

A behavioral mechanism underlying ecological divergence in the malaria mosquito *Anopheles gambiae*

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Disruptive selection mediated by predation on aquatic immature stages has been proposed as a major force driving ecological divergence and fostering speciation between the M and S molecular forms of the African malaria mosquito, *Anopheles gambiae*. In the dry savannahs of West Africa where both molecular forms co-occur, the S form thrives in temporary pools filled with rainwater, whereas the M form preferentially breeds in permanent freshwater habitats where predator pressure is higher. Here, we explored the proximal mechanisms by which predation may contribute to habitat segregation between molecular forms using progeny of female mosquitoes captured in Burkina Faso. We show that the S form suffers higher predation rates than the M form when simultaneously exposed to the widespread predator, *Anisops jaczewskii* in an experimental arena. Furthermore, behavioral plasticity induced by exposure to the predator was observed in the M form, but not in the S form, and may partially explain its habitat use and ecological divergence from the S form. We discuss the role of adaptive phenotypic plasticity in allowing successful colonization of a new ecological niche by the M form and highlight further research areas that need to be addressed for a better understanding of the ultimate mechanisms underlying ecological speciation in this pest of major medical importance. *Key words*: adaptation, *Anopheles gambiae*, behavior, habitat divergence, mosquito, notonectidae, phenotypic plasticity, predation, speciation. [*Behav Ecol* 21:1087–1092 (2010)]

Phenotypic plasticity is the ability of an individual (or a genotype) to produce different phenotypes in different environments (Via et al. 1995; Agrawal 2001). As such, phenotypic plasticity provides a mechanism by which species can tolerate wide environmental variation, and may therefore contribute to the successful colonization of a new environment, before adaptive evolution is possible (Baldwin 1896; Robinson and Dukas 1999; Sexton et al. 2002; Yeh and Price 2004). The plastic changes in phenotype in response to environmental heterogeneity may be adaptive or not, depending on whether the induced phenotype has higher fitness in the environment in which it is expressed (Via et al. 1995; Price et al. 2003; Ghalambor et al. 2007). Any mechanism that reduces the risk of predation in a predator-rich environment increases survivorship and therefore is potentially adaptive (Skelly 1994;

Relyea 2001). Natural selection might then act on relevant genetic variation to further increase fitness and foster phenotypic and genetic divergence from the ancestral gene pool, a process known as genetic assimilation (Via et al. 1995; Price et al. 2003; Ghalambor et al. 2007). Alternatively, plasticity itself might be under selection, when populations are exposed to variable environments and no single phenotype has higher fitness across all environments (Via and Lande 1985; Ghalambor et al. 2007). Predator-induced phenotypic plasticity has received much attention, and many animal species have been shown to alter their activity, microhabitat use, morphology, and/or life history in response to predators (Ball and Baker 1996; Wellborn et al. 1996; Relyea 2001, 2003; Schmidt and Van Buskirk 2005). Predation has been shown to play a key role in structuring communities (Sih et al. 1985; Wellborn et al. 1996) and can have a direct impact on prey species range and distribution along an environmental gradient (Holt and Barfield 2009). Moreover, predation has long been implicated as a major selective force for the evolution of several morphological and behavioral characteristics of animals (Lima and Dill 1990; Vamosi 2005) and can drive

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adaptive trait divergence among lineages (Mikolajewski et al. 2006; Nosil and Crespi 2006). In the process, predation may ultimately drive diversification and speciation (Nosil and Crespi 2006; Langerhans et al. 2007; Gallet et al. 2009). In this study, we investigated the proximal mechanisms by which predation on aquatic immature stages may contribute to habitat segregation between 2 nascent species of the African malaria mosquito, *Anopheles gambiae* sensu stricto (Diptera: Culicidae).

The mosquito *Anopheles gambiae* s.s. (hereafter, *An. gambiae*) is the major vector of human malaria throughout sub-Saharan Africa. Recent molecular and population genetics studies have led to the recognition of 2 "molecular forms" within *An. gambiae*, known as the M and S forms (della Torre et al. 2001, 2002) among which gene flow appears to be highly restricted (Wondji et al. 2002; della Torre et al. 2005). Microarray studies found that the genomic divergence between molecular forms is clustered into 3 small DNA regions near the centromere of all 3 independently assorting chromosomes which together encompass approximately 3% of the genome, suggesting recent and still incomplete lineage sorting (Lehmann and Diabate 2008; Turner and Hahn 2010; White et al. 2010). The S molecular form is widespread throughout sub-Saharan Africa and is presumed ancestral, whereas the derived M form is restricted to West and Central Africa where it occurs in sympatry with the S form (see recent reviews by della Torre et al. 2005; Lehmann and Diabate 2008). Both the molecular forms are morphologically similar and share the same resources including vertebrate hosts, adult resting sites, and freshwater larval habitats. However, general ecological differences have been documented between the M and S forms (della Torre et al. 2005; Costantini et al. 2009; Simard et al. 2009). In the dry savannahs of West Africa, the M form preferentially breeds in permanent freshwater collections mainly resulting from human activity (e.g., agriculture and urbanization) and is reproductively active all year round, whereas the S form thrives in temporary breeding sites (e.g., rain-filled puddles, road ruts, and quarries) and is present during the rainy season only (Costantini et al. 2009). Adaptation to different larval habitats has been hypothesized to play a pivotal role in the speciation process (Coluzzi et al. 2002), but the ecological conditions that promoted speciation within *An. gambiae* have only recently been subjected to experimental study (Diabate et al. 2008; Manoukis et al. 2008). Biogeographic patterns of habitat segregation between the M and S forms were shown to conform to speciation by niche expansion (Costantini et al. 2009), providing support for ecological speciation (e.g., Schluter 2001, 2009). Moreover, recent field studies have suggested fitness trade offs apparently related to the length of hydroperiod in breeding habitats (Lehmann and Diabate 2008). Field transplantation experiments have shown that larvae of the S form develop faster than the M form in temporary water collections when predators are few, whereas larvae of the M form outcompete those of the S form in predator-rich environments (Diabate et al. 2005, 2008). We therefore considered the potential role of predation in defining differential selection pressures between M and S at their larval stage and ask specifically whether superior predator avoidance in the M form could explain its ability to colonize more complex long-lasting aquatic habitats where predator pressure on mosquito larvae was shown to be higher than in temporary puddles (Sunahara et al. 2002; Diabate et al. 2008).

The backswimmer, *Anisops jaczewskii* Hutchinson 1928 (Hemiptera: Notonectidae) is the most abundant and widespread predatory bug in permanent as well as temporary freshwater mosquito habitats in our study area in western Burkina Faso (Diabate et al. 2008). Notonectids were shown to have a major impact on aquatic invertebrate community structure (Blaustein et al. 1995; Blaustein 1998) and are

voracious predators of mosquito larvae (Sih et al. 1985; Eitam et al. 2002). Here, we simultaneously exposed larvae of the M and S form of *An. gambiae* to *A. jaczewskii* in an experimental arena to test whether attack rates differ between molecular forms. We found that the S form suffers a significantly higher predation rate than the M form. Notonectids act as ambush predators and detect their prey using visual stimuli and/or mechanosensory reception (e.g., Sih 1982; Scott and Murdoch 1983). Movement, therefore, in addition to increasing encounter rate, increases predation risk. We explored antipredator behavior in both molecular forms of *An. gambiae* by exposing single larvae to predation under similar experimental settings. We show that only the M form alters its behavior in the presence of the predator, by resting more. Hence, predator-induced behavioral plasticity in the M form may have been the proximal mechanism allowing for colonization of more permanent breeding habitats and may still contribute to the maintenance of larval habitat divergence between the molecular forms of *An. gambiae*.

MATERIAL AND METHODS

Mosquito collections

Mosquito larvae used in experiments were obtained from wild female *An. gambiae* collected indoors in 2 distinct locations in western Burkina Faso, West Africa, in August 2008. The M form of *An. gambiae* was captured in the village of Bama (lat 11°23'14"N, long 4°24'42"W) located 30 km northwest of Bobo Dioulasso. The village is surrounded by a 1200 ha of irrigated rice fields, where the M form predominates in collections of mosquitoes throughout the year (>95%; Diabate et al. 2002; Baldet et al. 2003). Females of the S molecular form were collected in Soumouso (lat 11°00'46"N, long 4°02'45"W), a typical village in the humid savannah area of western Burkina Faso located 50 km southeast of Bama. The S form of *An. gambiae* is dominant during the rainy season (June–November) and breeds in rain-filled puddles and quarries. Field-collected gravid and bloodfed *An. gambiae* females were placed individually in oviposition cups maintained under standard insectary conditions (temperature 28 ± 1 °C, 80 ± 10% relative humidity and 12:12 h light:dark) and provided with 5% glucose solution. After oviposition, females were identified to molecular form using a polymerase chain reaction–restriction fragment length polymorphism assay (Fanello et al. 2002). Progeny were pooled according to their mother's molecular form and raised in the insectary to the third instar. Larvae were starved for 24 h prior to the experiments to standardize hunger.

Predator collections

The predator *A. jaczewskii* (Hemiptera: Notonectidae) was collected in rice field irrigation canals in the village of Bama, where it was abundant (Diabate et al. 2008). Predators were caught using a plastic bowl and transferred to bottles for transportation to the insectary in Bobo Dioulasso. They were subsequently placed in individual plastic cups to avoid cannibalism (Sih 1982). Late fourth and fifth instar juveniles were used for experiments, after a 48-h starvation period.

Survival rate

Experiments were performed in 400-ml plastic cups filled with 200 ml of spring water. One specimen of *A. jaczewskii* was added per cup and constrained using an open-ended transparent plastic tube placed vertically in the cup (Kesavaraju et al. 2007). One larva (third instar) of each molecular form

was added per cup, and after a 5-min acclimation period, the tubes were slowly withdrawn, releasing the predator. The experiment ended when one larva was captured. The surviving larva was placed in 70% ethanol and subsequently identified using molecular diagnostic protocols (Fanello et al. 2002). Ten successive meals were offered to each of 15 predators (biological replicates) leading to a total of 150 observations. Survival rate for one molecular form was calculated separately for each of the 10 trials and was defined as the proportion of observations (of the 15 biological replicates) where the corresponding larva survived the predator attack. During each trial, time to grasp the prey was noted.

Because criteria for homogeneity of variance and normality were not met in these data sets, we performed nonparametric Wilcoxon tests to investigate differences in survival rate between molecular forms of *An. gambiae*. The survival of both forms was assessed by generalized linear models (GLMs) with a binomial link function using the software R v.2.9.1 (R Development Core Team 2009). The response was thus the survival probability of the M form (0 or 1), and the fixed effects were the meal rank (sequential trial number out of 10 successive meals offered to the predator), the time to grasp the prey, and their interaction. The best-fitting GLM was judged according to the Akaike information criterion (AIC). The significance of each fixed effect was then judged by comparing the most complete model with the simplified one using an analysis of variance (ANOVA). A Pearson correlation test was performed to assess the relationship between survival and meal rank.

Activity level

Behavior of third instar M and S larvae was recorded in predation and control treatments in 400-ml plastic cups filled with 200 ml of spring water. Predation treatment cups contained one specimen of *A. jaczewskii* and one larva of either the M or S form. Controls were similar but had no predator. As in the experiment above, open-ended transparent plastic tubes were used to isolate the predator in the predation treatment. Empty tubes were used in the control treatments. After 5 min of acclimation, the tubes were removed slowly from the cups, releasing the predator. Behavior of the mosquito larvae was recorded by instantaneous scan censuses (Martin and Bateson 2007) every 15 s for 7.5 min, or until the prey was captured, which resulted in a maximum of 30 observations per trial. Behavior was scored simultaneously in one predation

and one control treatment conducted with larvae of the same molecular form. Activity level was expressed as the proportion of observations a larva was engaged in any kind of movement, as opposed to resting during which no movement or feeding was observed (Jones 1954; Juliano and Reminger 1992; Grill and Juliano 1996; Kesavaraju et al. 2007). Activities included thrashing (larvae moving through the water via vigorous flexions of the entire body), filtering (larvae in open water, floating through the water propelled by movements of the mouth parts only), and browsing (larvae moving along the surfaces of the container, working their mouthparts along the surface). Activity levels were compared between forms and across treatments using a 2-way ANOVA with form, treatment, and their interaction as model effects. Twenty trials were conducted for each form. Only proportions based on at least 12 observations (i.e., >3 min) were used in this analysis to reduce error inherent in proportions based on very low sample sizes (Juliano and Reminger 1992; Kesavaraju et al. 2007).

RESULTS

Survival rate

Examination of mean survival rate across replicates for the M and S molecular forms of *An. gambiae* showed that the survival rate of the M form (mean = 0.613) was significantly higher ($W = 13\,800$, $P = 0.00009$) than the S form (mean = 0.387) when one larva of each form were simultaneously exposed to predation by *A. jaczewskii*. The simplest GLM (without interaction, AIC = 194.6) fits our data better than the complete model (AIC = 196.5) and identified meal rank as a significant factor affecting survival rate of mosquito larvae ($P = 0.005$). A logistic plot (Figure 1) shows that the survival rate of M form larvae increased with meal rank. Correlation between survival rate and meal rank was positive and statistically significant (correlation coefficient $r = 0.663$; $P = 0.036$). The effect of time to grasp the prey on survival rate was marginally significant ($P = 0.053$). As shown in Figure 2, the time to first capture increased with meal rank, and the linear regression of time to first capture with meal rank was significantly positive ($r = 0.56$, $P < 0.001$).

Activity level

ANOVA indicated no effect of molecular form on activity level ($F_{0.815}$, $P = 0.37$). However, a significant effect of treatment ($F_{10.487}$, $P < 0.002$) and interaction ($F_{5.424}$, $P = 0.022$) was

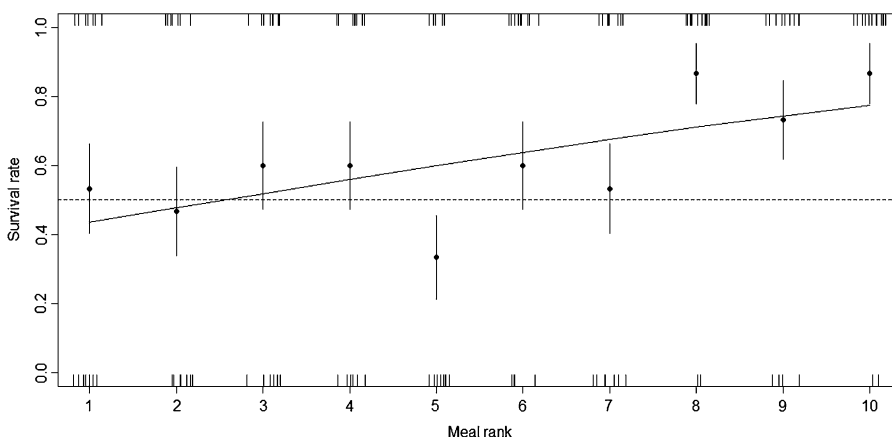
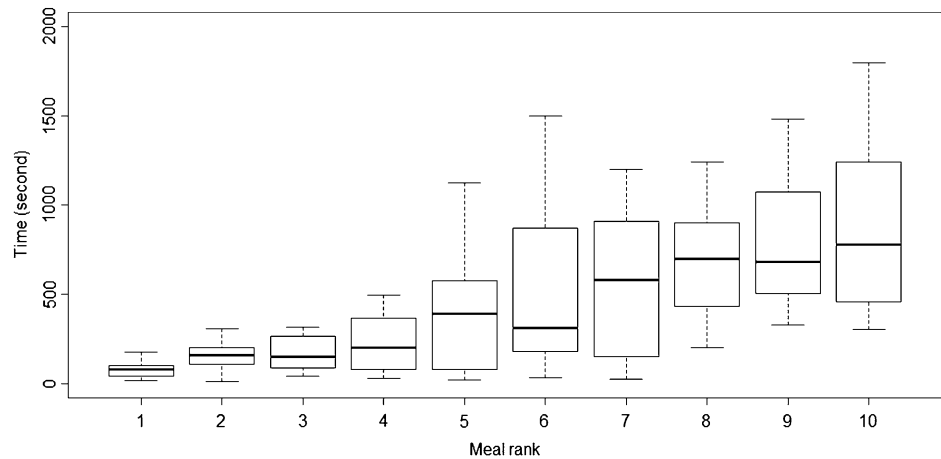


Figure 1
Mean survival rate of larvae of the M molecular form of *An. gambiae* exposed to 10 successive and successful attacks by the predator, *A. jaczewskii*. The predator was starved for 2 days prior to the experiment and was given the choice to feed on one larva of each molecular form of *An. gambiae*. Solid circles represent the mean survival rate across 15 biological replicates, and vertical bars show standard error of the estimate. Dashed line corresponds to a random choice ($P = 0.5$). Small vertical bars represent the survival data for each trial (M above, S below).

Figure 2

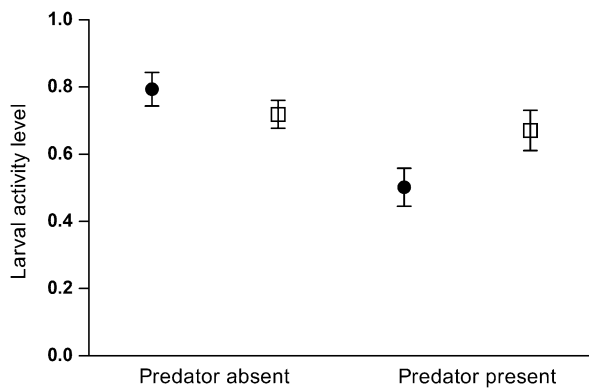
Time to first capture (in seconds) of one *An. gambiae* larva during 10 successive and successful attacks by the predator, *A. jaczewskii*. The box extends between the 25th and 75th percentile, and the median is denoted by a thick line. The whiskers extend up to the most extreme values.



observed, indicating that the effect of treatment depended on molecular form. Indeed, when exposed to the predator, M form larvae significantly reduced their activity level, whereas S form larvae did not (Figure 3).

DISCUSSION

The goal of this study was to evaluate divergent antipredator responses as a mechanism that may have promoted larval habitat segregation and ecological divergence between incipient species of the African malaria mosquito, *An. gambiae* (Diabate et al. 2008; Lehmann and Diabate 2008). It is known that permanent aquatic habitats, such as those preferred by the M form, generally harbor more complex insect communities and higher predator densities than ephemeral freshwater bodies that typically serve as larval development sites for the S form (Sunahara et al. 2002; Diabate et al. 2008). Based on the expectation that natural selection should strengthen antipredator responses by prey exposed to high predation pressure (Sih 1986), we compared vulnerability to predation in the M and S forms of *An. gambiae* on experimental exposure with the widespread aquatic predator, *A. jaczewskii*. In agreement with expectation, the results indicated that 1) the larval predation rate by *A. jaczewskii* was lower for M than for S and 2) the reduced predation rate on M form larvae could be explained by the significantly reduced activity level by M when exposed to the predator, a response not shown by S form larvae. Hence, predator-induced phenotypic plasticity ob-

**Figure 3**

Activity level (mean \pm standard error) of larvae (third instars) of the M (black circles) and S (white squares) forms in the absence or presence of the predator *A. jaczewskii* in an experimental arena.

served in the M form might have adaptive value, allowing larvae to reduce predation risk when exposed to high predation pressure characteristic of permanent larval development sites.

Selective predation by *A. jaczewskii* was evidenced only when the predator already fed on at least 6–7 mosquito larvae and starvation seems to alleviate any preference. Hence, increased selectivity with meal rank might reflect postingestive effects (e.g., satiation) in the predator. Hunger-dependent diet selection has indeed been documented in a number of animal species, including copepods (Demott 1989, 1993), *Daphnia* (Meise et al. 1985), or *Gambusia* mosquitofish (Bence and Murdoch 1986). Notonectids may therefore alter their selective feeding behavior in response to their own satiation level, with well-fed insects concentrating their attacks, to a greater degree than starved insects, on more easily detectable prey (e.g., with a higher activity level). However, it is likely that under field conditions, the level of feeding by notonectids would generally be sufficient for selective behavior to occur (Sih et al. 1985). Further studies on the level of feeding by wild notonectids in permanent versus temporary freshwater collections, together with finely tuned experimental investigations of the predator behavior (e.g., attack rate, prey handling time, and the proportion of prey eaten at various prey densities) are necessary to further investigate this issue. An alternative, not mutually exclusive, explanation for the apparent increase in the predator's preference for the S form may simply be the increased vigilance and predator avoidance behavior exhibited by the M form prey rather than a decision-based process in the predator (Pastorok 1980; Price and Paffenhöfer 1985). Indeed, the M form demonstrated predator-induced behavioral plasticity, whereas the S form did not, suggesting a better ability in the former to detect the predator and subsequently adopt less risky behaviors. It was shown for a number of mosquito species (e.g., *Culex pipiens* and *Ochlerotatus triseriatus*) that larvae are able to gauge predation risk using waterborne cues (Sih 1986; Juliano and Gravel 2002; Kesavaraju and Juliano 2004; Kesavaraju et al. 2007). Sih (1986) suspected "a combination of notonectid digestive enzymes and partially digested mosquito material" to be a possible cue used by *C. pipiens* larvae to modify their behavior in response to acute predation risk. More recent experiments further emphasized the role of chemical components from injured prey (i.e., alarm cues) and components from predators (i.e., kairomones) in inducing behavioral and physiological responses to predation in tadpoles and demonstrated that the combination of both types of cues were required to produce the full suite and amplitude of traits that are induced in response to acute

predation risk (Schoepfner and Relyea 2005). In our experiments, where 10 successive choice tests were conducted in the same cup without water replacement, it is likely that such chemicals would have accumulated. M form larvae might be more prone than their S form counterparts to detect these chemicals and respond more readily to the predation risk. It remains to be explored whether the M and S forms of *An. gambiae* are able to detect such chemicals to a similar extent in their aquatic environment and to adapt their response to these candidate cues.

The responses documented here are avoidance rather than escape responses. Avoidance occurs before a predator's attack begins, whereas escape occurs after the predator initiates an attack (Sih 1986; Lima and Dill 1990). Hence, avoidance might be both a more sophisticated and a more precise anti-predator response than the simple act of escaping when attacked, providing an adaptive advantage under high predation pressure. Predator avoidance responses have been shown to trigger long-term shifts in (micro)habitat use and activity level by prey, with major consequences for overall life history (e.g., Stein and Magnuson 1976; Peckarsky 1980; Jeffries and Lawton 1984). Here, we focused on activity level, but other behavioral responses, such as shifts in habitat use and differential ability to locate and use spatial refuges (Sih 1986, 1987), will need to be addressed to further characterize the antipredator response of M and S forms at the larval stage. Permanent freshwater habitats represent complex ecosystems that, in addition to diverse animal communities, also contain a variety of plant species that could act as refuges for mosquito larvae. A more comprehensive and comparative assessment of predator-induced phenotypic plasticity in the M and S forms (e.g., behavioral, physiological, and life-history traits; see Relyea 2001; Schmidt and Van Buskirk 2005) is likely to reveal additional differences that underlie larval habitat segregation and hence may have promoted ecological divergence and speciation within *An. gambiae*. Understanding the processes that generate and maintain biodiversity in the *An. gambiae* complex is of critical value given the tremendous importance of this mosquito for human health. The recognition of cryptic speciation within such medically important insects is paramount to vector control. Moreover, a clear understanding of the evolutionary forces that shape and maintain the genetic structure and phenotypic plasticity of these powerful disease vectors might be key to the development of more efficient tools for population monitoring and control (Fontenille and Simard 2004).

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