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Physiological Ecology

Overwintering in the Bamboo Mosquito *Tripteroides bambusa* (Diptera: Culicidae) During a Warm, But Unpredictably Changing, Winter

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Abstract

The bamboo mosquito, *Tripteroides bambusa* (Yamada) (Diptera: Culicidae), is a common insect across forested landscapes in Japan. Several studies have reported its overwintering as larvae and eggs, in both natural and artificial water containers. Nevertheless, it is unclear how sensitive this mosquito species is to changes in weather patterns associated with global warming. The El Niño event of 2015 through 2016 was one of the strongest on record and provided an ideal scenario for observations on the overwintering of the bamboo mosquito during a winter predicted to be unusually warm. Thus, we set oviposition traps in mid October 2015 and made weekly observations, from December 2015 to May 2016, on bamboo mosquito larval recruitment and pupation in Nagasaki, Japan. We found that larvae were pupating as late as the first week of January (prior records from the study site indicated mosquito pupation ended by mid-late October) and that pupation resumed in mid April (one month earlier than previous records at the study site). We also found that fourth instar larvae were able to survive in frozen oviposition traps following an extremely unusual snowstorm and cold spell and that recruitment of larvae from eggs happened after this unusual event. Our analysis suggested that overwintering and metamorphosis of the bamboo mosquito is sensitive to average and extreme temperatures, the latter measured by temperature kurtosis. Our results highlight the need to better understand changes in overwintering strategies in insects, and associated trade-offs and impacts on population dynamics, in light of climate change.

Key words: Schmalhausen's law, phenology, El Niño, climate change, diapause

Insects have developed different strategies to cope and survive the occurrence of extreme environmental conditions (Uvarov 1931, Janisch 1932, Bowler and Terblanche 2008). With the onset of climate change observations suggest that some new strategies to deal with extreme conditions could be evolving in insects and other invertebrates (Bowler and Terblanche 2008, Stoks et al. 2014). In mosquitoes (Diptera: Culicidae), from temperate latitudes, cold winter temperatures are associated with a diverse array of overwintering strategies. The two main mosquito overwintering mechanisms are diapause and quiescence, where the first has a preparatory phase triggered by environmental changes, while the latter does not have a preparatory phase and is a response that tracks environmental changes (Reisen et al. 2010). In mosquitoes, overwintering can occur as diapausing adults (Reisen et al. 2010), eggs (Tsunoda et al. 2015), or as quiescent larvae (Hoshi et al. 2014), quiescent adults (Reisen et al. 2010) or a combination of mechanisms depending on the developmental stage, e.g., diapausing eggs and quiescent larvae (Mori

et al. 1985), nevertheless none of these strategies include pupae (Denlinger and Armbruster 2014). Research on mosquitoes species from North America has shown that temperature could control quiescence in mosquito larvae (Sims 1982) and that both temperature and daylength could play a major role on triggering and terminating diapause in eggs (Shroyer and Craig 1983), and likely underpin observed patterns of mosquito phenology (Bradshaw and Holzapfel 2001, Bradshaw et al. 2004). These different strategies seem to have evolved independently numerous times in mosquitoes (Denlinger and Armbruster 2014) and they can drive the entomological risk of disease transmission by regulating the seasonal abundance of mosquito vectors (Ruiz et al. 2010, Shand et al. 2016), which is associated with vector-borne disease transmission risk (Smith et al. 2014). Moreover, rapid diapause evolution has been observed during the invasion and range expansion of important mosquito vectors of disease across environmental gradients (Urbanski et al. 2012). However, the major question remains: How much will diapause timing change in light of

climate change? For example, it has been observed that some mosquito species that overwinter as adults drastically change reproductive diapause patterns and gonotrophic activity during warm winters (Reisen et al. 2010), and important phenological changes could be expected for species that overwinter through quiescence, since physiological mechanisms are likely regulated by temperature (Reisen et al. 2010, Stoks et al. 2014).

The bamboo mosquito, *Tripteroides bambusa* (Yamada), is a common treehole mosquito species across temperate and subtropical East Asia (Tanaka et al. 1979). The overwintering of this species occurs as diapausing eggs and quiescent fourth instar larvae (Makiya 1968, Mori et al. 1985, Mogi 1996, Sunahara and Mogi 1997b). The immature stages of the bamboo mosquito frequently co-occur with immature stages from several mosquito species with medical importance, including two major globally invasive species, the Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae), and the Asian bush mosquito, *Aedes japonicus* (Theobald) (Diptera: Culicidae), as documented by numerous studies in Japan (Nakata et al. 1953, Bekku 1954, Kurihara 1958, Matuo 1961, Kurashige 1963, Makiya 1968, Moriya 1974, Zea Iriarte et al. 1991, Tsuda et al. 1994, Mogi 1996). It has also been suggested that *T. bambusa* might outcompete medically important species, like *Ae. albopictus*, when larval feeding resources are scarce (Sunahara and Mogi 1997a). Previous studies have also suggested that dry spells are a major mortality hazard for overwintering larvae of *T. bambusa* (Sunahara and Mogi 1997b), and that higher temperatures might drive an earlier

pupation start (Mogi 1996). Nevertheless, little attention has been given to the role that temperature might play on the overall pupation phenology in *T. bambusa* and its overwintering survival as larvae, or the recruitment of new larvae from overwintering eggs, especially during abnormally warm winters. Moreover, *T. bambusa* has population dynamics in ovitraps similar to what has been observed in natural treeholes of similar size. In both ovitraps and small treeholes (under 500 ml) *T. bambusa* reaches similar densities and co-occurs with a similar set of mosquito species (Zea Iriarte et al. 1991, Tsuda et al. 1994), thus making this species an excellent model to study overwintering ecology in semifield conditions that standardize larval habitats with the use of ovitraps attached to trees, rendering feasible the test of hypothesis about temperature impacts on mosquito overwintering during unusual winters.

Abnormally, warm winters in Japan are associated with the El Niño phenomenon (Zhang et al. 1996), a phenomenon that also increases the variability of weather patterns (Patz et al. 2002). During 2015 through 2016, a major El Niño event developed (McPhaden 2015, Levine and McPhaden 2016), thus allowing the prediction of an unusually warm winter. Indeed, in Nagasaki City (Japan), where *T. bambusa* is a common mosquito species (Omori et al. 1952, Tsuda et al. 1994, Hoshi et al. 2017), and where a meteorological station has been collecting data since the end of the 19th century (Fig. 1) weather records showed that the 2015/2016 winter rainfall (Fig. 1A) was similar to the median season, but temperature (Fig. 1B–D) was unusually high, especially minimum temperature
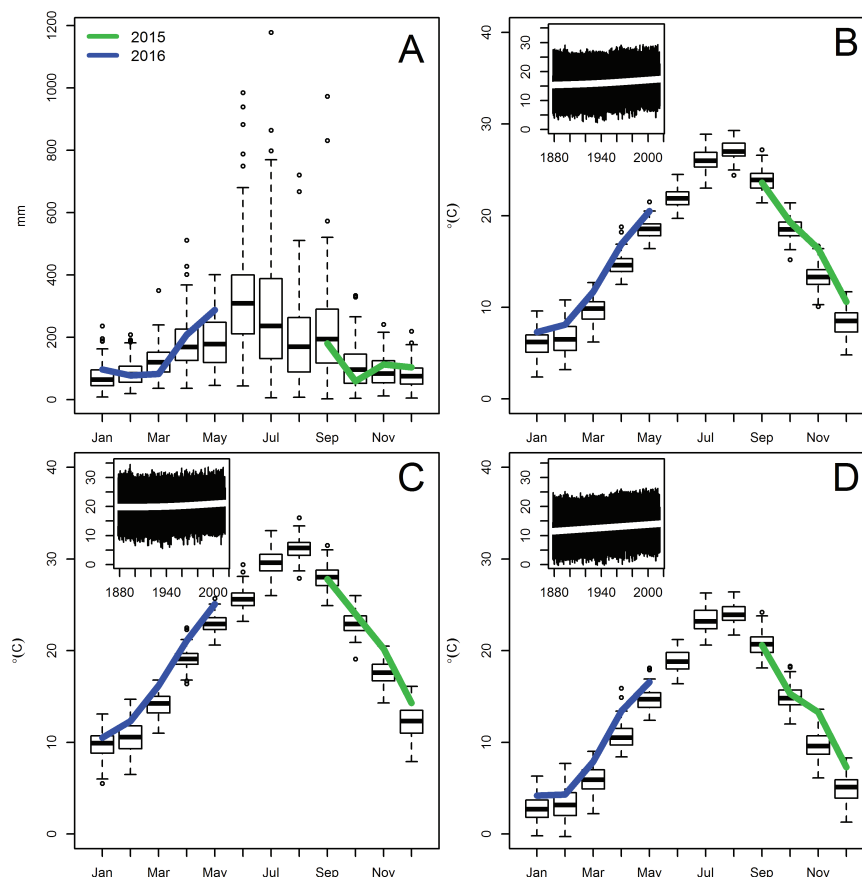

Fig. 1. Seasonal climate and the 2015 through 2016 fall, winter, and spring weather of Nagasaki, Japan: (A) rainfall, (B) mean temperature, (C) maximum temperature, and (D) minimum temperature. In (A to D), monthly histograms are built using historical weather records between January 1878 and December 2016. Inset panels in (B, C, and D) show the warming temperature trends in Nagasaki from 1878 to 2016. The inset legend of (A) indicates the color coding used for temperatures between September 2015 and May 2016.

(Fig. 1D). Previously, in our study area, *T. bambusa* pupation has never been recorded after the end of October, in years where winter temperatures were not abnormally hot in Nagasaki city (Mori et al. 1985) but also in Saga city (Sunahara and Mogi 1997b), at a similar latitude. The first pupation has been previously reported for mid-May (Mori et al. 1985, Sunahara and Mogi 1997b), an observation also made in the more southern Kagoshima city (Makiya 1968). Nevertheless, similar studies on the more southern islands of Tanegashima and Yakushima showed the first pupation of *T. bambusa* to occur in mid-March (Mogi 1996), suggesting that temperature, probably through faster larval development via the accumulation of degree days, might play a role on regulating quiescence in *T. bambusa* fourth instar larvae. Thus, the abnormal warm winter of 2015 through 2016 provided an ideal scenario to test the hypothesis that warmer temperatures may modify overwintering patterns in *T. bambusa*. Given previous observations about the phenology of this species in field and 'semifield' conditions, we evaluated changes in the end and beginning of *T. bambusa* pupation and provided detailed information about the impact that temperature and its variability measured as Standard Deviation (SD) and kurtosis had on the survival of quiescent fourth instar larvae and the recruitment of larvae from quiescent eggs.

Materials and Methods

Study Site and Mosquito Sampling

Our study was conducted in Nagasaki city, where Rainfall is seasonal having a unimodal peak in the summer around June and July and reaching a minimum during the winter in December and January (Fig. 1A). Similarly, mean temperature is highly seasonal, reaching a peak in July and August (Fig. 1B), and similar patterns are observed for the maximum (Fig. 1C) and minimum temperatures, where minimum temperatures rarely go below 0°C (Fig. 1D). It is worth highlighting that over the last 136 years, there has been some considerable warming at Nagasaki, with mean temperatures raising about 2°C (Fig. 1B), the trend being slightly larger for minimum temperature (Fig. 1D).

Nagasaki city is the capital of Nagasaki Prefecture, located in western Kyushu island (Fig. 2B), western Japan (Fig. 2A). In Nagasaki city, we conducted our study at the Nagasaki University Medical Campus in Sakamoto (Fig. 2C). To sample immature *T. bambusa* mosquitoes during their overwintering we placed seven ovitraps across the Campus (Fig. 2D). We set seven ovitraps given the longitudinal nature of our study, which ensured that enough data were collected for a statistically powerful analysis (Chaves 2010), especially considering that only three covariates were measured locally at each ovitrap. The location of ovitraps was constrained by the presence of trees whose height was above 12 m, explaining the larger concentration of traps in the southern side if our study area were a small secondary forest patch, locally known as 'Gubiro ga Oka', is present (Fig. 2D). Each ovitrap consisted of a 350-ml transparent plastic cup (Daiwa Bussan Co. Inc, Gojisho, Japan) covered on the outside with 45-mm wide black tape (Daiso Co., Hiroshima, Japan). Each ovitrap had a 5-mm drainage hole to ensure that mosquitoes were not lost due to the overflow of water contents, and also that each ovitrap did not collect more than 330 ml of water. Traps
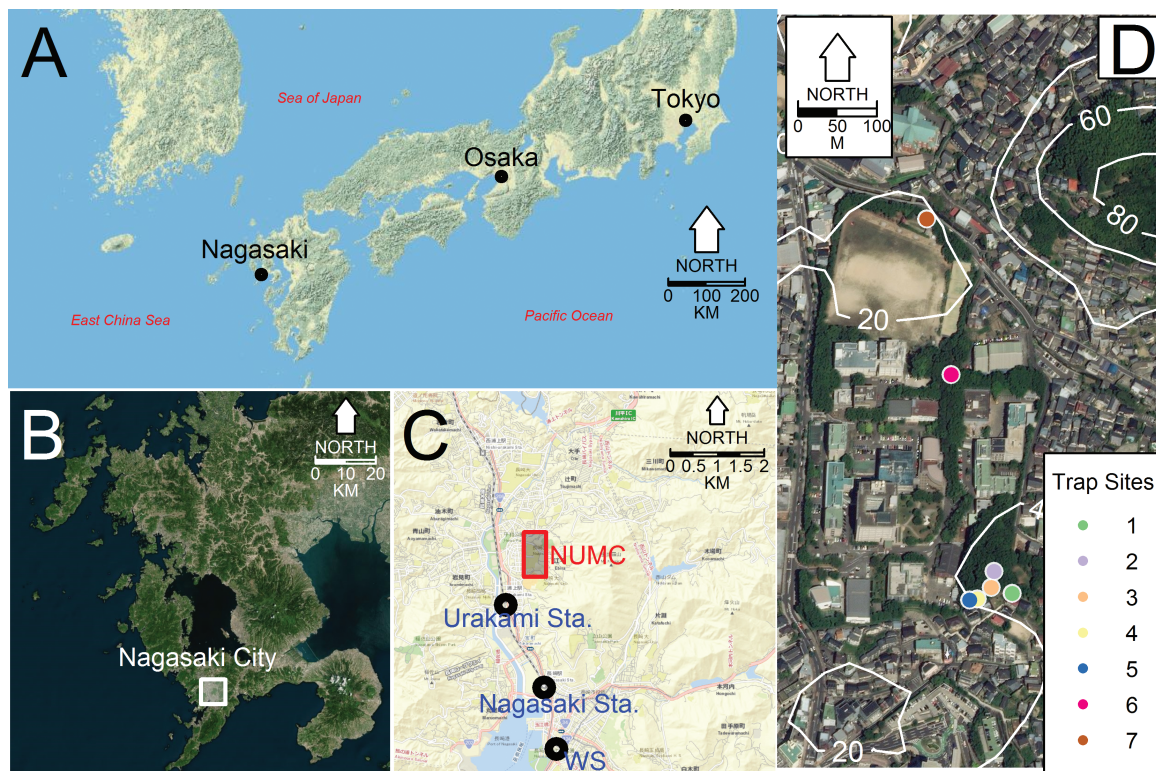

Fig. 2. Study Site. (A) Nagasaki is located in Kyushu island, western Japan. For reference the locations of Osaka and Tokyo, the two major cities in Japan, are also indicated in the map. (B) Nagasaki city is in southwestern Nagasaki prefecture, Western Kyushu island, the city core is highlighted in the map. (C) Nagasaki University Medical Camps (NUMC) is highlighted in this map, which magnifies the Nagasaki city core highlighted in (B). Nagasaki and Urakami train stations are presented as nearby reference points, and the location of the Nagasaki Weather Station (WS) is also indicated. (D) Trap locations inside NUMC. This map magnifies the area highlighted in (C). Each trap is color coded, see insect legend for details. Contour lines indicate elevation, in m, above the sea level and were derived from an ASTER digital elevation model for the studied area (http://gdem.ersdac.jspacesystems.or.jp/).

were covered in black, because it has been shown that *T. bambusa* preferentially oviposits in dark containers (Toma and Miyagi 1983). Traps were set at 1.2-m height, because it is known that *T. bambusa* oviposits in ovitraps at this height (Zea Iriarte et al. 1991, Chaves et al. 2015). Traps were deployed on 17 October 2015, coinciding with the end of seasonal adult activity for *T. bambusa* (Hoshi et al. 2017). This date was chosen to see if mosquitoes were still ovipositing this late in the season of an unusual warm fall (Fig. 1) and to minimize variability in the age of eggs that could hatch during the winter, while maximizing the probability of having diapausing eggs (Mori et al. 1985). On 17 October 2015, 150 ml of mineral water were added to each ovitrap. On 13 December 2015, we started to monitor each ovitrap weekly, recording whether they had small larvae, i.e., first, second, and third instar, the number of fourth instar larvae (i.e, larvae longer than 5 mm) and the number of pupae. Note that we waited a couple of months to gather observations on larval abundance, in order to ensure that increases in the number of larvae was due to the recruitment from larvae that hatched from diapausing eggs, since there was the possibility that females could have kept laying nondiapausing eggs late during the fall because of the unusually warm temperatures (Mori 1976). In addition, we did not count small larvae due to the great variability in density of these stages, which renders their censing unreliable in the field (Tsuda et al. 1994). Large larvae, i.e., fourth instar of species different from *T. bambusa*, as well as all pupae we found alive when sampling, were removed and raised to adults in a 25°C insectary for morphological identification of adults using the taxonomic key by Tanaka et al. (1979). We also recorded each week the water volume at each ovitrap and on 20 December placed a weather logger, Hobo model UA-002-64 (Onset Co., Bourne, MA) next to each ovitrap, which recorded light incidence in lumen per square feet and temperature in degree Celsius every 30 min for the duration of the study, which ended on 14 May 2016, the time recorded in a few studies as the start of pupation (Bekku 1954, Makiya 1968, Sunahara and Mogi 1997b) and the beginning of adult *T. bambusa* activity (Hoshi et al. 2017). Daily Temperature data from the Nagasaki city weather station and temperature records from the weather loggers were summarized weekly. More specifically, we estimated weekly mean, minimum, maximum temperature, as well as, the standard deviation and kurtosis for each of these series. This was done, given that in previous studies, we have observed that weather variability, measured as SD and kurtosis, are often fundamental to understand the impact of a changing environment on the abundance of mosquitoes (Chaves et al. 2011a, 2012, 2014; Chaves 2016). For rainfall, we generated a cumulative weekly time series from daily records from the weather station. Data on water volume from the ovitraps were used as collected, while we also estimated weekly cumulative light incidence from the loggers.

Statistical Analysis

Data analysis was done in two stages, first doing an analysis for the combined observations from all ovitraps at the study site, and then considering observations from each ovitrap.

For the combined observations analysis, we used the 23-wk-long time series for the proportion of ovitraps that had water and larvae and for the total number of fourth instar *T. bambusa* larvae across all ovitraps. These two time series underwent the same analysis protocol, which started with the inspection of the autocorrelation function (ACF) and partial autocorrelation function (PACF). These two functions graphically depict the correlation of temporal observations for all the time series (ACF) and for consecutive time lags (PACF), thus providing essential information on the potential lack of independence between temporal observations (Chaves 2010) that

needs to be considered when fitting a time series model (Shumway and Stoffer 2011). With information from the ACF and PACF, we fitted autoregressive models for each of the time series and used the coefficients from these models to prewhiten the weekly weather time series data generated from daily records of the Nagasaki city weather station. Prewhitening is a filtering process that removes autocorrelation structures from ancillary time series, thus avoiding the erroneous identification of correlation between time series just because they have a similar autocorrelation structure (Shumway and Stoffer 2011). We then used residuals from the autoregressive models and the prewhitened time series to estimate cross-correlation functions (CCFs), which are functions that graphically depict the correlation between two variables at different time lags (Shumway and Stoffer 2011). Based on the information from the CCFs and the PACF, we then fitted generalized linear models with an appropriate distribution (Faraway 2006) that had an autoregressive component and that included all lagged covariates found as significant with the CCFs. The resulting models from the previous step were then simplified using a process of backward elimination, where covariates are left out one at a time and models with an equal number of parameters are compared through their AIC (Akaike Information Criterion), a metric that compares model fit with different combinations and/or number of parameters (Faraway 2004). In each step, the model with the lowest AIC is then selected until no further simplifications are possible (Kuhn and Johnson 2013).

For the analysis considering data from each individual ovitrap, we employed regression trees (Olden et al. 2008). Regression trees are a set of rules that predict an outcome when a series of conditions in a set of covariates are met, thus recreating patterns in a response variable as function of multiple covariates (Faraway 2006). Here, we used regression trees to predict the probability that a trap was positive for small larvae, large larvae, or pupae. In this analysis, we fitted three models, where the response was a weekly time series that indicated whether an ovitrap had small larvae, large larvae, or pupae. In the analysis for the small larvae, we considered only environmental variables as covariates. These included the weekly time series measured at each trap: water volume (in ml), cumulative light intensity (in lumen/ft^2), mean, maximum and minimum temperature (in °C), as well as the SD and kurtosis of each temperature time series. For the large larvae (fourth instar) besides the set of environmental covariates employed in the tree for small larvae, we also considered the presence of small larvae during the previous week in the ovitrap. The tree for the pupae included the set of environmental covariates, the abundance of large and presence of small larvae during the previous week. We chose this analysis technique given its power to capture nonlinear relationships between the response and the covariates (Faraway 2006), the lack of assumptions about spatial or temporal independence (Chaves 2010) and the built-in process of variable selection where covariates that are not useful to the creation of predictive rules do not appear in the final model (Olden et al. 2008).

Results

Figure 3 shows weekly time series for the whole study site. Figure 3A shows the proportion of ovitraps that had water, and small or any type of *T. bambusa* larvae, all of which fluctuated through the study period. Here, it is worth highlighting that following a major snowstorm and cold spell at the end of seventh week of the study, there were no small larvae in any of the ovitraps in the eighth week. During the study period, the average (±SD) proportion of ovitraps with water was 0.91 ± 0.18, and the proportion with small larvae
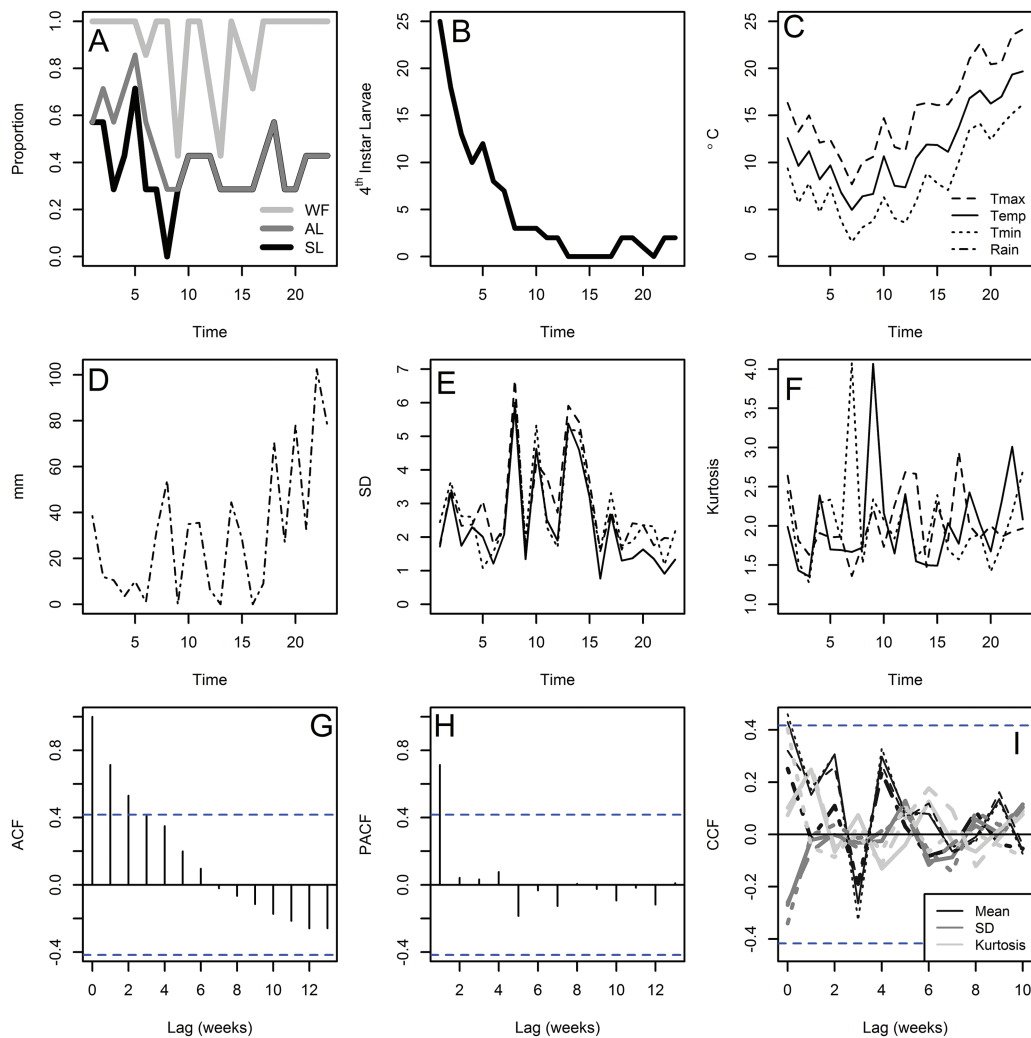
Fig. 3. Site-wide weekly time series. (A) Proportion of traps with water (WF), with any stage (AL), and small *T. bambusa* larvae (SL). (B) Total number of fourth instar *T. bambusa* larvae across the seven trap locations. (C) Temperature, minimum, mean, and maximum. (D) Rainfall. (E) SD of the temperature and rainfall time series. (F) Kurtosis of the temperature and rainfall time series. (G) ACF for the total number of fourth instar *T. bambusa* larvae. (H) PACF for the total number of fourth instar *T. bambusa* larvae. (I) CCFs between the total number of fourth instar *T. bambusa* larvae and temperature (mean, minimum, and maximum) and rainfall average, SD, and kurtosis estimates. Data in (C) and (D) come from the Nagasaki city weather station. For line coding in (C, E, F, G, and I), refer to the inset legend in (C). For color coding in (I), refer to its inset legend. Blue dashed lines in (G, H, and I) refer to the 95 % confidence limits where correlations are within what is expected by random.

0.38 ± 0.15 or any type of larvae 0.45 ± 0.16. Note that in week 8 after observations started none of the traps had small larvae, but the fraction of traps with small larvae quickly recovered to around 40% by the 10th week of the study, an indication of the continuous recruitment of larvae from eggs during the winter. Also, after the 10th week of the study, the lines for small larvae (SL) and all larvae (AL) overlap. Figure 3B shows the total number of large larvae, which continuously decreased through the study period, becoming 0 on the 13th week of the study, when some of the traps became dry (Fig. 3A). The decrease observed in larvae was due to both mortality as fourth instar larvae and to pupation, for example, by week 4 after we started to monitor larval density, we counted seven dead pupae and found three pupae alive while sampling, which assuming no further recruitment of fourth instar larvae from small larvae, implies a 38% (10 out of 26 fourth instar larvae, the maximum number of larvae counted across the census, on the first day of recorded observations) pupation rate in December. Nevertheless, there was likely some recruitment of fourth instar larvae as suggested by the

increase from 10 to 12 fourth instar larvae between weeks 4 and 5 of the recorded period (Fig. 3B). During the study period, the average (±SD) number of large *T. bambusa* larvae per ovitrap was 5.00 ± 6.68, and we counted a total of 115 larvae, when adding the totals from each weekly census. However, three larvae (out of seven) survived the freezing temperatures following the snowstorm and cold spell of the seventh week. Figure 3C shows the temperature, which reached a minimum during the seventh week of the study when the snowstorm and cold spell happened. Figure 3D shows rainfall during the study period. As expected, the number of ovitraps without water (Fig. 3A) followed rainfall dynamics, reaching local minima when there was no rainfall. Similarly, the number of fourth instar larvae became 0 (Fig. 3B) following a period of low rainfall (Fig. 3D), when some traps dried. Figure 3E shows the SD of temperature (mean, maximum, and minimum), all of which peaked during the seventh week, while Fig. 3F shows the kurtosis, which peaked on week 7 for the minimum temperature and in week 10 for mean temperature. Fig. 3G and H, respectively, show the ACF and PACF of

Table 1. Parameter estimates for the best Poisson generalized linear model explaining total fourth instar *T. bambusa* larvae abundance from all ovitraps

Parameter	Abundance increase	Estimate	SE	*z*-value	*Pr(>|z|)*
Intercept	—	−0.518	0.4112	−1.261	0.207
Fourth instar larvae abundance in the previous week (n_{t-1})	1.131	0.123	0.0127	9.678	<2e−16*
Minimum temperature kurtosis (Kurtosis Tmin)	1.533	0.427	0.1484	2.878	0.004*
AIC		86.747			
AIC FM		88.743			
ΔAIC		1.996			

AIC is the Akaike information criterion of the model; AIC FM is the AIC of a full model that also considered minimum temperature, and ΔAIC is the AIC difference between the full model and best model.

*Statistically significant (*P* < 0.05).

the large larvae abundance time series, which is typical of a first-order autoregressive process, implying that fourth instar *T. bambusa* larvae abundance is associated with itself during the previous week. Figure 3I shows the cross-correlation function between large larvae and the environmental variables, showing that the abundance was significantly (*P* < 0.05) correlated with minimum temperature and its kurtosis without a lag. Here, it is also important to consider that temperature time series summarize data for the day when abundance was recorded and the 6 d prior to its recording.

Using information from the correlation functions for the number of fourth instar *T. bambusa* larvae, we fitted a Poisson generalized linear model to explain the abundance of large larvae as function of itself in the previous week and minimum temperature and its kurtosis. We chose a Poisson distribution given that our response variable were counts (Faraway 2006). Through the process of model selection, we found that abundance with a 1-wk lag and minimum temperature kurtosis was enough to explain the abundance of large larvae, since this model minimized the AIC (Table 1). We also found that the Poisson distribution was a sensitive choice (residual deviance = 28.696, df = 19, $P(\chi 2) = 0.07$), thus ruling out the use of overdispersed count distributions, like the negative binomial distribution. Regarding parameter estimates, we found that for each additional larva, the week before the number of larvae increased by 13 and 53% for each additional unit of minimum temperature kurtosis (Table 1). Figure 4 shows a surface based on parameters from the fitted model. Figure 4 also illustrates the model goodness of fit (indeed the deviance in the response was reduced by 76%), and how the dynamics drastically changed after the snowstorm and its associated cold spell, as well as the crash in the population (when fourth instar *T. bambusa* larvae disappeared) when no rainfall was recorded.

The proportion of positive traps to any kind of *T. bambusa* larvae also showed an ACF (Supp Fig. 1A [online only]) and a PACF (Supp Fig. 1B [online only]), similar to the one for the number of large *T. bambusa* larvae, which is typical of a first-order autoregressive process. Nevertheless, unlike what was observed for the number of large larvae, this time series was not significantly associated with any of the covariates for the tested time lags (Supp Fig. 1C [online only]), suggesting that processes associated with the colonization of ovitraps were related to local ovitrap conditions.

Figure 5 shows data collected from each individual ovitrap, from the 3rd (27 December 2015) to the 23rd (14 May 2016) weeks of the study. Figure 5A shows the number of fourth instar larvae and alive pupae at each trap. It is worth highlighting that the highest number of larvae were in trap 6. Pupae were observed alive as late as 27 December 2015 and 3 January 2016, although the pupae did not emerge as adults in the laboratory, and joined other seven dead pupae found in the ovitraps by 3 January (four in ovitrap 6 and one each in ovitraps 4, 3, and 5, not shown in Fig. 5A). Similarly,
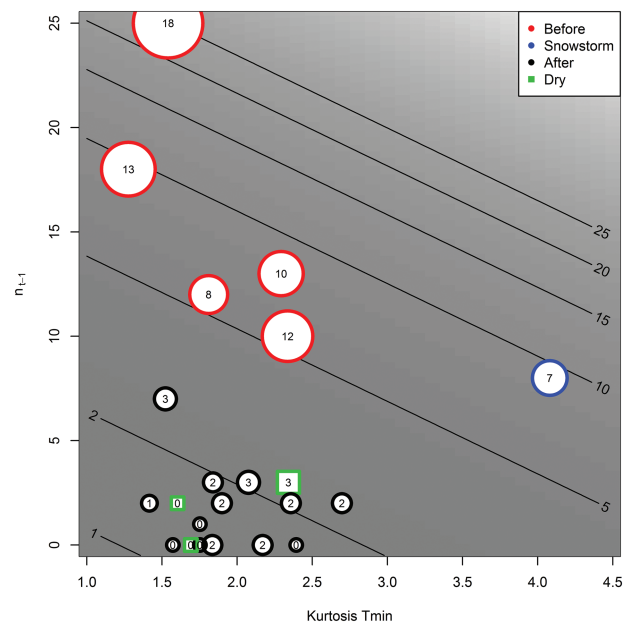

Fig. 4. Surface for the best Poisson generalized linear model explaining the weekly abundance of fourth instar *T. bambusa* larvae. The *x*-axis shows the kurtosis of the minimum temperature (Kurtosis Tmin) and the *y*-axis shows the abundance in the previous week (n_{t-1}). Contour lines indicate the fitted values and marks, whose size is proportional to the number of recorded larvae (indicated at the center of each mark). A guide for color coding of markings is presented in the inset legend, where 'before' and 'after' indicate, respectively, the abundance prior to, and after, the 27 January 2016 'snowstorm' and dry the weeks were no rainfall was recorded in Nagasaki city.

in trap 6, we saw the first pupation during week 20 of our study (23 April 2016). Trap 2 never had any kind of aquatic mosquito. In week 19, traps 1 and 6 had, respectively, three and one fourth instar larvae of *Ae. albopictus*, while in week 20, trap 7 had one larva of *Ae. albopictus*. Figure 5B shows the water volume collected by each oviposition trap, showing that traps 1 and 7 were dry twice, traps 2 and 5 became dry once, but stayed dry for 2 weeks, and trap 4 became dry in week 13, coinciding with the extinction of fourth instar larvae from the study site (Fig. 3B), and that there was a high variability in the water volume across ovitraps. Figure 5C shows the light intensity reaching the traps which was highest in trap 7 and some traps had a spike in week 10, coinciding with the time when these traps became dry. Figure 5D shows the average weekly temperature, which was very homogenous across all traps. Figure 5E shows the maximum weekly temperature at each ovitrap. Maximum temperature was highest in trap 7 throughout the study, and it was also high in trap 1 at the start of the experiment and on
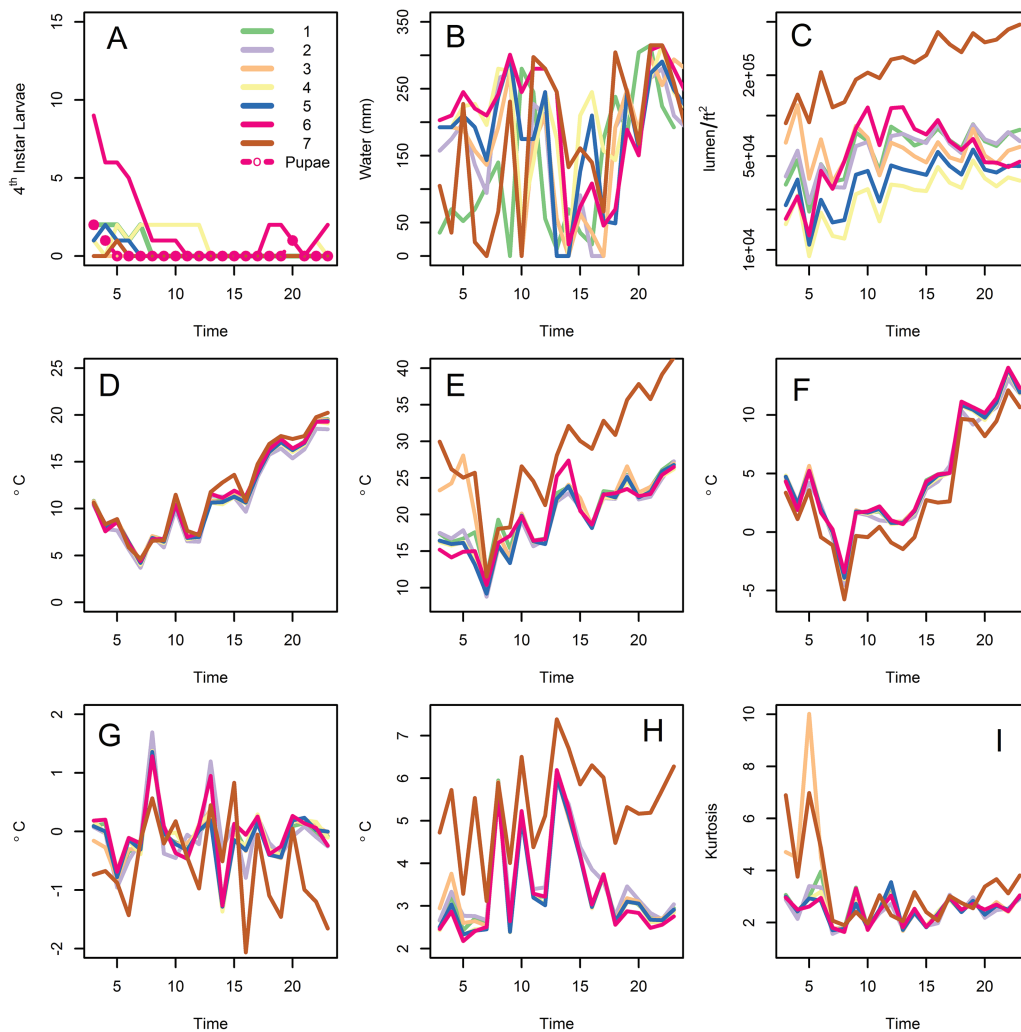
Fig. 5. Individual traps weekly time series: (A) abundance of fourth instar larvae and pupae of *T. bambusa,* (B) water volume, (C) light, (D) mean temperature, (E) maximum temperature, (F) minimum temperature, (G) difference between the mean and median temperature, (H) SD of the temperature, and (I) kurtosis of the temperature. The inset legend of (A) shows the color codes for the different traps.

week 14 for trap 6, when this trap became dry (Fig. 5B). Figure 5F shows that minimum temperatures were quite homogeneous across all ovitraps, being just slightly lower in trap 7, and also shows that the cold spell following the snowstorm of week 7 was synchronous across all traps in week 8, when small larvae disappeared from all ovitraps (Fig. 3A). Figure 5G shows the difference between the median and mean temperature for all ovitraps, where trap 7 shows a behavior clearly different from all other ovitraps. Figure 5H shows temperature SD for all ovitraps, which was highest in trap 7 during the whole study period. Figure 5I shows the temperature kurtosis for each ovitrap. It is worth highlighting that at the start of the study, kurtosis was larger in traps 1 and 7, and there were synchronous peaks for kurtosis, for all traps, in weeks 8 and 13, the first one corresponding to the cold spell that followed the snowstorm, whereas the second one corresponds to a week where the difference between maximum (Fig. 5E) and minimum (Fig. 5F) temperatures was large. The different patterns observed in ovitrap 7 could be related to being placed on an isolated tree.

The regression tree explaining the occurrence of small larvae in the ovitraps is presented in Fig. 6. In regression trees, variables at more basal nodes are the most important to make predictions, in this case the weekly cumulative light intensity. Coefficients at each node

are followed to the left branch, indicating that weekly cumulative light intensity below 88,710 lumen/ft^2 (light < 8.871e + 4) follows the node branching at (temperature SD = TSD ≥ 2.644), while a light above 88,710 lumen/ft^2 (light > 8.871e + 4) follows the node branching at (TSD < 7.054). In general, this regression tree indicates that light and high temperatures were factors increasing the probability that at any time an ovitrap was positive for small larvae. In the tree, both temperature SD and kurtosis are important factors for predicting the occurrence of larvae in a bound fashion, neither being too high nor too low.

Figure 7 shows the regression tree explaining the occurrence of large larvae in the ovitraps. The most important factor explaining their presence was temperature SD and then temperature kurtosis and light. In contrast with what was observed with the small larvae, a relatively high temperature SD was associated with ovitraps having large larvae. Relatively high temperature kurtosis, i.e, more leptokurtic weekly temperature distributions were, in general, associated with the presence of fourth instar larvae.

Figure 8 shows the regression tree explaining the occurrence of pupae in the ovitraps. For pupae presence, the most important factor was water, and then, in general, pupae occurrence was more likely at higher temperatures.
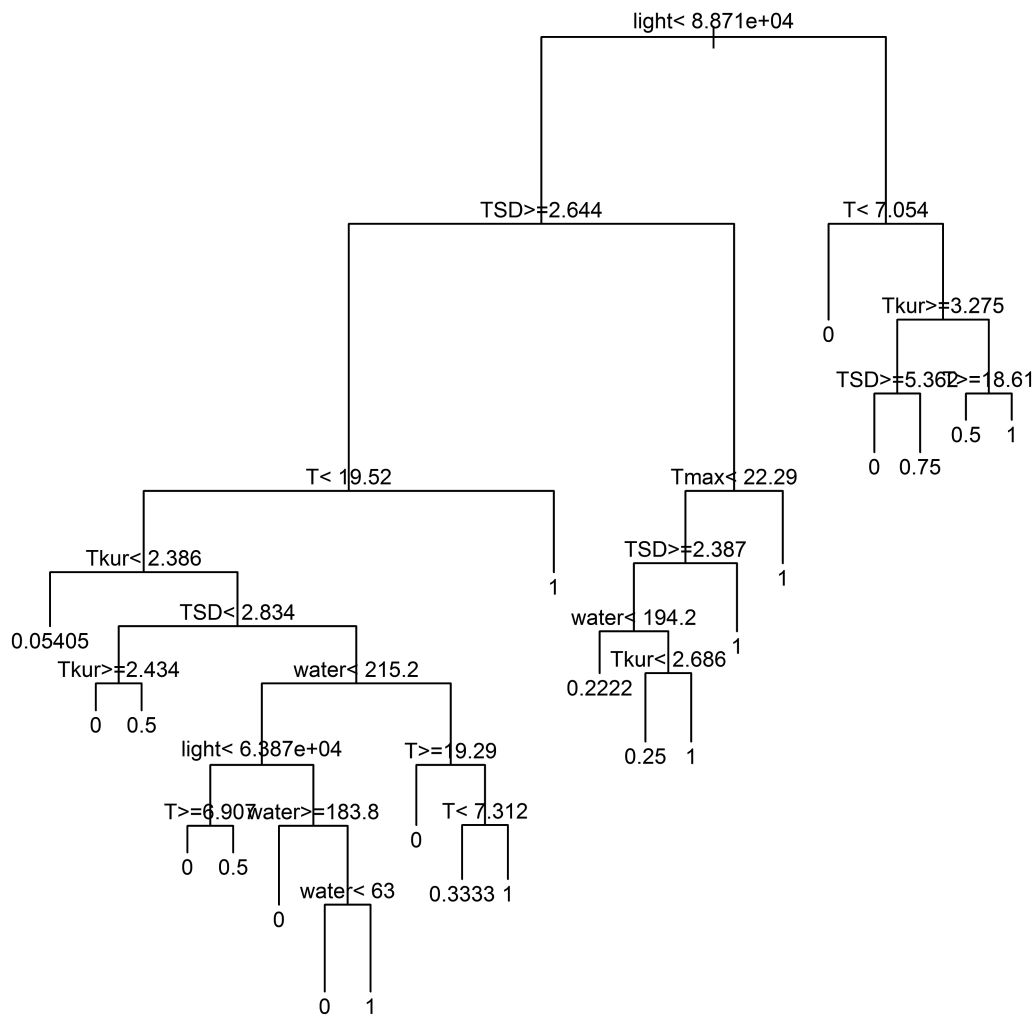
Fig. 6. Small (first to third instars) *T. bambusa* larvae presence regression tree model. Values in the terminal nodes indicate the probability of finding small larvae in a trap. Variables were measured weekly and include light, measured in lumen/ft^2, mean temperature (*T*), maximum temperature (T_{max}), SD of the temperature (TSD), kurtosis of the temperature (T_{kur}), and water level, measured in ml (water). Temperature measurements were in °C. When fitting the tree all predictions were cross-validated and the minimum split size, i.e., minimum number of observations per node, was 7.

Discussion

Our observations further support the suitability of ovitraps as good experimental units for studying larval mosquito overwintering ecology of container mosquito species. Both the percent of ovitraps with water (~90%) and the number of ovitraps with *T. bambusa* (~40%) were similar to previous observations for this species in treeholes during fall and winter months at Nagasaki (Tsuda et al. 1994). Our observations also document that fourth instar larvae of *T. bambusa* are able to survive in frozen containers for at least 48 h, the duration of the cold spell (with temperatures below freezing) during the turn from the 7th to the 8th week of our study, where almost 50% of the large larvae survived (three out of seven). Similar observations have been made on *Orthopodomyia alba* Baker, *Orthopodomyia signifera* (Coquillet), and *Anopheles barberi* Coquillet, all treehole species from the Great Lakes region of North America (Copeland and Craig Jr 1990), and also in *Wyeomyia smithii* Coquillet (Diptera: Culicidae) (Zani et al. 2005), where larvae can survive freezing conditions, and the survival increases with larval developmental stage. The relative high survival of large *T. bambusa* larvae was contrasting with the survival observed in the small larvae, all of which died, illustrating the increased environmental autonomy, or resistance, of

individuals with a more advanced development, i.e., the large larvae, to extreme environmental conditions (Janisch 1932, Bar-Zeev 1958, Bowler and Terblanche 2008). In that sense, it is worth highlighting that extreme cold temperatures have similar impacts as extreme high temperatures (Chaves et al. 2011b), since both have a differentially stronger mortality impact on younger larvae. Although it has been frequently reported that mosquitoes can survive in containers whose surface gets frozen (Denlinger and Armbruster 2014), it would be interesting to understand the special conditions that made possible the survival of larvae during the unusual freezing experienced in our study area, where temperatures below 0°C are rare (Isida 1969), an scenario different from that of the Great Lakes of North America with long and cold winters (Copeland and Craig Jr 1990), even more since it occurred against the background of an unusually warm winter (Fig. 1). Our analysis suggested that larval density and temperature variability, measured by temperature kurtosis, might play a role, but further observations are required to understand if survival is related, for example, to a density-dependent behavior to avoid freezing. Similarly, it will be interesting to evaluate the impacts of the mortality we observed on autogeny levels at the population level. None of the spring pupae from our ovitraps came from fourth instar larvae in quiescence from the fall, and it has been observed
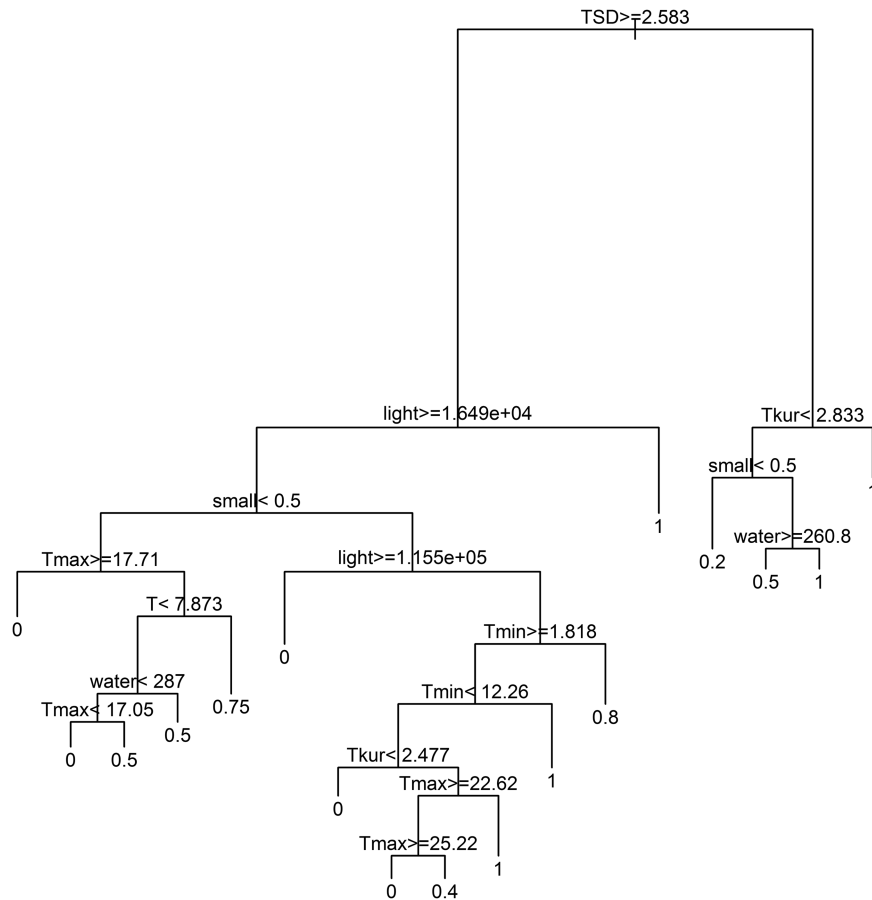
Fig. 7. Large (fourth instar) *Tripteroides bambusa* larvae presence regression tree model. Values in the terminal nodes indicate the probability of finding large larvae in a trap. Variables were measured weekly and include the presence of small larvae the previous week (small), light, measured in lumen/ft^2, mean temperature (T), maximum temperature (T_{max}), minimum temperature (T_{min}), kurtosis of the temperature (T_{kur}), and water level, measured in ml (water). Temperature measurements were in °C. When fitting the tree all predictions were cross-validated and the minimum split size, i.e., minimum number of observations per node, was 7.

that autogeny in *T. bambusa* is more common in females emerging from overwintering larvae that enter quiescence in the previous fall (Mori 1976).

A novel observation was the late pupation of *T. bambusa* fourth instar larvae, which occurred as late as the last week of December and the first week of January, which goes well beyond what has been previously reported, mid- to late-October (Mori et al. 1985, Sunahara and Mogi 1997b), and this late pupation is probably a maladaptive response to warming, since all pupae died before emerging into adults. Similarly, the first pupation occurred 1 month earlier, in mid-April, than what has been previously observed in Nagasaki (Mori et al. 1985) and Saga (Sunahara and Mogi 1997b), and quite interestingly despite the high mortality of fourth instar larvae, recruitment of larvae from diapausing eggs was continuous during the winter, and pupation started earlier, despite pupating individuals came from larvae that hatched during the winter. Thus, our results suggest that warming might be altering the overall phenology timing of *T. bambusa*, and despite what seems maladaptive response to temperature for pupation, the continuos recruitment of larvae from diapausing eggs allowed the survival of the population and a earlier pupation than what has been previously recorded for this species at the study site (Mori et al. 1985), and similar (Sunahara and Mogi 1997b) or even lower (Makiya 1968) latitudes.

Our observations also suggest that *T. bambusa* might be a good model species to understand molecular and physiological

mechanisms behind diapause (Denlinger 2002), since our results are suggestive of temperature regulated quiescence in larvae and a seemingly temperature-dependent termination of egg diapause. In that sense, it would be also interesting to evaluate the trade-off of different diapause mechanisms across mosquito species under climate change. For example, *Ae. albopictus*, the only species co-occurring with *T. bambusa* in our study, overwintered exclusively as diapausing eggs, probably minimizing mortality by unusual weather events when compared with *T. bambusa*. Thus, different winter diapause strategies can lead to different mosquito densities at the start of the adult mosquito active season, which, in turn, might be fundamental to understand mosquito outbreaks and the entomological risk of disease transmission (Chaves et al. 2012, 2014).

Regarding the importance of temperature for breaking quiescence in fourth instar larvae; i.e., for triggering pupation, the regression tree analysis suggested that its mean and maximum value were important, probably because physiological processes at this stage are triggered when certain temperature thresholds are experienced by insects (Janisch 1932). In contrast, the presence of small and large larvae was more sensitive to having environments with an appropriate level of kurtosis, i.e., not too leptokurtic, i.e., with low variation around the median value, but with extreme changes, not too platykurtic, with a large variation around the median, thus making extreme changes less rare. This result is in accordance with Schmalhausen's law (Chaves and Koenraadt 2010, Chaves 2017) which postulates that resilience
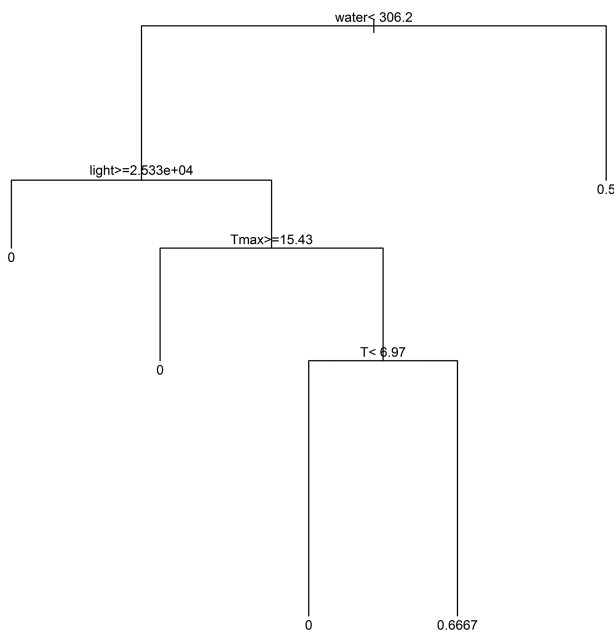
Fig. 8. *T. bambusa* pupae presence regression tree model. Values in the terminal nodes indicate the probability of finding pupae in a trap. Variables were measured weekly and include, water level, measured in ml (water), light, measured in lumen/ft^2, mean temperature (T), and maximum temperature (T_{max}). Temperature measurements were in °C. When fitting the tree all predictions were cross-validated and the minimum split size, i.e., minimum number of observations per node, was 7.

in organisms to cope with changes along any dimension of existence is decreased when forced towards the limits of existence in any of its dimensions of existence. Thus, it is not only that mortality increases, or more generally that fitness decreases, with extreme environmental conditions but also with the frequency of occurrence of such extreme environments, something measured by the kurtosis. Indeed, our data showed that unexpected warm temperatures had the detrimental impact of leading to pupae that subsequently died, and the sudden cold spell had a major impact on the massive mortality of small and large larvae by freezing. However, our results also highlight how impacts of warming temperature on insects are unlikely restricted to those induced by raising temperature on accelerating physiological processes but also to the stress that switching from leptokurtic to platykurtic environments impose in organisms.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

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