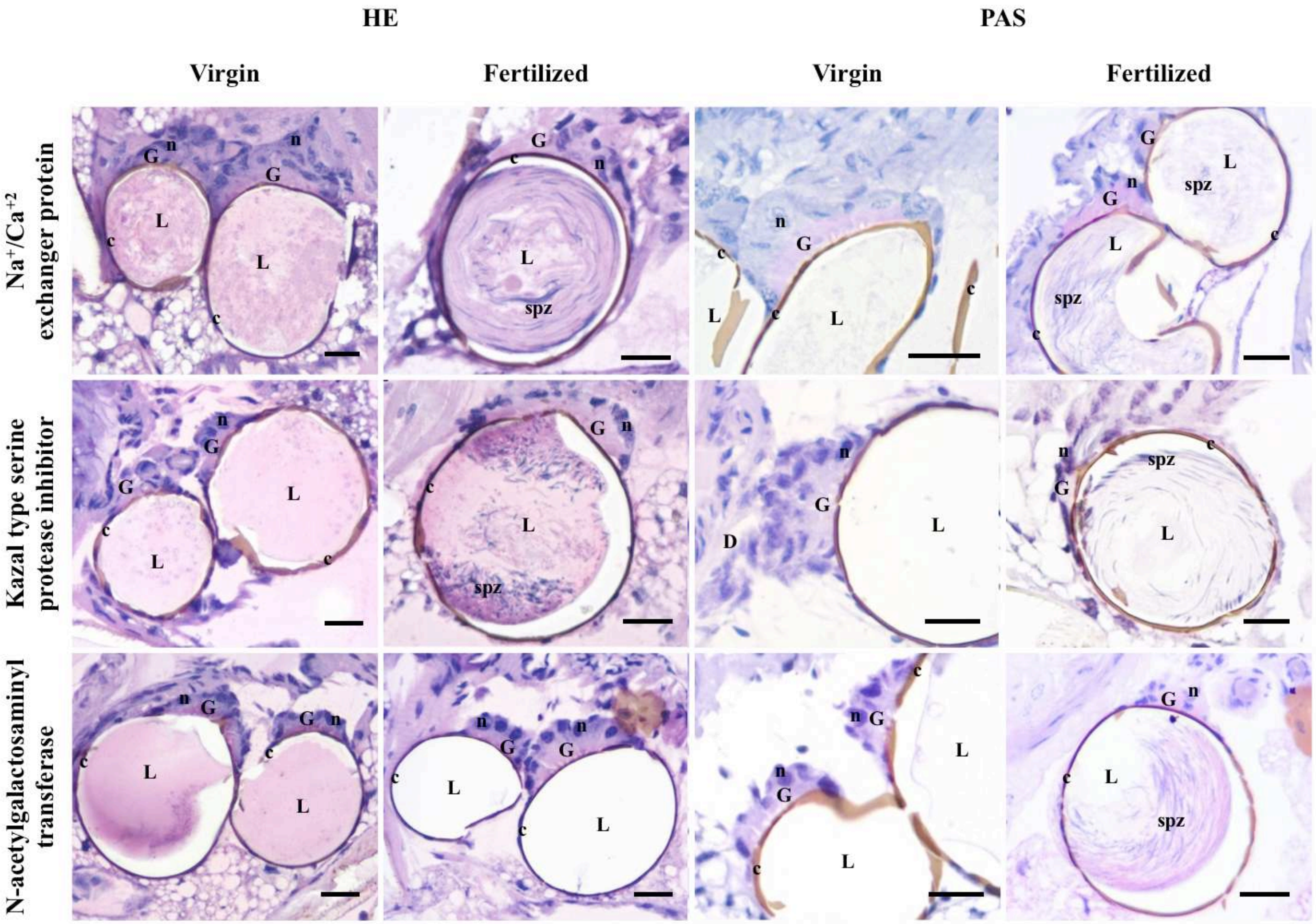


Fig. 14: Histological sections of *Aedes aegypti* spermathecae after the knockdown in virgin and fertilized spermathecae. The knocked-down treatments can be compared with EGFP (control) from Fig 12. Sections were stained with HE (left) and PAS reaction (right). (L) reservoir lumen, (G) spermathecal gland, (n) cell nucleus, (spz) spermatozoa, (c) reservoir cuticle. Bar: 10 µm.

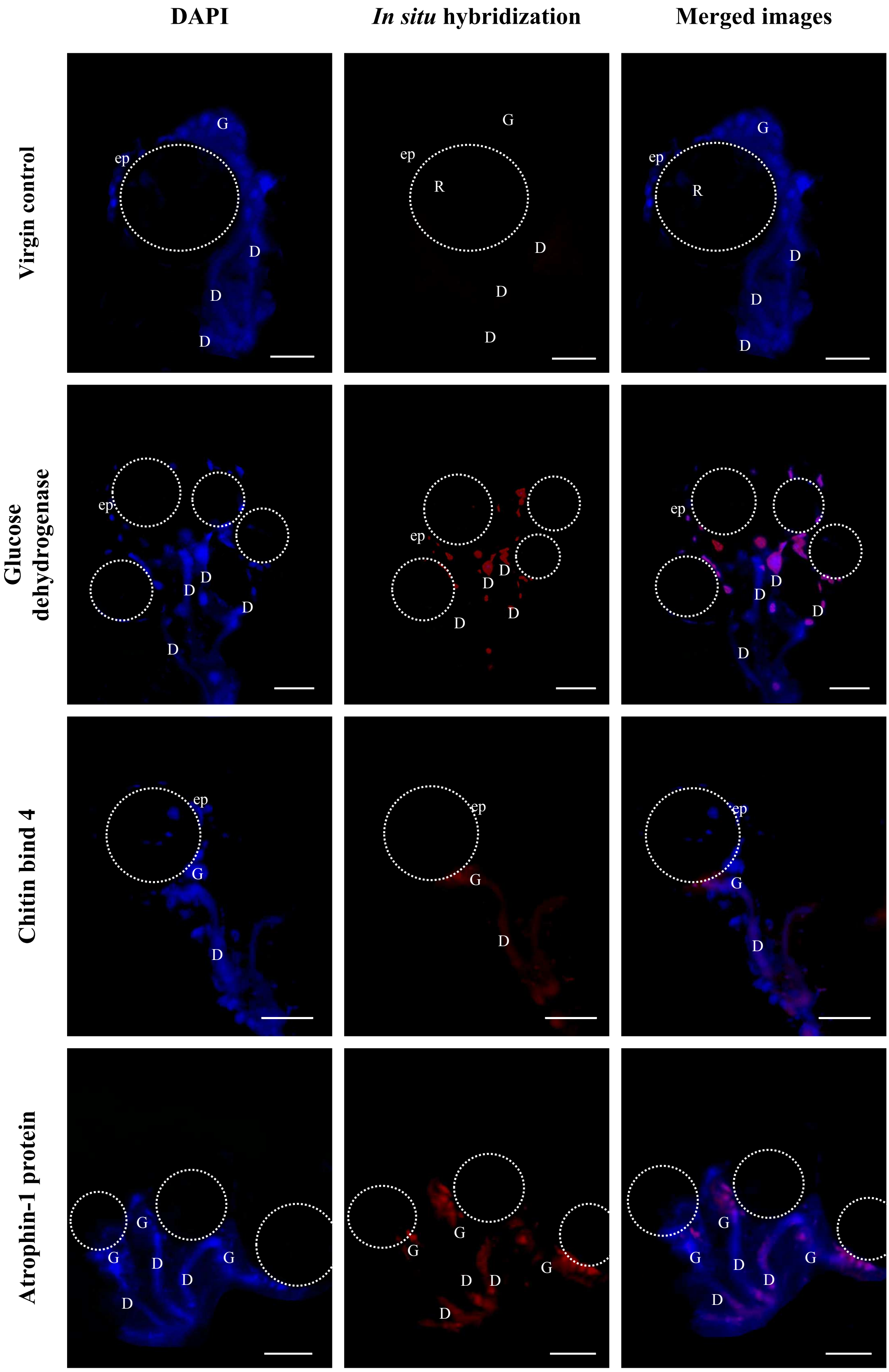


Fig. 15: Detection of gene transcripts in the whole mounts of spermathecae of *A. aegypti* by *in situ* hybridization with red RNA probes and DAPI (blue). (D): spermathecal duct, (G): spermathecal gland, (dc): spermathecal duct cells, (ep): epithelial cells, dotted area: spermathecal reservoir. Bar: 20 mm.

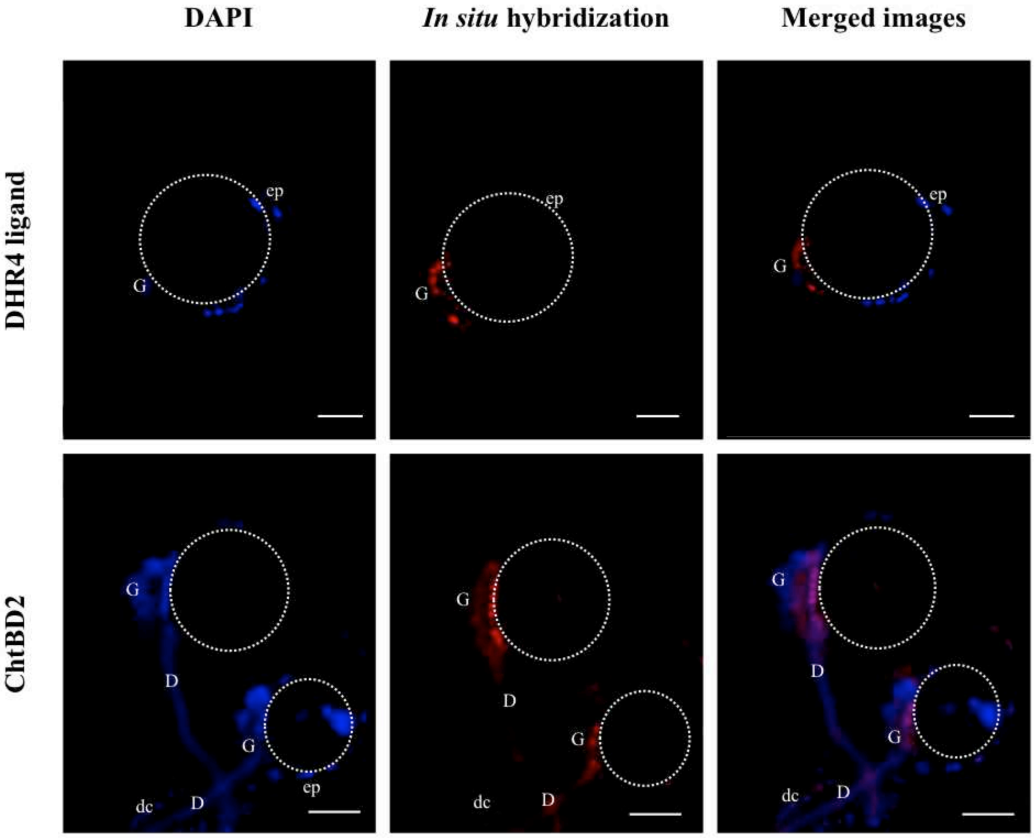


Fig. 16: Detection of gene transcripts in the whole mounts of spermathecae of *A. aegypti* by *in situ* hybridization with red RNA probes and DAPI (blue). (D): spermathecal duct, (G): spermathecal gland, (dc): spermathecal duct cells, (ep): epithelial cells, dotted area: spermathecal reservoir. Bar: 20 mm.

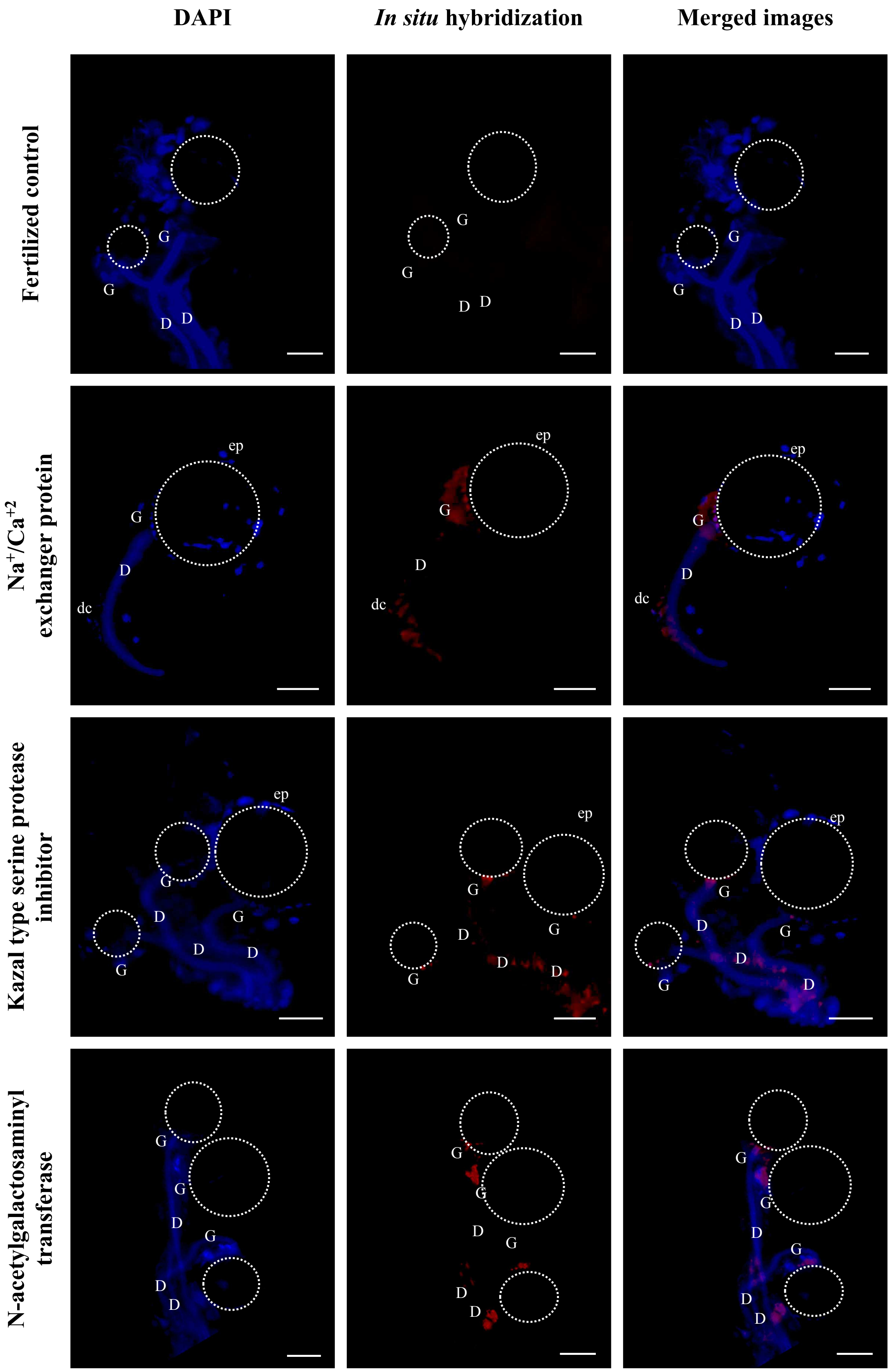


Fig. 17: Detection of gene transcripts in the whole mounts of spermathecae of *A. aegypti* by *in situ* hybridization with red RNA probes and DAPI (blue). (D): spermathecal duct, (G): spermathecal gland, (dc): spermathecal duct cells, (ep): epithelial cells, dotted area: spermathecal reservoir. Bar: 20 mm.

The Gld also acts in metabolic pathways related to sperm allocation, gametes utilization, and eggs development, reinforcing its importance for the female reproductive success. Those pathways are not isolated, but they are connected by an intricate net, sharing common intermediates (Iida & Cavener 2004). Despite Gld importance, a reduction in the levels of Gld did not result in huge effects in the females knocked-down. Considering that the energy metabolism is fundamental for the female survival, we may assume that should be an interplay/interconversion of intermediates among other energy pathways compensating the Gld absence avoiding any catastrophic effect for Gld knocked-down females (Briegel 1990, Kunieda et al. 2006).

For the *in situ* hybridization, different from what was found in *D. melanogaster* in which the Gld mRNA was detected only in spermathecal ducts (Cox-Foster et al. 1990, Schiff et al. 1992), our data showed that in the *A. aegypti* Gld mRNA is distributed along the whole spermatheca. This evidence corroborates the spermathecal role in the sperm metabolism maintenance inside of its organ.

Chitin is a major component of the cuticle of the spermathecae of *A. aegypti*. The spermathecal reservoir is lined by a thick cuticle, which is continuous with the cuticle with the spermathecal duct, isolating the sperm from the direct contact with the hemolymph (Curtin & Jones 1961, Clements & Potter 1967, Pascini et al. 2012). Despite chitin importance for organ structure, its role in the gametes maintenance is not well understood. Based on this, two chitin-related genes presenting highly transcript number in both virgin and fertilized spermathecae were chosen for dsRNA experiments to study their role in the maintenance of sperm within the spermathecal reservoir. One chosen gene was the Chitin bind 4 (Ae-187521), highly expressed in virgin spermathecae, and the other was the ChtBD2 (Ae-88956), highly expressed in fertilized spermathecae.

There was a survival reduction in knocked-down females for Chitin bind 4. This could be an evidence that the encoded protein is related with the cuticular protection, turning the females more susceptible to the environmental damage (Arakane et al. 2011, reviewed by Muthukrishnan et al. 2012). The knocking down of ChtBD2 affected the weight of injected females, however, the specific metabolic pathway affected was not identified. This effect could be related to the disruption of the synthesis of the chitin scaffold of the peritrophic matrix, to the female nutrients storage or even related to female development (reviewed by Merzendorfer & Zimoch 2003 and by Muthukrishnan et al. 2016). In addition, the oviposition rate of knocked-down females was reduced, however, the laid eggs were in higher number than the control. These eggs were longer but with a reduced area. Despite this modified morphology, it did not affect the fecundity of dsRNA-injected females. Likely for Chitin bind 4, the eggs are long after the knocking down of ChtBD2, but it did not affect the fecundity.

The transcripts for both Chitin bind 4 and ChtBD2 were detected in the spermathecal gland, in the region close to the glandular ductules by the which, the glandular cells communicate with the reservoir lumen. The signal can be associated with the glandular cells or epithelial cells, or either both, because these two group of cells that compounds the spermathecal gland are physically associated (Clements & Potter 1967, Pascini et al. 2012, Pascini et al. 2013). In addition, the glandular ductule is internally covered by a thin layer of chitin continuous with the reservoir cuticle, supporting the hypothesis that the proteins that have an affinity for chitin are expected to be extracellularly secreted proteins into the extracellular space by exocytosis (Jasrapuria et al. 2012, reviewed by Muthukrishnan et al. 2012).

Atrophin (Atro) is a wide family of transcription regulators, which controls the negative regulation of Epidermal Growth Factor Receptor (EGFR) acting as a negative regulator of the EGFR controlling the imaginal discs development. Atro acts as a transcriptional co-repressor in multiple developmental processes in *Drosophila* (Charroux et al. 2006, Karres et al. 2007, reviewed by Wang and Tsai 2008, Davis et al. 2013). The high expression levels of Atrophin-1 protein (Atro-1) in virgin spermathecae suggests that it should play role in the preparation of the spermathecal environmental to receive the sperm. After the mating, the Atro-1 transcripts are not seen in the fertilized spermathecae. It can be inferred that Atro-1 is related with the control of other vital processes in *A. aegypti* females, once that knocked-down females for this gene had a reduced survival as showed in *B. germanica* (Rubio et al. 2013). According to our results, Atro-1 it is not related to female reproductive process, once the phenotype of females (i.e., weight, blood-meal intake, and fecundity and fertility rates) was not changed after the dsRNA injection. In addition, as in the other tested genes, the eggs were longer when compared to the control, but it did not affect the egg-hatching.

A weak fluorescence signal for Atro-1 was detected in the apical portion of the cells of the spermathecal gland, probably related with the glandular ductule opening, communicating with the spermathecal reservoir. The lower signal intensity corroborates to what was described for *D. melanogaster*, in which the expression levels of Atro-1 reduced after the emergence (Zhang et al. 2002).

Transcripts for nuclear receptors are highly expressed in virgin spermathecae. Nuclear receptors (NRs) are ligand-regulated transcription factors, encoded by a large gene superfamily, related with steroid signaling pathways, which are involved with the insect development (reviewed by Owen & Zelent 2000, Sullivan & Thummel 2003, Cruz et al. 2009, Ribeiro et al.

2016). In addition, in *M. sexta*, the expression of NR (MHR4) is related with spermatogenesis in early spermatid differentiation phase, in the testis (Weller et al. 2001).

The DHR4 ligand transcripts were only found in the virgin spermathecae. The reduction of expression of this gene after the mating indicates that this gene can be related with the female behavior changes after the mating in terms of reduced receptivity to males, blood-seeking behavior and later trigger the egg development (Findlay et al. 2008). Furthermore, in *A. aegypti* some NRs are related to vitellogenesis, which involves a massive production of yolk protein precursors (YPPs) by the fat body and their subsequent internalization into the oocyte to support embryonic development (Cruz et al. 2009, Gulia-Nuss et al. 2015).

The knocking-down for the DHR4 ligand can arrest the pupal development of insects (Weller et al. 2001, Sullivan & Thummel 2003, King-Jones et al. 2005, Cruz et al. 2009, Mané-Padrós et al. 2012). In *A. aegypti* adults, the knocking-down for DHR4 ligand resulted in a loss of weight before and after the blood meal. We believe that this alteration could be related with the energy storage by the fat body as described in *D. melanogaster* DHR4 mutants, which resulted in smaller and lightweight individuals with short larval development. (King-Jones et al. 2005). Despite the weight reduction and egg morphology, the DHR4 ligand knocking-down did not affect the female survival or its fecundity. The DHR4 ligand did not cause costs in adults of *A. aegypti* females; it seems to be crucial during immature development (King-Jones et al. 2005, Cruz et al. 2009).

The expression of DHR4 ligand in *A. aegypti* spermathecae was detected in the glandular portion, close to the reservoir cuticle, which may be related with regulating the secretion flow in the glandular ductules to the spermathecal reservoir. This result is consistent to what was previously described during *D. melanogaster* metamorphosis, in which DHR4 protein can be found primarily located in the nucleus of fat body and salivary glands cells, regulating its function as a transcription factor (King-Jones et al. 2005).

We identified a great number of transcripts related to the general enzymatic activity in the spermathecomes. Part of these transcripts were characterized as the coding sequences of N-acetylgalactosaminyl transferase (GlcNAc) described in *A. aegypti* as part of the UDP-N - acetylglucosamine (UDPGlcNAc) pathway (Kato et al. 2016). The UDPGlcNAc are involved in protein-sugar contact addition, belonging to the chitin biosynthesis, degradation, modification, and glycosylation. Chitin is a linear polymer of N-acetylglucosamine (GlcNAc), an essential element of the exoskeleton in insects, also found in the peritrophic matrix (PM), in the procuticle, tracheae and at muscle attachment points (Asensio et al. 2000, reviewed by Merzendorfer & Zimoch 2003, Kato et al. 2006, Zhang et al. 2012). The GlcNAc participates in the conversion of Glucosamine-6-phosphate in N-acetylglucosamine-6-phosphate, requiring the activity of several enzymes including N-acetyltransferase, which shares the glycolytic pathway leading to the formation of polymeric chitin from the amino sugar monomers (reviewed by Merzendorfer & Zimoch 2003 and Muthukrishnan et al. 2016).

Surprisingly, the GlcNAc transcripts were only detected in the spermathecae of virgin *A. aegypti* females. Once the gametes are stored, in the fertilized spermatheca, the GlcNac number of transcripts are reduced. The chitin metabolism might be active in virgin females and not in fertilized one to allow the cuticle final set before the sperm allocation. Besides GlcNac importance, the knocking-down for this enzyme did not reduce survival, as described after GlcNac depletion in *Tribolium castaneum* (Arakane et al. 2011). In addition, our injected females presented a higher weight before the blood meal, which did not last after the blood-feeding, and no change over the spermathecal morphology that could cause reduced sperm maintenance was noticed too after the knocking-down.

There was a reduction in the number of females that laid eggs after GlcNac knocking-down, but the egg number laid for each prolific female was higher than the control. Besides the egg production reduction in part of females, the knocking down of GlcNac did not imply in reduced female fecundity, suggesting that something could be affected during the egg development but do not persist over the progeny. These effects were not strong as described in *T. castaneum*, in which the GlcNac down-regulation resulted in loss of appetite, or inability to feed or to digest, resulting in starvation, and complete cessation of oviposition and death (Arakane et al. 2011). Considering that GlcNac participates in the synthesis of chitin, and the GlcNac silencing reduced oviposition rate, we can infer that GlcNac is related to the egg shell (chorion) synthesis in *A. aegypti*. The chorion is formed by a chitin meshwork, known as serosal cuticle, providing mechanical protection, prevents egg dehydration and facilitates fertilization and embryo respiration (Mathew & Rai 1975, Mansur et al. 2014, Gulia-Ness et al. 2015).

The knocking-down of GlcNac did not change the phenotype of spermathecae or in their sperm allocation capacity. However, our results corroborate those described for females of *Rhodnius prolixus* (Heteroptera) knocked-down for chitin synthase, in which the oviposition rate in 60% and resulted in eggs with deformed morphology. As in *R. prolixus* (Mansur et al. 2014), the eggs laid by *A. aegypti* females knocked-down for dsGlcNac did not hatch.

The GlcNac transcripts were detected in the spermathecal gland and in the spermathecal duct glandular cells, but with a low signal intensity. The transcripts corresponding to GlcNAc enzyme can participate in the interconversion of the chitin substrates that surround the lumen of the spermathecal ducts. The chitin metabolism could be important for the maintenance of the secretory ductules that allow the glandular secretion releasing towards reservoir lumen (reviewed by Merzendorfer & Zimoch 2003; Muthukrishnan et al. 2016).

The AeSigP-109183 coding sequence was the most representative transcript of fertilized spermathecae. These transcripts corresponded to Kazal type serine protease inhibitor (KSPI). The KSPI is a family of proteins that prevents unwanted proteolysis acting as a substrate analogue that binds competitively through its reactive site (by the non-covalent binding), forming a relatively stable proteinase–proteinase inhibitor complex. (reviewed by Laskowski & Kato 1980, Campos et al. 2004, Qian et al. 2015). As one of the most important protease inhibitors, KSPI play regulatory roles in the processes that use serine proteinases in insects (reviewed by Laskowski & Kato 1980). KSPI also plays role as anticoagulant factor present in the midgut of *Triatoma infestans* (Triatomine) during blood meal acquisition and digestion. In this case, the KSPI infestin 4 showed inhibitory activities to trypsin, plasmin, and other intermediates of the coagulation cascade (Campos et al. 2004). The most well characterized KSPI in *A. aegypti* is the trypsin Inhibitor AaTI of the salivary glands (Santos et al. 2007). Previous studies suggested that AaTI may prevent the blood clotting during the blood feeding or blood digestion in the midgut (Watanabe et al. 2010). In addition, AaTI was also found widely distributed in the carcass, suggesting that it may act as endogenous proteases as an antimicrobial protection. The AaTI transcripts was also found in larvae and pupae whole body, suggesting other functions during the mosquito's development (Watanabe et al. 2010).The high expression of KSPI in fertilized spermathecae suggests that KSPI should be related to the immune response against any venereal transmission of pathogens present in the male ejaculate. Additionally, the secretion of KSPI in the spermathecal milieu could avoid damage to sperm (reviewed by Laskowski & Kato 1980, Rimphanitchayakit & Tassanakajon 2010).

There were no changes in the spermathecal morphology in knocked-down females for KSPI in comparison to the control. Surprisingly, these knocked-down females laid a higher amount of eggs, however these eggs were smaller than the control and only 40% of them were viable. This 60% progeny reduction suggests that the KSPI is related with egg antimicrobial protection. The reduction of KSPI expression could let the embryo more susceptible to damage, reducing fecundity (Colpitts et al. 2011). Curiously, the KSPI knocked-down females presented a higher weight before and after the blood meal compared to the control. This weight gain can be related to the KSPI role in the inhibition of development (Briegel 1990). The protease activity can be enhanced by KSPI knocking down in adult mosquitoes, allowing the body structuration to persist longer, providing the mass gain (reviewed by Laskowski & Kato 1980, Watanabe et al. 2010).

The KSPI expression predominates along the spermathecal duct, mostly present at its begging portion close to the common oviduct opening. Considering that KSPI is related to antimicrobial activity, the expression of KSPI should be stablished a chemical barrier, protecting sperm during their journey within the duct lumen against pathogens (Watanabe et al. 2010, Colpitts et al. 2011, Kim et al. 2013, Qian et al. 2015).

Transcripts for the coding sequence (AeSigP-66427) related with the Na^+/Ca^{2+} exchanging also was detected in a high number in the spermathecome. The RT-PCR showed that this gene is expressed in fertilized spermathecae, with no significate expression in the virgin organs. Furthermore, these transcripts are found in the spermathecal content of fertilized spermatheca. The high expression values of the Na^+/Ca^{2+} exchanger in fertilized spermathecae compared with the reservoir content may be the result of an additive effect caused by the mating plus the presence of the sperm inside of the reservoir which should trigger the Na^+/Ca^{2+} exchanger expression (reviewed by Werner & Simmons 2008).

Based on the function attributed by the *in silico* analysis, we propose that Na^+/Ca^{2+} exchanger should act in the ionic homeostasis of the *A. aegypti* spermathecae. In order to understand in which process(es) the encoded Na^+/Ca^{2+} exchanger protein is related, we proceeded with the knocking-down of this gene. Surprisingly, after dsRNA injection, the female phenotype was changed in respect to the blood-feeding rate of females, body weight after the blood meal, fertility and sperm motility. Aiming to confirm these deleterious effects, two other independent experiments were performed, achieving the same results.

The enormous reproductive potential of mosquitoes is largely known and depends on the ability of the female to find a host, take and digest a blood meal, develop eggs, escape from predators and finally find a suitable site for oviposition. This complex reproductive behavior leads the mosquito survival, enhancing the transmission of pathogens transmission (Briegel 1990, Bowen 1993, Farjana & Tuno 2013). The reduction of the expression of Na^+/Ca^{2+} exchanger protein resulted in the reduction in the number of females that took the blood meal (only 50% compared to the control). We were unable to identify which specific pathway this molecule is related, however, our data reinforce the importance of the ionic homeostasis for the host seeking. In addition, the host-seeking behavior seems not to be regulated by just one pathway, but also by an interplay with ovary signals, being disrupted in ovariectomized females of *Aedes atropalpus* (Diptera: Culicidae) (Bowen et al. 1993).

There is a positive correlation between body size and amount of ingested blood and egg production in *A. aegypti* females (Hagedor et al. 1973, Briegel 1990, Farjana & Tuno 2013, Gulia-Nuss et al. 2015). *A. aegypti* females can store 69-86% of the ingested protein, 16-46%

of lipids, while carbohydrates are mobilized nearly completely and stored (Briegel 1990). The Na^+/Ca^{2+} knocking-down inhibited the ovary development and the ovaries of knocked-down females resemble like the ones found in the not blood-fed females. This evidence indicates that the blood meal nutrients acquisition is disrupted by the knocking down, resulting in no egg development. Furthermore, once that a reduction in the Na^+/Ca^{2+} exchanger protein resulted in females with the same weight before and after the blood-feeding, it indicates that there was a damage in the fat body nutrients absorption/mobilization, suggesting that the ionic homeostasis should be one of the key mechanisms along the egg development.

The oocytes accumulate yolk protein precursors, which uptake is directly regulated by Na^+/K^+ ATPases (Pan et al. 1969, reviewed by Raikhel & Dhadialla 1992). In addition, Ca^{2+} ionic channels play an important role in Vg uptake regulating the stimulation of Vg uptake as a response to Juvenile Hormone induction (reviewed by Taylor 1987, Roth & Porter 1964, reviewed by Raikhel & Dhadialla 1992). Any damage over this ionic basis could result in egg Vg storage reduction, impairing the egg development.

The knocking down for Na^+/Ca^{2+} exchanger protein affected sperm motility compared to the control and stopped it completely seven days after the dsRNA injection, while in the females of the control group sperm was actively swimming. Due to the importance of the ionic channels for cell metabolism, we believe that the disruption of ionic homeostasis of spermathecae could lead to the blockage of the sperm movements and then sterilize mosquito females. Supporting this hypothesis, the ion unbalances in the spermathecal milieu in *Apis mellifera* (Hymenoptera: Apidae), it was shown that the hypertonic environment of the honey bee spermatheca due to high concentrations of Na^+ and K^+ ions resulted in non-motile sperm when the ionic concentration becomes lower, the sperm motility was restored. The hypertonic environment of the spermatheca maintained by these cations may enable the stored spermatozoa to avoid the loss of intracellular materials, mostly ions; representing a physiological-biochemical protective mechanism. Moreover, it was found that concentration of the Na^+, K^+, Ca^+, and Mg^+ cations were higher in spermathecae than in semen, seminal plasma or drone hemolymph, suggesting a complete separation of ionic environment between spermatheca and hemolymph (Davey 1958, Lensky & Alumot 1967, Verma 1973).

Within the spermathecal reservoir, the *A. aegypti* sperm are isolated from the rest of the female body, avoiding any hemolymph contact by a thick cuticle that surrounds internally this organ. The disruptive effect provided by the Na^+/Ca^{2+} exchanger protein reduction suggests the existence of an ionic basis for the control of sperm motility and longevity established within the spermathecal reservoir that can interfere with fecundity.

As for other treatments, the knocking down for Na^+/Ca^{2+} did not affect the spermathecal morphology. However, the staining of spermathecae sections with PAS reaction revealed the staining for neutral polysaccharides in the gland was stronger in comparison to the control, probably related with the accumulation of components due to the impairment of the normal Na^+/Ca^{2+} transport.

5. Final considerations and conclusions

In this study, the global gene expression profile of the spermathecae of the vector mosquito *A. aegypti* was generated, aiming to shed light over the molecular mechanisms behind the long-term sperm storage in females. We have provided a catalog of spermathecal transcripts of *A. aegypti* for virgin and fertilized females (spermathecomes). We were able to decipher the repertoire of molecules (messengers RNA and proteins) that were synthesized by spermathecae of virgin and fertilized *A. aegypti* females, which allow us to know in detail the effects of spermathecal filling in the gene expression of the organ. However, those findings are just the tip of the iceberg along the tiny and well-regulated processes that perpetrates the *A. aegypti* reproductive success and consequently, culminates in its vectorial competence.

The *in silico* analysis revealed that the panorama of gene expression varies according to the two physiological conditions. For example, the virgin female invests a huge amount of energy in preparing the organ to receive the sperm. Based on the differences on the profile of expression we have chosen some candidate genes with a relative higher number of transcripts to be tested *in vivo* by the RNAi technique. This method provided functional analyses of expressed genes related to the long-term storage of gametes within the spermathecae. Eight CDS of different functional groups or categories were chosen for our dsRNA assays, the effects of their knocking down for the sperm viability and female fecundity were studied separately. Based on what is already described on the literature, were chosen eight coding sequences with the functions related with the energy metabolism, chitin components, transcriptional regulation, hormonal signaling, enzymatic activity, antimicrobial activity and ionic homeostasis.

The knocking-down effects depend on the knocked down gene and provided some notorious results, showing different deleterious effects in the female survival, blood-feeding behavior egg production and fecundity. In addition, we were able to determine some of the ionic bases of the sperm motility, disrupting the whole progeny production process by the knocking down of Na^+/Ca^{2+} exchanger.

Blood-feeding mosquitos cause countless negative impacts on the economy and public health for being vectors of several pathogens of vertebrates. The high reproductive output is a determining factor for vector capacity of *A. aegypti* that is ensured by a single mating event, which is enough to get the sperm to fertilize the eggs during the female reproductive life. Mosquito females have a highly efficient mechanism for maintaining the viability of sperm within them (Rogers et al. 2008). This ability is attributed to the functions of the spermatheca in creating a suitable environment through physical protection and nutrient supply (Jones & Wheller 1965, Klowden & Chambers 2004, Pascini et al. 2012, reviewed by Pascini & Martins 2017). There is considerable interest in blocking or reducing mosquito high reproductive output in order to control their populations (Catteruccia et al. 2005). In this sense, the spermatheca arises as a target organ for population control of mosquitos because the disruption of its functions could decrease sperm viability, or even fecundity (reviewed by Pascini & Martins 2017).

Finally, a better understanding of mechanisms that guarantee the storage of sperm in the spermathecae will unravel potential targets for the reduction of vector populations in the field, therefore, may contribute to the control of burden diseases such as malaria and dengue. Based on that, blocking the expression of specific genes may reduce or interfere with the fertility (Rogers et al. 2008, Thailayil et al. 2011).

Tools for genetic editing such as CRISPR/Cas9 and the Transcription Activator-Like Effector Nucleases (TALENs) are a useful system to generate site-specific mutations, allowing the development of transgenic mosquito strains (Kistler 2015, Li et al. 2017). By genetic editing, can be generated beneficial phenotypes which can be used as new control strategies for vector mosquitoes. The modified strains can be used for population suppression, by reducing the number of competent vectors in the target population; or population replacement, reducing the vectorial capacity of some or all of the mosquitoes in the target population (Basu et al. 2015). The advances in the production of transgenic mosquitoes together with availability of the sequenced genome (Nene et al. 2007) and detailed gene expression data (Akbari et al. 2013) make the *A. aegypti* an ideal tool for genetic studies, with a critical role for the mosquitoes' pathogen transmission inhibition (Aryan et al. 2014, reviewed by Alphey 2014 and Adelman & Tu 2016).

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