Silencing of the *Hsf* gene, the transcriptional regulator of *A. gambiae* male accessory glands, inhibits the formation of the mating plug in mated females and disrupts their monogamous behaviour

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Discovering the molecular factors that shape the mating behaviour and the fertility of the mosquito *Anopheles gambiae*, the principal vector of human malaria, is regarded as critical to better understand its reproductive success as well as for identifying new leads for malaria control measures. In *A. gambiae* mating induces complex behavioural and physiological changes in the females, including refractoriness to subsequent mating and induction of egg-laying. In other insects including *Drosophila* a group of proteins named Accessory gland proteins (Acps), produced by males and transferred with sperm to the female reproductive tract, have been implicated in this post-mating response. Although Acps represent a set of promising candidates for unravelling the mating physiology, their role in inducing behavioural changes in mated *A. gambiae* females remains largely unknown. In this work, we demonstrate that a down-regulation of a large fraction of Acp genes via silencing of the Acp regulating transcription factor *Hsf*, abolishes the formation of mating plug in mated females and fails to induce refractoriness of mated female to subsequent inseminations. A significant fraction of females mated to *Hsf* silenced males (66%) failed to receive the mating plug though seminal fluid had been transferred as documented by the presence of spermatozoa in the female sperm storage organ. Furthermore, nearly all females (95%) mated to HSF-silenced males were re-inseminated when exposed to males carrying EGPF marked sperm. Our findings provide evidence showing that Acp genes regulated by the transcription factor HSF play a key role in the function of the male accessory glands.

Keywords: Reproductive Biology, Malaria, Vector-control, Fertility, Post-mating Behaviour, Receptivity to Remating, Mating Plug, *Anopheles gambiae*

Abbreviations

Acps	accessory gland proteins
MAGs	male accessory glands
HSFs	heat shock factors
HSEs	Heat shock sequence elements (HSF binding sites)
TF	Transcription factor

Introduction

The molecular mechanisms underlying the transfer of the seminal fluid proteins and the behavioural changes induced in female of *Anopheles gambiae* mosquitoes are not well understood. Nevertheless, unravelling such mechanisms is fundamental for understanding the factors influencing the reproductive success of the species, and hence its ability to reach high density and transmit malaria.

The identification of the molecules that regulate mosquito reproduction is crucial to target mosquito fertility, and may enhance the range of possible approaches and interventions to achieve eradication of vector populations. In many animal species including insects, different classes of seminal fluid proteins are transferred to females along with sperm during mating.[1] In different insect species these proteins known as Accessory gland proteins (Acps), are produced in in the male accessory glands (MAGs). Experimental observations mainly gathered in *Drosophila* have shown that Acps induce a variety

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of effects on the female's reproductive physiology including the induction of oogenesis, ovulation and oviposition,[1,2] accompanied by a markedly reduced propensity to remate.[1,2] Additionally, Acps play a key role in the storage and utilization of sperm and in sperm competition.[1,2] Though Acps have been identified in numerous insect species including fruit flies,[3–6] beetles,[7] honeybees,[8] butterflies,[9,10] crickets,[11,12] and medflies,[13] only recently they have been found in the mosquito vectors *A. gambiae*[14,15] and *Aedes aegypti*.[16] In the mosquito *A. gambiae*, bioinformatics and experimental investigations revealed the presence of 80 Acp genes with high tendency to cluster together, mainly into two distinct chromosomal locations.[14,17,18] Similarly to what has been observed in other species,[1] in *A. gambiae* mating induces transcriptional changes in the reproductive organs, the immune system and the brain of the female mosquito, that finally leads to egg fertilization and inhibition to subsequent mating.[19] However, unlike *Drosophila*, discrepancies between approaches and results have been observed in attributing a function to MAGs. In particular, intra-thoracic and intra-abdominal injections of MAG extracts, crosses with hybrid or spermless males showed conflicting results and did not yet clarify the role of Acps in inducing behavioural changes in mated females.[20–23] Only recently, functional studies have unravelled the role of HSF in regulating the transcription of Acp genes and showed how *Hsf* silencing resulted in a marked reduction of Acp transcripts that was associated to a significant impairment in the male reproductive success: Acp-silenced males generated a reduced number of progeny when mated with wild-type females, females mated with these males showed a significant reduction in the number of progeny resulting from a combined reduction in oviposition and hatching rate.[17] The observation that one of the Acps, a species-specific transglutaminase is involved in the formation of the mating plug in *A. gambiae* male mosquitoes provides further function evidence on the role of these male proteins in regulating fertility.[15,24] This transitory gelatinous structure, that is deposited in the atrium of the female and digested a few hours after mating, has been suggested to be implicated in forming a physical barrier to spermatozoa delivered upon subsequent mating[25,26] as well as in preventing sperm loss from the female storage organs.[27] In this work, we investigate the role of how silencing of the transcription regulator of Acps modulates post-mating behaviours in *A. gambiae.* Dissecting the molecular function of male seminal fluid proteins bears important implications for understanding the underlying molecular mechanisms regulating mating response as well as for identifying novel targets for vector control in order to reduce mosquito populations in the wild either through chemical or genetic intervention.[28]

Materials and Methods

Mosquito Rearing

Experiments were performed using laboratory-maintained *A. gambiae* G3 strain. For each experiment, to avoid uncontrolled mating, males and females were separated in the pupae stage and sorted in separate cages. Male and female mosquitoes were fed *ad libitum* a 10% sucrose solution.

Mating Analysis by isolation of mating couples

Mating experiments were done using pairs collected using 4-days-old insects as detailed previously[17] the main parameters being the number of males per cage (75), the female group size introduced in the male cages (10 females) and the cage dimensions ($18 \times 18 \times 18$ cm, Acrilong; Perugia, Italy). Single mating couples were collected using 50 ml Falcon tubes (BD Biosciences, Heidelberg Germany). Each female was euthanized using CO_2. Mated females were dissected in PBS to ascertain both the presence of sperm and the mating plug in the spermathecae and in the atrium respectively. Fresh dissected spermathecae and atria were placed onto a slide and covered with a coverslip to analyse the presence of sperm and mating plug within. The presence of sperm was assessed using a Nikon Eclipse TE2000-U microscope (x100 view; Nikon, Tokyo, Japan).

RNA interference

To target the *Hsf* isoforms (*Hsf1*, *Hsf2* and *Hsf3*) needed for silencing Acps in males, the *dsHSF123* construct[17] was used. In brief, to target the *Hsf* isoforms, a specific region of 277 bp of the AGAP011082 gene was used as template to create the *dsHSF123* construct. The *dsHSF123* fragment was obtained by enzymatic digestion using EcoRV and *Pst*I (89 and 361 bp from the beginning of the full-length sequence).

As control, we synthesized a *dsLacZ* construct directed against an unrelated bacterial sequence. The dsRNA constructs were generated following an established protocol:[29] the fragments were cloned into pLL10, and injected with 69 nl of each specific dsRNA (3 μg) within 24 h of eclosion. During injection, mosquitoes were anesthetized using CO2.

Transgenic A. gambiae lines

Transgenic male mosquitoes containing the reporter construct pBac{3xP3RFP}Vas2GFP (Vas2GFP construct)[30] were used for remating experiments. In brief, the Vas2GFP reporter construct is designed to direct eGFP germline expression throughout development. In addition the construct contains the 3xP3-RFP cassette as visual marker. The RFP signal generated by the 3XP3-RFP transformation marker

allows robust larval screening for expression of fluorescent reporter transgenes.

Remating Assay

Male (wild type and transgenic) and female pupae were sexed and placed in separate cages. Adult male and female mosquitoes were fed 10% sucrose ad libitum. Three separate experiments were performed depending on the males used for the first mating (*dsHSF123, dsLacZ* or wild-type (WT) mosquitoes). Four days after RNAi silencing (*dsHSF123 and dsLacZ*) or emergence (WT), mating experiments were done as previously described. Mated females from each experimental group were separated from males and allowed to recover for 48 h (time required for mating plug degradation). After this period, females were placed in new cages with 2-fold excess of transgenic males to test for subsequent mating events. Mosquitoes were allowed to remate for 48 h. Thereafter female mosquitoes were blood-fed on CD1 mice (Harlan Laboratories S.r.l., Udine, Italy) and placed in single polystyrene oviposition cups for the oviposition assay (described below). Two replicate experiments were performed for *dsHSF123, dsLacZ* and WT experimental groups to estimate for multiple reinsemination events.

Oviposition Assay

Females mated to either WT, *dsLacZ* or *dsHSF123*-treated males and subsequently tested for remating with pBac{3xP3RFP}Vas2GFP males were blood fed and placed in single oviposition cups containing a water reservoir covering the bottom of the glass and filter paper strips for egg's hydration and to allow larvae at hatching in water.[23] The newborn progeny were counted and evaluated for the presence of fluorescence signal using a Nikon Eclipse TE2000-U microscope (x100 view; Nikon, Tokyo, Japan). Reinsemination events are indicated by the presence of RFP[+] fluorescent progeny.

Statistical procedures

To discriminate between the propensity of Acp-silenced male mates to subsequent mating and the basal occurrence of polyandry in nulliparous *A. gambiae* mosquitoes reared in laboratory cages, the presence or absence of the mating plug and the presence or absence of sperm we used the contingency table test. Contingency table analyses data where the outcome is categorical, and where there is one independent (grouping) variable that is also categorical. In our case we built a table for each experiment type where the grouping variables are the type of the individuals (wild type, *dsLacz*, and *dsHSF123*) while the categorized outcomes were the presence or absence of the mating plug, the presence or absence of sperm, or the presence or absence of remating (depending on the considered experiment). The

significance of the difference between the two examined proportions between the experimental and control groups was assessed using the Pearson's chi-squared test. The analysis was done in Microsoft Excel 2007.

The Mann-Whitney U test with $p<0.05$ was used to compare the absolute number of transgenic and wild type progeny generated in the control and experimental groups. The study sample fulfilled the requirement to utilize the Mann-Whitney test: i) the control and experimental group were regarded as independent, ii) the number of cases to be compared is sufficient. Furthermore this test does not require a specific distribution of values (e.g. Gaussian) and can be performed using two groups with different numbers of individuals (although similarity gives better performance). The analysis was done using Minitab v16. To display the differences between the progeny of the control and experimental groups we used the boxplot diagrams using Minitab v16.

Results

HSF-silenced males fail to induce female refractoriness to further inseminations

To investigate whether changes in the levels of Acp gene expression had an effect on female propensity of remating, we conducted *in vivo* experiments by RNA interference-mediated knockdown in adult males aimed at selectively targeting HSF isoforms. As previously shown[17] HSF-silencing in male mosquitoes is associated with the down-regulation of 50% of the known Acp gene repertoire. To this aim we used the *dsHSF123* dsRNA construct directed against a conserved region present in the *Hsf1*, *Hsf2* and *Hsf3* isoforms. As a control we used two different groups, namely, wild type male mosquitoes and mosquitoes injected with the construct *dsLacZ* directed against an unrelated bacterial sequence. We conducted three independent mating experiments where virgin females were at first mated to *dsHSF123*- *dsLacZ* silenced and WT control males. Mated females, from each experimental group, collected from single mating couples were isolated and allowed to recover for 48 h (time required for mating plug degradation). The females were then placed in new cages with 2-fold excess of transgenic males (pBac{3xP3RFP}Vas2GFP)[30] and allowed to remate for 48 h. After blood feeding the mosquitoes were placed in single polystyrene oviposition cups for the oviposition assay. The experiment was designed to discriminate between the tendency of females to further mating as a consequence *Hsf* silencing and the basal occurrence of polyandry in nulliparous *A. gambiae* mosquitoes reared in laboratory cages.[31] In two independent experiments, a total of 22 females collected from mating pairs with *dsHSF123*-treated males and subsequently placed with transgenic males, produced 21 egg batches, of these one hatched and
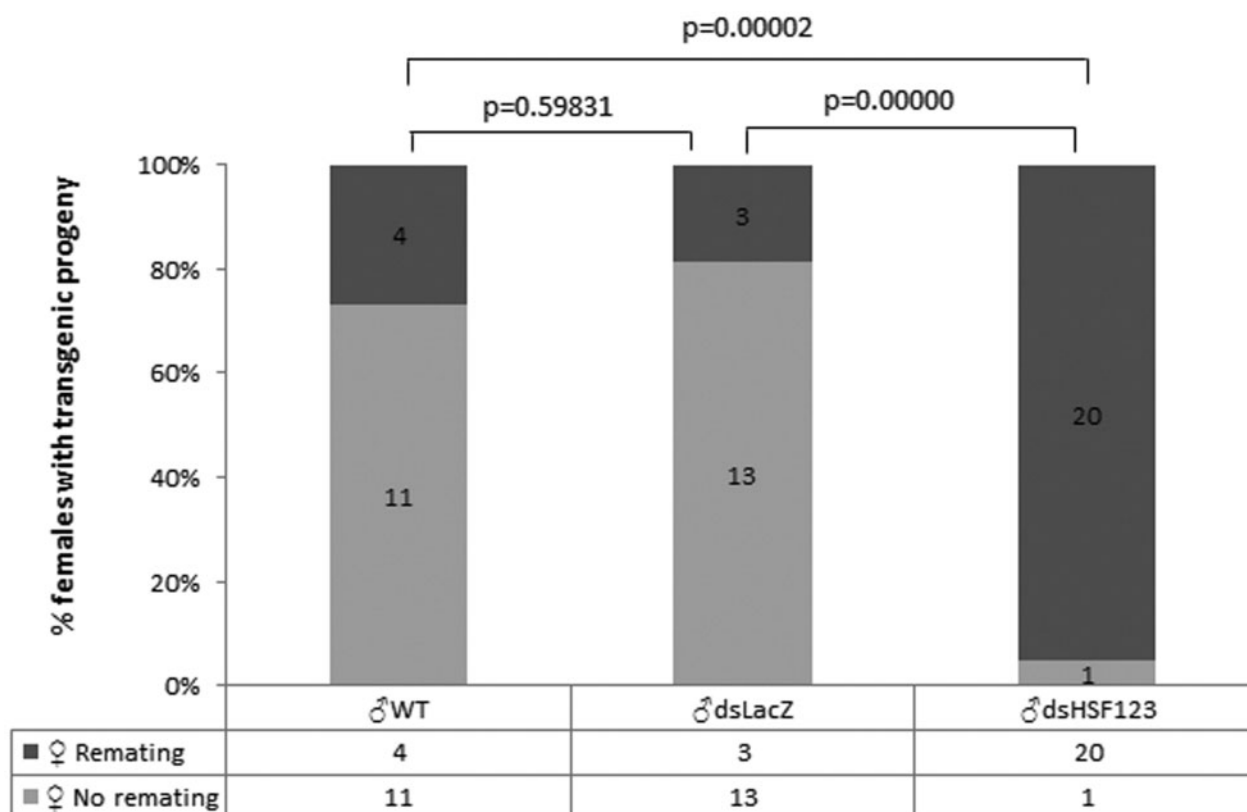
Figure 1 Females mated to *Hsf*-silenced males are not refractory to subsequent mating. Virgin females were mated to *dsHSF123*-males (experimental group) and two control male groups (*dsLacZ* and WT). Females collected from mating pairs were allowed to recover for 2 days and then exposed to transgenic males for other 48 h. Subsequently, females were blood fed and let to oviposit. Histograms represent the number of females that after being exposed to transgenic males generated transgenic progeny. The differences between experimental and control groups were statistically significant with p<0.001 as assessed using the contingency χ^2 test.

produced only wild-type progeny, 11 produced only transgenic progeny, while the remaining 9 produced both wild-type and transgenic progeny. In total, 20 egg batches (95.23%) produced transgenic progeny, indicating that insemination from second mating had occurred with high frequency (Figure 1). The control groups gave different results: in the the *dsLacZ* group, a total of 16 females were collected from the two consecutive mating and all of them produced egg batches that hatched; Only three females (18.75%) out of 16 produced a mixed wild type and transgenic progeny, in agreement with basal reinsemination rates[31] while the remaining 13 females (81.25%) generated as expected 100% wild type progeny. In the second control group (wild type males), 4 out of 15 females (26.66%) produced mixed progeny while the remaining 11 (68.75%) generated only wild type mosquitoes (Figure 1). We performed a contingency test to evaluate if the difference in the progeny composition between the experimental and control groups was significant (Figure 1). This analysis showed a statistically significant difference amongst the groups (contingency test, *dsHSF123*-*dsLacZ*, P=0.00000; *dsHSF123*-WT, P=0.00002; *dsLacZ*-WT, P=0.59831). To better estimate the difference between control and experimental groups we

also assessed whether there was a variation in the absolute number of transgenic and wild type progeny. The two distributions were compared with the Mann-Whitney U test with p<0.05. The null hypothesis (samples belong to the same distribution) was rejected with p=0.0000 (*dsHSF123*-*dsLacZ*) and p=0.00002 (*dsHSF123*-WT) confirming that a significant difference exists in the number of transgenic and wild type progeny obtained in control and experimental groups (Figure 2). No difference (p = 0.59831) was found between the two control groups (*dsLacZ*-WT) (Figure 2), indicating that the observed remating events in these mosquitoes are consistent with the basal polyandry frequency found in laboratory reared mosquitoes.[31]

HSF regulated Acp genes influence mating plug formation

We further investigated whether the disruption of the female monogamous behaviour could be attributed to altered mating plug transmission as a consequence of Acp down-regulation. To this aim, we conducted three types of mating experiments where virgin females were mated to *dsHSF123*-males and to *dsLacZ* and WT control males. When *dsHSF123*-injected males were mated with virgin females, mating couples were collected and a total of 20 out of 30 females (66.66%) mated to Acp-silenced males failed to
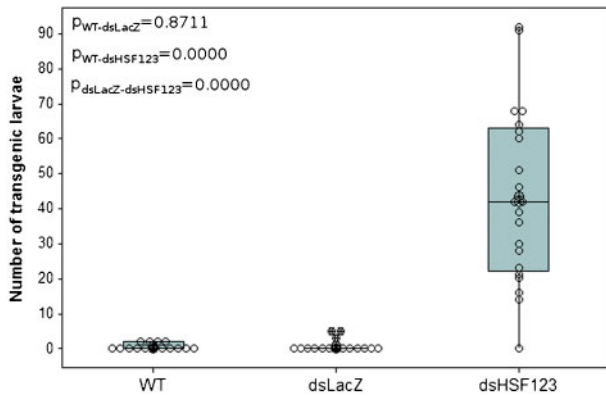
Figure 2 Comparison of absolute number of progeny obtained from secondly mated females. The box plots show the number of larvae (circle) produced by individual females mated with either *dsHFS123* or *dsLacZ* treated males. Female mated with wild type males are also included (WT). Females mated with *Hsf*-silenced males produced significantly higher transgenic offspring (second mating) than mates with *dsLacZ*-treated control males. The data represent two independent biological replicates. The average (crossed circle) and median values of produced larvae are indicated (*dsHSF123* group: median=42; *dsLacZ* group: median=0 and for the WT group: median=0). These differences were statistically significant with p=0.00000 as assessed with the Mann-Whitney U test.

receive a mating plug, compared to 5 out of 32 (15.62%) females mated to *dsLacZ* control, and to 9 out 33 (27.27%) females mated to wild type control males (Figure 3). These differences were statistically significant (contingency test, *dsHSF123-dsLacZ*, P= 4.23E-05; *dsHSF123*-WT, P=1.72E-03) thus confirming the important function of seminal fluid proteins for the mating plug formation.[5] Whereas, no significant difference was found between the number of mating plugs received by the control groups *dsLacZ* and WT mated females (contingency test, *dsLac* –WT, P=0.253) as detailed in Figure 3. These findings are in agreement with previous observations showing that the transglutaminase gene (AG AP009099), is involved in mating plug formation though the penetrance of the *Hsf* silencing phenotype is much more dramatic (67% against 15%) suggesting the involvement of additional yet unidentified seminal fluid genes.[15]

Role of mating plug in sperm retention in the female storage organ

We also assessed whether the function of the mating plug in ensuring sperm storage or preventing sperm
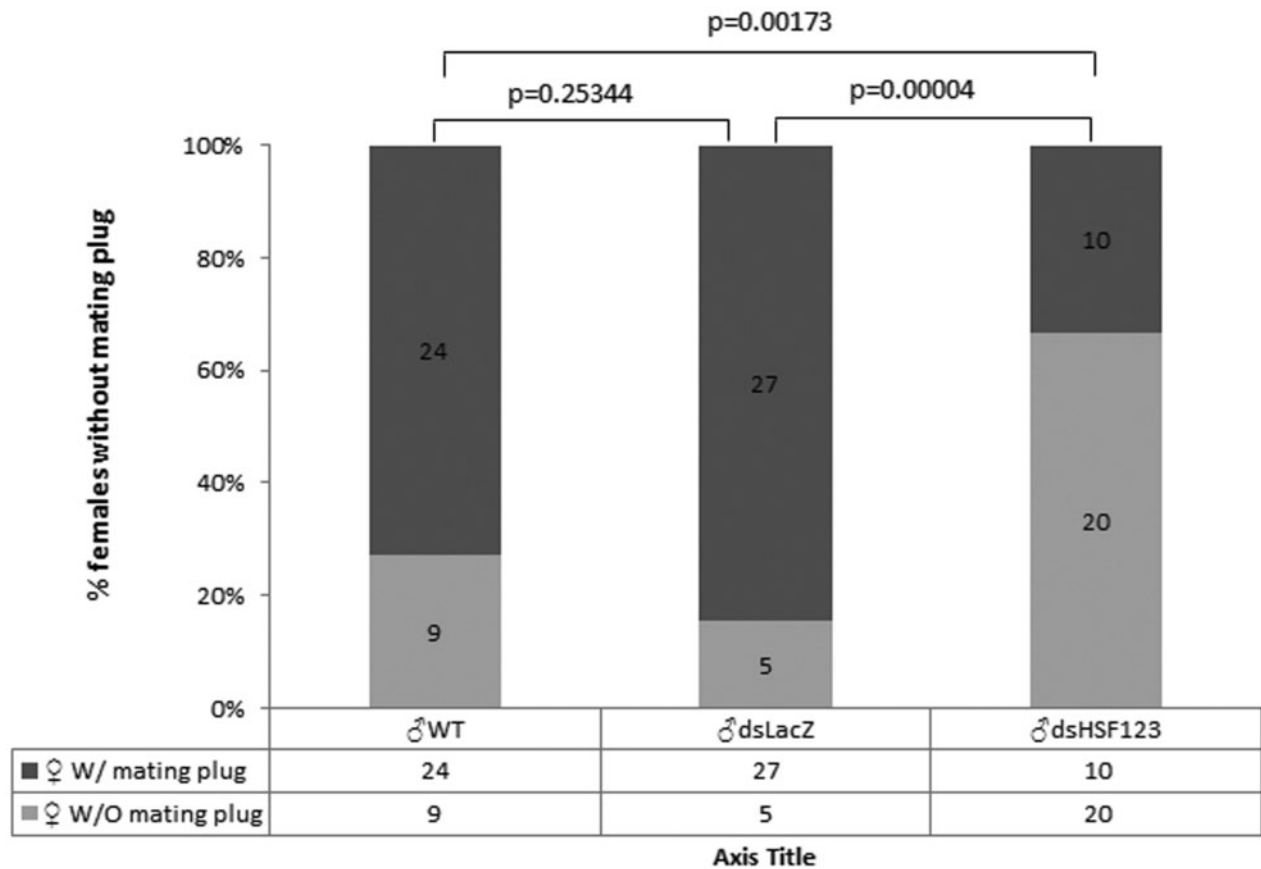
Figure 3 Females mated to *Hsf*-silenced males fail to receive mating plug. 4 days old virgin females were mated with *dsHSF123*-males (experimental group) and with the two control groups (*dsLacZ* and WT). Mating couples were collected and females were euthanized using CO_2 and dissected to ascertain the presence of the mating plug in the atria. The data represent two independent biological replicates. Each histogram represents the total number of females without the mating plug versus the total number of females present in that group. A total of 30 females mated with *dsHSF123* males, 32 females mated to *dsLacZ* and 33 females mated with WT males were considered. The differences between experimental and control groups were statistically significant with p<0.001 as assessed using the contingency χ2 test.
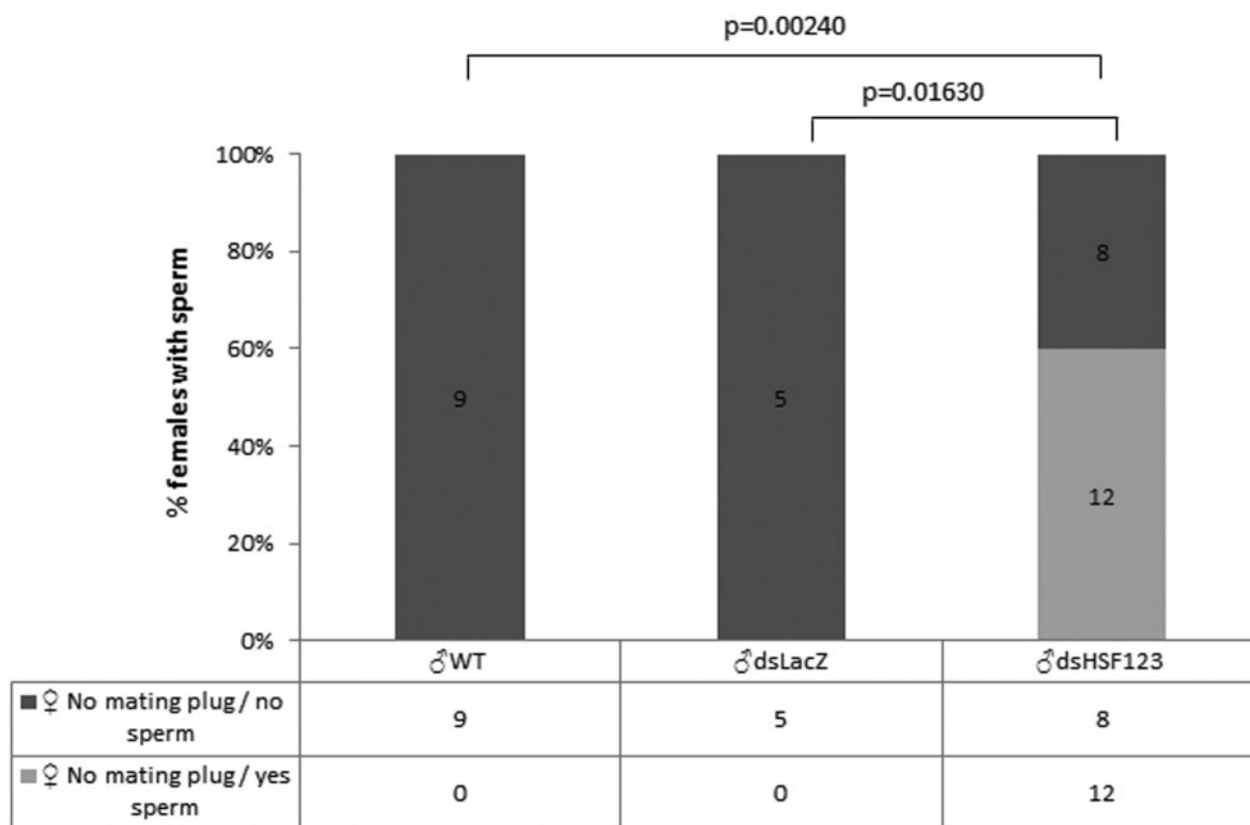
Figure 4 Sperm retention and mating plug. 4 days old virgin females were mated to *dsHSF123*-males (experimental group) and two control male groups (*dsLacZ* and WT). Histograms represent the total number of females without the mating plug in which the presence of sperm has been seen versus the total number of females in that group. A total of 30 females mated with *dsHSF123* males, 32 females mated to *dsLacZ* and 33 females mated with WT males were considered. The differences between experimental and control groups were statistically assessed using the contingency χ2 test.

loss from the spermatheca is preserved in females mated with *Hsf*-silenced males and lacking the mating plug. Our data show that most of the females that failed to receive the mating plug, had sperm cells in the spermatheca as demonstrated by microscopic analysis (Figure 4). In these females, sperm were not found in other parts of the reproductive tract. In contrast, all females mated to control groups that failed to receive the mating plug, did not show sperm cells in the storage organs indicating that transfer of seminal fluid had not occurred in these cases. The statistically significant difference between experimental and control groups (contingency test, *dsHSF123*-*dsLacZ*, P=1.63E-02; *dsHSF123*-WT, P=2.40E-03) do not support a function of the mating plug in storing sperm in sperm storage organs.

Discussion

In this work, we demonstrate that females mated to males injected with an *Hsf* silencing construct, previously reported to have 50% of the annotated MAG expressed genes down-regulated,[17] also showed a marked propensity to further mating with male mosquitoes resulting in successful secondary inseminations. This phenotype is quite dramatic, 95.23% of females previously mated to *Hsf*-silenced males are

receptive to further inseminations when compared to two different control groups (*dsLacZ* 18.75% and WT males 26.22%). The majority of such mated females generated only offspring from the second mating as detected by a transgenic marker while the remaining 45% generated a mixed progeny from the first and second mating. The females of the control groups generated mainly progeny from the first mating, mixed progeny was observed but consistent with the anticipated reinsemination rates observed in laboratory cages but never reaching values close to 100%. These findings would suggest that the seminal fluid of *Hsf*-silenced males lacks Acps that are needed for turning off female receptivity to further mating, in agreement with the observation that in these mosquitoes a large proportion of the Acp repertoire is down-regulated and therefore insufficient to prevent secondary mating events. Accordingly, the MAGs of *dsHSF123* silenced males lack AGAP009352 and AGAP012681 transcripts, orthologs of the *Drosophila* Acp70A,[17] the sex peptide (SP) known to reduce receptivity to subsequent mating in mated females.[1] To date the SP is the only Acp being known to decrease female receptivity in *D. melanogaster*.[32] Several investigations reported that females mated to males lacking the SP mated

more promptly than females mated to males with normal Acp content.[32,33] The high proportion of progeny from the second mating that we observed in females mated with *Hsf*-silenced males could be the result of a combined effect of reduced egg fertilization and embryo survival. In *Drosophila*, it has been observed that Acps play a role in sperm competition,[34] specifically, in sperm defense, that is the ability of the spermatozoa already present in a female to resist displacement by a subsequent insemination. The generalized down-regulation of Acp genes transcription in *dsHSF123*-males possibly also accounts for a reduction of the competitiveness of the sperm received during the first mating. As observed by Gilchrist and Partridge[35] the displacement increases if the second male provides the female with both sperm and Acps. We next investigated whether the increased female receptivity to remating was associated with an altered mating plug transmission and with consequent anomalies in sperm storage. Interestingly, when *dsHSF123*-injected males were mated with virgin females, the majority of mated females failed to receive a mating plug, compared to the 15.62% and 27.27% cases observed for females mated to *dsLacZ* and wild type control males. This result is in line with the observation that a HSF regulated Acp gene coding for a transglutaminase is known to be involved in mating plug formation and to function in sperm storage and egg fertilization in *A. gambiae*.[15] However contrary to previous reports[15] our findings do not support the notion that the function of mating plug is to ensure spermatozoa storage via mechanical obstruction. On the other hand is difficult to envisage how this would work given that the sperm is stored in the spermatheca several weeks while the plug disappears after few days. Our results seem to be consistent with the hypothesis that the mating plug in *A. gambiae*, may provide a physical barrier to spermatheca access from second mating. As *Anopheles* female mosquitoes only mate once in their lifetimes, they rely on a single set of Acps and sperm for their reproductive success. The observation that males in which Acp genes have been down-regulated via silencing of transcription factor *Hsf* do not induce mating refractoriness in females has significant implications for understanding the role of Acp genes in *A. gambiae* reproductive physiology and provide the rationale to search for new targets to manipulate the fertility of this mosquito species.

Acknowledgments

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