

MOSQUITO BIOLOGY

Evolution of sexual traits influencing vectorial capacity in anopheline mosquitoes

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The availability of genome sequences from 16 anopheline species provides unprecedented opportunities to study the evolution of reproductive traits relevant for malaria transmission. In *Anopheles gambiae*, a likely candidate for sexual selection is male 20-hydroxyecdysone (20E). Sexual transfer of this steroid hormone as part of a mating plug dramatically changes female physiological processes intimately tied to vectorial capacity. By combining phenotypic studies with ancestral state reconstructions and phylogenetic analyses, we show that mating plug transfer and male 20E synthesis are both derived characters that have coevolved in anophelines, driving the adaptation of a female 20E-interacting protein that promotes oogenesis via mechanisms also favoring *Plasmodium* survival. Our data reveal coevolutionary dynamics of reproductive traits between the sexes likely to have shaped the ability of anophelines to transmit malaria.

Anophelines are the only mosquitoes capable of transmitting human malaria, a disease that affects hundreds of millions of people and causes approximately 600,000 fatalities annually (1). The vectorial capacity of different anopheline species, a metric of their ability to transmit the *Plasmodium* parasites that cause malaria, is governed by multiple elements of mosquito biology. These include longevity, blood-feeding preferences, immune responses to the parasites, and mosquito population densities, the latter largely determined by the mosquito's reproductive success (2). Across most anopheline species, females are monandrous, with reproduction relying on a single mating event that generally occurs in swarms (3). Our knowledge on the reproductive biology of this mosquito genus derives principally from studies in *Anopheles gambiae*, the primary malaria vector. In this species, males coagulate seminal secretions produced by the male accessory glands (MAGs) into a mating plug that is transferred to females during mating, delivering also large titers of the steroid hormone 20-hydroxyecdysone (20E) (4, 5). Sexual transfer of 20E has profound effects on female physiology as it induces a cascade of events that render *A. gambiae* females refractory to further copulation while increasing egg produc-

tion and triggering egg laying (5, 6). The mating-induced increase in oogenesis is mediated by vitellogenic lipid transporters that are also regulated by lower levels of 20E produced by the female after a blood meal (7). Interestingly, these lipid transporters facilitate *Plasmodium* development by reducing the parasite-killing ability of the mosquito immune system, providing a link between reproductive and immune processes (8). Additionally, the 20E-regulated monandrous mating system positively affects female longevity—a key feature of malaria transmission given the lengthy *Plasmodium* cycle within the mosquito—by decreasing predation risks associated with swarming behavior (3) and avoiding the fitness costs associated with multiple matings in other insects (9–11). Therefore, 20E in *A. gambiae* modulates multiple physiological processes that are highly relevant to the mosquito's competence to transmit malaria: reproductive success, parasite development, and longevity.

Mating plug formation and 20E synthesis in the MAGs are not observed in other mosquito genera or in the fruit fly *Drosophila melanogaster* (3, 5, 12), suggesting that these traits are potential targets of sexual selection that have either been lost by other insects or gained in anophelines. Given their fundamental relation to fitness, ejaculate characters are often subject to rapid change driven by coevolutionary interactions between the sexes (13, 14). Importantly, the evolution of novel reproductive traits in males that induce adaptive changes in female physiology may also affect pathogen transmission in insect vectors of disease (15). Phylogenetic analyses of relevant sexual traits can therefore offer insights into how male-female coevolutionary dynamics influence the vectorial capacity of different species.

To determine how selection has shaped the macroevolution of reproductive traits that also impact malaria transmission, we set out to estab-

lish the evolutionary trajectories of the mating plug and male 20E in anophelines, taking advantage of the recent completion of genome assemblies for 16 *Anopheles* species (16). We first characterized the occurrence of mating plug formation among nine geographically dispersed species, which span the three subgenera (*Cellia*, *Anopheles*, and *Nyssorhynchus*) that include the most relevant malaria vectors and comprise a range of evolutionary distances from *A. gambiae* (16). We performed mating experiments on laboratory-reared colonies, dissected female reproductive tracts from each of the nine selected species, and assessed whether females had received a mating plug. We observed considerable divergence in plug phenotypes (Fig. 1). Three African species (the previously characterized *A. gambiae*, plus *A. arabiensis* and *A. funestus*) and one Indian species (*A. stephensi*) had a solid, highly coagulated plug; three east and southeast Asian species (*A. farauti*, *A. dirus*, and *A. sinensis*) and one European species (*A. atroparvus*) exhibited a less compact, more amorphous structure—likely reflecting reduced levels of coagulation—whereas no plug was detected in the New World species *A. albimanus*.

We next assessed whether 20E is produced in the MAGs of the same species and identified significantly different hormone levels through an enzyme immunoassay on MAGs from virgin males ($P < 0.0001$) (Fig. 2A). Although all species with a fully coagulated plug produced high titers of 20E (above 200 pg/MAGs), two species with partial plug coagulation, *A. dirus* and *A. farauti*, had intermediate hormone levels (<100 pg/MAGs), and negligible amounts (<20 pg/MAGs) were found in *A. sinensis* and *A. atroparvus* (intermediate plugs) and in the plugless *A. albimanus*. Sexual transfer of 20E was determined in five species for which we could reliably isolate mating events and dissect females shortly after copulation (Fig. 2B). All species transferred approximately half of the 20E content from their MAGs, with the exception of *A. arabiensis*, which invested the full 20E complement, suggesting a possible divergence of mating strategy in this important vector. This observed variation in hormone sexual transfer led us to assess the role of 20E in inducing postmating responses in other anophelines. Similar to results previously obtained in *A. gambiae* (6), 20E injections in virgin *A. arabiensis* females, a species with high levels of male-transferred 20E, triggered both oviposition and refractoriness to mating (Fig. 2, C and D). Conversely, these postmating responses were not induced in *A. albimanus*, as expected given the lack of 20E transfer by males (Fig. 2, C and D). Consistent with these findings, a mating-induced increase in egg development was observed in *A. arabiensis* and *A. stephensi* but not in *A. albimanus* (fig. S1).

To determine whether mating plug transfer and 20E synthesis in the MAGs are ancestral or derived traits within the anophelines, we next performed ancestral state reconstructions using maximum parsimony. As outgroups, we selected two related dipterans, *D. melanogaster* and *Aedes*

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aegypti, for which sufficient genomic information is available to build our phylogeny (fig. S2). Phylogenetic hypotheses about species relatedness

were based on maximum likelihood analysis of 4829 single-copy orthologs. Parsimony reconstructions indicated that the most recent common

ancestor of the species included in our phylogeny was likely one in which males were incapable of coagulating seminal secretions and did not

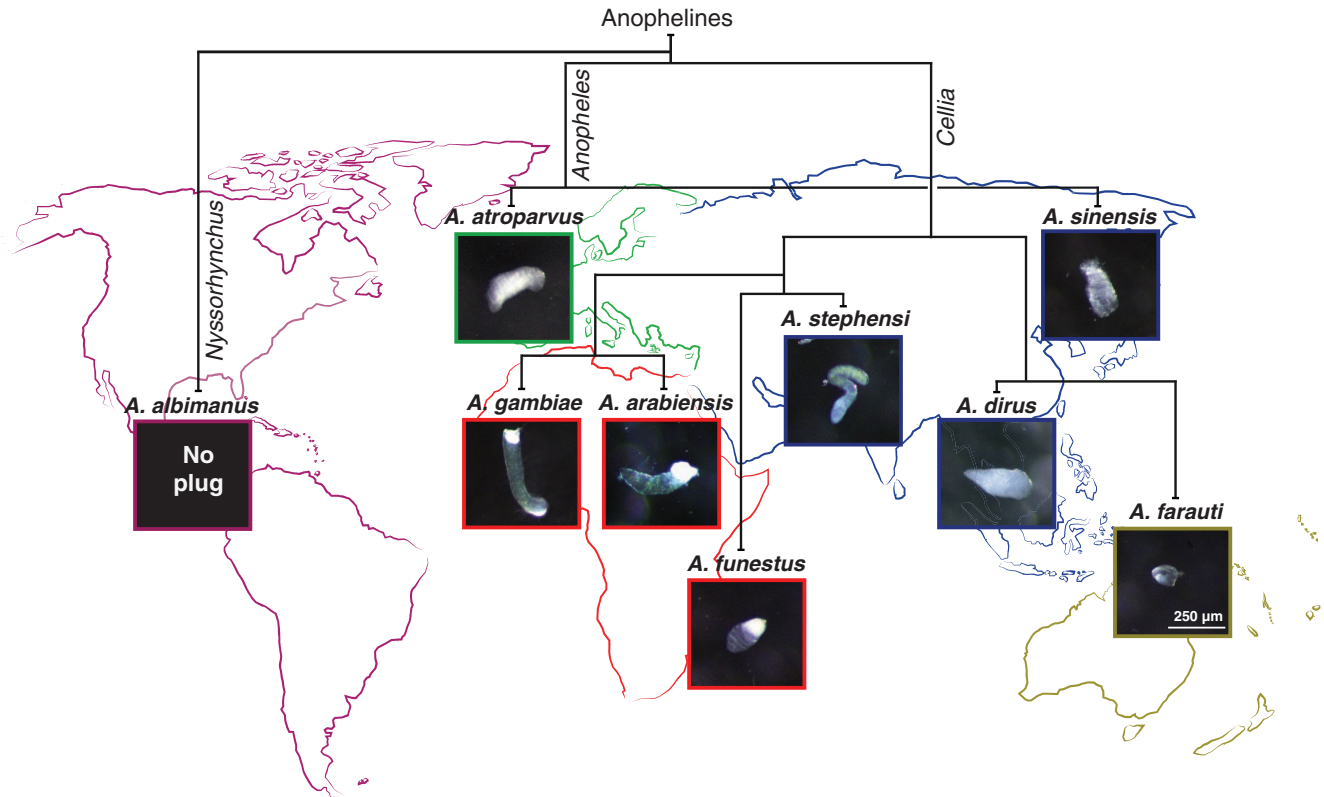


Fig. 1. Mating plug phenotypes across anophelinae. Considerable divergence in mating plug phenotypes is observed across nine geographically dispersed anopheline species that span three major subgenera (vertical branch labels). The African *A. gambiae*, *A. arabiensis*, and *A. funestus* and the Indian *A. stephensi* species transfer a structured, fully coagulated plug, whereas the east and southeast Asian species *A. farauti*, *A. dirus*, and *A. sinensis*, along with the European species *A. atroparvus*, present a less coagulated, amorphous plug phenotype. No mating plug transfer was recorded in the New World species *A. albimanus*. Species are placed according to their approximate geographical distribution, and the scale bar present in *A. farauti* applies to all. For accurate branch lengths, see fig. S2.

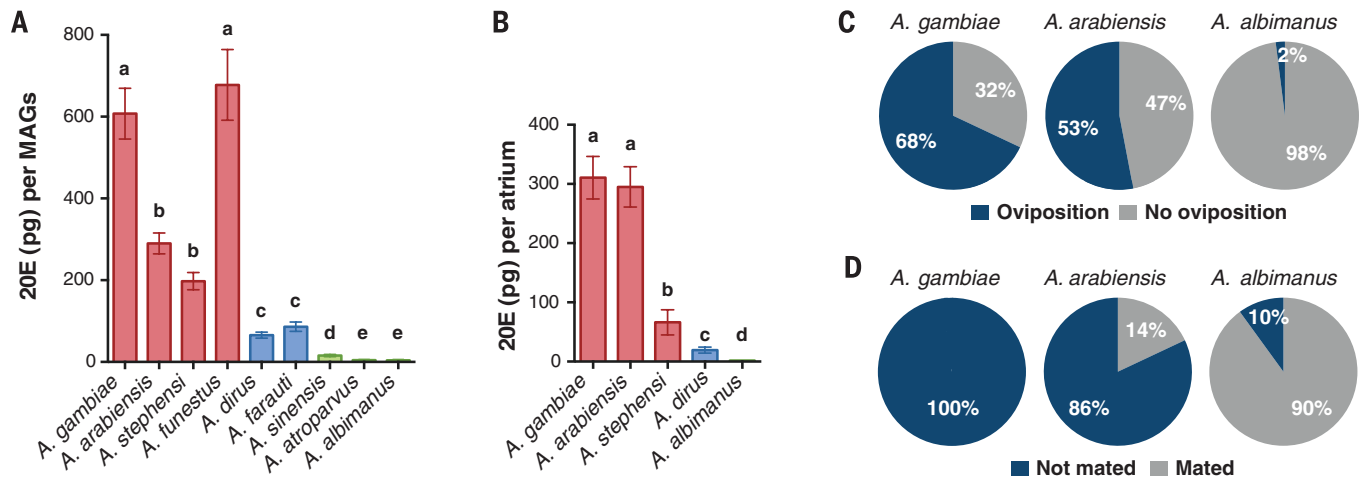


Fig. 2. Synthesis and transfer of 20-hydroxyecdysone (20E) and its effect on oviposition and refractoriness to mating. (A) 20E levels in male accessory glands (MAGs) differ significantly across the nine anophelinae [analysis of variance (ANOVA) $F_{8,73} = 227.8$, $P < 0.0001$]. Species are color-coded according to 20E levels (high, red; intermediate, blue; negligible, green). (B) 20E levels in the female atrium from five selected species dissected 1 hour postmating. *A. gambiae* and *A. arabiensis* receive significantly higher levels of 20E from males compared with the other species analyzed (ANOVA $F_{4,32} =$

80.52, $P < 0.0001$). In both (A) and (B), results are presented as mean values \pm SEM, and letters indicate post hoc significance (Tukey's post hoc test). (C and D) 20E injections in virgin females induce oviposition after blood feeding (C) and refractoriness to mating (D) in a species that sexually transfers 20E (*A. arabiensis*, $P < 0.0001$), but not in 20E-less *A. albimanus* ($P = ns$) when compared with ethanol-injected controls (Fisher's exact test). *A. gambiae* results are taken from previously published data (6). Both oviposition and refractoriness data are presented as percentage relative to control females.

synthesize the steroid hormone 20E in their MAGs (Fig. 3). From this ancestor, the ability to form a plug did not evolve until after the *A. albimanus* lineage split from the remainder of the *Anopheles* (node A). Our analysis indicates that plug formation began as a partially coagulated

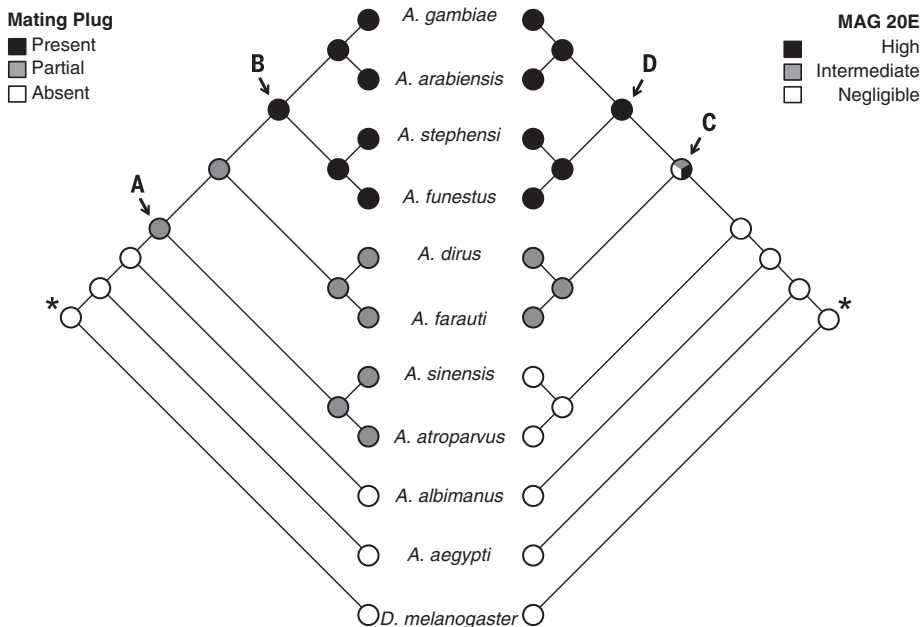
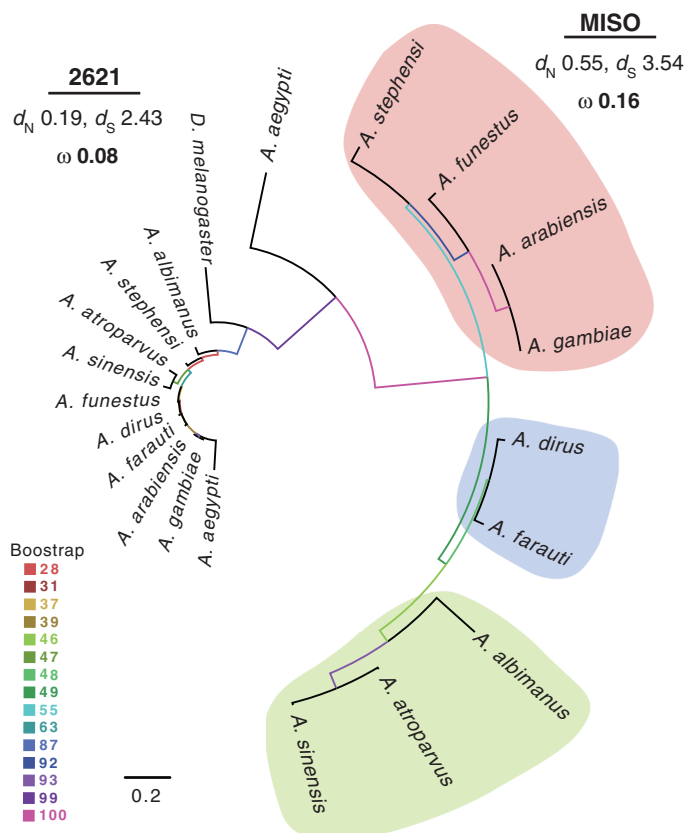


Fig. 3. Maximum parsimony multistate ancestral state reconstructions of mating plug and 20-hydroxyecdysone (20E) characters. Mating plug phenotypes (left) and 20E titers (right) from nine anopheline species and two related dipteran species (*A. aegypti* and *D. melanogaster*) were each coded as multistate categorical characters. Reconstructions reveal that the most common ancestor to all species (indicated by *) was one that lacked a mating plug and did not produce 20E in their male accessory glands (MAGs). Labeled nodes (A to D) are discussed in the text.

Fig. 4. Phylogenetic analysis of MISO and AGAP002621.

A maximum likelihood phylogeny of protein sequences from nine anophelines and two outgroup dipteran species (*D. melanogaster* and *A. aegypti*) using randomized accelerated maximum likelihood (RaxML). Across the anophelines, high sequence conservation (shorter branch lengths) is seen in the MISO paralog AGAP002621 (2621), whereas greater divergence, reflected in a higher d_N and ω ratio, is exhibited by MISO. Colors are coded according to 20E levels in Fig. 2A.



structure, with subsequent elaboration into a fully coagulated plug occurring at node B, the common ancestor of *A. gambiae*, *A. arabiensis*, *A. stephensi*, and *A. funestus*. The production of 20E was not likely to start until node C (where all states are equally parsimonious), suggesting that 20E synthesis in the MAGs evolved shortly after plug coagulation. High titers of 20E occurred at node D, representing the same common ancestor in which full plug coagulation arose. No reversions to the plugless and 20E-less states have occurred in these extant species. The remarkable congruence in ancestral state reconstructions of plug coagulation and 20E levels was confirmed with character correlation and independent contrast analysis, which indicates strong support for a model of correlated evolution between these two characters (independent contrasts, $r = 0.86$, $F_{1,10} = 26.65$, $P = 0.0006$).

Given our finding of correlated evolution between male ejaculate characters and the observed cross-species increase in egg development as a consequence of 20E transfer, females are predicted to exhibit a pattern of parallel (or reciprocal) evolution of the reproductive traits that translate male 20E into such a fitness benefit (17–19). To explore this prediction, we performed an evolutionary analysis of the female reproductive protein Mating-Induced Stimulator of Oogenesis (MISO), which to date is the only fitness-increasing female factor known to interact with sexually transferred 20E (5). MISO in *A. gambiae* is strongly induced by 20E specifically in the atrium (uterus) (5). Upon induction, the interaction of MISO with 20E leads to an increase in the expression of vitellogenic lipid transporters after blood feeding, causing a boost in oogenesis by a mechanism also known to promote *Plasmodium* development (5, 8). We created a maximum likelihood protein phylogeny using the orthologs of MISO (AGAP002620) and its adjacent paralog (AGAP002621) in the anopheline species and the *Aedes* and *Drosophila* outgroups studied above. Although the paralog AGAP002621 was considerably conserved, MISO was highly divergent and evolved more rapidly than AGAP002621 [phylogenetic analysis by maximum likelihood (PAML) analysis: ratio of the number of nonsynonymous to synonymous substitutions (d_N/d_S) MISO, 0.16; d_N/d_S AGAP002621, 0.08] (Fig. 4). Interestingly, among the species producing high titers of 20E, *A. funestus* showed a greater degree of sequence similarity to *A. gambiae* than to *A. stephensi* (Fig. 4), more consistent with 20E levels (Fig. 2A) than with phylogenetic distance across these species (fig. S2). Moreover, *A. albimanus*, which diverged some 100 million years ago from the rest of the anophelines (16) (fig. S2), surprisingly grouped with *A. sinensis* and *A. atroparvus* (Fig. 4), the other two species with negligible 20E levels (Fig. 2A). Taken together, these data indicate that MISO may be the product of gene duplication from its ancestral paralog and thus subject to relaxed evolutionary constraints promoting acquisition of novel functions (20), potentially driven by divergence in male 20E levels. Additional elements of female physiology

are likely to have evolved in response to these substantial changes in ejaculate characteristics. As an example, *A. gambiae* females synthesize significantly lower levels of 20E after blood feeding compared with *A. albimanus* (7, 21), indicating a possible adaptation of female 20E to levels transferred by the male. The observed effects of the male 20E-MISO interaction in regulating egg development suggest that the evolution of sexually transferred 20E will have influenced other blood-feeding-induced processes, with possible consequences for parasite transmission. Notably, a role for ecdysone in mediating protozoan parasite development has been reported in a number of insect species [reviewed in (22)], including other vectors of human disease (23).

Our phylogenetic approaches combined with phenotypic analyses of multiple reproductive traits provide considerable insight into a group of important disease vectors. Multiple key entomological parameters that directly affect malaria transmission are influenced by the diverse functions of sexually transferred 20E: mosquito densities via MISO-mediated increased oogenesis (5); parasite development through the expression of lipid transporters that protect *Plasmodium* from the mosquito immune system (8); and longevity due to reduced mating-associated fitness costs (9–11). Consequently, divergent sexual transfer of 20E across anophelines may have shaped their ability to transmit this deadly disease, and, intriguingly, all four species that transfer large levels of 20E are major malaria vectors originating from Africa and India, the regions of highest malaria burden (7). By demonstrating correlated evolution in male ejaculate characters and parallel changes in female physiology implicated in vectorial capacity, we reveal coevolutionary dynamics likely to have fundamentally influenced disease transmission to humans.

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and R.M.W. analyzed the data. S.N.M., E.G.K., A.S., and F.C. wrote the manuscript. S.N.M., E.G.K., and A.S. contributed equally to this study. All gene sequences are freely available via given gene identifiers from VectorBase (www.vectorbase.org). The single-copy ortholog sequences used to produce the species phylogeny are available via OrthoDB (<http://cegg.unige.ch/orthodbmoz2>). Protein sequence alignments employed for the species and MISO-AGAP002621 phylogenies are available via DRYAD: doi:10.5061/dryad.6f576.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 and S2
References (24–32)

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CLIMATE CHANGE

Atlantic and Pacific multidecadal oscillations and Northern Hemisphere temperatures

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The recent slowdown in global warming has brought into question the reliability of climate model projections of future temperature change and has led to a vigorous debate over whether this slowdown is the result of naturally occurring, internal variability or forcing external to Earth's climate system. To address these issues, we applied a semi-empirical approach that combines climate observations and model simulations to estimate Atlantic- and Pacific-based internal multidecadal variability (termed "AMO" and "PMO," respectively). Using this method, the AMO and PMO are found to explain a large proportion of internal variability in Northern Hemisphere mean temperatures. Competition between a modest positive peak in the AMO and a substantially negative-trending PMO are seen to produce a slowdown or "false pause" in warming of the past decade.

Distinguishing between forced and unforced variability in climate is critical for assessing the impact of anthropogenic forcing on temperature, drought, hurricane activity, weather extremes, and other climate phenomena. The North Atlantic and North Pacific oceans are the key drivers of internal variability in Northern Hemisphere temperatures on multidecadal time scales, but there is substantial uncertainty in their relative contributions to the observed variability. We applied a new semi-empirical method that uses a combination of observational data and a large ensemble of coupled climate model simulations to assess the relative roles of both forced and internal variability in the Northern Hemisphere over the historical period.

The Atlantic Multidecadal Oscillation (AMO) (7) is the leading mode of internal variability in North Atlantic sea surface temperature (SST) on multidecadal (~50 to 70 years) time scales (2–4). The Pacific Decadal Oscillation (PDO) (5, 6) is

the leading mode of North Pacific internal SST variability but, as defined, consists of at least two distinct signals, one roughly bidecadal with a ~16- to 20-year period and the other multidecadal with a ~50- to 70-year period (4, 7–9). The PDO and AMO time series typically are defined in terms of the temporal pattern of temperature change in the north-central Pacific and North Atlantic, respectively. The multidecadal component of the PDO may in part be related to the AMO [although centered in the Atlantic, it appears (2, 3) to project at least weakly onto the Pacific] and in part reflective of low-frequency variability related to the El Niño–Southern Oscillation (ENSO) and its extratropical response (10–16). We distinguish the multidecadal component from the conventionally defined PDO by terming it the "PMO," and we term the multidecadal component of internal Northern Hemisphere mean temperature variability the "NMO."

Prior methods used to define these internal variability components and their influence on Northern Hemisphere temperature include (i) a simple linear detrending of the mean North Atlantic SST time series (17–21), (ii) estimating the forced trend based on regression of North Atlantic SST against global mean SST and

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Evolution of sexual traits influencing vectorial capacity in anopheline mosquitoes

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Mating plugs promote malaria parasites

Males of some of the malaria-transmitting mosquitoes "plug" females after copulation to stop interloping males from mating. The mating plug also delivers a steroid hormone into the female uterus. This hormone pulse promotes egg production and stimulates egg laying. It also curbs the mosquitoes' immune responses, which allows parasites such as malaria to develop unhindered. Mitchell *et al.* discovered that plugs are a recent evolutionary acquisition (see the Perspective by Alonzo). South American anopheline mosquitoes lack these plugs altogether, whereas African and Indian species have complex plugs replete with hormones. It is unlikely to be a coincidence that the most elaborate mosquito plugs are also found in regions where malaria transmission rates are highest.

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