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***Caudal* is a negative regulator of the *Anopheles* IMD Pathway that controls resistance to *P. falciparum* infection**

April M. Clayton, Chris M. Cirimotich¹, Yuemei Dong, and George Dimopoulos[#]

W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA

Abstract

Malaria parasite transmission depends upon the successful development of *Plasmodium* in its *Anopheles* mosquito vector. The mosquito's innate immune system constitutes a major bottleneck for parasite population growth. We show here that in *Anopheles gambiae*, the midgut-specific transcription factor *Caudal* acts as a negative regulator in the Imd pathway-mediated immune response against the human malaria parasite *P. falciparum*. *Caudal* also modulates the mosquito midgut bacterial flora. RNAi-mediated silencing of *Caudal* enhanced the mosquito's resistance to bacterial infections and increased the transcriptional abundance of key immune effector genes. Interestingly, *Caudal*'s silencing resulted in an increased lifespan of the mosquito, while it impaired reproductive fitness with respect to egg laying and hatching.

Keywords

Anopheles; mosquito; *Plasmodium*; innate immunity; *Caudal*; Imd pathway

1. INTRODUCTION*

Malaria, caused by the *Plasmodium* parasite, affects approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against *Plasmodium* and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission (Cirimotich et al., 2011a).

Malaria transmission between humans depends on the successful completion of the parasite's lifecycle in the mosquito. This journey begins with the ingestion of *Plasmodium* gametocytes by the female mosquito through a blood meal. The *Plasmodium* male and

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[#]Corresponding author. Address: W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA. Tel.: 1-443-287-0128. Fax: 1-410-955-0105. gdimopou@jhsp.edu.

¹Present address: Battelle Memorial Institute, 1001 Research Park Boulevard # 400, Charlottesville, VA 22911, USA

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***Abbreviations:** *Cad*, *Caudal*; *Pf*, *Plasmodium falciparum*; Imd, immune deficiency; RNAi, RNA interference; dsRNA, double-stranded RNA; CFU, colony-forming unit; ROS, reactive oxygen species; Cp, carboxypeptidase; ANK, ankyrin; TEP1, thioester-containing protein 1; FBN9, fibrinogen immunorectin 9; APL1, *Anopheles Plasmodium*-responsive Leucine-rich repeat protein 1; APL2, *Anopheles Plasmodium*-responsive Leucine-rich repeat protein 2; LRIM1, Leucine-Rich Immune Molecule 1; AMPs, antimicrobial peptides; *Cec-1*, *Cecropin-1*; *Cec-3*, *Cecropin-3*; *Def-1*, *Defensin-1*; *Gam*, *Gambicin*.

female gametocytes differentiate in the mosquito's midgut lumen, where fertilization produces zygotes. The zygotes are transformed into motile ookinetes that invade and migrate across the midgut epithelium at roughly 18-36 h post-ingestion. Once the ookinetes have reached the basal side of the midgut epithelium, they transform into oocysts and subsequently undergo several rounds of mitosis. The oocysts then rupture, releasing thousands of sporozoites into the mosquito hemocoel about 14 days after the infectious blood meal. The sporozoites migrate through the mosquito hemolymph and invade the salivary glands. During the female *Anopheles*' next blood meal, these *Plasmodium* sporozoites are injected into the human host, thereby completing the sexual cycle within the mosquito vector (reviewed in (Levashina, 2004; Vlachou et al., 2006)).

The innate immune system of *Anopheles*, the vector's main line of defense against parasites, fungi, bacteria, and viruses, is engaged at multiple stages of *Plasmodium* infection (Cirimotich et al., 2010; Levashina, 2004; Meister et al., 2004; Michel and Kafatos, 2005). Much insight concerning this innate immune defense has been gathered from studies in *Drosophila* (reviewed in (Aggarwal and Silverman, 2008; Meister et al., 2004)). The mosquito's anti-*Plasmodium* and antibacterial defenses are largely controlled by the Toll and Imd (immune deficiency) NF- κ B immune signaling pathways. Infection-responsive activation of the Toll and Imd pathways ultimately leads to the nuclear translocation of the NF- κ B transcription factors *Rel1* and *Rel2*, respectively. These transcription factors are negatively regulated in the cytoplasm by *Cactus* and *Caspar*, respectively. Elicitation of the Toll and Imd pathways causes the degradation of *Cactus* and *Caspar*, allowing the *Rel* factors to enter the nucleus and transcriptionally activate immune effector genes such as antimicrobial peptides (AMPs) and other factors. Through alternative splicing, the *Rel2* gene produces a full-length form (*Rel2-F*) that includes the carboxyl-terminal ankyrin (ANK) and death domains, and a shorter form (*Rel2-S*) lacking such domains. The *Rel2-S* form is constitutively translocated to the nucleus, where it regulates the transcription of immune genes (Meister et al., 2005).

While the Toll pathway has been shown to defend against *P. berghei* and *P. falciparum*, the Imd pathway has emerged as the most effective pathway in the defense against the human malaria parasite *P. falciparum* (Frolet et al., 2006; Garver et al., 2012; Garver et al., 2009; Mitri et al., 2009). Specifically, investigations from our lab demonstrated that depletion of *Caspar*, a negative regulator of *Rel2*, results in an Imd pathway-mediated immune defense that confers almost complete refractoriness to *P. falciparum* in three major *Anopheles* malaria vector species: *An. gambiae*, *An. stephensi*, and *An. albimanus*. Depletion of the Toll pathway negative regulator *Cactus* resulted in a significantly greater resistance to infection with the rodent malaria parasite *P. berghei* (Garver et al., 2009). Our previous work has also shown that these two *Plasmodium* spp. elicit diverse innate immune responses at the gene transcript level (Dong et al., 2006a). Previous studies conducted by us and by others have identified a diverse repertoire of anti-*Plasmodium* immune effectors regulated by *Rel2*, including APL1, TEPI, LRRD7 (APL2), FBN9, and LRIM1 (Blandin et al., 2004; Dong et al., 2006a; Fraiture et al., 2009; Meister et al., 2005; Mitri et al., 2009; Povelones et al., 2009; Riehle et al., 2008; Rottschaefer et al., 2011). We have also substantiated and characterized the potency of the Imd pathway in the anti-*Plasmodium* defense through the use of genetically modified immune-enhanced *Anopheles* mosquitoes that express blood meal-inducible *Rel2* in both the midgut and fat body tissues (Dong et al., 2011). The transient activation of this transgene resulted in almost complete resistance to the human malaria parasite at a negligible fitness cost, prompting further investigation of this system as an innovative malaria control strategy (Dong et al., 2011). We have also shown that the natural bacterial flora of the mosquito midgut elicits a basal level of immune activity mediated by AMPs and other effector genes that act against *Plasmodium* and thus primes the mosquito for infection (Dong et al., 2009).

The potency of the Imd pathway in the anti-*P. falciparum* response warrants further dissection in light of recent studies (Dong et al., 2011; Garver et al., 2012; Garver et al., 2009). There are, for example, only a few studies detailing the regulation of *Rel2* once it has been translocated to the nucleus. Here, we show that the transcription factor *Caudal* (*Cad*) is an antagonist of *Rel2*, and thus a negative regulator of the Imd pathway in the *Anopheles* mosquito. While our studies support previous work performed in adult *Drosophila*, we show for the first time a role for *Cad* in the immunity of the adult *Anopheles* mosquito to *Plasmodium* infection and its role in influencing Anopheline fitness.

Findings in *Drosophila* identify *Cad* as a homeobox transcription factor that regulates posterior and intestinal development of the *Drosophila* embryo, and it has also been shown to be required for embryogenesis in nematodes, other arthropods, and vertebrates (Epstein et al., 1997; Hunter and Kenyon, 1996; Mlodzik et al., 1985; Mlodzik and Gehring, 1987; Schoppmeier et al., 2009; van den Akker et al., 2002; Whittle et al., 1986; Wilson et al., 2010). *Cad* in adult *Drosophila* is predominantly expressed in the posterior midgut, where it is involved in the maintenance of midgut-specific gene transcription (Choi et al., 2008; Ryu et al., 2004). Ryu and colleagues (Ryu et al., 2008) showed that silencing *Cad* led to the overexpression of certain AMPs and a modulation of the midgut microbiota. Their study emphasized the antagonistic relationship between *Cad* and *Relish*, a relationship that ensures a balance between immune defense and microbiota homeostasis in the gut (Charroux and Royet, 2010; Leulier and Royet, 2009; Muyskens and Guillemin, 2008; Royet, 2011; Ryu et al., 2008).

In our study, RNAi-mediated silencing of *Cad* specifically compromised *P. falciparum* development in the gut tissue, suppressed the midgut microflora, and enhanced resistance to systemic bacterial infections, most likely by causing an increased transcriptional abundance of AMPs and other effector genes. Interestingly, *Cad* silencing resulted in increased longevity, but it impaired the mosquitoes' fecundity and fertility.

2. MATERIALS AND METHODS

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Commercial anonymous human blood was used for parasite cultures and mosquito feeding, and informed consent was therefore not required. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

2.2. Mosquito rearing

An. gambiae Keele strain mosquitoes were maintained under laboratory conditions at 27°C and 80% humidity with a 12 h day-night cycle. Larvae were reared on cat food pellets and ground fish food supplement. Adult mosquitoes were maintained on 10% sucrose and fed on mouse blood (mice were anesthetized with ketamine) for egg production (Benedict, 1997).

2.3. RNA isolation, quantitative real-time PCR (qRT-PCR), and RNA interference (RNAi)-mediated gene silencing

RNA was extracted and quantified (in triplicate samples) in different tissues (whole mosquito, gut, and fat body) using the RNeasy kit (Qiagen), and cDNA was prepared using the oligo(dT₂₀) primer and the Invitrogen Superscript III reverse transcriptase according to standard methodology. The quantitative real-time PCR (qRT-PCR) and RNAi gene-silencing assays were carried out according to (Dong et al., 2006a), and the ribosomal

protein *S7* gene was used for normalization of the cDNA templates. The fold change in the gene expression and the gene silencing efficiency (from the RNAi assays) were calculated according to the standard $E^{\Delta\Delta C_t}$ method (Pfaffl, 2001) when both primer efficiencies of the GOI (gene of interest) and the ribosomal *S7* gene were equal. The primer efficiencies were determined as described in (Pfaffl, 2001).

Several *An. gambiae* immune genes were screened for either anti-*Plasmodium* or antibacterial defense activity using RNA interference (RNAi) in wildtype mosquitoes. For these assays, the mRNA for the specific gene was selectively depleted from the adult female mosquitoes using established RNAi methodology (Blandin et al., 2002). The dsRNA injection assay of different genes was repeated three independent times with at least 50-80 mosquitoes in each experiment; the *GFP* dsRNA-injected mosquitoes served as controls. The RNAi gene silencing efficiencies were determined at 2-3 d post dsRNA injection for all genes tested and compared to the *GFP* dsRNA-injected control mosquitoes. The primers used for dsRNA synthesis and silencing verification, together with the gene silencing efficiencies, are presented in Supplementary Table S1. Additionally, a time course of the gene silencing efficiency of *Caudal* is represented in Supplementary Figure S1.

2.4. *P. falciparum* and *P. berghei* infection assays

To determine anti-*Plasmodium* activity, mosquitoes were fed on NF54 *P. falciparum* gametocyte cultures (0.01-0.05%, 0.05-0.1%, or 0.1-0.5% gametocytemia) (provided by the Johns Hopkins Malaria Institute Core Facility) through artificial membranes at 37°C or on a *P. berghei* ANKA-infected Swiss Webster mouse (at 19°C) (Dong et al., 2006a). The adult mosquitoes were starved for 8 to 12 h prior to feeding to ensure engorgement. To determine oocyst numbers, unfed mosquitoes were first removed, and the remaining mosquitoes were incubated for a further 7 days at 27°C or 13 days at 19°C for *P. falciparum* or *P. berghei*, respectively. Midguts were dissected out in PBS, stained with 0.2% mercurochrome, and examined using a light-contrast microscope (Olympus). Three biological replicates were used in each experiment.

A previously described method (Cirimotich et al., 2011b) was used to determine the sporozoite loads in the salivary glands of the infected mosquitoes. In brief, salivary glands were dissected; individual glands were then placed in Eppendorf tubes with 120 µl of PBS and homogenized (on ice). The homogenate was centrifuged at 8,000 rpm for 10 min, and approximately 90 µl of the supernatant was removed. The sporozoites were resuspended in the final 30 µl of PBS, and 10 µl of this suspension was placed in a Nuebauer counting chamber and counted after 10 min with a Leica phase-contrast microscope at 400x magnification.

The dot plots of the oocyst and sporozoite numbers in each gut epithelium and salivary gland, respectively, for each treatment were generated using the GraphPad Prism5 software, along with the median value indicated. Statistical significance indicated on the dot plots was derived from the Mann-Whitney Test. Additionally, when necessary, the Kruskal-Wallis test was performed with a Dunn's multiple comparison post-test. See Supplementary Table S2 for statistical analyses and prevalence rates of oocyst or sporozoite loads in dsRNA-treated mosquito midguts or salivary glands, respectively. Statistical significance was denoted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

2.5. Characterization of proliferated midgut microbial flora and Bacterial challenge survival assays

Isolation and colony-forming unit (CFU) enumeration of endogenous gut bacteria from 2-3 d old *Cad* and *GFP* dsRNA-treated mosquitoes were performed as previously described

(Dong et al., 2009). Briefly, mosquitoes were surfaced-sterilized by dipping them in 100-70% EtOH for 2 min and then rinsing them with 1X PBS twice for 1 min each. Midguts were dissected from each individual mosquito over a sterile glass slide containing a drop of 1X PBS, then transferred to a microcentrifuge tube containing 150 μ l of sterile 1X PBS, and macerated for 30 sec. Three 10-fold serial dilutions were then plated on LB agar and kept at room temperature for 48 h. Initial isolation was based on morphology, color, and size of the colony and was followed by molecular identification via 16S rDNA sequencing. Primers used to amplify the 16S rDNA were: Forward, AGAGTTTGATCCTGGCTCAG; Reverse (degenerate), TACGGYTACGCTTGTTACGACT (Cirimotich et al., 2011b).

Survival assays were conducted following bacterial challenges of *Cad*-, *Cactus*-, *Caspar*-, and *GFP* dsRNA-injected mosquitoes according to established protocols (Antonova et al., 2009; Dong et al., 2006a) with minor modifications. In brief, at 3-4 d post-injection, mosquitoes were injected in the thorax via a nano-injector (Nanoject, Drummond) with 69 nl of bacterial suspension (OD₆₀₀ 3.5 of *E. coli* –approximately 5×10^{15} cells/mL- or OD₆₀₀ 0.8 *S. aureus*-approximately 7×10^{15} cells/mL). For negative controls, 69 nl of 1X PBS was injected into control and experimental group mosquitoes. Dead mosquitoes were counted daily over a 7 d period after challenge with bacteria or 1X PBS. Approximately 20-30 mosquitoes were used for each group of injected mosquitoes, and three replicates were performed for all experiments. Survival patterns were determined using Kaplan-Meier survival analysis, and a log-rank test was used for significance evaluation with the GraphPad Prism5 software as described in (Dong et al., 2006b). To clarify, the overall significant trend refers to the log rank test result of the Kaplan-Meier analysis. The log rank test compares survivorship among experimental groups to the control group. The analysis used did not measure if the experimental groups were significant from each other. See Supplementary Table S3 for Kaplan-Meier survival analyses of dsRNA-treated mosquitoes after systemic bacterial infections.

2.6. Longevity, fecundity, and fertility assays

For the longevity assays of mosquitoes maintained on 10% sucrose solution only, approximately 30 to 80 3-4 d old adult female *Cad* and *GFP* dsRNA-treated mosquitoes were kept in a wax-lined cardboard cup at 27°C with 70% humidity. In a second cohort, the mosquitoes' survival rate was also monitored by providing a single naïve human blood meal to 3-4 d old dsRNA-treated mosquitoes, which were then maintained on a 10% sucrose solution. Alternatively, a cohort of 3-4 d old dsRNA-treated mosquitoes were provided with a *Pf*-infected blood meal (0.01-0.05% gametocytemia) and then maintained on a 10% sucrose solution. Three independent experiments were performed for each cohort, and all cohorts were monitored daily for survival. Monitoring continued until all mosquitoes had perished. The survival percentage represents the mean survival percentage for all three biological replicates as described in (Dong et al., 2011; Garver et al., 2009). Kaplan-Meier survival analysis and log-rank tests were performed as previously described and are available in Supplementary Table S4.

For the fecundity assays, approximately 40-90 3-4 d old *Cad* and *GFP* dsRNA-injected and uninjected mosquitoes were allowed to feed on human blood through an artificial membrane feeder for 30 min. The fed mosquitoes were transferred to individual vials (one mosquito per vial) outfitted with cotton and with moistened filter paper at the bottom of the vial. Individual chambers were incubated under normal rearing conditions. Eggs oviposited on filter paper were counted after 2 d using light microscopy. Female mosquitoes that did not produce eggs on day 2 were maintained and re-examined on day 3. After each count, eggs were submerged in a standard larval pan for rearing according to standard methods. First- to second-instar larvae were counted to determine the larval hatch rate. The fecundity and

larval hatch-rate assays were performed on three biological replicates, and the number of eggs laid by each female and the respective hatch rates were used to calculate mean values. Statistical significance was determined using the Mann-Whitney test. Statistical analyses are available in Supplementary Table S5.

3. RESULTS

3.1. Caudal is a midgut-specific transcriptional regulator of immune effector genes

In *Drosophila*, *Cad* is a developmental transcription factor as well as a midgut-specific antagonist of the Imd pathway transcription factor *Relish* (Ryu et al., 2008; Ryu et al., 2004). Because the innate immune signaling pathways are highly conserved between *Drosophila* and *Anopheles* (Waterhouse et al., 2007), we hypothesized that *Cad* (Gene ID: AGAP009646) might play a similar role in *An. gambiae*. To test this hypothesis, we assayed *Cad* transcript abundance in the adult female mosquito midgut, fat body, and remaining tissue (carcass). Consistent with the expression pattern observed in adult *Drosophila* (Choi et al., 2008; Ryu et al., 2008; Ryu et al., 2004), we saw an almost 18-fold higher abundance of *An. gambiae* *Cad* transcripts in the midgut than in the fat body and a ~100-fold higher abundance than in the carcass (Fig. 1A).

We next hypothesized that mosquito *Cad* also negatively regulates gene expression by the Imd pathway transcription factor *Rel2*. To test this hypothesis, we analyzed mRNA levels of the *Rel2*-regulated antimicrobial peptide (AMP) genes *Cecropin-1* (*Cec-1*), *Cecropin-3* (*Cec-3*), *Defensin-1* (*Def-1*), and *Gambicin* (*Gam*) and the anti-*Plasmodium* effector genes *FBN9* and *LRRD7* at 12 h and 24 h in the midgut following RNAi-mediated silencing of *Cad* as compared to mRNA levels in *GFP* dsRNA-treated mosquito midguts (Fig. 1B). This panel of effector genes represents the three major classes of AMPs and anti-*P. falciparum* effectors, and they are all regulated by microbial challenge (Blandin et al., 2002; Dimopoulos et al., 1997; Dong et al., 2006a; Kim et al., 2004; Luna et al., 2006; Meister et al., 2005; Vizioli et al., 2000; Vizioli et al., 2001a; Vizioli et al., 2001b; Zheng and Zheng, 2002) (Fig. 1B). An approximately 2-fold increase in the transcript abundance of *Cec-1*, *Cec-3*, and *Def-1* was observed at 12 h after *Cad* silencing, while the abundance of *Gam* transcripts decreased by almost 2-fold. At 24 h after *Cad* silencing, we observed a 2- to 3-fold change in the abundance of *Def-1*, *Cec-1*, *Cec-3*, and *Gam* transcripts. The anti-*Plasmodium* effectors *FBN9* and *LRRD7* displayed a ~2-fold increase in transcript abundance at 12 h after *Cad* silencing, but they were suppressed to almost the same degree at 24 h after silencing. The down regulation observed for these two effectors may be explained by complex temporal transcriptional regulation. Additionally, as a transcription factor, *Cad* may function in multiple pathways. Similar dynamic profiles of immune gene regulation has been observed in which certain effectors are up regulated while others are down regulated at a specific time point in previous studies where components of the Imd-*Rel2* pathway were manipulated in *Anopheles* (Dong et al., 2011; Garver et al., 2009).

3.2. Caudal controls *P. falciparum* but not *P. berghei* infection

Since *Cad* is a midgut-specific transcriptional modulator of immune effector genes (Fig. 1B), we subsequently investigated the effect of its silencing on the mosquito's susceptibility to *P. berghei* and *P. falciparum* infection of the midgut. The median number of *P. falciparum* oocysts in *Cad*-silenced mosquitoes was significantly lower (~3-fold) than in *GFP* dsRNA-injected controls at 7 days after feeding on a gametocyte culture (Fig. 2A). However, the median number of oocysts in *Cad*-silenced mosquitoes infected with the rodent parasite *P. berghei* (Fig. 2B) was not significantly different from that of control *GFP* dsRNA-treated mosquitoes.

Our data indicate that the negative regulation of innate immune responses by *Cad* affects *Plasmodium* development in a pattern similar to the Imd pathway (Fig. 2A, B) (Dong et al., 2011; Garver et al., 2012; Garver et al., 2009; Mitri et al., 2009). To further investigate *Cad*'s relationship with the Imd and Toll pathway-mediated defenses against *Plasmodium* infection of the midgut tissue, we co-silenced *Cad* with factors of these two pathways and assessed the effect of this silencing on parasite infection (Fig. 2C, D). Co-silencing of the Toll pathway transcription factor *Rel1* with *Cad* did not influence the effect of *Cad* silencing on *P. falciparum* infection (Fig. 2C). In contrast, co-silencing of the Imd pathway transcription factor *Rel2* reverted the effect of *Cad* silencing on mosquito resistance to *P. falciparum* infection, indicating an antagonistic relationship between these factors and implicating *Cad* as a negative regulator of the Imd pathway (Fig. 2C). The influence of *Cad* silencing on the resistance to *P. falciparum* followed a similar pattern to that produced by depleting *Caspar*, a negative regulator of the Imd pathway upstream of *Rel2*-mediated transcription (Garver et al., 2012; Garver et al., 2009) (Fig. 2D). Co-silencing of *Cad* with *Imd* also minimized the effect of *Cad* silencing on *P. falciparum* infection, further corroborating a role for *Cad* as a negative regulator of the Imd pathway (Fig. 2D). We do note that interpretation of RNAi co-silencing assays needs to be done cautiously since co-silencing phenotypes can depend on a variety of parameters such as gene silencing efficiency and mRNA and protein turnover. Further information on the statistical analyses and prevalence rates are provided in Supplementary Table S2.

To gain a better understanding of *Cad*'s influence on the mosquito's overall susceptibility to *P. falciparum*, we investigated sporozoite loads in the salivary glands of *Cad*-silenced mosquitoes (Fig. 2E). We saw a ~3-fold decrease in *P. falciparum* sporozoite load in the salivary glands of *Cad*-silenced mosquitoes relative to control mosquitoes (Fig. 2E). It is important to note that the data presented in Fig. 2A and Fig. 2E is not from the same infection experiment (supplemental information provided in Supplementary Table S2). Although silencing of *Cad* did not render mosquitoes completely refractory to *P. falciparum* at high infection intensities (Fig. 2A-2E), it is important to note that laboratory infection levels are much higher than those in nature. We have previously shown that manipulation of the Imd components *Caspar* and *Rel2* can render mosquitoes refractory when infection levels are similar to those observed in the field (Dong et al., 2011; Garver et al., 2012).

3.3. Caudal -mediated immune activation potentiates transgenic resistance to *Plasmodium*

We have shown that genetic manipulation of the Imd pathway can confer resistance to *P. falciparum* infection through the transient overexpression of *Rel2* (Dong et al., 2011). Our findings suggest that RNAi-mediated silencing of *Cad* could be used to further potentiate the anti-*Plasmodium* response produced by the overexpression of the transgene *Rel2*. To investigate this possibility and to better understand the function of *Cad* in modulating *Rel2*-controlled anti-*Plasmodium* activity, we assessed the impact of *Cad* silencing in transgenic *An. stephensi* mosquitoes that overexpressed *Rel2* after a blood meal in the midgut (the Cp-carboxypeptidase-line) (Dong et al., 2011). We performed these assays at infection intensities similar to those observed in the field (Cohuet et al., 2006). Silencing the *An. stephensi* *Cad* gene resulted in a significantly lower median number of oocysts in wildtype as well as transgenic mosquitoes when compared to *GFP* dsRNA-treated control mosquitoes (Fig. 2F). Interestingly, *Cad* silencing rendered both WT and transgenic mosquitoes refractory to *P. falciparum* infection as a measure of median oocyst load. These results demonstrate that *Cad* plays a conserved role in the anti-*P. falciparum* defense across two malaria vector species and that it can further boost the anti-*P. falciparum* activity conferred by transgenic *Rel2* overexpression. Hence, *Cad* can potentially be used to increase resistance to *P. falciparum*, and it may therefore represent a promising immune factor for use in

developing novel malaria control strategies based on genetically engineered *Plasmodium*-resistant mosquitoes.

3.4. Caudal regulates the proliferation and species composition of the mosquito's midgut microbiota

While the mosquito midgut microbiota is beneficial and necessary for the insect, it can become detrimental if it over proliferates, and it must therefore be kept in check by the innate immune system (Cirimotich et al., 2011c). The Imd pathway has been shown to play an active role in this process as a key regulator of immune responses in the *Drosophila* and mosquito gut (Buchon et al., 2009; Meister et al., 2009), and we have already shown that *Cad* influences AMP transcript abundance by negatively regulating the Imd pathway, a finding that suggests an active role for *Cad* in the suppression of the midgut microbiota (Fig. 1B). In the present study, we also investigated the influence of *Cad* silencing on the prevalence and species composition of the midgut microbiota. We first focused on LB-culturable bacteria, and we observed that silencing *Cad* produced a significant decrease (by ~6-fold) in the average midgut bacterial load when compared to *GFP* dsRNA-treated control mosquitoes, and it resulted in the modification of the bacterial species composition (Fig. 3A; Supplementary Tables S6 and S7). Specifically, a decrease in overall species diversity (based on *16S* rDNA sequences) from seven to three Gram-negative bacterial species was observed upon *Cad* silencing.

To provide information concerning the influence of *Cad* on the majority of the total microbiome, we also investigated the result of *Cad* silencing on bacterial load by conducting qRT-PCR analysis of *16S* rRNA gene expression in the midgut over a time course of 12, 24, and 48 h after *Cad* dsRNA treatment compared to control mosquitoes treated with *GFP* dsRNA. Our data showed (Fig. 3B) that *16S* rRNA abundance was decreased at 12 (~8-fold), 24 (~2-fold), and 48 h (~12-fold) after *Cad* dsRNA injection. The diminished proliferation of the midgut microbiota in response to *Cad* silencing is likely attributed to the increased transcriptional abundance of *Rel2*-controlled AMP and other effector genes (Fig. 1B). Such dynamics could reflect the differential proliferation among, and competition between, different bacterial species in response to an enhanced immune response mediated by the silencing of *Cad*. Since silencing of *Cad* depletes the microflora, which has been shown to act negatively on *Plasmodium*, this decreased number of gut microbes may therefore partly offset the enhanced anti-*Plasmodium* immune defense upon *Caudal* silencing.

3.5. Caudal's role in the defense against systemic bacterial infections

We continued our investigations of *Cad*'s role in antibacterial defense by investigating its role in systemic bacterial infections. We assessed the survival of *Cad*-, *Cactus*-, or *Caspar*-silenced and *GFP* dsRNA-treated control mosquitoes after systemic infection with either *E. coli* (Gram-negative) or *S. aureus* (Gram-positive). The two negative regulators *Cactus* and *Caspar* were also included in these experiments, since the Toll pathway has been shown to be primarily involved in the defense against Gram-positive bacterial infections, while the Imd pathway primarily acts against Gram-negative as well as some Gram-positive bacteria (Cirimotich et al., 2010; Garver et al., 2009; Meister et al., 2005). We assayed mosquito mortality for 7 days after challenge via intra-thoracic injections with either bacteria or with 1X PBS (as a control--data not shown) (Fig. 3C, D). Silencing of the three immune factors after *S. aureus* challenge resulted in quite different mosquito survival patterns (Fig. 3C), and an overall significant trend was observed. As expected, *Cactus* silencing increased mosquito survival after Gram-positive bacterial infection. Interestingly, *Cad* silencing also potentiated the mosquito's ability to defend against Gram-positive bacterial challenge to a greater degree than *Caspar* silencing or *GFP* dsRNA treatment of mosquitoes. This could be

explained by the observed increase in elicitation of *Def-1* and *Gam* (Fig. 1B), which have previously been shown to participate in the defense against Gram-positive bacteria (*Def-1*) or against both bacterial classes and also *Plasmodium* (*Gam*) (Dong et al., 2006a; Vizioli et al., 2001b). Alternatively, the extended lifespan could reflect some other function of *Cad* that influences mosquito longevity (see section 3.6). While silencing of the three immune factors did not result in different survival patterns after *E. coli* challenge, we noted that the mosquitoes in the *Cad*-silenced group were able to survive longer after challenge than the other experimental groups or the controls (Fig. 3D). This difference could be due to the increased transcript abundance of *Cec-1* and *Cec-3*, which have been implicated in the defense against Gram-negative bacteria (Fallon and Sun, 2001; Kim et al., 2004; Luna et al., 2006; Vizioli et al., 2000; Zheng and Zheng, 2002). *Cad* confers resistance to bacterial infection possibly via increasing the transcript abundance of AMPs, a finding corroborated by other recent and relevant studies (Coggins et al., 2012; Dong et al., 2011). Since *Cad* is midgut-specific, our data also suggest that this tissue can play a role in systemic immune responses against pathogens in the insect's hemocoel.

3.6. Caudal's effects on mosquito fitness as a measure of longevity, fecundity, and fertility

Studies of the trade-offs between lifespan and immune defense have shown that mounting an immune response may accelerate aging in insects; chronic and sustained, but not acute and transient, *Relish*-dependent immune signaling reduces the lifespan of *D. melanogaster* and *Anopheles* (Dong et al., 2011; Garver et al., 2009; Libert et al., 2006; Robb and Forbes, 2006). To provide further insight on the biology of *Cad* in *An. gambiae*, we assessed the impact of its transient silencing on several facets of mosquito fitness. Specifically, we monitored longevity, fecundity (number of eggs per female), and fertility (number of eggs that hatch into larvae) in *Cad* and *GFP* dsRNA-treated mosquitoes. We assessed longevity in dsRNA-treated mosquitoes that had been maintained under one of the three following conditions: 1) on 10% sucrose only, 2) fed a naïve blood meal and then maintained on 10% sucrose, or 3) fed on a *Pf*-infected blood meal and then maintained on 10% sucrose (Fig. 4A). Surprisingly, the silencing of *Cad* increased the longevity of mosquitoes under all three conditions, with an overall significant trend. Particularly, *Cad* dsRNA-treated mosquitoes that had obtained a naïve blood meal and were then maintained on 10% sucrose survived longer than the other experimental groups (Fig. 4A). Such a conferred fitness advantage of a *Plasmodium*-resistant phenotype upon blood feeding is particularly interesting from a malaria control perspective. This increased longevity could be due to the fact that *Cad* results in a lower prevalence of specific midgut-associated bacterial species that may proliferate greatly after blood meal ingestion and negatively affect mosquito longevity, or it may be the result of some other unknown function of *Cad* that is not necessarily related to the immune system.

In contrast, silencing of *Cad* impaired both the fecundity and fertility of mosquitoes (Fig. 4B, C). Approximately 50% of the *Cad*-silenced mosquitoes failed to produce eggs after a naïve blood meal, and the overall egg production by this cohort was 33% and 45% less than that of the *GFP* dsRNA-treated and untreated mosquito control cohorts, respectively (Fig. 4B). Also, eggs produced by *Cad*-silenced mosquitoes displayed a 45% and 41% lower hatch rate than that of eggs produced by the *GFP* dsRNA-treated and untreated mosquitoes, respectively (Fig. 4C). *Caudal*'s impact on fitness is in contrast to the results of our previous studies on the *Imd* pathway, which did not indicate any such impairment of fecundity or fertility. Specifically, *Caspar*-silenced or *Rel2*-overexpressing mosquitoes showed no or only minimal differences in longevity, fecundity, and fertility compared to control mosquitoes (Dong et al., 2011; Garver et al., 2009). The differential effects of *Cad* silencing on fitness, as compared to those of other *Imd* pathway factors, may highlight the functional versatility of this factor in the mosquito, since *Caudal* was first identified as a gene involved

in *Drosophila* embryonic posterior development (Mlodzik et al., 1985; Mlodzik and Gehring, 1987).

With respect to embryogenesis, *Caudal* is expressed during *Drosophila* oogenesis and blastoderm cellularization (Mlodzik and Gehring, 1987). Also, maternal expression of *Caudal* in the ovarian follicles has been reported in the dipteran flies *Lonchopoda*, *Empis*, and *Haematopota* (Stauber et al., 2008). Previous work has highlighted *Caudal*'s role in regulating the specification and organization of the genital disc in *Drosophila*. The genital disc originates from a portion of the embryonic tail segments and eventually manifests as adult female or male genitalia structures and the analia structures (Chen et al., 2005; Freeland and Kuhn, 1996; Kuhn et al., 1995). Our collective knowledge of *Caudal*'s developmental functions in *Drosophila* and other dipteran insects points to the likelihood of similar roles for *Caudal* in *An. gambiae* and may also explain why silencing of this gene impairs egg production and larval hatching.

Interestingly, the effect of *Cad* silencing on adult mosquito fitness parameters is also significantly contrasted with those imposed by *Cad* silencing in adult *Drosophila* (Ryu et al., 2008). As compared to wildtype flies, *Cad*-silenced flies displayed a greater frequency of midgut cell apoptosis, which in turn negatively affected longevity (Ryu et al., 2008). The physiology of the hematophagous mosquito gut differs greatly from that of the fly, and this difference could explain the discrepancy of the results in the two insects. However, it would be interesting to investigate whether any apoptosis is occurring in *Cad*-silenced mosquitoes that could impose reproductive costs rather than being a detriment to the mosquitoes' lifespan. Previous studies in *Anopheles* have shown that immune elicitation in response to bacterial and *Plasmodium* infections negatively affects reproductive capability by causing cell death in the ovarian follicular epithelial cells, thereby decreasing oocyte development (Ahmed and Hurd, 2006; Ahmed et al., 2001; Hopwood et al., 2001). Pathogen infection-induced reactive oxygen species (ROS) have also been shown to impair reproduction by causing oocyte damage (DeJong et al., 2007; Molina-Cruz et al., 2008). It would therefore also be interesting to explore the relationship between ROS production and *Cad* silencing with respect to reproductive fitness.

The negative impact of *Cad* silencing on reproduction could alternatively be the result of a reallocation of energy during a time of need to combat infection. For example, in *Drosophila*, the fat body has been shown to reallocate resources in order to elicit an immune response to infection and, as a result, suppresses insulin signaling and diminishes nutrient stores and growth (DiAngelo et al., 2009). The dynamic shift between immunity and reproduction output observed in our study is consistent with a recent study by Rono and colleagues (Rono et al., 2010), which revealed that two nutrient proteins crucial for oocyte production, lipophorin and vitellogenin, interfere with the anti-*Plasmodium* effector TEP1. This group also showed that the Imd and Toll pathways inhibit the expression of vitellogenin in order to confer immune defense against *Plasmodium*. *Caudal* could play a key role in this molecular mechanism that determines the trade-off between the anti-*Plasmodium* response and oocyte development and production.

4. DISCUSSION

4.1. Caudal is a regulator of the Imd pathway-controlled transcription factor Rel2

In this study, we show that *Cad* is a midgut-specific antagonist of the Imd pathway transcription factor *Rel2*. Silencing of *Cad* resulted in a general increase of *Rel2*-regulated AMP and anti-*Plasmodium* effector gene mRNA abundance (Fig. 1B) in a manner similar to when the Imd pathway is activated through other means of molecular manipulation in *Anopheles* (Dong et al., 2011; Garver et al., 2009).

4.2. Caudal regulates Plasmodium species-specific defense

We demonstrate that *Cad* specifically controls resistance to *P. falciparum* and not *P. berghei* infection in a pattern similar to the Imd pathway (Fig. 2A-2D) (Dong et al., 2011; Garver et al., 2012; Garver et al., 2009; Mitri et al., 2009). Silencing of *Cad* decreased oocyst loads of the midgut and sporozoite loads in the salivary glands, thereby influencing the mosquito's overall vector competence for *P. falciparum* (Fig. 2E). We also show that RNAi-mediated silencing of *Cad* can further potentiate the anti-*Plasmodium* response produced by the overexpression of a recombinant *Rel2* in transgenic *An. stephensi* (Fig. 2F). This demonstrates that *Cad* plays a conserved role in the anti-*P. falciparum* defense across two malaria vector species. *Cad* may therefore represent a promising immune factor for use in developing novel malaria control strategies based on genetically engineered *Plasmodium*-resistant mosquitoes.

4.3. Caudal regulates microbial homeostasis of the midgut

Our studies show an active role for *Cad* in the regulation of the midgut microbiota (Fig. 3A, 3B). Particularly, *Cad* silencing resulted in a decreased prevalence and altered species composition of the midgut microbiota. The diminished proliferation of the midgut microbiota in response to *Cad* silencing is likely attributed to the increased transcriptional abundance of *Rel2*-controlled AMP and other effector genes (Fig. 1B). *Cad* also plays a role in the systemic antibacterial defense with regards to positively influencing survival against hemolymph bacterial infections (Fig. 3C, D).

4.4. Caudal's influence on mosquito fitness suggests functional diversity

We have previously shown that a transient activation of the Imd pathway-controlled *Rel2* did not significantly impact longevity, fecundity and fertility. In contrast, silencing of *Cad* increased the longevity (Fig. 4A) and impaired both the fecundity and fertility of mosquitoes (Fig. 4B, C). *Caudal*'s impact on fitness is in contrast to the results of our previous studies on the Imd pathway and suggests functional versatility of this factor in the mosquito. *Drosophila Caudal* was first identified as a gene involved in embryonic posterior development (Mlodzik et al., 1985; Mlodzik and Gehring, 1987), and *Cad*-silenced flies displayed a greater frequency of midgut cell apoptosis, which in turn negatively affected longevity (Ryu et al., 2008). The vastly different physiology of the hematophagous mosquito gut from that of the fly is likely to explain the differential functions of the two homologs. Additionally, the dynamic shift between immunity and reproduction output observed in our study is consistent with a recent study by Rono and colleagues (Rono et al., 2010), which revealed that two nutrient proteins crucial for oocyte production, lipophorin and vitellogenin, interfere with the anti-*Plasmodium* effector TEP1. This group also showed that the Imd and Toll pathways inhibit the expression of vitellogenin in order to confer immune defense against *Plasmodium*. *Caudal* could play a role in the molecular circuit that determines the trade-off between the anti-*Plasmodium* response and reproduction.

In sum, our findings show that *Anopheles Cad* functions in the Imd pathway to significantly modulate the mosquito's anti-*P. falciparum* defense. It is also a key factor in the mosquito's antibacterial defense and regulates the species composition and load of the mosquito midgut microbiota. Although we observed a fitness trade-off as a result of *Cad* silencing, the negative impact on mosquito fecundity and egg hatch rate may be overcome via an effective gene drive mechanism. The increased longevity may also allow mosquitoes to engage in additional gonotrophic cycles, and this may compensate for an impaired reproductive fitness compared to their wildtype counterparts (Hay et al., 2010; Marshall, 2009; Ward et al., 2011). In conclusion, our study encourages further investigation of *Cad*'s use as a tool for modulating the ability of mosquito vectors to transmit malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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RESEARCH HIGHLIGHTS

- *Caudal*-silenced mosquitoes have enhanced resistance to *P. falciparum* infection.
- *Caudal* regulates the mosquito midgut microbiota.
- Silencing of *Caudal* enhanced resistance to systemic bacterial infections.
- *Caudal*-silenced mosquitoes live longer but have impaired fecundity and fertility.

Fig. 1. Caudal expression in *Anopheles gambiae* and Immune-transcriptional profile upon Caudal-silencing

(A) Fold difference in transcript abundance of *Cad* in the midgut and fat body compared to the carcass (set at a basal level of 1) of wildtype female mosquitoes (ribosomal gene *S7* used as an internal control). (B) Midgut-specific transcript abundance of select AMP and anti-*Plasmodium* effector genes (with ribosomal gene *S7* used as an internal control) at 12 and 24 h post RNAi-mediated silencing of *Cad*. Each column and error bar represents the fold change and the standard error of the mean compared to *GFP* dsRNA-treated mosquito midguts. For both figures, three biological replicates were performed.

Fig. 2. Anti-*Plasmodium* defense in *Caudal*-silenced mosquitoes

P. falciparum oocyst (A) and *P. berghei* oocyst (B) loads in the midguts of *An. gambiae* mosquitoes silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). (C-D) *P. falciparum* oocyst loads in the midguts of *An. gambiae* mosquitoes silenced for *Cad* or *Caspar* (D) as well as mosquitoes simultaneously silenced for *Caudal* (*Cad*) and immune effector gene *Rel1* (C), *Rel2* (C), or *Imd* (D). (E) *P. falciparum* sporozoite loads in the salivary glands of *An. gambiae* mosquitoes silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). (F) *P. falciparum* oocyst loads in the midguts of *An. stephensi* non-transgenic (wt) and Cp transgenic mosquitoes silenced for *Caudal* (*Cad*) or injected with dsRNA *GFP* (control). For all figures, circles represent the number of parasites from individual mosquitoes, and horizontal lines represent the median number of parasites. Three

biological replicates were performed, and indicated statistical significance was derived from Mann-Whitney tests. The p -values are presented where *: $p < 0.05$ or $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$. Additional statistical analyses appear in Supplementary Table S2.

Fig. 3. Antibacterial responses in *Caudal*-silenced mosquitoes

(A) The midgut microbiota load was analyzed via colony-forming unit (CFU) assays in *An. gambiae* *Cad* and *GFP* dsRNA-treated mosquitoes. (B) Midgut-specific transcript abundance of microbial load (with the ribosomal gene *S7* used as an internal control) at 12, 24, and 48 h post RNAi-mediated silencing of *Cad* (the 0 h time point is also included). (C) Survival curves for *Cad*, *Cactus*, *Caspar*, and *GFP* dsRNA-treated mosquitoes systemically infected with *S. aureus* or (D) with *E. coli*. For both figures, survival was assessed for 7 days, and the curves indicate average percent survival. Statistical significance was observed for (C) but not for (D). For (A) and (B), columns and error bars represent mean values and the standard error of the mean. Three biological replicates were conducted. The Mann-Whitney test was used for (A), and statistical significance is indicated where *: $p < 0.05$ or $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$. For (C) and (D), Kaplan-Meier survival analysis was used with the log-rank (Mantel-Cox) test for significance evaluation. Supplementary information is provided in Supplementary Table S3.

Fig. 4. Fitness outcomes in *Caudal*-silenced mosquitoes

(A) Longevity studies in *Cad* and *GFP* dsRNA-treated mosquitoes were conducted under the following conditions: (1) mosquitoes maintained on 10% sucrose solution; (2) mosquitoes given a single naïve blood meal and then maintained on 10% sucrose; or (3) mosquitoes given a single *P. falciparum*-infected blood meal and then maintained on 10% sucrose. Survival was assessed until 100% mortality was reached. The curves indicate the average percent survival for each cohort. Kaplan-Meier survival analysis was used with the log-rank (Mantel-Cox) test for significance evaluation. Additional information is provided in Supplementary Table S4. (B) Fecundity analysis in *Cad* and *GFP* dsRNA-treated and untreated mosquitoes; the circles represent the number of eggs laid per female after a single naïve blood meal; the horizontal bars represent the mean number of eggs laid per female, and error bars represent the standard error of the mean. Data were subjected to the Mann-Whitney statistical test, and the plots are data from three independent biological replicates. Statistical significance is indicated where *: $p < 0.05$ or $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$. (C) Fertility analysis in *Cad* and *GFP* dsRNA-treated and untreated mosquitoes; hatch rates indicate the average percentage of eggs giving rise to first to second instar larvae. Error bars represent the standard error of the mean. The Mann-Whitney statistical test was used and

significance is indicated where *: $p < 0.05$ or $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$. Three biological replicates were conducted. See additional information in Table S5.