Journal of Thermal Biology 139 (2026) 104482

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Cold tolerance and egg diapause shape overwintering success in *Aedes koreicus* mosquitoes

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ARTICLE INFO

Keywords:
Dormancy
Egg hatching
Invasive mosquito
Temperature fluctuation
Thermal ecology

ABSTRACT

Aedes koreicus, a temperate mosquito native to East Asia, is rapidly expanding across Europe. While diapause is documented in this species, the combined effects of temperature and exposure duration on egg overwintering success remain poorly understood. We experimentally quantified the hatching success of diapausing and non-diapausing *Ae. koreicus* eggs exposed to five fluctuating cold regimes (ranging from 5 °C to −20 °C) for periods of 2 to 30 days, using a constant 5 °C control. Under control conditions, hatching success was similar between both egg types. However, cold exposure markedly reduced success, with non-diapausing eggs experiencing a significantly stronger decline (0.343 ± 0.029 SE) than diapausing eggs (0.487 ± 0.031 SE). Generalised linear beta-binomial models revealed a strong, nonlinear interaction between temperature, exposure duration, and egg type. Notably, diapausing eggs exhibited a hatching peak at intermediate subzero temperatures (−5 °C to −10 °C) and maintained higher success during prolonged cold exposure. Conversely, non-diapausing eggs were substantially more sensitive, showing sharper declines across the thermal gradient. These results align with the overwintering strategies of other temperate *Aedes* species, such as *Ae. albopictus*, and emphasize the necessity of considering both temperature and duration when assessing cold tolerance. Our findings provide essential parameters for phenological modeling and improve predictions of *Ae. koreicus* invasion potential in colder regions under variable climatic conditions.

1. Introduction

Aedes koreicus (Edwards, 1917) is a mosquito species native to continental eastern Asia, specifically China, Korea, and eastern Russia (Seok et al., 2024). First detected outside its native range in Belgium in 2008 (Versteirt et al., 2012), it has progressively expanded its distribution across several temperate European regions, with established populations in the Netherlands, Germany, Switzerland, Austria, Italy, Slovenia, Hungary and the Black Sea coast of the russian-occupied Crimean Peninsula and the adjacent Russian mainland (European Centre for Disease and Control, 2023). Although closely related to the more extensively studied *Aedes albopictus* (the Asian tiger mosquito), *Ae. koreicus* is proven to be a less competent vector of arboviruses (Ciocchetta et al., 2018; Höller et al., 2025; Jansen et al., 2021, 2022; Montarsi et al., 2015a), though it remains relatively understudied both in terms of its vector competence and life-history traits. Crucially, its bionomics and the role of temperature in shaping life-history traits of this species have only recently begun to attract attention.

Preliminary observational studies and habitat suitability models suggest that *Ae. koreicus* occupies a colder segment of the thermal niche compared to *Ae. albopictus*, with established populations in continental and alpine climatic regions (Arnoldi et al., 2022; Liu et al., 2023; Marcantonio et al., 2016; Marini et al., 2019). However, experimental data supporting this inference are limited. To date, only one laboratory-based study has comprehensively examined the bionomics of an Italian population of *Ae. koreicus* (Marini et al., 2019). Yet, a major question remains largely unexplored: how cold and freezing temperatures affect overwintering success at the egg stage, the main life stage capable of surviving winter conditions in temperate climates.

Diapause is a hormonally regulated state of suspended development and reduced metabolic activity that allows some mosquito species to survive under unfavourable conditions. Until recently, it was unclear whether *Ae. koreicus* eggs entered a true, hormonally regulated diapause or remained in a state of reversible quiescence. This distinction is critical, as diapause provides enhanced resistance to environmental extremes. We recently confirmed the presence of a facultative, photoperiod-induced diapause in *Ae. koreicus* eggs, with hatching rates significantly increasing only after approximately 100 days of dormancy

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https://doi.org/10.1016/j.jtherbio.2026.104482
Received 9 December 2025; Received in revised form 20 March 2026; Accepted 7 May 2026
Available online 7 May 2026
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(Marini et al., 2022). This finding aligns *Ae. koreicus* more closely with other invasive container-breeding *Aedes* mosquitoes, such as *Ae. albopictus*, whose eggs also exhibit a photoperiod-driven diapause enabling survival through cold winters (Denlinger and Armbruster, 2014).

Typically, diapause is associated with both behavioural and physiological modifications that enhance cold tolerance. For instance, *Ae. albopictus* diapause eggs have been shown to survive exposure to temperatures as low as −10 °C for short durations (12 and 24hrs; Thomas et al., 2012) and −5 °C for a month (Tippelt et al., 2020), with diapause-induced changes in chorion ultrastructure further improving resistance to desiccation and cold stress (Kreβ et al., 2016). Geographic origin, photoperiod exposure, and cold acclimation have all been shown to influence the overwintering success of container-breeding mosquitoes (Lacour et al., 2015; Tippelt et al., 2019, 2020).

Despite the overall trend of rising global temperatures, cold and freeze-thaw cycles remain a seasonal reality in much of the species' native and invasive ranges, especially in inner valleys and at higher elevations or latitudes. Thus, understanding the extent to which *Ae. koreicus* eggs, both diapausing and non-diapausing, can tolerate prolonged exposure to low temperatures is essential for predicting their persistence and further spread.

In this study, we investigate the response of *Ae. koreicus* eggs to fluctuating cold temperature regimes, focusing on the differences between diapausing and non-diapausing eggs, following a protocol similar to the one adopted in Tippelt et al. (2020). We aim to quantify how minimum temperature and exposure duration influence subsequent hatching success, thereby providing empirical data to inform predictions of the species' overwintering potential and population ecology under ongoing climatic change.

Based on current knowledge of temperate *Aedes* egg physiology, and building on previous evidence that *Ae. koreicus* develops faster than *Ae. albopictus* at colder temperatures and is predicted to be more abundant during the colder months of the breeding season (Marini et al., 2019), as well as field observations suggesting greater cold tolerance in this species (Montarsi et al., 2015b), we formulated three hypotheses. First (H1), we expected diapausing eggs of *Ae. koreicus* to show higher hatching success than non-diapausing eggs across a range of sub-zero temperatures and cold exposure periods, reflecting diapause-associated cold tolerance. Second (H2), we hypothesised that temperature and cold exposure periods would interact to produce a non-linear hatching response in diapausing eggs. In contrast, in non-diapausing eggs, we expected hatching success to decline more uniformly with colder and longer exposure. Third (H3), by integrating our experimental data with previously published results for *Ae. albopictus* (Tippelt et al., 2020), we tested interspecific comparison of cold tolerance and predicted that *Ae. koreicus* eggs would occupy a broader and colder thermal niche, maintaining higher hatching success across a larger fraction of the temperature–duration space. We tested these hypotheses using beta-binomial regression models fitted to hatching data from controlled fluctuating cold-exposure experiments.

2. Materials and methods

2.1. Establishment and maintenance of Aedes koreicus colonies

2.1.1. Non-diapausing colony

A laboratory colony of *Ae. koreicus* was established in 2020 from larvae and pupae collected in Castel Ivano municipality, Italy (46°02′N, 11°32′E; 360 m a.s.l). Mosquitoes were reared under controlled environmental conditions in a climatic chamber set at 21 ± 1 °C, 75 ± 5% relative humidity (RH), and a photoperiod of 16L:8D, including 1 h dawn and 1 h dusk transitions. Larvae were reared in 500 ml plastic cups containing 250 ml of dechlorinated water and fed daily with finely ground commercial cat food (Adult Fit 32™, Royal Canin®, Aimargues, France). Adult mosquitoes were maintained in 45 × 45 × 45 cm mesh cages (Bugdorm, MegaView Science Co., Ltd., Taiwan) and provided with cotton pads soaked in 10% sucrose solution ad libitum.

To promote mating behaviour and resting, a black fabric was placed along one wall of each cage, as recommended in (Watson et al., 2000). Females were blood-fed with cow blood twice a week for 1 h using a Hemotek® membrane feeding system (Hemotek Ltd., Accrington, England). Two ovitraps filled with grass infusion and lined with filter paper served as oviposition support.

2.1.2. Diapausing colony

A second colony for inducing diapause was established using larvae hatched from the non-diapausing colony eggs. Rearing conditions were the same as above, except that from the fourth larval instar (L4) onwards, individuals were maintained under a short-day photoperiod of 11L:13D with 1 h dawn and 1 h dusk. This photoperiod corresponds to a typical autumnal day length across temperate Europe, a season during which field populations of *Ae. albopictus* are known to produce a high proportion of diapausing eggs (Toma et al., 2003).

2.2. Egg storage and pre-experiment conditions

Eggs from both colonies were collected weekly and stored in plastic cups within loosely sealed polyethylene bags at 21 ± 1 °C and 75 ± 5% RH. Non-diapausing eggs were kept under a photoperiod of 16L:8D, while diapausing eggs were maintained under 8L:16D, a photoperiod routinely adopted for long-term laboratory storage of *Ae. albopictus* eggs that we assumed to be valid also for *Ae. koreicus* due to the lack of information for this species. As the photosensitive stages in this similar species are the parental pupa and adult rather than the egg (Wang, 1966; Imai and Maeda, 1976; Mori et al., 1981), diapause fate is determined prior to oviposition and is not affected by post-laying photoperiodic conditions.

2.2.1. Egg hatching trials

Eggs aged 2–3 months were used for both diapausing and non-diapausing treatments. Eggs from the same oviposition batch were divided into control and cold-treatment groups. Under a stereomicroscope, egg strips were visually inspected, and only eggs meeting the following criteria were selected for inclusion in the cold-treatment protocol: intact operculum (no signs of opening), absence of collapse, and overall healthy appearance of the chorion. For each group, three egg strip segments containing approximately 20 selected eggs were cut and placed into covered Petri dishes. The number of selected eggs per segment was recorded prior to treatment.

To reduce thermal shock, climatic chamber temperatures were gradually decreased by 5 °C per day until the starting experimental temperature (+5 °C) was reached.

To assess baseline egg hatching performance prior to cold exposure, a subsample of eligible eggs was drawn from each batch used in the cold treatments experiments. For each oviposition date, three strips of approximately 20 eggs per batch were incubated at 21 ± 1 °C with a standard food supplement (4 mg cat food per Petri dish) and monitored for hatching. Unhatched eggs were subsequently bleached to confirm embryogenesis and assess developmental stage independently of hatching outcome. For diapausing eggs, a second viability check was performed at 2.5–3 months post-oviposition to confirm diapause termination prior to experimentation. To formally test whether baseline hatching success differed between egg types or across batches assigned to different cold-treatment groups, we fitted a beta-binomial model to these control incubation data using glmmTMB (Brooks et al., 2017), specifying an interaction between the egg type (diapausing and non-diapausing) and the assigned cold-treatment group as a categorical factor. Since all subsamples were incubated at 21 °C, this factor serves as a batch identifier rather than an actual temperature treatment, and any significant effect should be interpreted accordingly.

2.3. Cold exposure protocol and hatching assay

We followed a protocol similar to the one adopted in (Tippelt et al., 2020), summarised in Fig. 1. Egg strips were exposed to five different fluctuating temperature cycles: from 5 °C to 0 °C, from 5 °C to −5 °C, from 5 °C to −10 °C, from 5 °C to −15 °C and from 5 °C to −20 °C. Each cycle followed a 24-h profile: 8 h at 5 °C, 4 h cooling to the minimum temperature, 8 h at the minimum temperature, and 4 h warming back to 5 °C. Eggs were exposed to these cycles for 2, 5, 10, 20, or 30 consecutive days. Environmental conditions were maintained at 75 ± 5% RH under constant darkness (0L:24D).

A constant 5 °C exposure served as a control treatment. For each temperature-duration combination (20 combinations in total), two to three replicate strips containing 20 eggs each were used, for a total of 15 strips (300 eggs) per condition. Cold exposures began with the mildest cycle (5 °C/0 °C), followed by increasingly extreme cycles.

Following cold treatment, Petri dishes with eggs were transferred to 21 °C and left inside a polystyrene container for 2–4 days to acclimate to warmer temperature (Thomas et al., 2012). Eggs were then immersed in 150 ml of dechlorinated water in plastic cups, maintained at 21 ± 1 °C, 75 ± 5% RH, and a 16L:8D photoperiod (with 1 h dawn/dusk transitions). Four milligrams of finely ground cat food were added to stimulate hatching. Egg papers were submerged for five consecutive days, then allowed to dry for two days. This wet–dry cycle was repeated two to three times. Emerging larvae were counted and removed daily.

Eggs that did not hatch by the end of the final cycle were considered non-viable and not further examined for viability or embryo development.

2.4. Data analysis

We evaluated the factors influencing egg hatching success using generalised linear models (GLMs) with a beta-binomial error distribution, implemented in glmmTMB (Brooks et al., 2017). The beta-binomial family was selected to account for overdispersion commonly observed in count-based hatching data. The response variable was specified as the number of hatched eggs out of the total number of selected eggs per replicate. Two model formulations were tested to address H1 and H2. Because eggs originated from distinct oviposition batches, we initially fitted beta-binomial mixed models including a random intercept for batch. In all cases, the estimated variance of the random effect was near zero and model fit, as assessed by AIC and likelihood-ratio tests, did not improve (ΔAIC <2). We therefore retained simpler models without random effects.

To assess species-level differences in cold tolerance (H3), we additionally retrieved and harmonised egg hatching data from a temperate strain of *Ae. albopictus* reported in (Tippelt et al., 2020), allowing direct quantitative comparison with our *Ae. koreicus* experimental results.

2.4.1. Model 1: treatments vs. control comparison (H1)

To directly test the effect of cold exposure treatments versus the control (constant temperature set at 5 °C) on the number of eggs hatched (H1), we implemented a GLM with a binomial error distribution and logit link function defined as follows:

$$\text{Hatched} / \text{Total} \sim \text{ExpSetting} \times \text{TypeEggs} \qquad \text{(Eq. 1)}$$

Here, the response variable is the number of hatched eggs out of the total number of selected eggs per replicate, ExpSetting is a categorical variable with two levels: control (baseline) and treatment (cold exposure periods), and TypeEggs is also a categorical variable distinguishing diapausing and non-diapausing eggs (with diapausing eggs set as the reference category).

2.4.2. Model 2: temperature response across egg types and cold exposure periods (H2)

To assess how temperature, egg type, and cold exposure time interact to influence hatching (H2), we modelled the number of hatched eggs as a function of a second-order polynomial of temperature, egg type, and cold exposure periods:

$$\begin{aligned} \text{Hatched} / \text{Total} \sim{} & \text{poly}(\text{Temperature}, 2) \times \text{TypeEggs} + \text{TypeEggs} \\ & \times \text{Period\_length} + \text{poly}(\text{Temperature}, 2) \times \text{Period\_length} \end{aligned} \qquad \text{(Eq. 2)}$$

In this model, poly(Temperature, 2) denotes a second-order polynomial of temperature to allow detection of potential non-linear (non-quadratic) relationships between temperature and hatching response, Period_length is a continuous variable referring to the duration (in days) of exposure to cold conditions, and TypeEggs is defined as in Eq. (1).

Both models described in Equations (1) and (2) were fitted using the *glmmTMB R* package (Brooks et al., 2017), which supports generalised linear models with a beta-binomial error distribution. Model selection was guided by the Akaike Information Criterion (AIC) and likelihood ratio tests (LRTs) to identify the most parsimonious model adequately describing the data. Model diagnostics were performed using the DHARMa package version 0.4.7 (Hartig, 2016), evaluating model

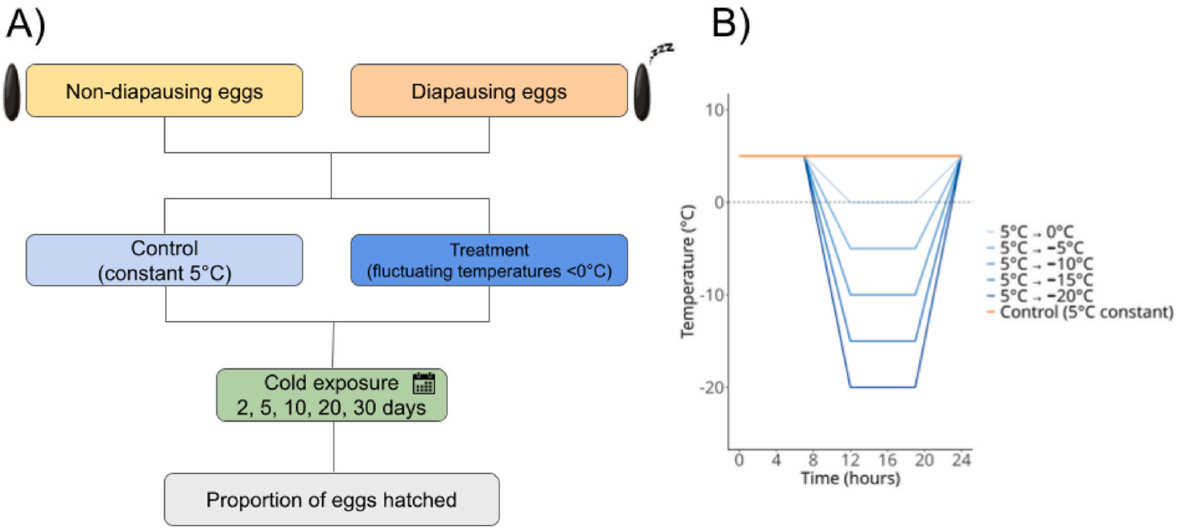

Fig. 1. (A) Flowchart illustrating the experimental design for cold exposure treatments in *Aedes koreicus* eggs. (B) Twenty-four-hour temperature cycles showing the thermal profiles applied in each treatment.

assumptions including potential zero inflation, the presence of outliers, and deviations from a uniform residual distribution.

To better visualise and interpret the outcomes of Eq. (2), Estimated Marginal Means (EMMs) of the proportion of hatched eggs were obtained from the fitted beta-binomial models using the *emmeans* package version 1.11.1 (Lenth and Piaskowski, 2017). EMMs represent model-based predictions of response probabilities averaged over the levels of other factors in the model, allowing direct comparison of treatment effects on the response scale. Predicted means and their 95% confidence intervals were computed on the logit scale and subsequently back-transformed to proportions (0–1 scale) for interpretation. For visualisation, EMMs were calculated across interpolated combinations of temperature (−20 to 0 °C), exposure period (2–30 days), and egg type (diapausing, non-diapausing), and plotted as heatmaps to illustrate the joint effects of thermal regime and cold exposure length on hatching success.

To assess the robustness of Eq. (2) model to the unbalanced experimental design, i.e., some treatment and control combinations were not performed, a sensitivity analysis was performed by refitting the beta-binomial model (Eq. (2)) to a reduced dataset excluding temperature–duration combinations that lacked replication across both egg types: specifically, −20 °C at 20 days, −15 °C at 2 and 30 days, and −10 °C at 2 days. The reduced model retained the same fixed effects structure, error family, and link function as the main model.

2.4.3. Model 3: interspecific comparison of cold tolerance between Aedes koreicus and Ae. albopictus (H3)

To assess species-level differences in cold tolerance, we extracted data from (Tippelt et al., 2020) by manually digitising Fig. 2 using WebPlotDigitizer (Rohatgi, 2025). This procedure allowed us to retrieve the proportion of eggs hatched for diapausing and non-diapausing eggs of a temperate *Ae. albopictus* strain collected in Freiburg, Germany, in 2015, under experimental conditions comparable to those used in our study.

We modelled the number of hatched eggs as a function of temperature, species identity, egg type, and cold-exposure duration using a second-order polynomial for temperature to account for nonlinear thermal responses:

$$\begin{aligned}\text{Hatched} \big/ \text{Total} \sim\ & \text{poly}(\text{Temperature}, 2) \times \text{Species} \\ & + \text{poly}(\text{Temperature}, 2) \times \text{TypeEggs} + \text{TypeEggs} \times \text{Species} \\ & + \text{Period\_length} \times \text{Species}\end{aligned} \qquad \text{(Eq. 3)}$$

where Species is a categorical variable distinguishing *Ae. albopictus* (reference level) and *Ae. koreicus*, and all other terms follow the definitions used in Eqs. (1) and (2). Estimated marginal means (EMMs) of hatching probability were subsequently computed from the fitted beta-binomial model to facilitate visualisation and interpretation of species-specific cold-tolerance patterns.

All analyses were performed in R version 4.5.1 (R Core Team, 2025). Data manipulation and visualisation were conducted using the *tidyverse* package suite (Wickham et al., 2019). The raw data and the script used for the analyses are available on GitHub at https://github.com/danddr/koreicus_cold_exp.

3. Results

Overall, the hatching success under control conditions was comparable between diapausing and non-diapausing eggs (0.568 ± 0.029 SE and 0.585 ± 0.030 SE, respectively). In contrast, exposure to cold treatments markedly reduced hatching success, with non-diapausing

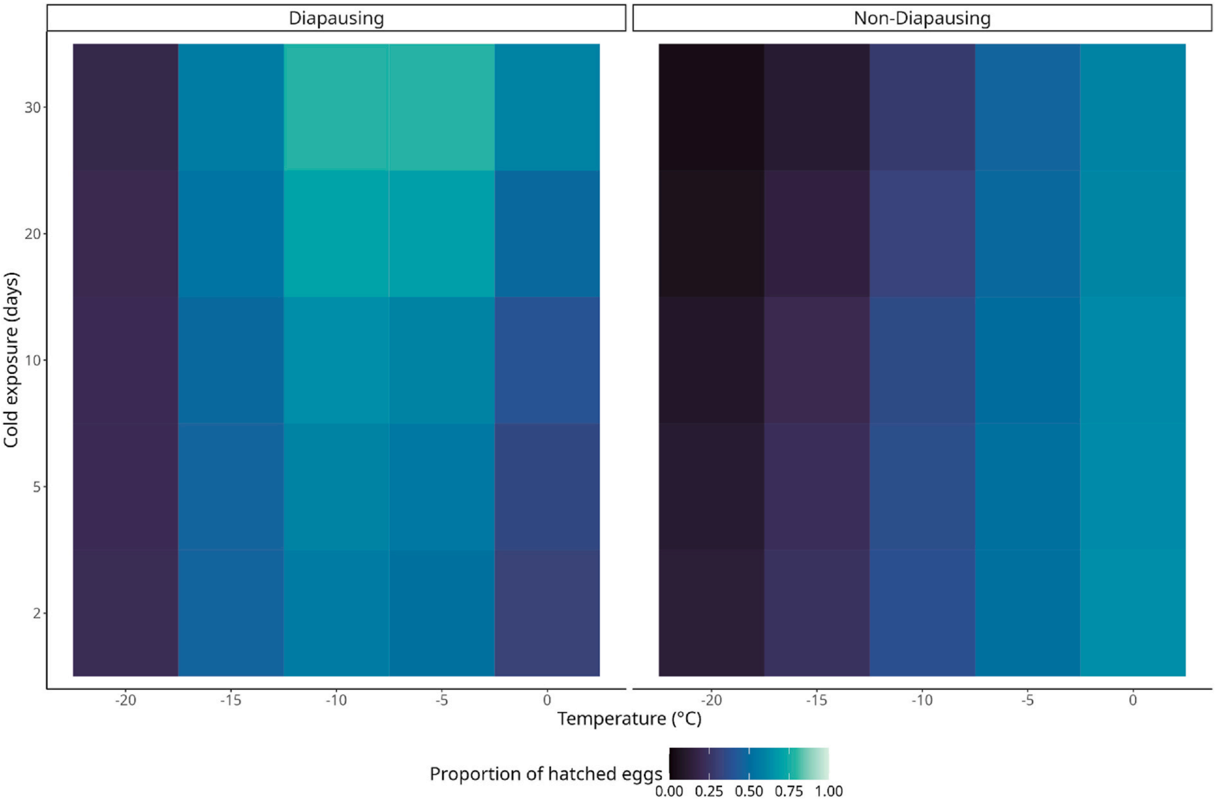

Fig. 2. Model-predicted hatching probability of *Aedes koreicus* eggs (Eq. (2)) as a function of temperature and cold exposure duration, derived from estimated marginal means (EMMs) of the beta-binomial model. Predictions are shown continuously across the full experimental temperature range (−20 to 0 °C) based on the fitted quadratic temperature term; note that empirical data were collected at five discrete temperature levels (0, −5, −10, −15, −20 °C) and that values at intermediate temperatures represent model interpolations. Colours represent predicted hatching probability, with warmer tones indicating higher hatching success.

Table 1
Mean (± standard error, SE) hatching success of *Aedes koreicus* eggs hatched under control and cold-temperature treatments across five cold exposure periods (2, 5, 10, 20, and 30 days). Eggs were classified as Non-Diapausing or Diapausing, and each combination of temperature and experimental setting is shown. Values represent the mean ± SE of replicates; missing combinations (NA) indicate treatments not performed.

Egg type	Temperature (°C)	Experimental setting	2 days	5 days	10 days	20 days	30 days
Non-Diapausing	−20	Control	-	-	-	-	-
		Treatment	0.184 ± 0.018	0.183 ± 0.073	0.165 ± 0.030	0.000 ± 0.000	0.066 ± 0.020
Non-Diapausing	−15	Control	-	-	-	-	-
		Treatment	0.115 ± 0.018	0.167 ± 0.073	0.052 ± 0.030	0.129 ± 0.014	0.076 ± 0.043
Non-Diapausing	−10	Control	0.667 ± 0.142	0.686 ± 0.168	-	-	-
		Treatment	0.416 ± 0.042	0.217 ± 0.044	0.273 ± 0.039	0.277 ± 0.054	0.266 ± 0.104
Non-Diapausing	−5	Control	0.552 ± 0.180	0.781 ± 0.074	0.663 ± 0.044	0.576 ± 0.019	0.493 ± 0.076
		Treatment	0.682 ± 0.115	0.663 ± 0.069	0.648 ± 0.105	0.713 ± 0.095	0.503 ± 0.061
Non-Diapausing	0	Control	0.681 ± 0.092	0.592 ± 0.069	0.455 ± 0.078	0.450 ± 0.029	0.424 ± 0.065
		Treatment	0.621 ± 0.086	0.629 ± 0.059	0.429 ± 0.071	0.479 ± 0.162	0.628 ± 0.098
Diapausing	−20	Control	-	-	-	-	-
		Treatment	0.176 ± 0.068	0.235 ± 0.102	0.115 ± 0.068	-	0.134 ± 0.038
Diapausing	−15	Control	-	-	-	-	-
		Treatment	-	0.433 ± 0.083	0.567 ± 0.060	0.901 ± 0.051	-
Diapausing	−10	Control	-	-	-	-	-
		Treatment	-	0.784 ± 0.039	0.480 ± 0.069	0.748 ± 0.080	0.580 ± 0.202
Diapausing	−5	Control	0.535 ± 0.043	0.617 ± 0.069	0.481 ± 0.023	0.721 ± 0.062	0.684 ± 0.010
		Treatment	0.434 ± 0.093	0.514 ± 0.052	0.553 ± 0.042	0.679 ± 0.041	0.798 ± 0.075
Diapausing	0	Control	0.383 ± 0.044	0.467 ± 0.044	0.432 ± 0.023	0.690 ± 0.096	0.670 ± 0.162
		Treatment	0.317 ± 0.033	0.368 ± 0.165	0.401 ± 0.012	0.573 ± 0.023	0.581 ± 0.083

eggs showing a stronger decline (0.343 ± 0.029 SE) than diapausing eggs (0.487 ± 0.031 SE). The overall mean ± SE hatching success for each temperature–exposure combination are reported in Table 1.

Some treatment and control combinations were not performed (indicated as NAs in Table 1) due to logistical constraints and variability in egg availability. Experimental prioritisation was necessary to ensure sufficient replication across key temperature regimes, as egg production varied between batches. In addition, maintenance of the diapausing colony during the winter months occasionally limited the number of egg batches, contributing to unbalanced replication across some treatments.

Baseline hatching success was broadly comparable across egg batches and between diapausing and non-diapausing eggs incubated at 21 °C prior to cold exposure (Fig. SM1; Table SM1). The beta-binomial model revealed no significant difference in hatching success between diapausing and non-diapausing eggs ($p = 0.175$), and no significant batch-level effects across most cold-treatment groups (all $p > 0.10$). The only exception was the batch assigned to the 0 °C cold-treatment group, which showed marginally higher baseline hatching success compared to the reference batch ($p = 0.025$); this is interpreted as a batch effect rather than a biologically meaningful result, given that all subsamples were maintained under identical standard incubation conditions. Overall, these results confirm adequate and comparable baseline egg hatching perfomance across batches and egg types prior to cold exposure.

Table 2
Summary of the beta-binomial model testing the effect of cold treatment and egg type on hatching success of *Aedes koreicus* eggs (Eq. (1)). Estimates (β) are on the logit scale with standard errors (SE), Wald *z*-values, *p*-values, and 95% confidence intervals.

Term	Estimate (β)	Std. Error	z value	p-value	95% CI
(Intercept)	0.326	0.152	2.15	**0.032**	0.029, 0.624
Treatment	−1.025	0.189	−5.43	**<0.001**	−1.395, −0.655
Diapausing eggs	−0.079	0.224	−0.35	0.725	−0.518, 0.360
Treatment × Diapausing eggs	0.716	0.277	2.59	**0.010**	0.174, 1.259

3.1. Effects of cold treatments and control on the number of hatched eggs (H1)

The beta-binomial model (Eq. (1)) evaluated the effect of cold-exposure treatment (vs. control) in interaction with egg type (diapausing vs. non-diapausing) on the number of hatched eggs (Table 2). The overall model provided a good fit to the data (AIC = 1168.3; dispersion = 4.92), indicating that moderate over-dispersion was adequately captured by the beta-binomial error structure.

Cold treatment significantly reduced hatching success across egg types ($\beta = -1.025$; $p < 0.001$), confirming a strong negative effect of cold exposure. The main effect of egg type alone was not significant ($\beta = -0.079$; $p = 0.725$), indicating similar baseline hatching rates between diapausing and non-diapausing eggs under control conditions. However, a significant interaction between treatment and egg type ($\beta = 0.716$; $p = 0.010$) revealed type-specific responses to cold exposure, indicating that diapausing eggs were less negatively affected by cold treatment than non-diapausing eggs, consistent with their greater physiological tolerance to low temperatures.

Model-based predicted probabilities (Table 3) confirmed the observed pattern: hatching success decreased sharply under cold treatment in non-diapausing eggs (from 0.581 ± 0.152 SE to 0.33 ± 0.112), whereas the reduction was comparatively mild in diapausing eggs (from 0.562 ± 0.165 SE to 0.482 ± 0.117). Model fit was further assessed using simulated residuals from the DHARMa package: no evidence of zero inflation ($p = 0.512$), outliers ($p = 0.680$), or deviation from uniform residual distribution ($p = 0.671$) was detected, indicating an adequate fit of the beta-binomial model to the observed hatching data.

Table 3
Model-based predicted hatching probabilities (±95% confidence intervals) derived from the beta-binomial model testing the effect of cold treatment and egg type on hatching success of *Aedes koreicus* eggs (Eq. (1)).

Egg type	Experimental setting	Predicted probability (95% CI)	Std. Error
Non-diapausing	Control	0.581 (0.507, 0.651)	±0.152
	Treatment (cold)	0.332 (0.285, 0.382)	±0.112
Diapausing	Control	0.562 (0.481, 0.639)	±0.165
	Treatment (cold)	0.485 (0.428, 0.542)	±0.117

Table 4
Summary of the beta-binomial model (Eq. (2)) evaluating the effects of temperature, egg type, exposure duration and their interaction on the hatching success of *Aedes koreicus* eggs.

Term	Estimate (β)	SE	z value	p-value	95 % CI
(Intercept)	−0.429	0.151	−2.84	**0.005**	−0.725, −0.133
poly(Temperature, 2) 1	1.710	1.470	1.16	0.245	−1.170, 4.590
poly(Temperature, 2) 2	−6.500	1.470	−4.42	**<0.001**	−9.380, −3.620
Non-Diapausing eggs	−0.055	0.208	−0.26	0.792	−0.461, 0.352
Exposure period (days)	0.026	0.010	2.70	**0.007**	0.007, 0.045
Temp$_1$ × Non-Diapausing eggs	8.200	1.660	4.94	**<0.001**	4.940, 11.500
Temp$_2$ × Non-Diapausing eggs	5.710	1.560	3.66	**<0.001**	2.650, 8.770
Non-Diapausing eggs × Exposure period	−0.048	0.013	−3.68	**<0.001**	−0.073, −0.022
Temp$_1$ × Exposure period	0.198	0.081	2.45	**0.014**	0.040, 0.356
Temp$_2$ × Exposure period	−0.069	0.077	−0.89	0.372	−0.220, 0.082

3.2. *Effects of temperature, egg type, and cold exposure duration on the number of hatched eggs (H2)*

The beta-binomial model (Eq. (2)) revealed a non-linear relationship between temperature and hatching success, modulated by both egg type and exposure periods (Fig. 2; Table 4).

There was no significant difference in overall hatching success between diapausing and non-diapausing eggs ($\beta = -0.055$; $p = 0.792$). In contrast, cold exposure periods (*Period_length*) had a small but significant positive effect on hatching probability ($\beta = 0.026$; $p = 0.007$), indicating that longer cold exposures slightly increased the likelihood of hatching across temperature regimes.

The first and second-order terms of the polynomial transformation of temperature indicated a pronounced unimodal relationship with hatching success. The linear component was not statistically significant ($\beta = 1.71$; $p = 0.245$), whereas the quadratic term was highly significant and negative ($\beta = -6.50$; $p < 0.001$). Strong and statistically significant interactions were detected between temperature and egg type, suggesting that thermal sensitivity differed markedly between diapausing and non-diapausing eggs. Both the linear ($\beta = 8.20$; $p < 0.001$) and quadratic ($\beta = 5.71$; $p < 0.001$) interaction terms were significant, indicating that hatching success of non-diapausing eggs increased more steeply with temperature and followed a more pronounced unimodal pattern than in diapausing eggs.

The interaction between egg type and cold exposure periods was negative and significant ($\beta = -0.048$; $p < 0.001$), indicating that a longer cold exposure is more detrimental for non-diapausing eggs than for diapausing ones. Additional interactions involving temperature and cold exposure periods were also detected: a positive effect of the linear temperature term ($\beta = 0.198$; $p = 0.014$) suggested slightly enhanced hatching at moderate temperatures with longer exposures, while the quadratic interaction was not significant ($\beta = -0.069$; $p = 0.372$).

There was no evidence of zero inflation ($p = 0.488$), and the dispersion test indicated no significant over- or under-dispersion ($p = 0.600$). The uniformity test confirmed that residuals followed the expected distribution ($p = 0.990$), supporting overall model adequacy, while the bootstrap outlier test detected four potential outliers ($p = 0.060$), a frequency slightly above the expected rate but not statistically significant.

The sensitivity analysis conducted on the balanced subset of the data yielded results fully consistent with Table 4: the signs and significance of all key coefficients were retained (Table SM2), confirming that the main conclusions are robust to the removal of unbalanced temperature–duration combinations.

Overall, these results quantify how *Ae. koreicus* egg hatching success is shaped by a non-linear thermal response, moderated by both egg physiological state and exposure periods, with diapausing eggs exhibiting greater resilience to prolonged cold exposure. Model-based predicted probabilities derived from the same model (Fig. 2) confirmed this pattern, showing that hatching probability for diapausing eggs peaks at intermediate temperatures (−10 °C to −5 °C) and increases with cold exposure time, while non-diapausing eggs displayed a monotonic hatching decline towards colder temperatures.

3.3. *Interspecific comparison of cold tolerance between Aedes koreicus and Ae. albopictus (H3)*

Across all treatments, *Ae. koreicus* exhibited substantially higher hatching success than *Ae. albopictus*, as reported in (Tippelt et al., 2020). In *Ae. albopictus*, the mean egg hatching success was low and similar between diapausing (0.161 ± 0.039 SE) and non-diapausing eggs (0.155 ± 0.025 SE). In contrast, *Ae. koreicus* showed markedly higher hatching success in both egg types, with diapausing eggs reaching 0.487 ± 0.031 SE and non-diapausing eggs 0.343 ± 0.029 SE. These raw values already indicate a pronounced species-level difference in cold-related hatching performance, with *Ae. koreicus* consistently outperforms *Ae. albopictus* across egg types.

Consistent with these observations, the beta-binomial model (Eq. (3)) revealed strong species-level differences in thermal response (Table 5). Across all temperature and cold exposure combinations, *Ae. koreicus* had significantly higher hatching probabilities than *Ae. albopictus* ($\beta = 3.21$; $p < 0.001$), confirming its overall superior performance under cold conditions. Non-diapausing eggs hatched less successfully than diapausing eggs regardless of species ($\beta = -0.690$; $p = 0.020$), whereas cold exposure periods had no detectable main effect on

Table 5
Summary of the beta-binomial model (Eq. (3)) evaluating the effects of temperature, species, egg type, and cold-exposure periods on the eggs hatching success in *Aedes koreicus* and *Ae. albopictus*.

Term	Estimate (β)	SE	z value	p-value	95 % CI
Intercept	−3.252	0.642	−5.06	**<0.001**	−4.513, −1.987
poly(Temperature, 2)_1	36.87	10.14	3.64	**<0.001**	16.96, 56.71
poly(Temperature, 2)_2	−13.52	5.88	−2.30	**0.021**	−24.99, −2.05
Species (*Ae. koreicus*)	3.211	0.656	4.90	**<0.001**	1.924, 4.498
Non-diapausing egg	−0.690	0.298	−2.32	**0.020**	−1.274, −0.107
Exposure period (days)	−0.0003	0.0121	−0.03	0.977	−0.024, 0.023
poly(Temperature, 2)_1 × *Ae. koreicus*	−32.41	10.20	−3.18	**0.001**	−52.39,-12.43
poly(Temperature, 2)_2 × *Ae. koreicus*	6.44	5.83	1.10	0.269	−4.98, 17.86
poly(Temperature, 2)_1 × Non-diapausing egg	8.46	1.94	4.37	**<0.001**	4.67, 12.25
poly(Temperature, 2)_2 × Non-diapausing egg	3.66	1.81	2.02	**0.043**	0.11, 7.20
Species (*Ae. koreicus*) × Non-diapausing egg	0.056	0.321	0.17	0.863	−0.573, 0.684
Species (*Ae. koreicus*) × Exposure period	0.005	0.014	0.33	0.740	−0.022, 0.031

hatching ($p = 0.977$).

Clear species-specific differences in thermal sensitivity were evident from the significant temperature × species interaction. For *Ae. albopictus*, the linear temperature term was strongly positive ($\beta = 36.87$; $p < 0.001$) and the quadratic term negative ($\beta = -13.52$; $p = 0.021$), indicating a steep unimodal temperature response with very low hatching at cold extremes. In *Ae. koreicus*, however, the linear temperature slope was markedly attenuated ($\beta = -32.41$; $p = 0.001$), resulting in a much flatter thermal curve. This implies that *Ae. albopictus* hatches only near a narrow thermal optimum, while *Ae. koreicus* maintains relatively high hatching success across a broader and colder thermal range. The quadratic temperature × species interaction was not significant, suggesting that differences arise primarily from slope steepness rather than curvature.

Temperature also interacted strongly with egg type. In non-diapausing eggs, both the linear and quadratic components of the temperature interaction were significant ($\beta = 8.46$, $p < 0.001$; $\beta = 3.66$, $p = 0.043$), indicating a more pronounced unimodal response and generally lower cold tolerance compared with diapausing eggs. In contrast, the species × egg type interaction was not significant ($p = 0.863$), showing that the relative difference between diapausing and non-diapausing eggs is comparable in both species. Likewise, species did not differ in their response to cold exposure periods ($p = 0.740$).

Model-derived predicted probabilities (Fig. 3) corroborated these findings, highlighting the weaker cold-resilience of *Ae. albopictus* relative to *Ae. koreicus* (Fig. 2).

As we did before, we evaluated model adequacy using DHARMa simulation-based diagnostics. The dispersion test showed that residual variability was fully consistent with model expectations $p = 0.992$), and the zero-inflation test indicated that the observed number of zeros did not differ from that predicted under the fitted model ($p = 0.520$). The bootstrap outlier test detected only a single marginal observation among 195 data points ($p = 1.000$), falling within the expected range of sampling variability. Finally, the uniformity test confirmed that the scaled residuals followed the theoretical distribution ($p = 0.407$).

4. Discussion

In this study we estimated the hatching success of *Ae. koreicus* eggs across a range of controlled cold exposure, durations and fluctuating thermal regimes. Unsurprisingly, our results support H1 and H2: diapausing *Ae. koreicus* eggs consistently outperformed non-diapausing eggs under cold exposure, and showed an apparent optimum under intermediate subzero temperatures and prolonged exposure, showing that *Ae. koreicus* egg hatching success is influenced not only by temperature but also by the duration of their exposure to cold. These findings align with previous studies on other temperate *Aedes* species, such as *Ae. albopictus,* and provide new experimental evidence for the cold tolerance strategies of *Ae. koreicus*, expanding upon our earlier work (Marini et al., 2022). Additionally, our observational and modelling results support H3, showing that *Ae. koreicus* eggs, regardless of the physiological state, display markedly greater cold tolerance than temperate *Ae. albopictus* populations (Thomas et al., 2012; Tippelt et al., 2020; Won and Choi, 2025). This advantage arises from both a higher baseline probability of hatching and a thermal niche shifted towards lower temperatures over which successful hatching can occur, including under prolonged exposure to sub-zero temperatures.

In line with what is known about diapause in *Ae. albopictus* (Lacour et al., 2015; Thomas et al., 2012), diapausing eggs of *Ae. koreicus* displayed considerable resilience to prolonged exposure to low and freezing temperatures. In contrast, non-diapausing eggs exhibited reduced hatching success under cold treatment, particularly as exposure

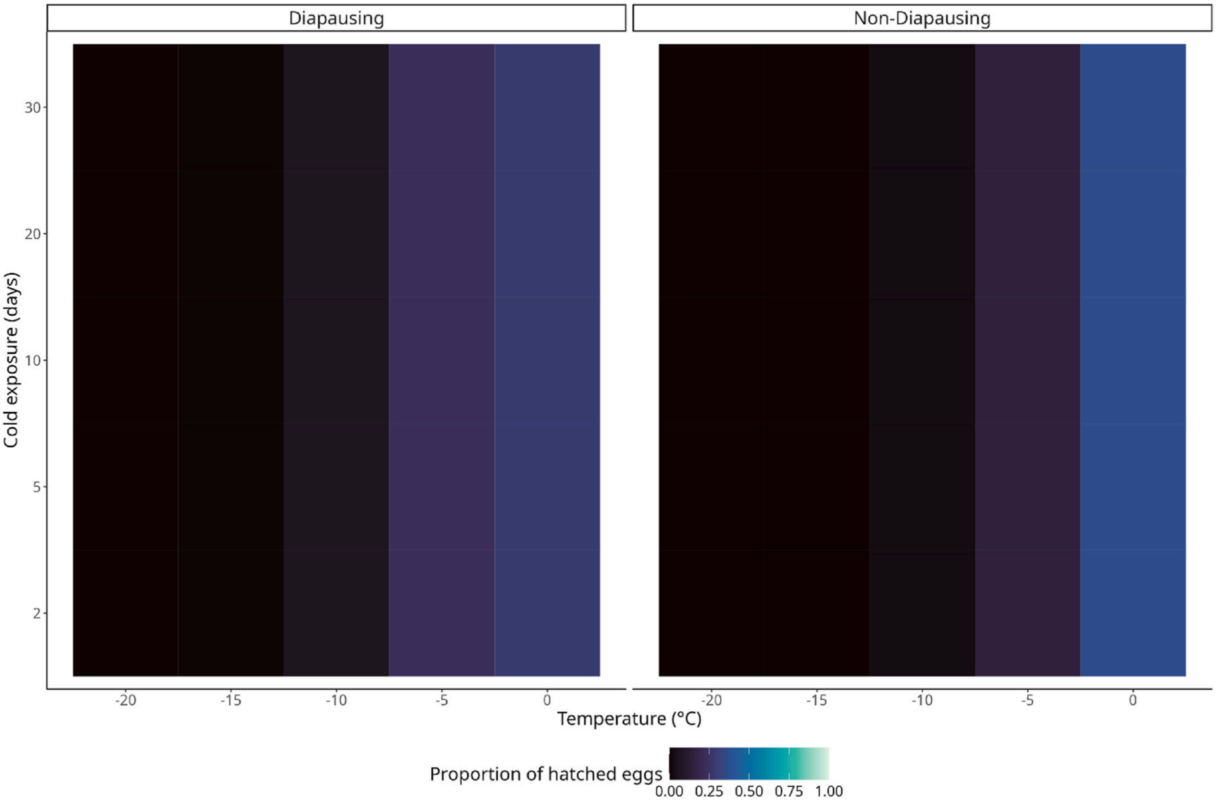

Fig. 3. Model-predicted hatching probability of *Aedes albopictus* eggs (Eq. (3)) as a function of temperature and cold exposure duration, derived from estimated marginal means (EMMs) of the beta-binomial model. Predictions are shown continuously across the full experimental temperature range (−20 to 0 °C) based on the fitted quadratic temperature term; note that empirical data were collected at five discrete temperature levels (0, −5, −10, −15, −20 °C) and that values at intermediate temperatures represent model interpolations. Colours represent predicted hatching probability, with warmer tones indicating higher hatching success.

time increased. This difference in cold tolerance reflects fundamental physiological differences between the two egg types and reinforces the ecological role of diapause as a survival strategy in temperate climates (Denlinger and Armbruster, 2014; Diniz et al., 2017). While models of insect overwintering and survival often rely on threshold temperatures or cumulative degree-day metrics, our results show that time spent under cold conditions significantly influences egg viability. Diapausing eggs exhibited increased hatching success with longer exposure periods, consistent with the hypothesis that they respond to favourable external stimuli only after a fixed interval has elapsed (Denlinger and Armbruster, 2014).

We note that egg selection for eligibility was based on external morphological criteria and did not allow direct confirmation of embryo presence; a small number of non-viable eggs may therefore have been included, which could marginally affect hatching success estimates. More broadly, our study assessed egg survival solely through hatching response, an ecologically relevant endpoint, but one that does not resolve the underlying physiological mechanisms. Future work could address this by examining embryonic development directly, assessing chorion integrity, or quantifying metabolic and gene expression changes associated with diapause and cold tolerance. Complementing laboratory approaches with field-based overwintering studies would further allow validation of these findings under naturally fluctuating conditions (Tippelt et al., 2019), where additional stressors such as desiccation and microbial exposure may interact with thermal effects in ways that controlled experiments cannot fully capture.

Additionally, we highlight that the marginally higher baseline hatching success observed in the batch assigned to the 0 °C treatment group (Table SM1) suggests that hatching proportions estimated by the model (Eq. (2)) may be slightly overestimated for that group, as the higher intrinsic viability of those eggs could have contributed to the observed hatching response independently of the thermal treatment. However, we consider this unlikely to affect the main conclusions of the study: the most striking differences in cold tolerance between diapausing and non-diapausing eggs were detected at sub-zero temperatures (−5 to −20 °C), where no batch-level viability differences were identified, and the biological signal is therefore unlikely to be confounded by pre-existing variation in egg quality.

The high cold tolerance observed in diapausing eggs also has clear implications for the species' invasion ecology. The ability to withstand extended periods of freezing temperatures suggests that *Ae. koreicus* is well-adapted for establishment in higher latitudes and elevation regions, where *Ae. albopictus* is less competitive, even under future climate scenarios where winter conditions may remain cold but become more variable or shortened in duration (Dumont et al., 2025). This supports field reports of *Ae. koreicus* persistence in Alpine foothills and continental areas characterised by cold winters (Andreeva et al., 2021; Arnoldi et al., 2022; Baldacchino et al., 2017; Montarsi et al., 2015b, 2022). Beyond filling a fundamental knowledge gap, our results carry important implications for vector surveillance and control in temperate regions. The strong interaction between egg type and cold-exposure duration indicates that diapausing eggs of *Ae. koreicus* can persist longer under winter conditions and hatch more synchronously once temperatures rise. This pattern implies that *Ae. koreicus* may generate earlier and more concentrated early-season emergence pulses than *Ae. albopictus*, potentially anticipating the onset of the mosquito biting season (Baldacchino et al., 2017; Montarsi et al., 2015b). Such a shift has consequences for entomological surveillance campaigns, as current surveillance programs often rely on assumed overwintering limits and may therefore initiate sampling too late or terminate monitoring too early, underestimating the true distribution, establishment, and seasonal dynamics of this species.

From the operational perspective, these findings can be of particular interest in areas where multiple invasive *Aedes* species co-occur. Standard low-cost surveillance tools (e.g. ovitrap monitoring and stereomicroscopic egg counts) cannot reliably distinguish egg morphology among *Ae. koreicus*, *Ae. japonicus*, and *Ae. albopictus*, complicating the interpretation of early-season egg detections (Anicic et al., 2023). Misidentification may obscure species-specific phenology, especially if *Ae. koreicus* begins hatching earlier than the other species. As shown in recent assessments of exochorion pattern recognition, even trained personnel often struggle to reliably differentiate *Ae. japonicus* from *Ae. koreicus* using routine optical methods (Anicic et al., 2023). This uncertainty may lead to an underestimation of *Ae. koreicus* population densities and delayed detection of local establishment or expansion. An integrated monitoring strategy that couples field surveillance with model-based forecasting would substantially improve early detection, risk assessment, and the timing of targeted interventions. In particular, incorporating species-specific cold-tolerance parameters into quantitative models of life-cycle dynamics and habitat suitability could greatly enhance predictive power (Arnoldi et al., 2022; Da Re et al., 2022; Marcantonio et al., 2016; Marini et al., 2019).

5. Conclusions

This study provides novel quantitative data and evidence that *Ae. koreicus* diapause and cold tolerance are both time- and temperature-dependent, while non-diapausing eggs are far more vulnerable to cold exposure. These findings support a more refined understanding of overwintering ecology in invasive *Aedes* species and provide essential quantitative data for improving phenological and distribution models. As *Ae. koreicus* continues to expand its range in Europe and other temperate regions, understanding the interplay between diapause, cold exposure, and hatching success will be central to predicting its ecological and public health impacts.

Declaration of generative AI use

During the preparation of this work, Daniele Da Re used ChatGPT 5.1 to check the grammatical consistency and flow of the text of the first draft. After using this tool, all the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Daniele Da Re: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Daniele Arnoldi:** Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. **Annapaola Rizzoli:** Conceptualization, Writing – review & editing. **Giovanni Marini:** Conceptualization, Investigation, Methodology, Writing – review & editing.

Declaration of competing interest

We have nothing to declare.

Acknowledgments

Daniele Da Re was supported by the Marie Sklodowska-Curie Actions - Postdoctoral fellowship Nr. 101106664. The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2026.104482.

Data availability

The raw data and the scripts used for the analyses are available on GitHub at https://github.com/danddr/koreicus_cold_exp. The experimental dataset will also be deposited in the *AedesTraits* database (https://zenodo.org/records/17752818. https://doi.org/10.5281/zenodo.15149903) to ensure long-term preservation and discoverability.

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