Knockdown of ecdysone receptor in male desert locusts affects relative weight of accessory glands and mating behavior

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Abbreviations

20E: 20-hydroxyecdysone; E: ecdysone; EcR: ecdysone receptor; MAG: male accessory gland; RXR: retinoid-X-receptor; USP: ultraspiracle

Abstract

Locusts have been known as pests of agricultural crops for thousands of years. Recently (2018-2021) the world has faced the largest swarms of desert locusts, *Schistocerca gregaria*, in decades and food security in large parts of Africa and Asia was under extreme pressure. There is an urgent need for the development of highly specific bio-rational pesticides to combat these pests. However, to do so, fundamental research is needed to better understand the molecular mechanisms behind key physiological processes underpinning swarm formation, such as development and reproduction. The scope of this study is to investigate the possible role(s) of the ecdysteroid receptor in the reproductive physiology of male *S. gregaria*. Ecdysteroids and juvenile hormones are two important classes of insect hormones and are key regulators of post-embryonic development. Ecdysteroids are best known for their role in moulting and exert their function via a heterodimer consisting of the nuclear receptors ecdysone receptor (EcR) and retinoid-X receptor (RXR). To gain insight into the role of SgEcR and/or SgRXR in the male reproductive physiology of *S. gregaria* we performed RNAi-induced knockdown experiments. A knockdown of *SgEcR*, but not *SgRXR*, resulted in an increased relative weight of the male accessory glands (MAG). Furthermore, the knockdown of these genes, either in combination or separately, caused a significant delay in the onset of mating behavior. Nevertheless, the MAG appeared to mature normally and the fertility of mated males was not affected. The high transcript levels of *SgEcR* in the fat body, especially towards the end of sexual maturation in both males and females, represent a remarkable finding since as of yet the exact role of *SgEcR* in this tissue in *S. gregaria* is unknown. Finally, our data suggest that in some cases SgEcR and SgRXR might act independently of each other. This is supported by the fact that the spatiotemporal expression profiles of *SgEcR* and *SgRXR* do not always coincide and that knockdown of *SgEcR*, but not *SgRXR,* significantly affected the relative weight of the MAG.

Keywords: 20-hydroxyecdysone, ecdysteroid, insect, male accessory gland, reproduction, retinoid-x receptor

1. Introduction

In recent years (2018-2021) the largest outbreaks of desert locusts, *Schistocerca gregaria*, in decades have threatened food security in large parts of Africa and Asia (www.fao.org). The devastating effects of locust swarms, as well as the fact that neurotoxic insecticides with limited selectivity are massively sprayed to combat them, stress the need for more selective, eco-friendly locust management strategies, especially when considering the importance of securing global food production for future generations of humans (Cullen et al., 2017). However, a crucial first step is to gain more knowledge of the physiological mechanisms underlying key processes, such as reproduction, in this locust species.

During an insect's life cycle the interplay of two classes of insect hormones, ecdysteroids and juvenile hormone (JH), is of vital importance. Peaks in ecdysteroid levels trigger the moulting process in juveniles, while a strong reduction in JH levels during the final larval stage either initiates a final moult to adulthood in Hemimetabola, or a pupal stage in Holometabola (Riddiford, 2012). The active ecdysteroid in *S. gregaria* is 20-hydroxecdysone (20E). Ecdysone (E) is synthesized in the prothoracic gland (PG) through a series of enzymatic conversions catalyzed by a series of cytochromic P450 enzymes known as the *Halloween* genes and subsequently released into the hemolymph (Niwa and Niwa, 2014). In peripheral tissues, such as the Malpighian tubules, E is converted to 20E by the *Halloween* gene *Shade* (Marchal et al., 2012). In *S. gregaria* the PG persist in adults, although ecdysteroid production, as in other insects, is taken over by the reproductive organs (Simonet et al., 2004; Tawfik et al., 1997; Verlinden et al., 2009). The 20E receptor is a heterodimer of two nuclear receptors: ecdysone receptor (EcR) and ultraspiracle (USP)/retinoid-X-receptor (RXR) (Yao et al., 1993). USP occurs in holometabolan insects, while RXR is the ortholog of USP in hemimetabolan insects (Hill et al., 2013). After 20E binds to this receptor complex several downstream genes are regulated, such as the genes encoding ecdysone-induced proteins 74 and 75 (E74 and E75), broad-complex (Br-C), hormone receptors 39 and 3 (HR39 and HR3) and Blimp-1 (Agawa et al., 2007; Karim and Thummel, 1992; Huet et el., 1995).

It has been well established that both hormonal signaling systems remain present in adult insects and then play important regulatory roles in reproduction (Raikhel et al., 2005). In insects, JH and/or ecdysteroids stimulate sexual development, but their relative contribution to this regulation appears to be strongly species-dependent. In several dipteran and lepidopteran species, such as the fruit fly, *Drosophila melanogaster*, the silk moth, *Bombyx mori* and the yellow fever mosquito, *Aedes aegypti*, ecdysteroids are necessary for ovarian maturation (Gancz et al., 2011; Swevers and Latrou, 2003; Vogel et al., 2015). By contrast, in many other insects, including the locusts *Locusta migratoria*

(Lagueux et al., 1977) and *S. gregaria* (Gijbels et al., 2019), JH regulates ovarian maturation. On the other hand, ecdysteroid signaling appeared to be crucial for choriogenesis in *S. gregaria* (Lenaerts et al., 2019). Although these hormones and their signaling pathways have been extensively studied in adult females, their role remains largely understudied in males.

In adult male insects ecdysteroids have been shown to be crucial for male fertility and development of the reproductive system (Dalton et al., 2009; Ganter et al., 2011; Herndon et al., 1997; Ishimoto et al., 2009; Leiblich et al., 2019; Muramatsu et al., 2020; Sharma et al., 2017; Shinbo and Happ, 1989; Xu et al., 2020). In insects, spermatozoa produced by the testes are stored in paired seminal vesicles, which are surrounded by the male accessory glands (MAG). The MAG secretions are essential for male fertility as they are important for sperm maintenance, sperm transfer, spermatophore formation and in some species affect female behavior and physiology (Avila et al., 2011; Gillot, 2003). The role of ecdysteroids in adult males has been mostly studied in *D. melanogaster*, where a reduction in ecdysteroid levels or *EcR* caused an increase in male-male courtship behavior (Dalton et al., 2009; Ganter et al., 2011). Additionally, a knockdown of *EcR*, but not *USP*, resulted in smaller, underdeveloped MAG and rendered males infertile (Sharma et al., 2017). A limited number of reports is available for other species. An ecdysteroid peak in the pupal stage of *B. mori,* stimulates MAG growth (Shinbo and Happ, 1998). MAG protein synthesis and sperm production were affected in the red flour beetle, *Tribolium castaneum,* after an RNAi-induced knockdown of several nuclear receptors that play a role in the 20E-induced signaling cascade (E75 or HR38 and E78 or HR39, respectively) (Xu et al., 2020). Muramatsu et al. (2020) suggested that higher ecdysteroid titers in males of the Japanese mealybug, *Planococcus kraunhiae,* drive male development and cause extreme sexual dimorphism. These studies contributed to the increasing evidence supporting an important role of ecdysteroids in male reproductive physiology, and more specifically MAG development and activity. In contrast, in various insect species, including *S. gregaria,* JH has been shown to stimulate MAG development (Gassias et al., 2021; Holtof et al., 2021; Ismail and Gillot, 1995; Parthasarathy et al., 2009; Okamoto et al., 2009). It has been suggested that a functional divergence occurred in the nuclear receptors that form the ecdysone receptor complex, *EcR* and *USP*/*RXR* (Bonneton et al., 2003; Hayward et al., 2003; Hult et al., 2011; Jones et al., 2013; Nowickyj et al., 2008), and this may perhaps explain some of the functional differences that are observed between Diptera/Lepidoptera and other insect species. This again stresses the importance of expanding the existing knowledge regarding the role of these ecdysteroid receptor components from mostly dipteran and lepidopteran model organisms towards other insect species.

In the current study, we addressed this knowledge gap by investigating the possible role of EcR and RXR in male reproductive physiology of the desert locust, *S. gregaria*. We performed an RNAi-

mediated knockdown of both components of the ecdysteroid receptor complex and analyzed phenotypic effects on growth of the testes and MAG, display of mating behavior and reproductive success.

2. Materials and methods

2.1 Insects

Desert locusts (*Schistocerca gregaria gregaria*) were kept under crowded conditions at a controlled temperature (30°C ± 1°C), relative humidity (40-60%) and photoperiod (a daily 14 h:10 h light:dark cycle). In addition, during this photoperiod incandescent light bulbs (40 W) generated a temperature gradient within the cages. Locusts were allowed daily to feed *ad libitum* on fresh cabbage leaves, while their diet was also supplemented with rolled oats. Adult females deposited their eggs in pots filled with slightly moistened turf-sand mixture. Pots were collected weekly and then transferred to empty cages, where eggs were allowed to hatch. Before starting with the described experiments, newly moulted locusts were collected to obtain cohorts of developmentally synchronized animals. Males and females were kept together until day 8 of the adult stage (AdD8) (as sexual maturity is normally reached at AdD10-AdD12). To obtain virgin males at the start of the mating observation experiment, all females were removed from the cages at AdD8. The rationale behind the selection of sampling moments during the fifth nymphal stage (N5) was previously described by Verbakel et al. (2021). Approximately 3 days prior to ecdysis the inter-wing distance first exceeds 1.8mm (N5IW). Next, the body weight of the animals peaks (N5WP) and subsequently drops (N5WD) approximately 2 and 1 days prior to ecdysis, respectively.

2.2 Characterization of MAG development

All dissections were performed under a SZ2-ST binocular microscope (Olympus) and images were taken using a mounted HD-Pro VC.3038 camera (Euromex). MAG were dissected from males on different time points of the N5 and the adult stage, from N5D0 till AdD11. MAGs were assessed for yellowing of the adipose tissue surrounding the seminal vesicle, presence of secretion in the white glands (Suppl. Fig. 1). Additionally, the seminal vesicle was dissected and the presence of spermatozoa was confirmed using a DM IL LED microscope (Leica). MAG were weighed using a Genius ME215p analytical balance (Sartorius) and weights were divided by the total body weight and expressed as a percentage resulting in the accessory gland somatic index (AGSI). The complete MAG complex was collected for protein extraction as described in 2.6.

2.3 RNA extraction and cDNA synthesis

Tissues for RNA extraction were dissected and collected in MagNa Lyser Green Beads (Roche) and immediately transferred to liquid nitrogen and subsequently stored at -80°C. Tissues were homogenized in a Magna Lyser Instrument (Roche) at 6500 rpm for 30 s. Total RNA was extracted from the homogenate using the RNeasy Lipid Tissue Kit (Qiagen). Possible genomic DNA

contamination was eliminated using an on-column DNAse digestion (RNAse-free DNAse, Qiagen). The concentration of the resulting RNA extracts was determined by means of a Nanodrop spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Inc.). RNA extracts were stored at -80°C.

For cDNA synthesis, 500 ng of RNA was diluted to 6 µl in Milli-Q water (Millipore) and reverse transcribed using the PrimeScriptTM RT reagent kit (Takara, Invitrogen Life Technologies) according to the manufacturer's protocol. The resulting cDNA was diluted 10-fold in Milli-Q water (Millipore) and stored at -20°C.

2.4 Quantitative RT-PCR

The primers for RT-qPCR were previously validated by Lenaerts *et al*. (2019) (Suppl. Table 1). Reference genes were selected from a pool of 7 candidate household genes using the geNorm software as described by Van Hiel *et al*. (2009). For measurements of samples derived from MAG, *ubiquitin* (*ubi*) and *ribosomal protein 49* (*RP49*) were selected; for analyzing the tissue distribution, *elongation factor 1-alpha* (*EF1α*) and *RP49* were selected. All reactions were performed in duplicate and each reaction consisted of 5 µl Fast SYBR Green Master Mix (Applied biosystems), 0.5 µl forward primer, 0.5 µl reverse primer and 4 µl of cDNA. A no-template condition containing 4 µl of Milli-Q water (Millipore) without cDNA was included as a negative control. A calibrator sample was added to each plate to control for variability between plates. All reactions were run on a StepOne System (ABI Prism, Applied Biosystems) using the following programme: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s.

2.5 RNA interference

2.5.1 Double-stranded RNA (dsRNA) synthesis

Two non-overlapping dsRNA constructs for *SgEcR* (*dsEcR1* and *dsEcR2*), *SgRXR* (*dsRXR1* and *dsRXR2*) and one construct for *GFP* (*dsGFP*) were produced using the MEGAscript ® RNAi kit (Ambion) as previously described by Lenaerts *et al*. (2016). This procedure of dsRNA production is based on a high-yield *in vitro* transcription reaction from a user-provided DNA template with flanking T7 promoter sequences. Therefore, forward and reverse primers flanked by the T7 promoter sequence were used in a PCR reaction with REDTaq DNA polymerase (Sigma-Aldrich) to amplify a fragment of the target gene. Amplicons were separated by agarose gel electrophoresis (1.2% agarose gel containing GelRedTM (Biotium) and further purified (GenElute^{TM} Gel Extraction Kit, Sigma-Aldrich). These fragments were further cloned (TOPO® TA cloning kit for sequencing, Invitrogen) and sequenced to confirm the amplicon sequence (Sanger sequencing, LGC genomics, Berlin, Germany).

The resulting plasmids were subsequently used as templates for *in vitro* transcription. The dsRNA concentration was determined as described in 2.3. The dsRNA was further diluted to 40 ng/µl in *S. gregaria* Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) to avoid osmotic shock effects upon *in vivo* injection, and stored at -20°C.

2.5.2 dsRNA injection

Locusts were injected with dsRNA diluted in 10 µl of Ringer using a Hamilton syringe. The dsRNA was injected into the hemocoel by entering the needle pointing towards the thorax between the first and second abdominal segment. Animals were marked on their prothoracic tergite with different colors of nail polish to distinguish the experimental conditions. The first injection was given one day after the final moult (AdD1) and additional injections were given on AdD5 and AdD9 to ensure the maintenance of knockdown throughout the period of sexual maturation. In the case of the copulation experiment (2.5.4) a fourth injection at AdD13 was given. Initially animals were injected with either 10 µl of a mixture containing 400 ng of *dsEcR1* and *dsRXR1* or 400 ng of *dsGFP* (control) (Fig. 4). Subsequently, to study the observed phenotype in more detail *EcR* and *RXR* were targeted separately and 400 ng of *dsGFP*, *dsEcR1*, *dsEcR2*, *dsRXR1* or *dsRXR2* in 10 µl of Ringer solution were injected (Fig. 5). Injected males were either weighed and dissected to study the male reproductive system at AdD12 (2.5.3) or introduced to a female starting from AdD9 to study the male's reproductive capacity and mating behavior (2.5.4).

2.5.3 Dissection and tissue collection

Virgin males were weighed and subsequently sacrificed for tissue collection on AdD12. MAG and testes were dissected and weighed as described in 2.2 and collected in Ringer solution. Excess Ringer was removed by placing the tissue on a paper towel. Testes and MAG weights were divided by the total body weight and expressed as a percentage resulting in the gonadosomatic index (GSI) and the accessory gland somatic index (AGSI), respectively. The testes and one random lobe of the MAG complex were collected in MagNa Lyser Green Beads (Roche) and RNA was extracted as described in 2.3. The second lobe of the MAG complex was collected for protein extraction as described in 2.6.

2.5.4 Copulation experiment

In our colony, males usually start showing mating behavior between AdD10 and AdD12. In this experiment, we monitored the first display of mating behavior by *dsGFP*-, *dsEcR*- and/or *dsRXR*-injected virgin males when introducing them to virgin females. Monitoring started on AdD9. In the morning of AdD9, after feeding, all males and females were individually separated in small cages 1 h

prior to the experiment. At the start of the experiment each virgin male was introduced to one randomly selected female of the same age. All couples were kept together for 2 h and every successful mating was recorded. A copulation was considered successful when the male had mounted the female and their genitals were interlocked. All animals that did not successfully copulate were returned to their original cages, keeping virgin males and females separated. This monitoring experiment was repeated daily with the remaining virgin males.

Locust couples that had mated were separated after 24 h. Then the males were sacrificed and the females were provided with a long cylinder-shaped pot containing a slightly moistened sand-turf mixture in which females deposit their eggs. These pots were checked daily, and if eggs were present they were counted and incubated in a separate cage. The appearance of hatchlings was monitored daily and all hatchlings derived from each individual female were counted. Hatching success was determined by dividing the total number of hatchlings by the total number of eggs per female.

2.6 Protein extraction

Upon dissection, MAG were rinsed three times with ice-cold phosphate-buffered saline (PBS) (8 g/l NaCl, 0.2 g/l KCl, 0.144 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH=7.4) and excess PBS was removed. Next, 500 µl of ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS dissolved in 25 mM Tris buffer, pH 7.6) were added. Prior to adding the RIPA buffer cOmplete protease inhibitor cocktail (Roche) was added according to the manufacturer's recommendations (1 tablet per 50 ml of extraction buffer). Samples were immediately transferred to dry ice and stored at -20 °C. Subsequently, tissues were homogenized using a teflon micropestle attached to a drill and sonicated 3 times for 5 s. During the extraction procedure, homogenates were kept on ice to avoid protein degradation. Finally, samples were centrifuged for 10 min at 4 °C and 16.000 x g and the resulting supernatant was transferred to a new tube. The concentration of protein extracts was measured using the BCA (bicinchoninic acid assay) assay as described by Walker (1994). Protein concentration was normalized to MAG weight to obtain the relative protein content of the MAG.

2.7 Statistical analysis

For statistical analysis GraphPad Prism 6 (GraphPad Software Inc.) was used. To compare AGSI and relative protein content between different time points a one-way ANOVA with Tukey post-hoc test was performed (3.1). For all RT-qPCR experiments relative transcript levels were first log-transformed before performing an unpaired Student's t-test or one-way ANOVA with Holm-Šidák post-hoc test (3.3.1). To compare *EcR* and *RXR* transcript levels in different tissues between immature and mature males multiple t-tests with Bonferroni correction were performed (3.2). The mating behavior was compared between groups by performing a log-rank (Mantel-Cox) test followed by a Gehan-Breslow-

Wilcoxon test with Bonferroni correction to make pairwise comparisons (3.3.3). To compare the means of other dependent variables (weight, AGSI, GSI, relative protein content, egg clutch size and hatching rate) a one-way ANOVA with Holm-Šidák post-hoc test was used.

3 Results

3.1 Male accessory gland development

The maturation of the MAG during the N5 and the adult stage was studied as described in 2.2 (Fig. 1). At the start of the N5 stage, the MAG were small in size, as reflected by their low AGSI. The AGSI increased towards the end of the N5 stage, and continued to increase further during the adult stage. At N5WD the AGSI was significantly higher than in the early N5 stage (N5D0-N5D4) (one-way ANOVA with Tukey post-hoc test, $p<0.0110$), and in the adult stage the AGSI reached its highest levels at AdD9 and AdD11 when compared to all other time points (one-way ANOVA with Tukey post-hoc test, $p<0.0001$). The relative protein content of the MAG was low in the N5 stage and did not increase until AdD5, where AGSI was significantly higher compared to the N5 stage (one-way ANOVA with Tukey post-hoc test, $p<0.0062$). The relative protein content continued to increase and was significantly higher on AdD9 compared to all other time points (one-way ANOVA with Tukey post-hoc test, $p<0.001$), with exception of AdD11.

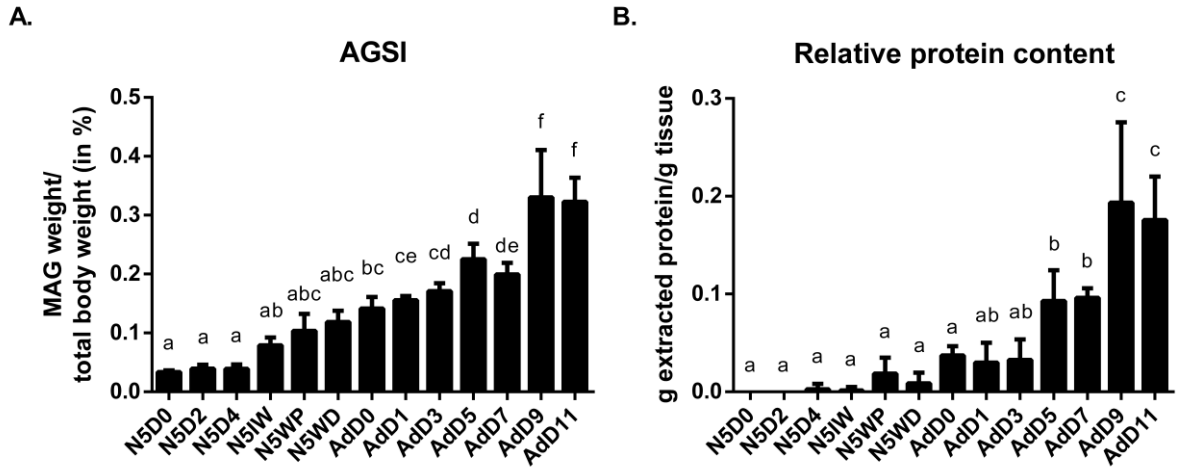

Fig. 1: MAG development during the last nymphal (N5) and the adult stage. The accessory gland somatic index (AGSI) (A.) and the relative protein content of the MAG (B.) were measured as indicators of sexual maturation. Measurements were taken starting on the day animals moulted to the N5 stage (N5D0) till day 11 of the adult stage (AdD11). Data are represented by bar graphs and error bars indicate the standard deviation (n=5). Statistical significance ($p<0.05$) is indicated by (a combination of) distinct characters (a-f in panel A; a-c in panel B); data marked with the same character are not significantly different. **A:** The AGSI was calculated by dividing the weight of the MAG complex by the total weight and is expressed as a percentage. **B:** The relative protein content of the MAG was determined by a BCA assay on whole MAG protein extracts and normalized to the MAG weight. Abbreviations mentioned at the X-axis: Ad: adult locusts; N5: fifth instar nymphs; IW: inter-wing distance; WP: weight peak; WD weight drop; D0-11: number of days in the given stage.

Throughout the entire N5 stage, as well as in young adults, the MAG had a pale white color and the tubular glands were transparent. This changed around AdD3, when the first white secretions were visible within the white glands (Suppl. Fig. 1). The first secretions originated at various locations in the white glands. As animals mature, the secretions inside the glands extended further downwards until the entire lumen of the tubules was filled with the secreted fluid, which occurred between AdD7 and AdD9. During this time period the tissue surrounding the seminal vesicle turned from an off-white or pale yellow color to a bright yellow color, comparable to that of the fat body. At AdD7 the first spermatozoa were visible within the seminal vesicle and at AdD9 we observed that the seminal vesicle was filled with semen in all males that we examined. This time point coincides with the start of the yellowing of the cuticle in crowd-reared males. Desert locust males were becoming sexually mature between AdD9 and AdD12 of the adult stage, as demonstrated by their first display of mating behavior in this period.

3.2 Tissue distribution of *SgEcR* and *SgRXR* transcripts

A transcript profile was obtained for *SgEcR* and *SgRXR* in different male locust tissues using RT-qPCR (Fig. 2). Transcript levels of these genes were measured in brain (B), abdominal ganglia (AbG), testes (T), male accessory glands (MAG) and fat body (FB) of sexually immature (AdD3) and mature (AdD15) adult males. Both genes were expressed in both regions of the central nervous system (CNS), as well as in peripheral tissues including testes and MAG. Relative *SgEcR* transcript levels were low in adult testes but higher in male fat body, where they further increased significantly upon sexual maturity (multiple t-tests with Bonferroni correction, $p=0.00579$). On the other hand, relative *SgRXR* transcript levels in fat body remained low, while *SgRXR* transcript levels were significantly higher in MAG of sexually mature males compared to immature ones (multiple t-tests with Bonferroni correction, $p=0.000204$).

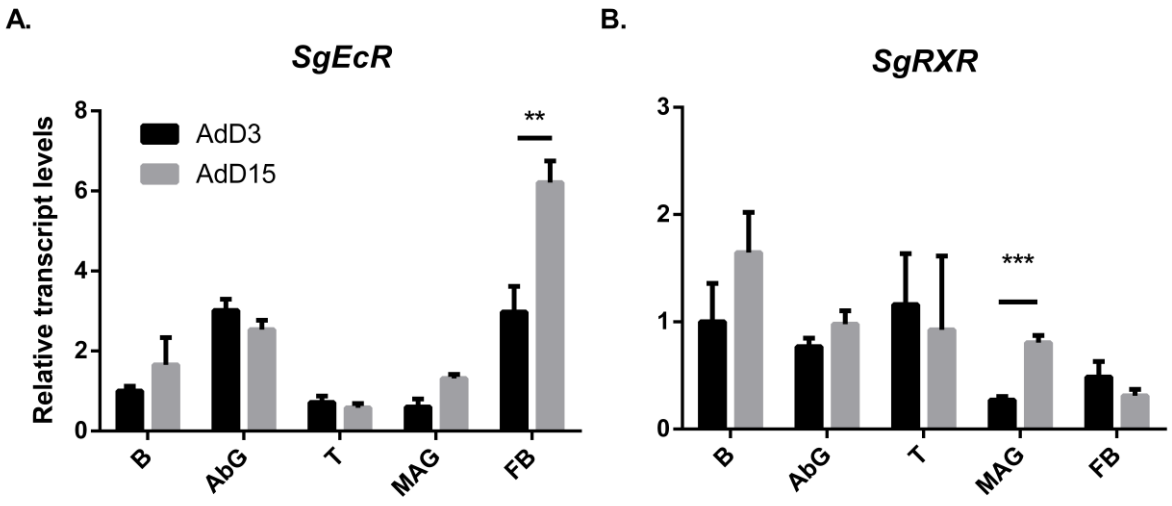

Fig. 2: Tissue distribution of *SgEcR* and *SgRXR* transcripts. Relative transcript levels of *SgEcR* (A) and *SgRXR* (B) were determined by RT-qPCR in different tissues of both immature (AdD3, black bars) and sexually mature (AdD15, grey bars) *S. gregaria* males (B: brain, AbG: abdominal ganglia, T: testes, MAG: male accessory glands, FB: fat body). Data are represented by bar graphs and error bars indicate the standard deviation (n=5). Transcript levels were normalized using *elongation factor 1-alpha* (*EF1α*) and *ribosomal protein 49* (*RP49*) as reference genes. Relative transcript levels of *SgEcR* and *SgRXR* are significantly higher in the fat body and MAG respectively of sexually mature males (unpaired student t-test with Bonferroni correction, ds*EcR*: p=0.00579, dsRXR: p=0.000204). Statistical significance is indicated with an asterisk (**: p≤0.01, ***: p≤0.001).

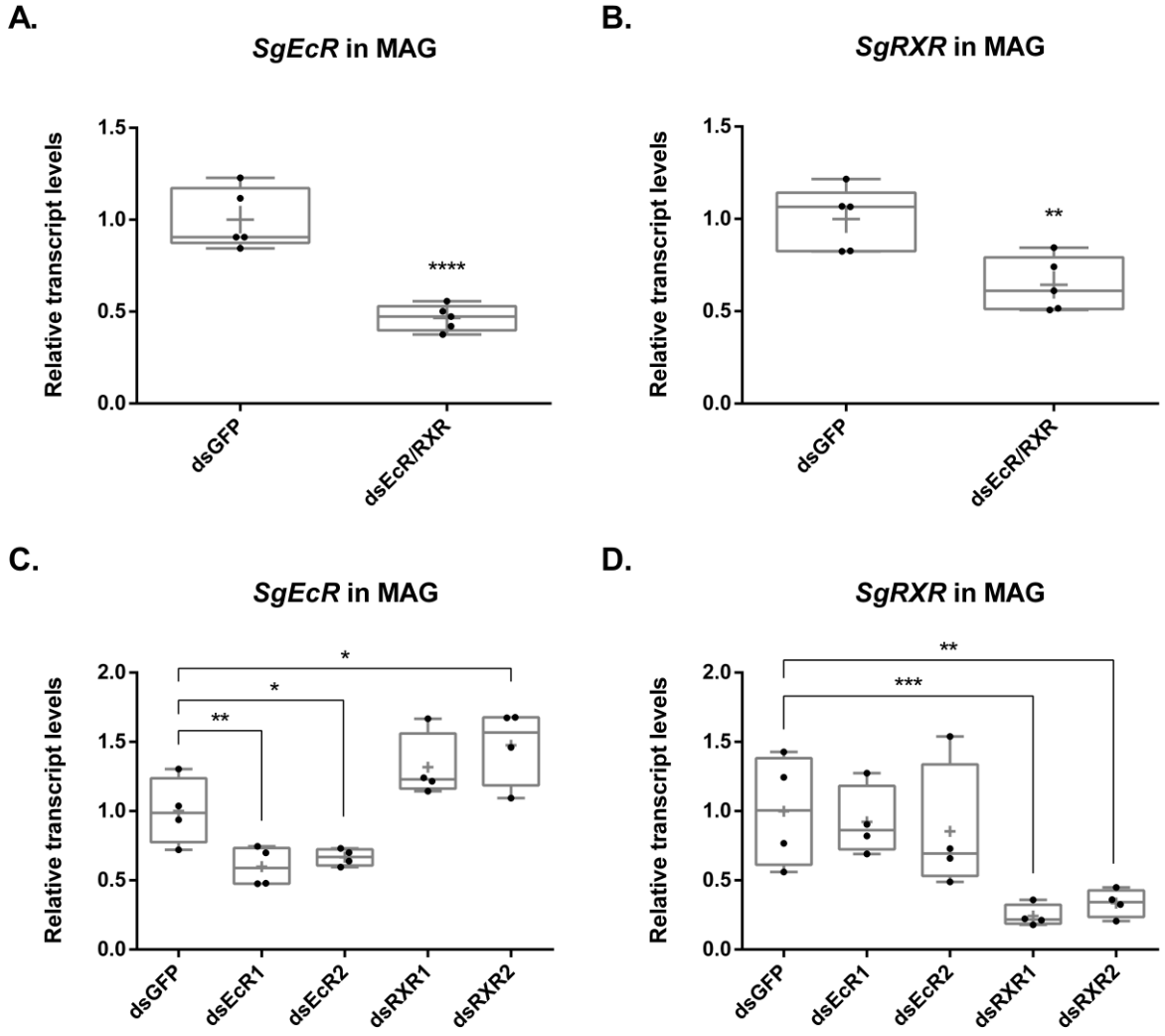

Fig. 3: *SgEcR* and *SgRXR* transcript levels in MAG of dsRNA injected locusts. Relative transcript levels of *SgEcR* and *SgRXR* in the MAG were determined by RT-qPCR after injection of a combination of *dsEcR* and *dsRXR* (A, B) or *dsEcR* (C) or *dsRXR* (D) separately. Data are represented by boxplots containing the upper and lower quartile, while the whiskers indicate the minimum and maximum. The median and mean are indicated by the grey line in the center of the box and the grey cross respectively. Statistical significance is indicated with an asterisk (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001). **A**: Relative transcript levels of *SgEcR* were significantly lower after injection of *dsEcR*/*dsRXR* (unpaired student t-test, p<0.0001). **B**: Relative transcript levels of *SgRxR* were significantly lower after injection of *dsEcR*/*dsRXR* (unpaired student t-test, p=0.0083). **C**: Relative transcript levels of *SgEcR* were significantly lower after injection of *dsEcR*1, ds*EcR*2 or ds*RXR*2 (one-way ANOVA with Holm-Šidák's post-hoc test, *dsEcR1*: p=0.0094, *dsEcR2*: p=0.0367, *dsRXR2*: p=0.0367) compared to ds*GFP*. **D**: Relative transcript levels of *SgRXR* were significantly lower after injection of *dsRXR* (one-way ANOVA with Holm-Šidák's post-hoc test, *dsRXR1*: p=0.0004, *dsRXR2*: p=0.0031) compared to *dsGFP*.

3.3 RNAi-induced knockdown of *SgEcR* and *SgRXR*

3.3.1 *SgEcR* and *SgRXR* transcript levels in MAG of dsRNA injected locusts

In the case of a double knockdown (*dsEcR*/ds*RXR*) transcript levels of both *SgEcR* and *SgRXR* were significantly lower in the MAG compared to transcript levels of the corresponding gene in the *dsGFP* (control) condition (unpaired Student t-test, *SgEcR*: $p<0.0001$, *SgRXR*: $p=0.0083$) with a reduction at transcript level of 53.43% (SgEcR) and 35.63% (*SgRXR*) (Fig. 3). When adult male locusts were injected with a *dsEcR* or *dsRXR* construct separately, the relative transcript levels of, respectively, *SgEcR* and *SgRXR* were significantly reduced in the accessory glands when compared to *dsGFP* (one-way ANOVA with Holm-Šidák post-hoc test, *SgEcR: dsEcR1*, $p=0.0094$; *dsEcR2*, $p=0.0367$; *SgRXR:* dsRXR1, $p=0.0004$; *dsRXR2*, $p=0.0031$). The reductions at transcript level amounted to 40.03% (*dsEcR1),* 33.32% (*dsEcR2)*, 75.69% (*dsRXR1*) and 66.45% (*dsRXR2*). Additionally, transcript levels of *SgEcR* significantly increased upon injection of *dsRXR2* (47.62%, $p=0.0367$), but not *dsRXR1* (31.74%; $p=0.0581$). By contrast, *SgRXR* transcript levels were not affected by injection of *dsEcR*.

3.3.2 Analysis of males after knockdown of *SgEcR* and/or *SgRXR*

Males injected with *dsEcR*/*dsRXR* had a significantly higher AGSI when compared to control animals (unpaired Student t-test, $p=0.0040$) (Fig. 4C). A higher AGSI was also measured after injection of only *dsEcR* (*dsEcR1*: $p=0.0249$; *dsEcR2*: $p=0.0328$), but not *dsRXR* (Fig. 5C). No significant differences were observed in total body weight, GSI or relative protein content of *dsEcR* and/or *dsRXR* injected animals (Fig. 4 and 5). In addition, we also monitored the males' reproductive development, based in part on our observations made on untreated males (described under 3.1). Males across all conditions displayed normal yellow coloration of their cuticle, arising between AdD10 and AdD12. Spermatozoa were present in the seminal vesicles of these males, which was checked in a subset of animals in each condition. Also, no differences in the timing of the appearance of white secretions were observed.

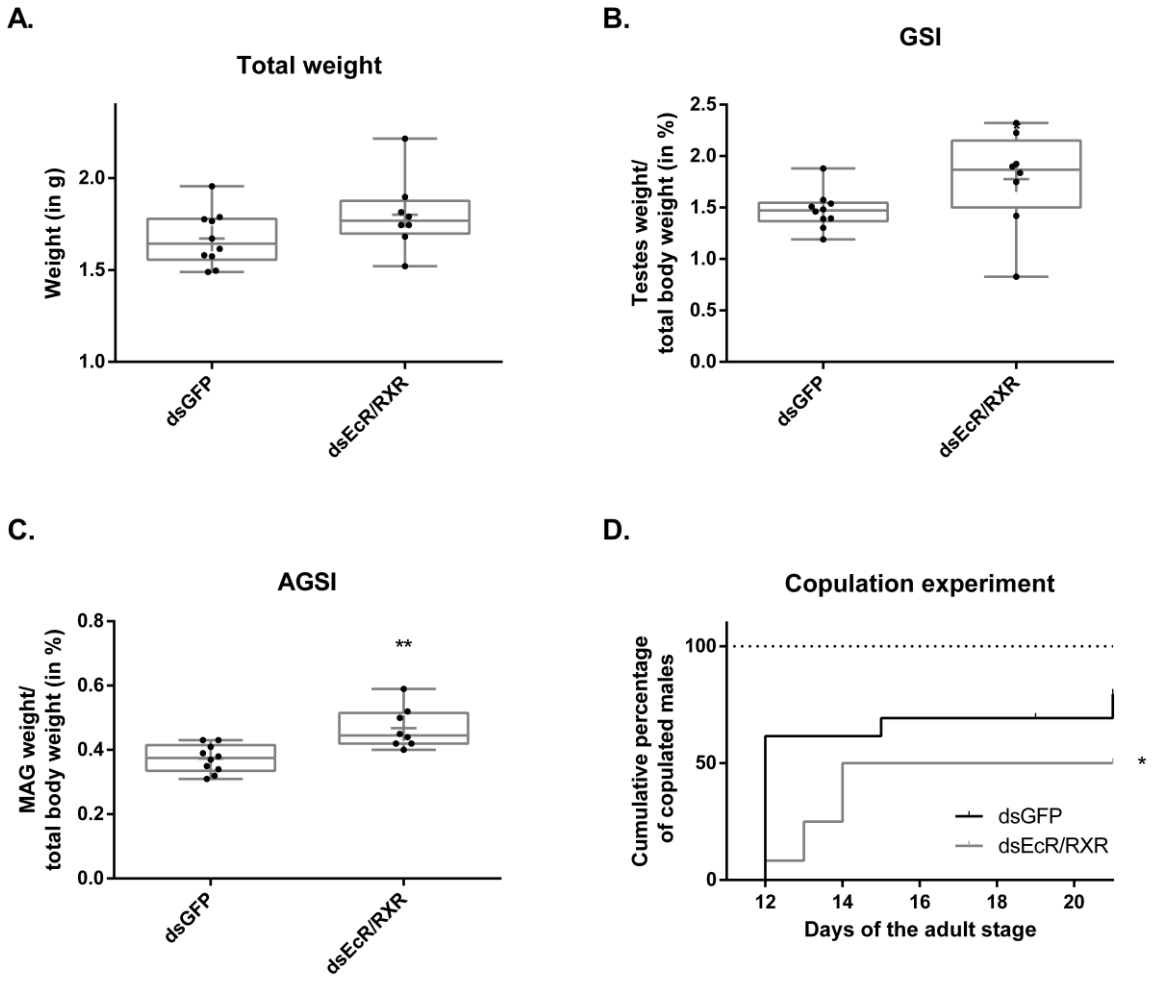

Fig. 4: Effects of an RNAi-induced knockdown of a combination of *SgEcR* and *SgRXR* on the male reproductive system and mating behavior. Locust were injected with either *dsGFP* (control condition) or a mixture of *dsEcR* and *dsRXR* and were subsequently either dissected at AdD12 (A-C, *dsGFP*: n=10, *dsEcR*/*dsRXR*: n=8) or introduced to a female at AdD9 (D, *dsGFP*: n=13, *dsEcR*/*dsRXR*: n=12) . Data are represented by boxplots containing the upper and lower quartile, while the whiskers indicate the minimum and maximum. The median and mean are indicated by the grey line in the center of the box and the grey cross respectively. Statistical significance is indicated with an asterisk (**: p≤0.01). **A**: The entire animal was weighed to obtain the total weight (in g). **B**: The gonadosomatic index (GSI) is calculated by dividing the testes weight by the total weight expressed as a percentage. **C**: The accessory gland somatic index (AGSI) is calculated by dividing the weight of the MAG complex by the total weight expressed as a percentage. The AGSI was significantly higher in animals injected with *dsEcR/dsRXR* compared to control animals (unpaired student t-test with Welch's correction, p=0.0040). **D**: The cumulative percentage of copulated males (y-axis) in function of age (days of the adult stage, x-axis). The 100% mark is indicated by a dotted line. The onset of mating behavior was significantly delayed in *dsEcR*/*dsRXR*-injected animals compared to the control condition (Gehan-Breslow-Wilcoxon test, p=0.0434). At least 50% of the *dsGFP*-injected males had already mated on AdD12, compared to AdD21 for animals injected with *dsEcR*/*dsRXR*. Animals that died during the experiment were censored and indicated by ticks on the graph.

3.3.3 Mating behavior and post-mating reproductive success

To investigate the possible *in vivo* effects of *SgEcR* and/or *SgRXR* knockdown, we monitored the sexual behavior and reproductive success of males. Upon injection of *dsEcR*/*dsRXR* (Fig. 4D; ds*GFP*, n=13; *dsEcR/dsRXR*, n=12) the first copulation was observed on AdD12 in both experimental and control condition. The onset of the first mating event was significantly delayed in *dsEcR*/*dsRXR* injected males compared to control males (Gehan-Breslow-Wilcoxon test, p=0.0434). Upon injection of only *dsEcR* or *dsRXR* (Fig. 5E and Suppl. Fig. 2; *dsGFP*, n=30; *dsEcR1*, n=21; *dsEcR2*, n=22; *dsRXR1*, n=21; *dsRXR2*, n=21) the first successful copulations were observed on AdD10 (control) and AdD12 (experimental conditions). From then on, their numbers cumulatively increased over time. At least 50% of the control males had already mated on AdD14, compared to AdD20 (*dsEcR2*), AdD17 (*dsRXR1*) and AdD18 (*dsRXR2*). At the end of the experiment (AdD27) 48% of *dsEcR1* injected males had mated. The onset of the first mating event was significantly delayed in *dsEcR* and *dsRXR* injected males, when compared to control males (Gehan-Breslow-Wilcoxon test, *dsEcR1*: p<0.0001; *dsEcR2*: p<0.0001; *dsRXR1*: p=0.0166; *dsRXR2*: p=0.0074), while there were no significant differences between two constructs targeting the same gene or between *dsEcR* and *dsRXR* constructs.

Regarding the mated females and their offspring, we did not observe any significant differences in the time between mating and oviposition (Suppl. Fig. 2A), the numbers of eggs per egg pod (Suppl. Fig. 2B), the time between oviposition and hatching (Suppl. Fig. 2C) or the hatching rate (Suppl. Fig. 2D) (oviposition and egg clutch size: *dsGFP*, n=18; *dsEcR1*, n=5; *dsEcR2*, n=11; *dsRXR1*, n=14; *dsRXR2*, n=8; hatching and hatching rate: *dsGFP*, n=16; *dsEcR1*, n=5; *dsEcR2*, n=11; *dsRXR1,* n=12; *dsRXR2*, n=7).

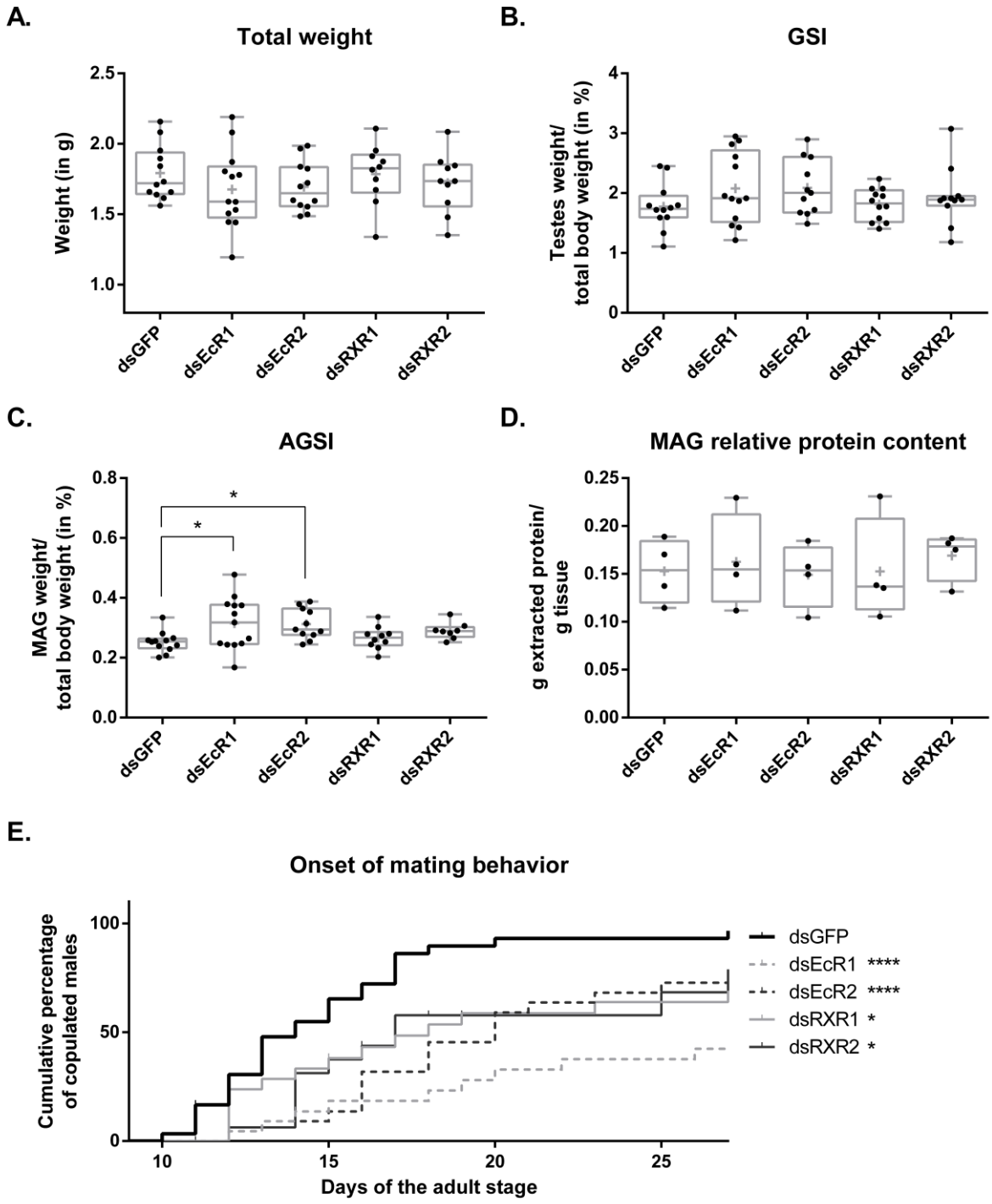

Fig. 5: Effects of an RNAi-induced knockdown of *SgEcR* and *SgRXR* separately on the male reproductive system and mating behavior. Locusts were injected with either *dsEcR*, *dsRXR* or *dsGFP* (control condition) and were subsequently either dissected at AdD12 (A-D, n=15) or introduced to a female at AdD9 (E, n=30). Data are represented by boxplots containing the upper and lower quartile, while the whiskers indicate the minimum and maximum. The median and mean are indicated by the grey line in the center of the box and the grey cross respectively. Statistical significance is indicated with an asterisk (*: p≤0.05, ****: p≤0.0001). **A**: The entire animal was weighed to obtain the total weight (in g). **B**: The gonadosomatic index (GSI) is calculated by dividing the testes weight by the total weight expressed as a percentage. The AGSI was significantly higher in animals injected with *dsEcR1*

and *dsEcR2* compared to control animals (one-way ANOVA with Tukey's post-hoc test, *dsEcR1*: p=0.0249, *dsEcR2*: p=0.0328), which was not the case for *dsRXR*-injected animals. **C**: The accessory gland somatic index (AGSI) is calculated by dividing the weight of the MAG complex by the total weight expressed as a percentage. **D**: The relative protein content of the MAG was determined by a BCA assay using whole MAG protein extracts and normalized to the MAG weight. **E**: The cumulative percentage of copulated males (y-axis) in function of age (days of the adult stage, x-axis). Males were introduced to females at day 9 of the adult stage (*dsGFP*, n=30; *dsEcR1*, n=21; *dsEcR2*, n=22; *dsRXR1*, n=21; *dsRXR2*, n=21). The 100% mark is indicated by a dotted line. The onset of mating behavior was significantly delayed in *dsEcR*- and *dsRXR*-injected animals compared to the control condition (log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test, *dsEcR1*: p<0.0001, *dsEcR2*: p<0.0001, *dsRXR1*: p=0.0166, *dsRXR2*: p=0.0074) while no significant differences were found between two constructs targeting the same gene or between *dsEcR* and *dsRXR* constructs. At least 50% of the *dsGFP* injected (control) males had already mated on AdD14, compared to AdD17 (*dsRXR1*), AdD18 (*dsRXR2*) and AdD20 (*dsEcR2*). At the end of the experiment (AdD27) only 48% of *dsEcR1* injected males had mated. Animals that died during the experiment were censored and indicated by ticks on the graph.

4 Discussion

4.1 Male sexual maturation

Sexual maturity in *S. gregaria* males is generally marked by the yellow coloration of the cuticle (in case of gregarious locusts) and the occurrence of mating behavior (Norris, 1954; Odhiambo, 1971; Pener and Simpson, 2009). However, the presence of functional semen (seminal fluid and mature spermatozoa) is a direct indicator of male sexual maturity and crucial to male fertility. Here, we characterized the development of the MAG, including observations of their development as well as weight, relative protein content and the presence of spermatozoa inside the seminal vesicle (Fig. 1). We consider AdD9 to be a pivotal time point in male sexual maturation, as at this moment the seminal vesicle was filled with spermatozoa and white secretions were visible throughout the white glands. Our observations mostly coincide with earlier findings (Gallois, 1989; Norris, 1954; Odhiambo, 1971; Quesada-Moraga and Santiago-Álvarez, 2001). However, Odhiambo (1971) observed that at AdD3 no secretions were visible and the secretory machinery of the opalescent gland was still underdeveloped. The first secretory activity in the opalescent gland at histological level was observed at AdD5. By contrast, we observed the first white secretions two days earlier (AdD3) (Suppl. Fig. 1) and at AdD9 the seminal vesicle of all males was filled with spermatozoa, which is a couple of days earlier compared to Norris' (1954) results. There are several possible explanations for these differences. Odhiambo (1971) discussed the development of the opalescent gland and it was stated that these results were representative of the other gland types, implying that no secretory activity was observed in the white glands at AdD3. Additionally, secretory activity was studied at 3 cross-

sections (basal, middle and apical), though it is unclear at what exact site these sections were made. In the current study, we observed the first white secretions at various sites of the white gland, which means it is possible that the first appearance of white secretions was missed by only looking at 3 different cross-sections described in literature (Odhiambo, 1971). Next, Norris (1954) stated that the binocular microscopes used at the time had a more limited magnification and the first appearance of secretions or spermatozoa was possibly missed. Finally, it is known that several factors, such as nutrition, temperature and humidity can heavily affect growth, development and sexual maturation of *S. gregaria* (Gündüz and Gülel, 2002). However, apart from an ambient temperature that was similar to the temperature at which our colony is kept, no additional information was provided (Norris, 1954; Odhiambo, 1971). In addition, it has been described that locust lab colonies are genetically diverse, which affects various phenotypic traits (Berthier et al., 2010).

Based on our data, we can conclude that growth of the MAG in *S. gregaria* occurs mainly between AdD3 and AdD9, after which the MAG have reached their maximal weight and relative protein content and the seminal vesicle is filled with semen. Shortly after, between AdD10 and AdD12, the cuticle of gregarious males turns bright yellow and they start displaying mating behavior (De Loof et al., 2010; Holtof et al., 2021; Sas et al., 2007).

4.2 Spatial expression profile

Transcript levels of *SgEcR* and *SgRXR* were measured in a variety of tissues of both immature and mature animals using RT-qPCR (Fig. 2). The expression profile of these genes has recently been studied in adult *S. gregaria* (Lenaerts et al., 2016; Lenaerts et al., 2019), though the only male tissues included were testes and MAG of animals at AdD10, at which point the seminal vesicle contains semen. However, the yellowing of the cuticle and the first mating behavior occurs between AdD10 and AdD12. We therefore expanded on this expression profile and compared tissues from sexually immature (AdD3) and mature males (AdD15). Both genes are expressed in the CNS as well as the fat body, gonads and MAG, in agreement with the tissue distribution described by Lenaerts et al. (2019). Transcript levels of *SgEcR* are remarkably high in the fat body and significantly increase during male sexual maturation. A similar increase was observed in females (Lenaerts et al., 2019), specifically towards the end of the first reproductive cycle. The fat body is crucial for the energy metabolism of insects and has additional roles in the synthesis of hemolymph proteins, endocrine regulation and immunity (Badisco et al., 2013). However, based on our results, the role of *SgEcR* in the fat body is as of yet unclear.

4.3 Effects of an RNAi-induced knockdown of *SgEcR* and/or *SgRXR*

A knockdown of *SgEcR* affected relative MAG weight (AGSI), which was not the case for a knockdown of *SgRXR*. Besides this heavy MAG phenotype, the MAG matured normally, secretions were visible around the same time in all conditions, and spermatozoa were present in the seminal vesicle. Interestingly, this MAG phenotype is contrary to what was observed in *D. melanogaster*, where an RNAi-mediated knockdown of *EcR*, but not *USP*, caused MAG to be smaller, underdeveloped and rendered males infertile (Sharma et al., 2017). Ecdysteroids were shown to regulate MAG development in some insects, such as *D. melanogaster* and *B. mori* (Leiblich et al., 2019; Sharma et al., 2017; Shinbo and Hap, 1989). By contrast, in most other insects, including *S. gregaria*, juvenile hormone is crucial for MAG development (Gassias et al., 2021; Holtof et al., 2021; Ismail and Gillot, 1995; Okamoto et al., 2009; Parthasarathy et al., 2009). The functional divergence of *EcR* in insects has been suggested to be a consequence of coevolution between *USP-RXR* and *EcR* (Bonneton et al., 2003).

The cause of the relative MAG weight increase upon knockdown of *SgEcR* is unclear. No other morphological or developmental defects were observed in the male reproductive system and males that successfully mated produced normal offspring. Furthermore, we did not detect any significant differences in relative MAG protein content. Several reports show that the development of testes and/or MAG in insects is affected by their nutritional status (Huck et al., 2021; Macartney et al., 2018; Xu et al., 2015). Most interestingly, in *T. castaneum*, the IIS-pathway has been shown to link nutritional status to MAG development and protein production (Xu et al., 2015). In this study, we observed heavier MAG, although we did not detect any significant differences in body weight or relative protein content of the MAG. However, the relative protein content of the MAG as an indicator of MAG activity doesn't provide detailed information on specific MAG secretions. It would be interesting to study the secretory profile of different MAG gland types. However, to do so, more data on the MAG secretions and the activities of different gland types in *S. gregaria* would be needed. Furthermore, EcR has been shown to play a regulatory role in fat body of various insects where it affects lipid metabolism (Kamoshida et al., 2012; Lu et al., 2018; Nascimento et al., 2021; Wang et al., 2017). However, the only report on the role of ecdysteroids in the fat body of adult males comes from *D. melanogaster*, where a mild inactivation of EcR specifically in the fat body increases lifespan (Tricoire et al., 2009). It has been suggested that EcR exerts its effect on lifespan by affecting the insulin/IGF-1 pathway (IIS), although the evidence for this is mainly indirect (Schwedes et al., 2011; Tricoire et al., 2009). Additionally, insulin-related peptides (IRPs) have an important role in coupling nutritional status to sexual development (Huck et al., 2021; Macartney et al., 2018; Xu et al., 2015). Therefore, we hypothesize that a knockdown of *Sg*EcR in *S. gregaria* males may possibly

have affected IRP production or signaling in the fat body, which in turn may have affected MAG growth. If so, this would suggest the existence of a functional interaction between EcR and the IIS-pathway in locust males.

Mating behavior and fertility were investigated in an *in vivo* experiment (Fig. 4D, Fig. 5E and Suppl. Fig. 2). A significant delay in the onset of mating behavior was observed for all knockdown conditions when compared to the control condition. However, based on our observations male fertility was not affected by the knockdown of *SgEcR* or *SgRXR*. Nevertheless, it is important to note that data of these parameters are only available for males that successfully mated. So, it is possible that the functional knockdown in these males was less effective. Effects of ecdysteroids on mating behavior have been reported in other insect species. An effect of ecdysteroids on male courtship behavior is well described in *D. melanogaster* (Dalton et al., 2009; Ganter et al., 2011; Ishimoto et al., 2009). Additionally, an endocrine disruptor of ecdysteroids caused olfactory disruption and affected mating behavior in the cotton leafworm, *Spodoptera littoralis* (Avilès et al., 2020). There are several possible causes for the observed delay in mating: 1) the *SgEcR*/*SgRXR* knockdown caused a delay in sexual maturation and therefore the display of male mating behavior was also delayed; 2) the knockdown affected locomotion; 3) the depletion of ecdysteroid receptor components directly affected male behavior, which was most likely occurring in the CNS. First, we believe that it is unlikely that the knockdown caused a delay in sexual maturation, because knockdown males at AdD12 appeared to have a normally developed reproductive system. Apart from the fact that MAG were heavier in *SgEcR* knockdown animals, semen was present in the seminal vesicle and MAG secretions were visible. Nevertheless, it cannot be excluded that the knockdown may have affected the secretion of specific MAG factors or any other processes related to sexual maturation that were not observable in our experiments. Next, we can also exclude the hypothesis that a locomotion defect affected mating behavior. Locusts were placed into an arena and were provided food or females at different ends of the arena. Knockdown males were as active as control males (our observations). A delay in the onset of mating behavior has previously been reported in *S. gregaria* and was caused by an RNAi-induced knockdown of *fruitless* (Boerjan et al., 2011). *Fruitless* codes for transcription factors that were shown to be involved in male mating behavior. In *D. melanogaster, fruitless* mutants show a greatly reduced copulation frequency (von Philipsborn et al., 2014). Furthermore, a reduction of ecdysteroid signaling in *fruitless*-expressing neurons increases male-male courtship behavior (Dalton et al., 2009; Ganter et al., 2011). It is plausible that also in *S. gregaria*, as in *D. melanogaster*, ecdysteroids play a role in regulation male mating behavior, perhaps by a functional interaction with *fruitless*. In conclusion, the fact that a knockdown of *SgEcR* or *SgRXR* causes significant delays in the onset of mating behavior, which would affect adult male fitness, adds to the existing knowledge that *SgEcR*

and *SgRXR* also are pesticide target candidates in juvenile stages (Lenaerts et al., 2016) and adult females (Lenaerts et al., 2019).

Finally, the function of EcR and USP/RXR as a heterodimer has been well documented. Nevertheless, two of our results suggest that *Sg*EcR and *Sg*RXR may act independently of each other in addition to forming a heterodimer. First, the transcript profiles differ between both genes. Most notably the transcript levels of *SgEcR* are significantly higher in the fat body of sexually mature males, in contrast to *SgRXR*. Second, a knockdown of *SgEcR* resulted in a higher AGSI, which was not the case for a *SgRXR* knockdown. It has previously been suggested that both EcR and USP/RXR can act independently of each other in other insects (Bodofsky et al., 2017; Costantino et al., 2008; Hill et al., 2013; Hult et al., 2011; Nowickyj et al., 2008; Schubiger and Truman, 2000; Sharma et al., 2017; Zhu et al., 2003). There are only a few reports from dipteran insects showing that EcR or USP/RXR form dimers with other partners than each other (Costantino et al., 2008; Sharma et al., 2017; Zhu et al., 2003). However, very little is known about possible roles of EcR and USP/RXR independently of each other in non-dipteran insects. In *L. migratoria* and the German cockroach, *Blattella germanica RXR* mRNA was detected in early embryogenesis before the appearance of *EcR* mRNA, which suggested a role of RXR independently of EcR (Maestro et al., 2005; Nowickyj et al., 2008). Future research on this topic, especially in non-dipteran species, is necessary to shed light on the various roles of EcR and USP/RXR in mediating ecdysteroid signaling and regulating other processes.

5. Conclusion

RNAi-mediated knockdown of *SgEcR*, but not *SgRXR*, resulted in an increased AGSI. Furthermore, the knockdown of both genes, either in combination or separately, caused a significant delay in the onset of mating behavior. Nevertheless, the MAG appeared to mature normally and the fertility of mated males was not affected. The high transcript levels of *SgEcR* in the fat body, especially towards the end of sexual maturation in both males (Fig. 2) and females (Lenaerts et al., 2019), represent a remarkable finding since as of yet the exact function of *SgEcR* in this tissue in *S. gregaria* is unknown. Finally, our data suggest that *Sg*EcR and *Sg*RXR might act independently of each other. This is supported by the fact that the spatiotemporal expression profiles of *SgEcR* and *SgRXR* did not always coincide and that knockdown of *SgEcR*, but not *SgRXR,* significantly affected AGSI.

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