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Fruitless RNAi knockdown in the desert locust, *Schistocerca gregaria*, influences male fertility

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ABSTRACT

In *Drosophila melanogaster*, the male-specific splice isoform of the *fruitless* gene (Fru^M) encodes a set of transcription factors that are involved in the regulation of male courtship and copulation. Recent insights from non-drosophilid insects suggest a conserved evolutionary role for the transcription factor Fruitless. In the desert locust, *Schistocerca gregaria* and the German cockroach, *Blatella germanica*, both orthopteran insects, a conserved functional role for *fruitless* has been proposed. *Fru* specific RNAi knockdown in the third nymphal stage of male *Schistocera gregaria* delays copulation initiation and results in reduced progeny. In order to identify the origin of the observed phenotypic effects following a *fruitless* RNAi treatment in the male, we show that the *fru* knockdown has no detectable effect on spermio- or spermatogenesis and on the transfer of spermatozoa during copulation. Nevertheless, it is clear that the male seminal vesicles contain significantly less spermatozoa after *fru* RNAi as compared to *gfp* RNAi controls. We conclude that a lowered male fertility, caused by the *fru* knockdown in male desert locusts may be the direct cause for the reduction of the progeny numbers in their naïve female copulation partners.

1. Introduction

The innate sexual behavior of male insects provides an attractive system to study how complex behavioral patterns are generated. In both invertebrate and vertebrate species, specific events preceding and following the actual copulation event determine the success of a healthy progeny. In dipteran insects, these important sex-specific behaviors are well studied in the fruit fly, *Drosophila melanogaster*. In this insect, transgenic manipulations designed to reverse the normal sex-specific patterns of *fruitless* (*fru*) expression, yielded phenotypes in which male flies behave like females and females like males. These results led to the suggestion that *fru* alone was both necessary and sufficient for the generation of sex-specific behavior patterns (Demir and Dickson, 2005; Manoli et al., 2005, 2006; Vrontou et al., 2006). Subsequent analysis of *fru*-related research showed that a minimum of *doublesex* (*dsx*) expression is required and some other genes may be involved as well (Rideout et al., 2010; Shirangi et al., 2006). Recent reviews summarize and discuss the coupling between *fruitless* and the *fruitless-doublesex* double-switch on one hand and *Drosophila* behavior on the other hand (Billeter et al., 2006; Dauwalder, 2008; Siwicki and Kravitz, 2009; Villella and Hall, 2008; Yamamoto, 2008).

The involvement and conservation of *fru* in both copulation and reproduction-related processes outside dipterans has been demonstrated by two research groups independently in two different orthopteran insect species. In the male German cockroach (*Blattella germanica*) a *fru* sequence was cloned and its expression in the male brain and testes was studied. *B. germanica fru* encodes a 350-amino acid protein with BTB and zinc finger domains typical for *fru* sequences. Upon RNAi-mediated knockdown of *fru*, males did no longer exhibit courtship behavior. So it has been concluded that *fru* is necessary for male sexual behavior in this cockroach and that the role of *fru* as master regulator of male sexual behavior has likely been conserved along insect evolution, at least from cockroaches to flies (Clynen et al., 2011). The functional analysis in the German cockroach showed that the spermatheca of female copulation partners of *fru* RNAi males did not contain any spermatozoids whereas all the females that copulated with control males had spermatheca loaded with spermatozoa. Accordingly, the *fru*-mated naïve female cockroaches hardly formed any ootheca (2 out 44) in contrast to the control females (Clynen et al., 2011). The question remains whether the observed phenotype is caused

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by a hampered or disturbed maturation of the spermatozoa or a sperm transfer deficit.

In the desert locust, *Schistocerca gregaria*, Boerjan et al. (2011) identified a partial *fru*-gene sequence from an EST database (Badisco et al., 2011). Quantitative real-time analysis of this sequence in adult locusts revealed the highest expression in the testes, male accessory glands and in the brain (and optic lobes). Starting *fru* specific RNAi knockdown in the third and fourth nymphal stage resulted in a significantly lower cumulative copulation frequency of the RNAi-treated animals compared to controls after 3 h of observation. In addition, the gonadosomatic index, a measure for sexual maturity, of RNAi treated males was decreased compared to the controls. Analysis of the egg pods resulting from a successful copulation event was hampered in this study because the small sample size was too small to perform appropriate statistics. Nevertheless, egg pods from females that mated with an RNAi-treated male appeared to be smaller and contained less fertilized eggs compared to egg pods from females who mated with control males. Starting injections in the fifth nymphal stage showed the complete opposite for the cumulative copulation frequency and gonadosomatic index of the testes. From this analysis in *S. gregaria* it has been concluded that already in the early nymphal phases of male desert locusts, *fru* starts to play an important role in the regulation of successful copulation in the adult. Also from this study, it remained elusive whether the observed phenotypical effect was caused by aberrant courtship behavior or by an altered composition of the sperm or seminal fluids (Boerjan et al., 2011).

In this manuscript, we confirm the involvement of *fru* in copulation success in *S. gregaria* using a larger sample size and address the question whether the observed phenotypes can be explained by a sperm transfer deficit from the testes to the seminal vesicles or rather by a disturbance of the spermio- or spermatogenesis.

2. Material and methods

2.1. Experimental animals

S. gregaria were reared under crowded conditions, representing the gregarious phase. All the animals are kept at a controlled temperature of 32 °C, a photoperiod of 14 h and a relative humidity between 40% and 60%. They are fed daily with fresh cabbage and dried oat. The crowded adult (parent) locusts are kept in cages of 38 × 38 × 38 cm with 100–200 individuals per cage. Deposited egg pods are transferred to a bigger rearing cage where >1000 nymphs hatch. Male and female second instar larvae were separated and reared under crowded conditions. The animals entered the experiment at day zero of the third nympal stage. Males were subjected to dsRNA mediated knockdown of *fru* while the females were left untreated. At day 20 of adulthood, the females were presented with a male for copulation.

2.2. Fruitless RNA interference in the desert locust

Third nymphal animals were injected with 10 μl of elution solution (MEGAscript RNAi Kit, Ambion) containing 2.5 μg of *fru* specific dsRNA constructed as described in Boerjan et al. (2011). Control animals were injected with the same amount of elution solution containing 2.5 μg of *gfp* specific dsRNA. *Gfp* specific dsRNA was contructed using this forward (CAC GTGA AGG TGA TGC TAC ATA CGG AA) and reverse (CCT ACG TAA TCC CAG CAG CAG TTA CAA AC) primer. Booster injections were given every 7 days to ensure a continuous knockdown.

2.3. Evaluation of the fruitless RNAi phenotype

2.3.1. Statistical confirmation of earlier described phenotypical characteristics

The scoring of the phenotype (i.e. the time needed for actual copulation, determination of the gonadosomatic index, clutch size and fertilization percentage) of the *fru* RNAi induced phenotype has been described in Boerjan et al. (2011). All statistical tests were performed in GraphPad Prism 4. Expression data, weights, GSI's, and egg numbers were analyzed with a Mann–Whitney *U* test and the differences in fertilization percentage of clutches was analyzed with a one-sided Fisher exact test.

2.3.2. Structural analysis of fru RNAi adult male testes

Dissection of testes from 20-day old males that were RNAi treated from the third nymphal stage onwards was followed by overnight fixation (1% paraformaldehyde in 0.1 M PBS, pH 7.4). Prior to dehydration of the tissue, the testes were rinsed three times for 5 min with 0.1 M PBS. Next, the tissues were subjected to a dehydration series (70% ethanol to 100% ethanol) followed by an overnight incubation in ethanol:xylol (1:1). Finally, the tissues were embedded in paraffin (overnight at 60 °C). Once embedded, the testes were transversally sliced (7 μm) on a microtome (Thermo Scientific, Microm HM360). Testes sections were subjected to a standard hematoxylin staining to visualize the nuclei.

2.3.3. Sperm quantitation in the seminal vesicle

One day after the copulation experiment, when it was assumed that the seminal vesicles are filled up again with sperm as suggested for Lepidoptera (Giebultowicz et al., 1989), the seminal vesicles were dissected from both accessory glands of both *fru and gfp* RNAi treated males. The number of spermatozoa was quantified as the total number of nuclear DNA as visualised by a DAPI staining (4′,6-diamidino-2-phenylindole; Sigma–Aldrich). In brief: the complete seminal vesicles were collected in 200 μl aqua destillata. The samples were sonicated (2 × 2 s, 4 °C; Soniprep 150 equipped with an exponential microprobe, MSE, New Jersey) to ensure a homogenous distribution of DNA. Next, the solution was transferred to a black 96-well plate and 2 μl of DAPI solution (0.5 g/l) was added. The fluorescence of DNA bound DAPI was measured during 0.1 s on a Mithras LB940 device (Berthold, excitation 355 nm, emission 460 nm). The data were normalized for the auto-fluorescence of DAPI. Because for each male both seminal vesicles were analysed separately, the mean of both values was used for comparison of the different conditions.

3. Results

3.1. Statistical analysis of the fru-RNAi phenotype

In Boerjan et al. (2011), the phenotyping of the clutch size and fertilization percentage did not allow appropriate statistics due to the limited number of animals that successfully deposited an egg batch. In order to be able to perform appropriate statistics, we started a new experiment with a higher number of animals for statistical analysis. Phenotypic characteristics such as the delay in copulation initialization and the lower gonadosomatic index (a measure for sexual maturation) for *fru* dsRNA treated animals were confirmed (data not shown). A sufficient number of data points was collected for statistical confirmation of the difference in clutch size and fertilization percentage and is shown in Figs. 1 and 2, respectively. The male *fru* RNAi treatment caused a significant decrease of approximately 30% and 38% for the female clutch size and fertilization percentage, respectively.