The ecdysone receptor regulates several key physiological factors in *Anopheles funestus*

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**Abstract**

**Background:** Malaria is a devastating disease, transmitted by female *Anopheles* mosquitoes infected with *Plasmodium* parasites. Current insecticide-based strategies exist to control the spread of malaria by targeting vectors. However, the increase in insecticide resistance in vector populations hinders the efficacy of these methods. It is, therefore, essential to develop novel vector control methods that efficiently target transmission reducing factors such as vector density and competence. A possible vector control candidate gene, the ecdysone receptor, regulates longevity, reproduction, immunity and other physiological processes in several insects, including malaria vectors. *Anopheles funestus* is a prominent vector in sub-Saharan Africa, however, the function of the ecdysone receptor in this mosquito has not previously been studied. This study aimed to determine if the ecdysone receptor depletion impacts *An. funestus* longevity, reproduction and susceptibility to *Plasmodium falciparum* infection.

**Methods:** RNA interference was used to reduce ecdysone receptor expression levels in *An. funestus* females and investigate how the above-mentioned phenotypes are influenced. Additionally, the expression levels of the ecdysone receptor, and reproduction genes lipophorin and vitellogenin receptor as well as the immune gene, leucine rich immune molecule 9 were determined in ecdysone receptor-depleted mosquitoes using quantitative polymerase chain reaction.

**Results:** Ecdysone receptor-depleted mosquitoes had a shorter lifespan, impaired oogenesis, were less fertile, and had reduced *P. falciparum* infection intensity.

**Conclusions:** Overall, this study provides the first experimental evidence that supports ecdysone receptor as a potential target in the development of vector control measures targeting *An. funestus*.

**Keywords:** 20E-signaling, African malaria vector, *Plasmodium falciparum*, RNA interference

**Background**

Malaria is a severe disease transmitted by female *Anopheles* mosquitoes infected with protozoan *Plasmodium* parasites. In 2019, a concerning 229 million malaria cases and 409,000 deaths were recorded [1]. Malaria is endemic to tropical and sub-tropical regions of the world. Africa is severely affected, with more than 90% of cases and mortalities occurring on the continent annually [1]. The main malaria vectors in sub-Saharan Africa are either members of the *Anopheles gambiae* complex or the *Anopheles funestus* group [2]. The *An. funestus* group comprises eleven African species, but contains a single significant malaria vector, *An. funestus* [3–6]. Due to its highly anthropophilic (preference for a human host) and endophilic (preference to rest indoors) nature, *An. funestus* is one of the main vectors in sub-Saharan Africa [2, 7].

Efforts to eradicate malaria have been implemented by the World Health Organization (WHO) in the form of
of The Global Technical Strategy for Malaria (GTS) 2016–2030 [8]. The GTS milestones aim to eliminate at least 90% of malaria by 2030 especially in countries that were most affected in the year 2015 [8]. Unfortunately, the 2020 milestones were not achieved due to several factors [9], emphasizing the need for novel and effective control strategies. Malaria is controlled by adopting strategies targeting either the Plasmodium parasite or Anopheles vector.

Anopheles mosquitoes are primarily controlled using two insecticide-based control interventions: indoor residual spraying (IRS) and long-lasting insecticidal nets (LLIN). These methods are widely employed and their popularity is attributed to their efficiency, cost-effectiveness and ease of implementation [10]. In Africa, LLINs and IRS have proved to be very effective forms of vector control, preventing malaria transmission by 68% and 13% respectively between the years 2000–2015 [11]. Unfortunately, vectors have undergone adaptations that have hindered efforts in malaria eradication. Specifically, An. funestus mosquitoes have developed genetic insecticide resistance mechanisms [12–17], physical changes that limit insecticide uptake [18] and behavioural adaptations, such as diurnal and outdoor feeding [19, 20], to evade the current insecticide based interventions. Intensified efforts in vector control are, therefore, required to achieve the WHO targets and potentially eradicate malaria in endemic areas. One solution could be to explore the development of alternative control methods.

There is a growing interest in genetically-based control methods due to the availability of Anopheles genomes [21, 22] and development of molecular techniques, such as gene editing by clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 technology [23] among others. The identification of potential genes involved in vector susceptibility to Plasmodium or other factors, such as vector longevity and reproduction would be ideal for genetically-based control interventions to reduce malaria transmission. One such gene is the ecdysone receptor (EcR). The EcR is a nuclear receptor that functions as a ligand controlled transcription factor [24]. The EcR binds to the Ultraspiracle protein (USP) and the 20-hydroxyecdysone (20E) hormone to form the EcR-USP complex [25–27]. Together the functional EcR-USP complex binds to a region of DNA either in the form of an inverted or direct repeat, termed the ecdysone response element (EcRE) to activate transcription of the target gene identified as an “early gene” [24, 28, 29]. Upon activation, these early genes subsequently regulate the expression of several late genes. This process is termed the 20E signalling pathway. Importantly, EcR via the 20E pathway regulates several physiological functions that ultimately affect malaria transmission in vector species [30].

Vector longevity, reproduction and susceptibility to Plasmodium are all factors that affect the density of vectors and/or their competence to transmit malaria [31]. The 20E signaling pathway influences the survival of vectors and treatment of vectors with 20E agonists reduced their longevity [32, 33]. The role that EcR depletion plays in the 20E pathway, i.e. impairing the pathway, by regulating vector longevity however has not yet been characterized in An. funestus. Manipulation of this hormone receptor has provided invaluable insights about the 20E signaling pathway in the mosquito. Egg development is hindered and a decrease in the number of eggs developed is observed in EcR depleted Aedes aegypti and An. gambiae mosquitoes due to decreased 20E signaling [34, 35]. Two genes, Lipophorin (Lp) and Vitellogenin receptor (VgR), regulated by the 20E pathway, largely regulate the reproductive processes vitellogenesis and oogenesis [35–39] therefore to determine if EcR regulates these genes through 20E signaling is of interest. Furthermore, EcR depleted An. gambiae also developed fewer P. falciparum oocysts in comparison to the controls [35]. Implying that functional EcR is required in the 20E signaling pathway, to induce a successful immune response in vectors against Plasmodium. An important gene associated with Plasmodium immunity, leucine rich immune molecule 9 (LRIM 9), regulated by EcR in An. gambiae was discovered to exhibit immunity against Plasmodium berghei in a previous study [40]. The current study therefore aimed to (i) characterize the role of EcR in longevity, (ii) explore its function in fertility, oogenesis and regulation of Lp and VgR genes and (iii) to determine its role in LRIM 9 regulation and immunity against P. falciparum, all in an important African malaria vector, An. funestus.

Methods

Biological material

A laboratory strain of An. funestus mosquitoes originating from Mozambique (FUMOZ) was used in this study. Female FUMOZ mosquitoes were used for all data collection purposes and were aged accordingly. The FUMOZ strain was reared in the Maureen Coetzee insectary at the Wits Research Institute for Malaria (WRIM) under standard insectary conditions at ±26 °C with ±80% humidity and a 12:12 day:night cycle [41].

Gametocytes were produced as per Reader et al. [42]. Briefly, PfNF54 gametocytes were induced from a highly synchronized (> 95%) asexual ring-stage population at 0.5% parasitaemia and 6% haematocrit. Medium was aspirated daily and replaced with fresh medium pre-warmed to 37 °C. Gametocyte health was monitored with microscopy of Giemsa-stained smears. Mature PfNF54
gametocytes (> 98% stage V, 1.5−2.5% gametocytaemia) were used to infect An. funestus.

RNA extraction, DNase I treatment, and cDNA synthesis
RNA was extracted using the TRizol™ Plus RNA Purification Kit (12183555, Invitrogen, USA) according to the manufacturer’s instructions. RNA quality control consisted of spectrophotometry using the Nanodrop One (ND-ONE-W, Thermo Scientific, MA, USA) to determine RNA concentration and purity as well as agarose gel electrophoresis to confirm RNA integrity. A DNase treatment with the TURBO DNA-free™ Kit (AM1907, Ambion, TX, USA) was conducted according to manufacturer’s instructions to remove any contaminating genomic DNA carried over from RNA extraction. Once RNA was free of genomic DNA, First strand cDNA synthesis was conducted with the iScript™ cDNA Synthesis Kit (1708891, BioRad, CA, USA).

Quantitative PCR
Each 1X qPCR reaction consisted of 5 µl of IQ™ SYBR super-mix (1708880, Bio-Rad, CA, USA), 300 nM of each primer, 1 µl of cDNA and nuclease free water to make up a 10 µl reaction. Cycling conditions consisted of an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation, annealing and extension at 94 °C for 20 s, annealing temperature determined by optimization for 25 s and 72 °C for 30 s respectively with a final extension step at 72 °C for 10 min. A melt peak analysis and a no-template control excluding cDNA were included to assess for specificity and contamination, respectively. Reference genes RPS7 and RPL19 were used to normalize samples. Primer sequences are listed in Table 1.

RNA interference
The starting material for the production of dsRNA molecules consisted of a plasmid containing GFP or for EcR, cDNA prepared from RNA using the iScript™ cDNA synthesis kit and subject to PCR to obtain a 484 bp amplicon. Total RNA for this purpose was extracted from female An. funestus at 24 h post-blood meal, since this is the peak of EcR expression [43]. Subsequently, to add T7 promoter sequences to the 5′ end of the GFP clone and EcR template, a PCR was conducted using the T7-containing RNAi primers and the GFP or EcR template. The T7-containing 544 bp GFP and 530 bp EcR templates were concentrated and purified with ethanol precipitation to a final concentration of 1 µg/µl. Double stranded RNA molecules homologous to the EcR and GFP templates were synthesised with the MEGAscript® RNAi Kit.

| Name                  | Sequence (5′-3′) | Tm (°C) | Amplicon size (base pairs) | Reference                      |
|-----------------------|-----------------|---------|---------------------------|-------------------------------|
| An. funestus EcR (forward) | GAT TCT TCC GAC GTA GTG TG | 60      | 484                       | Designed in this study       |
| An. funestus EcR (reverse)   | TCC TCG TTG GGT GAG TTA | 60      |                           |                               |
| An. funestus T7-EcR (forward) | TAA TAC GAC TCA CTA TAG GGA GAG ATT CTT CCG ACG TAG TGT G | 67      | 530                       | Designed in this study       |
| An. funestus T7-EcR (reverse) | TAA TAC GAC TCA CTA TAG GGA GAT CCT CGT TGG GTG AGT TA | 68      |                           |                               |
| T7-GFP (forward)   | TAA TAC GAC TCA CTA TAG GGA GAA CTT AAA CCG CCA CAA GT | 66.5    | 544                       | Designed by Ms. E. Ekoka (WRIM) |
| T7-GFP (reverse)   | TAA TAC GAC TCA CTA TAG GGA GAG GGT GTT GTG GTA GTG | 68.4    |                           |                               |
| qPCR EcR (forward) | GCC GGT AGC ACA AGT AAT AG | 60      | 130                       | Designed in this study       |
| qPCR EcR (reverse) | GAT CGA GCA TTC CGA CAG | 60      |                           |                               |
| LRIM9 (forward)   | CAG TTC TTC ACC GCA TAG TT | 60      | 117                       | Designed in this study       |
| LRIM9 (reverse)   | TTG TCG TCC AGG TAG AGT T | 60      |                           |                               |
| Lp (forward)      | GCT TCG ACA AGG TGT TAG AG | 60      | 104                       | Designed in this study       |
| Lp (reverse)      | AAG ACC AAG AGC GGT AGT | 60      |                           |                               |
| VgR (forward)     | TAC TTA CGG CGG GAC TTA T | 60      | 147                       | Designed in this study       |
| VgR (reverse)     | GGA GCT GAT CCT GTA TTA TGG | 60      |                           |                               |
| RPS7 (forward)    | TTA CTG CTG TGT ACG ATG CC | 60.4    | 134                       | Ameyna et al. [14]            |
| RPS7 (reverse)    | GAT GGT GTG CTG CTG GTT | 62.3    |                           |                               |
| RPL19 (forward)   | GAA ACA CCA ACT CCC GAC A | 60.2    | 223                       | Spillings et al. [6]          |
| RPL19 (reverse)   | TCA ACA GGC GAC GCA ACA CA | 62.3    |                           |                               |
When females were 10 days old they were isolated prior to dsRNA treatment. After injection, the females were placed back with males for the remaining two days to fulfill the 12 day mating period. Mosquitoes that had oviposited seven days post blood meal, they were dissected to determine their mating status and observe if eggs were retained in ovaries.

**Plasmodium infection assay**

*Anopheles funestus* females aged seven to ten days old (to allow for optimal mating and encourage blood feeding) were injected with 10 µg/µl of dsEcR or dsGFP as described previously. Ninety mosquitoes were included per replicate and a total of eight biological replicates were conducted. Subsequent to injection, the 10% sucrose solution was removed and replaced with distilled water to encourage blood feeding. Concurrently, mosquitoes were isolated for RNA extraction, DNase 1 treatment, cDNA synthesis and qPCR. Twenty-four hours after nanoinjection, mosquitoes were offered a *Pf* NF54 infected blood meal (>98% stage V gametocytes, 1.5–2.5% gametocytaemia, 50% (v/v) A + male human serum, Interstate blood bank Inc, Memphis, Tennessee, USA) for 40 min using a glass feeder. Unfed mosquitoes were discarded while fed mosquitoes were maintained on a 10% sucrose solution for eight days post feeding. After this time, mosquitoes were aspirated into ethanol to immobilize them and subsequently transferred to 1× PBS. Mosquito midguts were dissected 8 days post infection and stained on a microscope slide using 0.1% mercurochrome (M7011, Sigma, MO, USA) [44]. Midguts were subsequently viewed under a compound microscope between 20 and 40× magnification and the intensity and prevalence of oocysts in each midgut was counted.

**Statistical analysis**

All qPCR data were analysed using relative gene expression analysis as per 2−∆∆Ct method [45]. The 2−∆∆Ct values were subsequently log transformed and statistical significance was determined using an unpaired student’s t-test [46]. The Kaplan Meier survival curve was used for longevity analysed using the Log-Rank test to calculate
statistical significance (GraphPad Prism 8). Statistical analysis for the eggs oviposited, fertility and *P. falciparum* oocyst intensity were evaluated for normality using the Shapiro–Wilk test. If data were not normally distributed the Mann–Whitney test was used, otherwise an unpaired students t-test was used. To determine statistical significance for the number of ovaries containing mature eggs compared to those with immature follicles, a two tailed Fisher’s exact test was used. *Plasmodium falciparum* oocyst prevalence data was analysed using a Chi-squared test. For all analyses, *p* values < 0.05 were considered to be statistically significant.

**Results**

**Injection of dsEcR reduces EcR expression but does not influence Lp, VgR and LRIM 9 expression in An. funestus**

To assess different phenotypes associated with EcR depletion, *An. funestus* females were injected with dsRNA targeting EcR (dsEcR) alongside dsGFP as a control. Relative expression, as determined by qPCR, was used to confirm gene silencing. EcR was significantly downregulated (*t*(6) = 4.3, *p* = 0.0051, unpaired student’s t-test) with a mean expression ± SEM of −2.23 ± 0.32 fold in dsEcR injected samples compared to −1.50e−06 ± 0.40 fold confirming that the dsEcR injection significantly reduced EcR transcription (Fig. 1A).

Likewise, the expression of several other transcripts *Lp, VgR* and *LRIM 9* previously demonstrated to be involved in mosquito reproduction, or susceptibility to *Plasmodium* species [38, 40, 47], were measured. In dsEcR injected females, *Lp* expression was downregulated to a mean ± SEM of −1.58 ± 0.12 fold compared to 1.33e−06 ± 0.66 fold in dsGFP injected females (Fig. 1B). This difference was however not statistically significant (*t*(4) = 2.35, *p* = 0.0785, unpaired student’s t-test). Expression of *LRIM 9* was reduced to a mean ± SEM of −0.56 ± 0.19 fold in dsEcR injected females compared to dsGFP injected females with a mean ± SEM of −1.00e−06 ± 0.08 fold, however no statistical significance was observed (*t*(4) = 2.703, *p* = 0.0539, unpaired student’s t-test) (Fig. 1C). Moreover, VgR expression was depleted to a mean ± SEM of −0.84 ± 0.03 fold in dsEcR treated females compared to −6.67e−07 ± 0.36 fold in dsGFP treated females (Fig. 1D). This difference was not statistically significant (*t*(4) = 2.327, *p* = 0.0805, unpaired student’s t-test). No significant difference was observed in *Lp, LRIM 9* nor *VgR*, suggesting depletion of EcR does not affect expression of these genes in this study.

**EcR depletion results in decreased longevity in An. funestus**

To determine the effect of EcR depletion on *An. funestus* longevity, mortality was recorded daily in dsEcR and dsGFP treated females until all mosquitoes had succumbed. Mosquitoes injected with dsEcR did not survive past 20 days whereas those injected with dsGFP survived up to 37 days. There was a statistically significant difference in the probability of survival between the two groups (χ²(1, *N* = 240) = 30.18, *p* < 0.0001, Log rank test). The median survival (time taken to reach a survival of 50%) of dsGFP females was 14 (95% CI of ratio: 2.96–5.41) days compared to 3.5 (95% CI of ratio: 0.19 to 0.34) days for females injected with dsEcR. This translates to a 54% decrease in longevity in dsEcR injected mosquitoes (Fig. 2). No significant difference (χ²(1, *N* = 240) = 2.011, *p* = 0.1561, Log rank test) was observed between the dsGFP and uninjected control groups, suggesting that the nanoinjection procedure did not influence longevity (Additional file 4: Table S1).

**EcR depletion decreases reproductive success in An. funestus.**

To determine EcRs effect on fecundity, the number of eggs oviposited by mated females was counted in dsEcR and dsGFP treated females. Although a total number of 157 females were induced to lay eggs, the number of females that oviposited were low. Altogether, 9 dsEcR treated females and 19 dsGFP treated females oviposited. No significant difference was observed in the blood feeding rates between dsEcR and dsGFP groups (Additional file 3: Fig. S3). The mean number of eggs per female ± SEM oviposited in dsEcR treated females was 36.11 ± 4.01 compared to 48.11 ± 4.12 in dsGFP treated females (Fig. 3A). This decrease however, was not significant (*t*(26) = 1.813, *p* = 0.0814, unpaired student’s t-test), suggesting that EcR depletion does not affect the number of eggs oviposited. Additionally no statistical difference (*t*(91) = 0.1522, *p* = 0.8793, unpaired student’s t-test) was observed between the dsGFP and uninjected control groups, suggesting that the nanoinjection procedure had no effect on the number of eggs oviposited (Additional file 4: Table S1). Those mosquitoes that had not oviposited were dissected to observe if eggs were retained in the ovaries. Surprisingly, the majority of ovaries of dsEcR injected mated *An. funestus* females closely resembled that of virgin *An. funestus* females, containing immature and undifferentiated oocytes whereas the majority of dsGFP injected females ovaries contained mature eggs (Fig. 3B). In dsEcR injected females, 32% of mated females developed eggs whereas 89% of dsGFP injected females developed eggs. This difference observed was statistically significantly different (*p* < 0.0001, Fisher’s exact test) (Fig. 3C). This suggests that dsEcR injected *An. funestus* females typically develop fewer eggs compared to controls. Together these results substantiate that the depletion of EcR had a deleterious effect on oogenesis by preventing the development of oocytes into mature
eggs in *An. funestus*. No significant difference (*p > 0.9999, Fisher’s exact test*) was observed between the dsGFP and uninjected control groups, indicating the nanoinjection procedure did not affect the oviposition process (Additional file 4: Table S1). Although very few dsEcR injected females oviposited eggs (*n = 9*), fertility was also compared between the dsEcR and dsGFP treated mosquitoes by monitoring the hatching of the eggs.

![Graphs showing log transformed relative fold change normalized against reference genes for EcR, Lp, LRIM 9 and VgR genes in dsEcR treated female An. funestus compared to the dsGFP treated control. Depletion of EcR resulted in the significant downregulation of EcR. The Lp, LRIM 9 and VgR genes were not significantly downregulated upon depletion of EcR. Error bars indicate standard error of mean (SEM). ** denotes *p < 0.01* and ns = not statistically significant. At least three biological replicates were used for each gene.](image)
oviposited. The median percentage fertility (25–75% percentile) in dsEcR treated mated females was significantly lower at 69% (55–80%) compared to 86% (76–93%) in dsGFP treated mated females (t(26) = 3.169, p = 0.0039, unpaired student’s t-test) (Fig. 3D). These results indicate that EcR depletion results in An. funestus females that are less fertile than control females. Furthermore, no significant differences (t(46) = 0.5975, p = 0.5531, unpaired student’s t-test) existed between the dsGFP and uninjected control groups, corroborating that the nanoinjection procedure did not affect fertility in An. funestus (Additional file 4: Table S1). Taken together, results showed that EcR influences reproductive processes such as oogenesis and fertility in An. funestus.

EcR depletion decreases P. falciparum infection intensity in An. funestus.

The effect of EcR depletion on An. funestus infection by P. falciparum was determined by comparing the intensity of oocysts and the prevalence of infection between dsEcR-injected and dsGFP-injected mosquitoes. Oocyst intensity was significantly decreased by 50% in dsEcR treated An. funestus females with 3.80 ± 0.54 oocysts per midgut compared to dsGFP treated An. funestus females with 9.37 ± 1.17 oocysts per midgut (Mann–Whitney U = 1751, p = 0.0013, 8 biological replicates) (Fig. 4). EcR depletion significantly decreased the number of oocysts that developed in the midgut. Additionally, the mean prevalence of infected An. funestus females was 69.45% (± 6.05) in dsEcR injected An. funestus females compared to 80.46% (± 4.28) in dsGFP injected An. funestus females (Fig. 4). No significance in infection prevalence was observed between dsEcR and dsGFP treated females (χ² (1, N = 720) = 2.205, p = 0.1376, Chi-squared test), suggesting that EcR depletion does not influence the prevalence of infection by P. falciparum. These results corroborate that EcR influences P. falciparum oocyst development in An. funestus but does not affect the incidence of infection by P. falciparum. No significant difference was observed between the dsGFP and uninjected control groups for either the P. falciparum infection intensity (Mann–Whitney U = 2300, p = 0.5337) or prevalence (χ² (1, N = 720) = 0.2584, p = 0.6112, Chi-squared test), suggesting that the nanoinjection procedure did not influence these processes (Additional file 4: Table S1).

Discussion

This study provides the first experimental evidence that EcR plays a crucial biological role in the major vector species An. funestus. Vectors such as An. funestus among others require novel control strategies due to the increase in insecticide resistance which reduces the efficacy of current vector control interventions. The transcription factor EcR was identified as a potential gene to be investigated due to its pleiotropic effects discovered in mosquitoes [30]. The current study sought out to determine how 20E signaling, using EcR as a proxy, regulates several factors that influence vector density and competence in An. funestus.

Depletion of EcR in An. funestus reduced longevity by over a half compared to the control. Similarly, in other insect species such as Drosophila melanogaster, Nilaparvata lugens and Sitobion avenae, reduction of EcR using dsEcR resulted in decreased longevity [48–50]. Together these findings confirm that reducing transcription of EcR results in deleterious effects on longevity, corroborating that EcR is essential in the regulation of longevity. Longevity plays a major role in malaria transmission. Wild An. funestus can survive approximately 30 days [2] and in this time are able to complete the extrinsic incubation period (EIP) required for Plasmodium parasites to mature and become infectious to humans. Several factors such as temperature, vector species, parasite species, vector nutrition and survival can influence the EIP [51]. Defining the duration of the EIP is therefore difficult, however it typically ranges from 12 to 16 days [51–53]. Reducing EcR in An. funestus females could therefore decrease longevity to a point where the EIP cannot be completed in the field, thereby reducing malaria transmission. Additionally, decreased vector longevity can reduce the vector to human ratio, possibly reducing malaria transmission rates [31].

Malaria transmission and vector density are also impacted by vector reproduction. Moreover, ingestion of a blood meal (a prerequisite for anautogenous mosquito reproduction) increases the risk of Plasmodium
transmission. Two reproductive processes, fecundity and fertility were assessed after EcR depletion. Impaired oogenesis was observed after reduction of EcR levels in An. funestus. Consistent with these findings, other studies reported reduced follicle length [34] and a reduction in the number of eggs developed [35] after EcR depletion in Ae. aegypti and An. gambiae. Lp and VgR are two important proteins involved in lipid transport to oocytes and the uptake of the YPP vitellogenin providing nutrients for oocytes to develop.
respectively [35–39]. In the current study, depletion of EcR did not affect the expression of the Lp and VgR genes, however 20E signaling was confirmed to regulate Lp and Vg gene expression in other studies [35, 38, 54, 55]. However, these studies are not directly comparable due to differences in methodologies used and in addition discrepancy may be due to the complex interaction of several other genes in the 20E pathway suggesting that EcR is not solely responsible for the regulation of these genes.

The profound mechanism of gene regulation by EcR is not yet fully understood but studies have discovered several key elements of this process. Ecdysone responsive genes such as Vg i.e. those containing EcR binding sites, also contain binding sites for several other transcription factors which regulate gene expression differentially depending on temporal and spatial requirements [55]. Changes in gene regulation in these ecdysone responsive genes are brought about indirectly by EcR coupled with the action of other transcription factors [56]. However,
EcR can also directly regulate the specific temporal or spatial expression of genes [57]. Moreover, ecdysone responsive genes such as Lp are regulated differentially in mosquito species such as Ae. aegypti and An. gambiae through a currently unknown mechanism [35, 38]. The current study has limited information on gene regulation by EcR but the above studies confirm that future studies should concentrate on this process to provide a clearer understanding of this process in An. funestus.

Depletion of EcR significantly reduced fertility in An. funestus. A source of uncertainty arises from the small sample size in this study, however it is known that An. funestus is refractory to colonization due to low blood feeding rates, mating success and oviposition rates (Koekemoer, pers comm). This experiment should be repeated to provide more clarity. Nevertheless, results from the current study are noteworthy, a haem peroxidase (HPX15) was found to control fertility in An. gambiae [58]. This enzyme requires 20E for normal functioning [58] and providing a mechanistic basis for the role of EcR in fertility. Silencing HPX15 was found to decrease fertility in females over multiple gonotrophic cycles [58]. EcRs regulation of fertility could, therefore, be due to its function as a transcription factor of HPX15, but will have to be investigated in future.

Furthermore, EcR was found to control transcription of the immune molecule LRIM 9 in a study by Upton et al. [40]. Depletion of LRIM 9 with dsLRIM 9 in An. gambiae resulted in a threefold higher P. berghei oocyst intensity, validating the importance of LRIM 9 in providing immunity against P. berghei [40]. However, this was not evident when EcR was depleted in An. funestus as a decrease in P. falciparum oocyst intensity was observed instead. This discrepancy could presumably be due to a variance in the activity of LRIM 9 against different Plasmodium species studied. Similarly, it could be attributed to differences in the immune systems and regulation of Plasmodium infection between An. gambiae and An. funestus. Immune genes of An. funestus are genetically more similar to the Asian malaria vector Anopheles stephensi than An. gambiae due to evolutionary divergences in these vectors [59–61]. The contradiction in these results suggest a different mechanism is involved in decreasing the intensity of P. falciparum oocysts when EcR is knocked down in An. funestus. Similar to the current study, Werling et al. [35] observed a decrease in oocyst intensity when dsEcR treated An. gambiae was infected with P. falciparum. Strikingly, this decrease in P. falciparum oocysts was accompanied by larger, faster maturing sporozoites which was brought about by Lp [35]. Presently, limitations such as a decrease in the longevity of dsEcR injected females (in this study) limited the sample size of infected mosquitoes available 16–18 days post infection and hindered the investigation of faster maturing sporozoites in An. funestus. These findings should however be further investigated in An. funestus once limitations are overcome to determine if the reduction in oocyst intensity results in faster maturing sporozoites and a more rapid EIP for P. falciparum or if a different immune strategy is adopted by the different Anopheles species. If the latter is proven true, an RNAi based approach targeting An. funestus EcR will need to be taken into consideration. It will also be interesting to determine the length of time that that EcR remains depleted in An. funestus after dsEcR treatment as this is a key factor in the development for a potential RNAi based control method.

If it is found to be true that depleting An. funestus EcR in any way increases parasite infectivity, genetically based control methods, such as gene drives [62], paratransgenesis [63], transmission blocking vaccines [64] or simply any form of EcR gene modification cannot be considered. Additionally, it will be important to confirm these findings on wild An. funestus populations from different geographical areas. Fortunately, decreasing EcR levels is only one of the possibilities to target ecdysone signalling to reduce malaria transmission. Nonsteroidal 20-hydroxyecdysone agonists share structural similarities with 20E, hence they are able to competitively bind to the EcR-USP complex, amplifying 20E signalling and all of its effects [65]. Five 20E agonists, namely chromafenozide, fufenoizide, halofenozide, methoxyfenozide and tebufenozide are currently available as insecticides targeting agricultural pests [66, 67]. These 20E agonists are promising as potential malaria vector control strategies as they exhibit low toxicity to non-target organisms and penetrate the mosquito cuticle [66–68]. The efficacy of some of these compounds has also been demonstrated against several Anopheles vectors [32, 33, 69].

**Conclusions**

The current study provided some insight on the biological function of EcR in An. funestus as a regulator of longevity, oogenesis, fertility and susceptibility to P. falciparum. Further research is required to determine the mode of action of, and genes involved in, EcRs regulation of pathways governing longevity, reproductive success and importantly Plasmodium infectivity in An. funestus. For example, the complex interaction of EcR and the 20E signalling pathway can further be elucidated through next generation RNA sequencing. This will allow for the identification of the complete profile of early and late genes regulated by EcR, providing a holistic overview of this pathway. These genes can thereafter be examined to determine their functions in regulating mosquito physiological process that potentially target vector density and competence.
Subsequently, success in both laboratory and field based aspects of this research could result in approved EcR based vector control methods that will potentially reduce malaria transmission.

Abbreviations
20E: 20-Hydroxyecdysone; dsRNA: Double stranded RNA; EcR: Ecdysone receptor; GFP: Green fluorescent protein; IRS: Indoor residual spraying; LLIN: Long-lasting insecticidal nets; Lp: Lipophorin; LRIM 9: Leucine rich immune molecule 9; VgR: Vitellogenin receptor; WHO: World Health Organization.

Supplementary Information
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Additional file 1: Figure S1. Relative EcR expression levels in dsEcR injected An. funestus females compared to dsGFP injected An. funestus females. The EcR gene was knocked down in dsEcR injected An. funestus females as EcR expression levels were drastically reduced compared to the GFP control. Statistically significant knockdown was evident in dsEcR injected An. funestus females 24, 48 and 72 h after injection as EcR expression in dsEcR injected An. funestus females was 0.11 ± 0.006 (p < 0.05), 0.01 ± 0.001 (p < 0.01) and 0.2 ± 0.06 (p < 0.05) respectively when compared to the GFP injected control of 1. This data confirmed EcR knockdown in An. funestus females injected with dsEcR. Data is representative of 2 biological replicates and normalised using an average of RPS7 and RPL19 reference genes. Expression levels calculated using relative quantification method (ΔΔCt). At each time point statistical significance was assessed with the unpaired student’s t-test. *p < 0.05, **p< 0.01, ***p < 0.001. Error bars represent standard deviation.

Additional file 2: Figure S2. The highest mating success rate was achieved when An. funestus males and females are combined for 12 days after which no further increases are observed. The percentage mating success rate increased progressively until it reached its highest value of 62.2% after 12 days of mating. After this point, the mating success rate reached a plateau until day 20. Statistical significance was calculated using one-way ANOVA with Tukey’s post hoc analysis to correct for multiple comparison. Data represents the means of 3 biological replicates. Error bars represent standard deviation of means. ns = not statistically significant p > 0.05; * = p < 0.01; ** = p < 0.001; *** = p < 0.0001. (n) = number of females per time point across 3 biological replicates.

Additional file 3: Figure S3. Blood feeding rates did not differ between treatment groups. Insignificant differences amongst treatment groups confirmed that the blood feeding rates did not influence any changes observed in the phenotypes of dsEcR treated An. funestus females (p > 0.05). Statistical significance calculated using an unpaired student’s t-test. Error bars represent standard deviation. ns = not statistically significant p > 0.05.

Additional file 4: Table S1. Statistical significance between dsGFP and un.injected controls from the various biological assays conducted.

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Authors’ contributions
LLK and EE conceptualized the study. LLK, EE, LN and SM contributed to study design. LMB and JR provided PNF54 gametocyte cultures and contributed reagents/materials for PNF54 infection. SM, EE and LN performed the experiments. SM analysed the data. LLK, EE and LN supervised the project. SM wrote the first draft of the manuscript. All authors contributed to subsequent versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All in vitro experiments involving human blood donors and human malaria parasites hold ethics approval from the University of Pretoria Research Health Sciences Ethics Committee (506/2018) and Natural and Agricultural Sciences Ethics Committee (NAS180000094). The work abides by the Declaration of Helsinki principles.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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