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Environmental effects on bet hedging in *Aedes* mosquito egg hatch

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Abstract   We used a path analysis procedure to examine the influence of environmental effects on the egg hatching response of the container breeding mosquito, *Aedes triseriatus*. *A. triseriatus* eggs were collected from 22 different sites across the eastern US, and exposed to repeated hatch stimuli in the laboratory. The resulting data were used to construct hatch indexes for each site. Structural equation modeling was used to discriminate among hypotheses relating to the functional relationships between population hatch trait and local climatic conditions. The results suggest that the delayed hatch pattern is an adaptive bet-hedging strategy that allows the species to manage desiccation risks. The selected model indicates that environmental variables differentially affect the immediate and the delayed, long term hatch patterns. High temperatures directly reduce the number of egg hatching on the first stimulus, but only indirectly affect the delayed hatch pattern. Low precipitation and high variability in precipitation directly increase the delaying pattern. The hatch trait appears to be a critical adaptation that allows the species to occupy a broad range in east North America.

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Introduction

Transitions between relatively resistant and vulnerable life history stages have posed strong selective pressures on a wide variety of organisms, leading to the convergent evolution of strategies controlling the tempo and mode of developmental progression (Cohen 1966; Livdahl 1979; Fell 1995; Cáceres 1997; Martin 1999; Gutterman 2002; Hairston and Kearns 2002). Examples of these developmental transitions include seed germination, cyst emergence, or egg hatching into environments that range unpredictably between favorable and unfavorable. Despite favorable environmental conditions and developmental pre-paredness, emergence is often staggered (Evans and Dennehy 2005). In this way, a sig-nificant fraction of the offspring remains in a resistant stage, effectively reducing the risk of total reproductive failure in the event of a shift to unfavorable conditions (Seger and Brockmann 1987).

This bet-hedging strategy implies an evolutionary tradeoff between risk aversion and early reproduction. In the absence of compensatory factors, any strategy that reduces per capita growth rate will be selectively disadvantaged relative to strategies that maximize it (Evans and Dennehy 2005). In fact, some environmental phenomena, such as seasonal timing (e.g., Hatchwell 1991; Van Noordwijk et al. 1995; Ludsin and DeVries 1997) or predation (e.g., Turchin and Kareiva 1989; Williams et al. 1993; Hairston 1997), should impose direct selection for immediate, concurrent emergence. Thus, many organisms may experience competing selective pressures for both immediate and delayed hatch. How are these divergent selection pressures resolved? We approach this question using eastern treehole mosquitoes, *Aedes triseriatus* (Say). Females of this species lay their eggs in batches in treeholes above the water line in forests across an extended area in east North America (Darsie and Ward 2005; see Fig. S1 for species distribution). The eggs hatch when submerged in water after a rainfall and the larvae develop in the confined envi-ronment until pupation occurs and the adult emerges. Larval development time varies from weeks to months, depending on temperature, habitat quality, resources, and larval density (Keirans and Fay 1968; Fish and Carpenter 1982; Livdahl 1982). The larva, in contrast with the relatively durable egg (Sota and Mogi 1992), is not able to survive if the habitat dries out due to drought conditions.

However, some eggs in each batch do not hatch after the first inundation, but hatch progressively after subsequent hatch stimuli inundations (Livdahl and Koenekoop 1985). This strategy, which is common among container-breeding mosquitoes (Gillett 1955a, b), effectively scatters the egg cohort though time, and has been suggested as an evolutionary response to biological factors such as competition (Livdahl et al. 1984), and to physical factors such as desiccation (Khatchikian et al. 2009), or more generally, as an adaptation to unpredictable environmental conditions. In contrast, other factors such as shortened growing-season and predation may impose strong selection for immediate hatching. It is expected that all of these factors can shape population hatch patterns, but the relative influences of such ecological variables have yet to be ascertained.

Here we describe the use of path analysis via structural equation modeling (SEM) (Wright 1921) to test the fit of the data to a priori causal hypotheses of functional rela-tionships between ecological variables and hatching patterns of *A. triseriatus*. In previous work, statistical modeling has shown that two indices of hatch delay, the proportion of eggs that hatch immediately and an index that captures the overall delayed hatch patterns through multiple hatch stimuli, were strongly correlated with temperature and precipitation (Khatchikian et al. 2009). According to those multiple regression models, precipitation has a positive effect on immediate hatch, which is moderated by low temperatures within the

domain of existing conditions. Temperature and precipitation interacted to produce predictions of highest delay where temperature was highest and precipitation was lowest. We proposed that these different hatch patterns reflect a tradeoff between the risk of desiccation during the larval stage and selective pressure to complete the life cycle before the onset of winter.

We sought to identify causal relationships between environmental variables and hatch patterns to clarify our understanding of the evolutionary pressures affecting mosquito populations and to explore, more generally, how complex phenotypic variables evolve in the face of complex and potentially opposed selective forces. In this report, we examine both direct and indirect effects of environment on hatch parameters, defining indirect effects as the result of environmental effect on one parameter through previous effect on another hatch parameter. We use path analysis to test explicit hypotheses aimed at clarifying the nature and the strength of relationships between environmental variables and hatch parameters in *A. triseriatus* using an information theory framework. Specifically, we test five groups of hypotheses for understanding the relationships (i.e., effects) between environment and immediate and delayed hatch patterns in *A. triseriatus*; the hypotheses are explicitly represented in Fig. 1.

Materials and methods

Egg collection

Eggs were collected with oviposition traps (16 oz plastic cups containing a $10 \times 4 \times 1$ cm wooden slat and filled halfway with water) from 22 forested locations across a wide range of *Aedes triseriatus* (Say) habitats (see Fig. S1). The sites were selected to maximize precipitation range and minimize potential bias from temperature and precipitation association. At each site, 20 oviposition traps were deployed at ground level during May and then June 1999. Following 1 month's exposure, wooden slats were recovered and returned to the laboratory. From each site, ten 50 egg batches were created. Once a week for 9 weeks, egg batches were exposed to a hatch stimulus consisting of 24 h immersion in an aerated 0.5 g/l nutrient broth solution. Responses to this stimulus have been found to compare well with hatch rates obtained in field immersion (see Livdahl et al. 1984; Livdahl and Edgerly 1987). Resulting hatched larvae were growth to adulthood. Adults were identified using an identification key (Darsie and Ward 1981) to check against species contamination; two mixed-species batches were discarded from the analysis. The likelihood of misidentified species was rather low for various reasons, including the distinctiveness of *A. triseriatus* when compared to other species and the strong niche differentiation characteristic of the family. At the end of the ninth stimulus, remaining unhatched eggs were checked for presence of viable embryos and total numbers were adjusted accordingly. A detailed description of site selection process, egg maintenance, hatching, and larval rearing procedures is presented elsewhere (see Khatchikian et al. 2009).

Hatch indices

For each egg batch, three hatch parameters were calculated: the fraction of eggs that hatched after the first stimulus (immediate hatch), and two statistics (*a posteriori,* newly developed) that represent the delayed hatch pattern. The first one is the stimulus when 50%
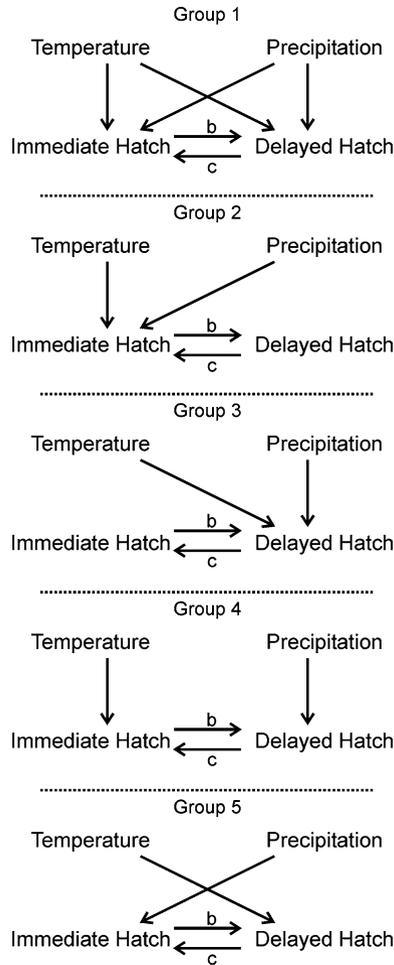
Group 1

Temperature   Precipitation

Immediate Hatch ⇄ Delayed Hatch
b
c

Group 2

Temperature   Precipitation

Immediate Hatch ⇄ Delayed Hatch
b
c

Group 3

Temperature   Precipitation

Immediate Hatch ⇄ Delayed Hatch
b
c

Group 4

Temperature   Precipitation

Immediate Hatch ⇄ Delayed Hatch
b
c

Group 5

Temperature   Precipitation

Immediate Hatch ⇄ Delayed Hatch
b
c

Fig. 1 Summary of path analysis models evaluated. Simplified representation of evaluated path analysis models for the five environment-hatch parameters hypotheses considered, each represented as a model group. *Group 1* direct temperature and precipitation effect on both hatch parameters. *Group 2* direct temperature and precipitation effects on immediate hatch. *Group 3* direct temperature and precipitation effects on delayed hatch parameters. *Group 4* direct temperature effect on immediate hatch and direct precipitation effect on delayed hatch parameters. *Group 5* direct temperature effect on delayed hatch parameters and direct precipitation effect on immediate hatch. In each group, models (*a*) have no effects among hatch parameters; models (*b*) have effects of immediate hatch on delayed hatch; models (*c*) have effects of delayed hatch on immediate hatch; and models (*d*) include effects in both directions, nonrecursive, equal to (b) + (c). For example, model 2b represents direct temperature and precipitation effects on immediate hatch and indirect effects of temperature and precipitation on delayed hatch parameters through a direct effect of immediate hatch

or more of the eggs in each batch have hatched (median stimulus), the second one is the delayed hatch dispersion in each batch, calculated as

$$-\sum_{i=2}^{9} p_i . \ln p_i$$

where p_i is the fraction of remaining unhatched eggs after first stimulus that hatch on stimulus i. When p_i is equal to zero, the term i takes the value of 0; thus, when all eggs hatch in the first stimulus the statistic takes the value of 0. The delayed hatch dispersion statistic takes a value of 0 when all eggs hatch in one single stimulus and takes its maximum value when equal egg fractions hatch in each stimulus (having a maximum value of 2.08 in the case of eight stimuli). For each collection site, one mean value was calculated for each statistic in order to avoid pseudoreplication artifacts.

Climatic data

We obtained climate data for the National Weather Service station closest to collection sites from the National Oceanic and Atmospheric Administration's National Climatic Data Center (accessible at ftp://ftp.ncdc.noaa.gov/pub/data/normals). Based on the 30 year (1960–1990) monthly maximum and minimum temperatures (°C) and precipitation (cm) averages for March to September, we calculated mean temperature, precipitation, and corresponding mean standard deviations (SD) for each weather station; the dataset, consisting of 231 egg batches, was reduced further to 19 locations due to shared proximity to weather stations.

Data analysis

We used multiple regressions within the context of path analysis. We tested each variable in the dataset for normality using the Shapiro–Wilk Test ($\alpha = 0.05$). Two parameters in the dataset failed the normality test, and transformations were made to correct them. Precipitation and SD precipitation values were transformed ($y' = 1-[1/y]$) to obtain normality. Pearson correlations between independent variables (temperature, SD temperature, precipitation, and SD precipitation) were calculated to identify covariant variables. Precipitation and SD precipitation were covariant ($P < 0.05$) and included in path analyses models. We used JMP 7.0 (SAS Institute 2007) for all statistical analyses unless stated otherwise.

We constructed the nested models' a priori topologies based on the five possible relationships of environment with hatch parameters considered, each one representing one group with four models. Group 1: direct temperature and precipitation effects on both hatch parameters. Group 2: direct temperature and precipitation effects on immediate hatch parameter. Group 3: direct temperature and precipitation effects on delayed hatch parameters. Group 4: direct temperature effect on immediate hatch parameter and direct precipitation effect over delayed hatch parameters. Group 5: direct temperature effect over delayed hatch parameters and direct precipitation effect over immediate hatch parameter. Within each group, four models were created representing different relationships between immediate and delayed hatch parameters, including both recursive and nonrecursive models: (a) models represent no effects between each hatch parameter; (b) models represent effects of immediate hatch over delayed hatch parameters; (c) models represent effects of delayed hatch parameters over immediate parameter; and (d) models include effects in both directions (nonrecursive models). In total, 20 models were evaluated (Fig. 1). A direct effect of median stimulus over delayed hatch dispersion was included as an additional constraint in all models. The best model was selected based on multiple criteria, including absolute fit and relative support in model comparisons. We evaluated the models with the χ^2 statistic for no differences comparing the tested model and saturated

model with the independence model. In addition, we set the following criteria for acceptable model fit: root mean square residual (RMR) values lower than 0.05 and comparative fit index (CFI) values above 0.95 (Schumacker and Lomax 2004). We used the ratio between the likelihood ratio χ^2 (CMIN) and degrees of freedom (df) to evaluate models for oversimplification, accepting values below 2. In order to resolve between multiple models reaching acceptable values, we compared the models using Akaike's Information Criterion (AIC, Akaike 1974) modified for small samples (AIC$_c$, Burnham and Anderson 2002, Angilletta et al. 2006), where lower values indicate better fit, as well as the goodness of fit index (GFI) and the adjusted goodness of fit index (AGFI), where higher values indicate better fit. In addition, in order to reveal potential contributions to the overall model, we examined whether the sequential removal of nonsignificant paths in the selected model improved the overall functionality of the model. After the first round of nonsignificant path removal, only the single path deletions that produced improvements in the model were kept and the process was iteratively repeated until no further improvements were possible. Path removal improvements were evaluated using the same fit criteria as above. Path analyses were performed using AMOS 7.0 (Arbuckle 2006), including specific paths for each hypothesis, explicit covariance between the exogenous variables precipitation and SD precipitation, and error terms for dependent variables. The χ^2 statistic used to compare models was included among the AMOS calculations. Path coefficients were calculated as standardized partial coefficients (mean = 0, variance = 1).

Results

Considering absolute fit criteria, only model 4b complied with all four criteria considered (χ^2 P value > 0.05, RMR < 0.05, CFI > 0.95, and CMIN/df < 2). This model also performed well with the relative fit criteria, presenting the lowest AIC$_c$, a relatively high GFI (value equal to 0.851, close to the highest value for all models, 0.879 for models 1b and 1c), and the highest AGFI. The AGFI could be interpreted as an alternative to the GFI index where it is adjusted for the number of parameters in the model. Considering the failure of other models to comply with CFI requirements and worse performance with the other relative fit criteria, model 4b was selected as the best description of the underlying biological processes. A summary of model fit parameters is presented in Table 1. The selected model (4b) indicates a direct temperature effect on immediate hatch parameter and a direct precipitation effect on delayed hatch parameters, with direct effects of immediate hatch on delayed hatch parameters. Temperature affects the immediate hatch directly and only indirectly affects the delayed hatch parameters.

The significant path coefficients in the selected model, model 4b (Fig. 2), suggest strong direct effects of precipitation and variability in precipitation on the delayed hatch response, specifically in the median number of hatch stimuli required to achieve a 0.5 or greater cumulative hatch fraction. High precipitation values decreased the number of required stimuli, while an increase in the variability of precipitation increases the number of required stimuli. The fraction hatching on the first stimulus (immediate hatch) is not affected by the precipitation patterns. Temperature directly affects the immediate hatch; increases in temperature decreases the number of eggs hatching on the first stimulus. Indirectly, an increase in temperature lengthens the dispersion pattern of the delayed hatch in subsequent stimuli. Summaries of total significant effects, both directly and indirectly, of environmental variables over hatch parameters appear in Table 2.

Table 1 Summary of model fit parameters

Environmental effect in model	Model	$P(\chi^2)$, df	RMR	CFI	CMIN/ df	AIC_c	GFI	AGFI
Direct effect of temperature and	1a	0.000, 7	**0.032**	0.397	6.664	−129.8	0.721	−0.114
Precipitation on both hatch parameters	1b	**0.076**, 5	**0.032**	0.925	**1.993**	−162.4	**0.879**	0.322
	1c	**0.076**, 5	**0.032**	0.925	**1.993**	−162.4	**0.879**	0.322
	1d*	–	–	–	–	–	–	–
Direct effect of temperature and	2a	0.000, 15	**0.022**	0.333	3.924	−133.5	0.675	0.393
Precipitation on immediate hatch	2b	**0.126**, 13	**0.012**	0.910	**1.454**	−169.5	0.811	0.593
	2c	**0.053**, 13	**0.023**	0.860	**1.706**	−166.2	0.774	0.513
	2d	**0.119**, 11	**0.048**	0.914	**1.514**	−167.7	0.805	0.504
Direct effect of temperature and	3a	0.000, 12	**0.034**	0.380	4.399	−133.6	0.714	0.332
Precipitation on delayed hatch	3b	**0.075**, 9	**0.042**	0.899	**1.735**	−164.8	0.824	0.454
	3c	**0.195**, 9	**0.032**	0.949	**1.371**	−168.1	0.864	0.577
	3d*	–	–	–	–	–	–	–
Direct effect of temperature on delayed hatch;	4a	0.000, 13	**0.022**	0.406	4.005	−136.3	0.711	0.377
Direct effect of precipitation on immediate hatch	4b	**0.275**, 11	**0.014**	**0.965**	**1.208**	**−172.1**	0.851	**0.622**
	4c	**0.173**, 11	**0.023**	0.936	**1.382**	−169.2	0.838	0.589
	4d	**0.178**, 9	**0.015**	0.944	**1.408**	−167.7	0.854	0.545
Direct effect of temperature on immediate hatch;	5a	0.000, 13	**0.015**	0.294	4.569	−129.0	0.688	0.320
Direct effect of precipitation on delayed hatch	5b	0.021, 11	**0.023**	0.825	2.049	−161.9	0.793	0.472
	5c	0.037, 11	**0.009**	0.854	**1.875**	−163.8	0.806	0.506
	5d*	–	–	–	–	–	–	–

Absolute fit criteria: $P(\chi^2)$, tests for differences comparing the tested model and saturated model with the independence model; df, degrees of freedom; RMR, root mean square residual; CFI, comparative fit index; CMIN/df, ratio between χ^2 statistic described above and degrees of freedom. Values reaching acceptable criteria are indicated in bold font. Relative criteria fit: AICc, Akaike's Information Criterion modified for small samples; GFI, the goodness of fit index; AGFI, adjusted goodness of fit index. Values presenting the best fit are indicated in bold font. Unidentified models are marked with *

The progressive deletion of nonsignificant paths in model 4b suggests that the effect of immediate hatch on median stimuli, and the effect of precipitation and SD precipitation on delayed hatch dispersion are not important for the model functionality. The deletion of these nonsignificant paths improved the overall model fit (χ^2 P value = 0.472, RMR = 0.012, CFI = 1.0, CMIN/df = 0.979, AIC$_c$ = −175.7, GFI = 0.852, AGFI = 0.703). The deletion of remaining nonsignificant paths (effect of SD temperature on immediate hatch and effect of mean stimuli on delayed hatch dispersion) did reduce the overall fit of the model.

An examination of non-selected models allows verification of some general trends in significant effects; all models that considered precipitation and SD precipitation direct effects on mean stimuli (models 1a,b,c, 3a,b,c, 4a,c,d) suggest the same effect as in the selected model (4b). Interestingly, some models that allow temperature direct effects on immediate hatch (1a,b, 2a,b, 4a) suggest the same effect as in the selected model (4b), but
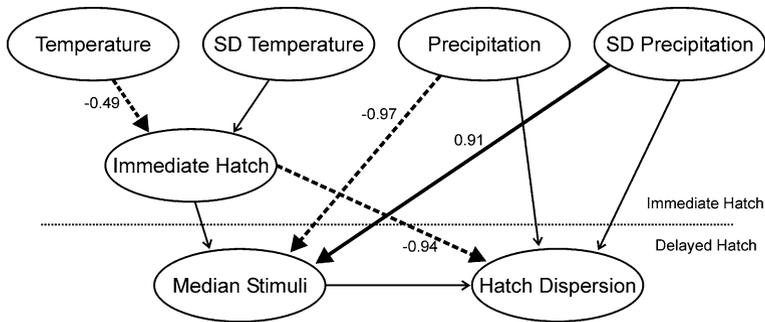
Fig. 2 Full representation of the selected path analysis model with greatest statistical support (model 4b); nonsignificant paths are shown with *thin lines*. Significant paths are shown with *thick lines* and *solid heads*, *solid lines* show positive effects, *dashed lines* show negative effects. Standardized regression coefficients (*direct effects*) are shown for significant paths

Table 2 Standardized regression weights of analyzed environmental variables over hatch parameters from the selected path analysis model with greater statistical support (model 4b, Fig. 1); these are total effects considering both direct and indirect effects

Environmental variable	Hatch parameter	Effect
Temperature	Immediate hatch	−0.494
	Delayed hatch dispersion	0.466
Precipitation	Median stimuli	−0.971
SD precipitation	Median stimuli	0.916

Only values significantly different from zero are considered

in those cases where delayed dispersion was allowed to affect the immediate hatch (1c, 2c,d, 3c, 4c,d) the temperature effect on immediate hatch was lost. In models that either immediate hatch or delayed hatch dispersion were allowed to affect each other (including nonrecursive models), significant effects were detected in all cases but one, model 4d, which reported a significant effect of immediate hatch on delayed hatch dispersion but a nonsignificant effect of delayed hatch dispersion on immediate hatch. Not surprisingly, the remaining nonrecursive models (1d, 3d, and 5b) were unidentified.

Discussion

A comprehensive analysis of the selected model (Fig. 2, see Table 2 for summary of effects) suggests that *A. triseriatus* populations from regions with low precipitation or a high level of variation in precipitation will present highly delayed, long term hatch responses. Interestingly, these precipitation variables did not affect the proportion of eggs that hatch in the first stimulus. Temperature was the only variable that was associated with the proportion of eggs hatching in the first stimulus. High temperatures were associated with reduced egg response to inundation, and indirectly, increased the spread of hatch response over hatch stimuli. The relationship of the latter with temperature is explained by the negative relationship between immediate hatch and delayed hatch dispersion detected in all models. These two hatch parameters are not directly related, as

the delayed hatch dispersion parameter depends on the proportion of egg hatching on different stimuli and not on the absolute number of unhatched eggs. Nevertheless, it should be noted that when a large fraction of the eggs hatch in the first stimulus, the few remaining unhatched eggs cannot spread their hatch over as many different stimuli. However, a similar argument could be made for median stimulus (which will take a value equal to one if the fraction hatching in the first stimulus reaches 0.5), but we observed no such relationship. Considering the SD temperature effect on overall model performance, it may play a marginal role in the immediate hatch response, although the effect is not significant.

These analyses allow predictions of how the hatch trait should respond in different conditions. In those populations facing low desiccation risks, where rainfall is abundant and/or consistent, most of the egg hatching occurs rapidly, in few stimuli, disregarding temperature effects. Concordantly, in high temperature situations, where few eggs hatch in the first stimulus, the majority do hatch on few subsequent stimuli. On the other hand, in desiccation prone habitats, high temperature could be playing a more relevant role as it would increase evaporation rates, and thus, risk. The combination of low precipitation, high variability in precipitation, and high temperature should be associated with a low fraction of eggs hatching in the first stimulus, with more repeated flooding events required to achieve high cumulative hatch levels. These results provide consistent support for the idea that delayed hatch is a critical evolutionary response to environmental conditions. Specifically, we propose that these results are consistent with a bet-hedging strategy that allows *A. triseriatus* populations to cope with intrinsic desiccation risk in its natural environment, effectively spreading risks over multiple instances as proposed by Khatchikian et al. (2009). Developmental delay caused by environmental stochasticity, in this context, can constitute a "cohort iteroparity" (after Wilbur and Rudolf 2006).

The path analysis approach allowed us to detect relationships that were previously unknown and provides a major expansion of the previously reported evolutionary role of the hatch trait. The statistical approach was used to test the fit of data to causal hypotheses regarding system functionality, in addition to the estimation of relative strengths of both direct and indirect environmental effects (sensu Shipley 2000). The analyses performed in this study, through the identification of specific effect of environmental variables, allowed the clarification of the somewhat conflicting predictions obtained with a multiple regression approach (Khatchikian et al. 2009). Nevertheless, we do not conclude that the selected model is the only possible model that explains the data, but rather is the best model among the nested models evaluated.

Further work should address several unknown effects of the hatch trait and environmental conditions in other life history parameters, such as egg diapause (Shroyer and Craig 1980) or larval diapause (Sims 1982). In addition, analyses of geographical population structure in the species (e.g., Munstermann et al. 1982; Beck et al. 2005) suggested low differentiation among populations. Concordantly, it could be proposed that the hatch trait evolves rapidly, in a manner similar to those of other life history traits (e.g., Sims 1985). Furthermore, the role of maternal influence on the hatch trait, if any, remains unknown, and the assessment of potential differences in maternal investment and/or parent-offspring conflicts is needed. Ideally, fitness performance of extreme strategies under different environmental scenarios would allow the development of testable mathematical models under a bet-hedging, ESS paradigm. We hope that this study will both spur further research on similar strategies in other groups, as well as in the *Aedes* hatch trait, considering that additional layers of complexity likely remain unresolved.

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