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Impact of temperature and nutrition on the toxicity of the insecticide λ-cyhalothrin in full-lifecycle tests with the target mosquito species *Aedes albopictus* and *Culex pipiens*

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Abstract The global spread of the Asian tiger mosquito *Aedes albopictus*, an urban pest as well as a vector for arboviruses, is a threat for public health. As control measures include the use of insecticides such as the pyrethroid λ-cyhalothrin, it is crucial to assess their efficiency and their potential impact on the biodiversity especially under climate change conditions. To evaluate the environmental risk, biotests are well established for non-target organisms but not yet for mosquitoes. We therefore developed a full-lifecycle biotest for mosquitoes kept under quarantine conditions based on the OECD guideline 219. Therewith we tested the effect of temperature and nutrition on the ecotoxicological response to λ-cyhalothrin on the mosquitoes *Ae. albopictus* and *Culex pipiens* by assessing sublethal and life history parameters. The efficiency of λ-cyhalothrin decreased in both mosquito species with increasing temperature and changed with feeding protocol. At effective concentrations for potential mosquito control in surface waters, λ-cyhalothrin poses a high risk for indigenous aquatic key role species inhabiting the same microhabitats. Those aspects should to be taken into account in vector control strategies.

Keywords Climate change · Population growth · Pyrethroid · Reproduction · Vector control

Key message

- We developed a not yet existing standardized chronic biotest for invasive vectors of human diseases like the Asian tiger mosquito *Aedes albopictus*.
- With this tool, we tested hypotheses about the species-specific response to an insecticide under multiple stressors like temperature and nutrition.
- We revealed a negative temperature toxicity relation, a food dependent efficacy and an environmental risk by applications of λ-cyhalothrin.
- These findings and the biotest itself are valuable complements in investigating mosquito control measures and for an interlaboratory comparison.

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Introduction

The Asian tiger mosquito *Aedes albopictus* (Skuse 1894) is an efficient vector of dengue, chikungunya fever viruses and potentially of 20 other mainly tropical arthropod-borne viruses as well as parasites like the canine heartworm *Dirofilaria immitis* (Shroyer 1986; Moore and Mitchell 1997; Genchi et al. 2009). Current estimates indicate that at least 284–528 million people are infected by dengue

viruses each year (Bhatt et al. 2013). The numbers have increased 30-fold in the last 50 years (World Health Organization 2009). This is mainly related to the rapidly expanding distribution of the key vectors of dengue viruses, *Aedes aegypti* and *Ae. albopictus* from their tropic/subtropic origin to temperate regions. The first records of *Ae. albopictus* on the European mainland date back to 1979 in Albania (Adhami and Reiter 1998) and 1990 in Genoa, Italy (Sabatini et al. 1990), and most of the northern and eastern Mediterranean region has since been colonized by this mosquito species (ECDC 2013). In Europe, a first outbreak of *Ae. albopictus*-transmitted chikungunya fever involving at least 250 patients was reported from the Emilia-Romagna region of Italy in 2007 (Angelini et al. 2007). Since then, the first isolated cases of autochthonous dengue virus transmissions very likely caused by *Ae. albopictus* have been reported from Croatia and France (Schmidt-Chanasit et al. 2010; La Ruche et al. 2010). In addition, mosquitoes native to temperate regions, such as *Culex pipiens*, the common house mosquito, are also potential vectors of arboviruses like Sindbis, Usutu and West Nile viruses and parasites like *Dirofilaria immitis* (Kettle 1984; Harbach 1988; Santa-Ana et al. 2007; Jöst et al. 2010).

Vector control measures against these mosquitoes often make use of pyrethroids like permethrin, deltamethrin and λ-cyhalothrin, organochlorines like DDT, and organophosphates like malathion (e.g., Angelini et al. 2007; Basilua Kanza et al. 2013) although in some current studies also natural repellents have been investigated (e.g. Suwansirisilp et al. 2012; Chio et al. 2013; Kulkarni et al. 2013). The pyrethroid λ-cyhalothrin (CAS-Nr.: 91465-08-6) is one of the most commonly used insecticides in vector control measures against mosquitoes (World Health Organization 1998). It is used for mosquito fogging and as a repellent on mosquito nets, cattle and in anthropogenic microhabitats like used tires (Schaefer et al. 1990; Curtis et al. 1996; Hewitt and Rowland 1999; Pettit et al. 2010). In addition, studies show the effectiveness of pyrethroids such as λ-cyhalothrin as an ultra-low volume (ULV) spray application against *Aedes* larvae in urban regions (Sulaiman et al. 1995). There are also reports that λ-cyhalothrin was routinely sprayed in receptacle breeding sites as a larvicide against *Ae. aegypti* in Australia in 2007, and this method was recommended for the control of *Ae. albopictus* in this region (Whelan et al. 2009). As λ-cyhalothrin is typically used in rice fields against pests like the rice water weevil, its direct use as a mosquito larvae control agent in this type of agriculture has also been discussed (Lawler et al. 2007). It is anticipated that, in the case of a mosquito-related epidemic in Europe, the use of pyrethroids such as λ-cyhalothrin is most likely (Stolz 2008). However, this would affect other species sharing the same microhabitat,

e.g., the indigenous mosquito and competitor *Cx. pipiens* as well as various invertebrate predators of mosquitoes (Carrieri et al. 2003; Lawler et al. 2007).

Due to the current anthropogenic climate change, the global average surface temperature has already increased by 0.65–1.06 °C between 1850 and 2012. The IPCC's Fifth Assessment Report considered a further increase of up to 1.5 °C over the next 100 years as likely for the most representative concentration pathways scenarios (RCPs) (Stocker et al. 2013). According to the van't Hoff Q_{10} concept, this temperature change will have a major impact on the population dynamics of poikilothermic invertebrates, for instance resulting in shorter generation times, increased seasonal activity, extension of geographical ranges, and increased chances for successful hibernation (Porter et al. 1991; Alto and Juliano 2001a, b). Moreover, it is known that a higher temperature often results in a higher sensitivity of aquatic invertebrates to toxicants in their environment (Cairns et al. 1975; Mayer and Ellersieck 1986; Heugens et al. 2001; Müller et al. 2012): Almost 70 % of 151 analyzed experiments showed a positive temperature–toxicity relationship (Heugens et al. 2001). For most insecticides, toxicity increased by factor 4.06 with an increase of 10 °C while commonly used organophosphates showed an even higher temperature dependence of their toxicity (factor 5.14; Mayer and Ellersieck 1986). One recent study showed a positive temperature–toxicity relationship of malathion in the mosquitoes *Culex restuans* and *Ae. albopictus* (Muturi et al. 2011). The classes of pyrethroids and pyrethrins, however, show ambiguous behavior (Mayer and Ellersieck 1986).

As of now there is no standardized full-lifecycle biotest for mosquitoes to adequately investigate the chronic toxicity of insecticides (Müller et al. 2013). To fill this gap and to further contribute to improving the comparability of experimental studies across laboratories, we propose an ecotoxicological full-lifecycle test for mosquitoes following the principle of the OECD guideline 219 for the testing of chemicals using the non-biting midge *Chironomus riparius* (OECD 2004). Based on the first published step for inter-laboratory comparisons of studies with mosquitoes, we used the recommended dynamic larval nutrition protocol according to Müller et al. (2013) as well as the static nutrition protocol recommended by the OECD guideline 219.

In this study we investigated (i) if there is a difference in the ecotoxicological response of the invasive tropic/subtropic and the mosquito species indigenous in temperate regions to the insecticide λ-cyhalothrin and (ii) the impact of higher temperatures on the ecotoxicological response to λ-cyhalothrin. By using two feeding protocols, we also aimed to answer the question (iii) whether different (but still sufficient) nutrition supply regimens alter the
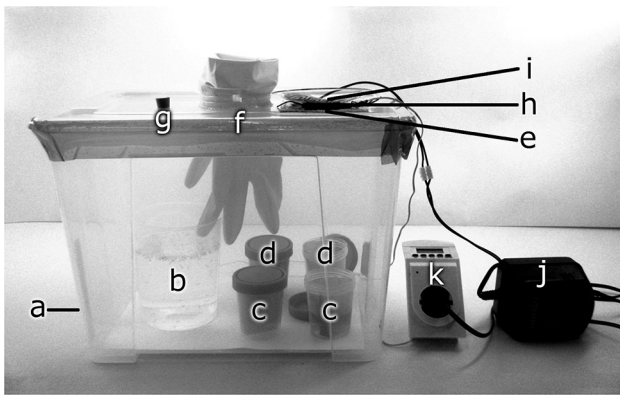
Fig. 1 Quarantine box for mosquito rearing and biotests. **a** 22 l box, **b** 1 l larvae vessel, **c** 100 mL glucose vessel, **d** oviposition vessel, **e** gauze-covered hole for air circulation and blood meal, **f** clove to manipulate the setup, **g** bung closing the hole to access the box, **h** blood meal, **i** heating foil, **j** power mains adapter, **k** thermo timer

ecotoxicological responses of these animals. Furthermore we evaluated whether the findings were important for vector control strategies in temperate regions under global climate change aspects and gave recommendations for stakeholders.

Materials and methods

Mosquito cultures and full-lifecycle test design

The long term lab strains used in this study (*Ae. albopictus* originally from Indonesia and *Cx. pipiens* f. *molestus*) were provided by Biogents AG (Regensburg, Germany) and bred in conditioning cabinets (MKKL 600, FLOHR instruments, Holland) at 25 ± 0.1 °C, 90 ± 0.7 % humidity and a photoperiod of 16:8 h light:dark cycle. In order to breed the two vector species under quarantine conditions and analyze the interactive effects of temperature, insecticides and nutrition during their full-lifecycle, a hermetically closed test system was established (Fig. 1).

In short, a 22 l plastic box (Samla, IKEA, Sweden) (Fig. 1a) was equipped with five plastic containers (VWR, Germany). A 1 l container was filled with 600 mL mosquito medium (1:1 mixed deionized and tap water) for larval development and insecticide and nutrient exposure (Fig. 1b). In addition, two 100 mL containers (VWR, Germany) were filled with 50 mL 2 % glucose solution in mosquito medium to feed imagines (Fig. 1c) and two others with 50 mL mosquito medium (Fig. 1d). These containers served also for oviposition and therefore were prepared with paper filters to facilitate sampling of the deposited eggs. Three holes were cut into the cover plate of the quarantine box (Fig. 1a). One hole was closed with metal gauze (1.4×1.6 mm mesh size) (Fig. 1e) to allow

for air circulation and supplying blood meals. A second hole was capped with a rubber glove (Fig. 1f) which was used to safely manipulate the experimental setup. The third hole was locked with a rubber bung (Fig. 1g) to adjust the water levels, to feed the larvae and to remove the eggs.

Experimental setup

The hatching of *Ae. albopictus* eggs was induced by exposing them to a mosquito hatch medium (1 g fresh baker's yeast [*Saccharomyces cerevisiae*], 0.5 g ascorbic acid as well as 0.5 g glucose dissolved in 1 l of heated [38 °C] mosquito medium) for 20 h at 25 °C air temperature. *Culex pipiens* egg floats were incubated in plain mosquito medium for 20 h at 25 °C. Larvae were fed with ground fish food (TetraMin flakes, Tetra, Germany) until emergence, either statically or dynamically. The static feeding protocol included eight administrations of 1 mg fish food larva^{-1} until pupation within 9 days at 30 °C, 11 days at 25 °C or 14 days at 20 °C. In contrast, the dynamically fed mosquito larvae received four administrations of 0.5 mg fish food larva^{-1}, three of 1 mg larva^{-1} and one of 1.5 mg larva^{-1} until temperature-dependent pupation occurred within 9–14 days (for details see Müller et al. 2013). The larval density used in our experiments was representative for natural breeding sites and below densities under which intraspecific competition has been observed within these conditions (Carrieri et al. 2003; Müller et al. 2013). The adult mosquitoes were supplied with 2 % glucose solution ad libitum as food. Female mosquitoes were additionally fed with 5 mL of reconstituted human blood (a 1:1 mixture of packed red blood cells and blood plasma kindly provided by the Institute for Transfusion Medicine and Immunohematology, Faculty of Medicine, Goethe University Frankfurt am Main) that was provided in a package made of desalted pig gut (Fig. 1h). To maintain a blood temperature of 37 ± 1 °C, the blood was warmed by a 12 W heating foil (Conrad Electronic SE, Germany; Fig. 1i) powered by a mains adapter (Voltcraft, Conrad Electronic SE, Germany; Fig. 1j) and controlled by a thermo-timer (TFA Dostmann GmbH & Co. KG, Germany; Fig. 1k).

For the experiments λ-cyhalothrin was applied in the form of the microcapsule formulation Karate® (Syngenta, Switzerland) due to its better comparability to the study of (Schroer et al. 2004). Furthermore, Karate® has a similar formulation like Demand® (Syngenta) registered for mosquito control measures and used e.g. in the study of Whelan et al. (2009). In the first concentration–response-experiments assessing the effects of λ-cyhalothrin on *Ae. albopictus* and *Cx. pipiens*, four replicates with 30 first instar larvae each were exposed to increasing λ-cyhalothrin concentrations at 20 °C under static nutrition and to single

static λ-cyhalothrin exposure. *Ae. albopictus* was tested at nine concentrations (nominal 0.01–1.0 µg λ-cyhalothrin L^{-1}) and *Cx. pipiens* at six concentrations (nominal 0.05–0.6 µg L^{-1}). Concentrations causing 10, 50 and 90 % mortality (LC_{10}, LC_{50} and LC_{90} values) were calculated for each species. A second test series was performed to assess the possible effects of increasing temperature on the eco-toxicological responses of *Ae. albopictus* and *Cx. pipiens*. At three different temperatures (20, 25 and 30 °C) and applying the dynamic nutrition protocol, three replicates with 30 first instar larvae each were exposed to the nominal LC_{10}, LC_{50} and LC_{90} (*Cx. pipiens*) and nominal LC_{50} (*Ae. albopictus*) that had been determined in the first experiment. LC_{10} and LC_{90} exposures were not performed with *Ae. albopictus* in this test series, due to limited biological material. A third test was performed to compare the nutritional impact on the temperature-dependent response of *Cx. pipiens* to λ-cyhalothrin. Here, *Cx. pipiens* first instar larvae (three replicates with 30 larvae each) were exposed to the LC_{10}, LC_{50} and LC_{90} at 20, 25 and 30 °C, but using the static nutrition protocol.

Sublethal endpoints and life history parameters

In order to assess the impact of λ-cyhalothrin on the lifecycle performance of mosquitoes, mortality (deduced by number of emergences) and adult wing length—which is known to be correlated to body size and weight (Nasci 1990; Briegel and Timmermann 2001)—were determined at the end of the experiments. The wing length was measured at the R_1 vein of adult females under a binocular (SZ61, Olympus, Japan) with a camera (KY-F754, JVC, Japan) using the software Diskus® (Version 5.50.14.58; C. H. Hilgers Technisches Büro, Germany). In addition, the number of egg floats per female, eggs per float, fertility of eggs, and released larvae per female were assessed as reproductive endpoints for *Cx. pipiens*. In order to assess the impact of λ-cyhalothrin on population dynamics, the mean finite rate of increase λ' was chosen due its common use in mosquito studies (Livdahl and Willey 1991; Léonard and Juliano 1995; Grill and Juliano 1996; Juliano 1998; Muturi et al. 2011) and was calculated by formula 1.

$$\lambda' = \frac{1}{N_0} \cdot e^\delta \cdot \frac{1}{e^{D+\epsilon/\delta}}$$

$$e \approx 2.71828183$$

$$\delta = \sum_x A_x f(w_x)$$

$$\epsilon = \sum_x x A_x f(w_x)$$

where,

λ' = mean finite rate of increase
N_0 = initial number of females per replicate
w_x = mean size of females eclosing on day x
$f(w_x)$ = fecunditysize relationship on day x
A_x = the number of females eclosing on day x
D = mean reproductive maturity time (oviposition)

Formula 1 is modified from Juliano (1998). The initial number of females (N_0) per replicate was 15 because the male–female ratio was assumed to be 1:1. In contrast to Juliano (1998), it was not necessary to calculate the fecundity-size relationship on day x $f(w_x)$ because the egg number of *Cx. pipiens* was counted in this study (dynamic nutrition experiment) and that of *Ae. albopictus* has been published by Delatte et al. (2009) for the three test temperatures. There is a lack of information about the individual fate of each female as we only considered whole cohorts, and therefore the term $\Sigma_x A_x f(w_x)$ was substituted by the survival rate A_x multiplied by the counted number of eggs. It was assumed that the time-dependent distribution of oviposition correlates with the distribution of the emergence of the cohort. Thereby it was possible to replace the term $\Sigma_x x A_x f(w_x)/\Sigma_x A_x f(w_x)$ either for *Cx. pipiens* by subtracting the mean 50 % emergence time (EMT_{50}) from the mean reproductive maturity time D (oviposition) or, for *Ae. albopictus*, by the mean of the pre-blood meal period summed up with the mean time of a gonotrophic cycle as determined by Delatte et al. (2009) for all three temperatures. λ' reaches from the values of 0 to $+\infty$ whereas $\lambda' = 1$ is the state of equilibrium of population growth and decline (Barnthouse et al. 2007).

Statistics

After log-transforming concentrations, a nonlinear regression was used to calculate LC_x values with the software Prism (Version 5.01, GraphPad Software Inc., USA). Prism was also used to compare the concentration–response-curves using Fisher's exact test. To identify significant differences between treatments, one- or two-factorial ANOVAs (Prism) and MANOVAs (software Statistica 7, StatSoft Inc., USA) were performed. Prior to running F tests, datasets were checked for outliers using Grubbs' test (GraphPad 2010). Subsequently, the homogeneity of variances was tested with Cochran's test (Statistica) at the 1 % level according to McGuinness (2002). If variances were heterogeneous, the respective dataset was transformed via box-cox transformation (software by Wessa 2008, script by NIST/SEMA-TECH 2006). Along with F tests, Bonferroni's post hoc test (Prism®) was performed in the case of ANOVAs and Tukey's post hoc test (honest significant differences; Statistica®) in the case of MANOVAs. All graphs were created with
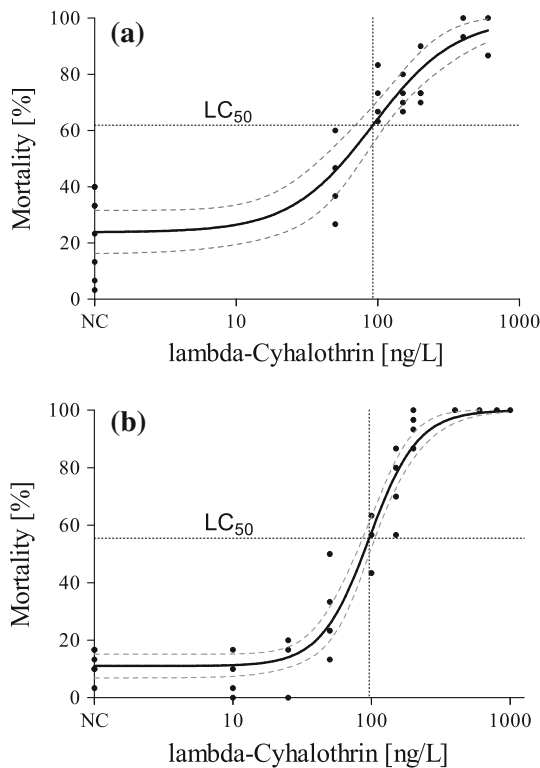
Fig. 2 Concentration-response-curve [mean ± 95 % confidence interval] for mortality [%] of **a** *Culex pipiens* and **b** *Aedes albopictus* after a full-lifecycle exposure to λ-cyhalothrin at static nutrition at 20 °C. NC (negative control): $n = 6$ ($\triangleq$ 180 larvae per treatment); insecticide treatments: $n = 4$ ($\triangleq$ 120 larvae per treatment)

Prism and pictures where edited with GNU Image Manipulation Program (GIMP) V2.7.

Results

Ecotoxicological response of two mosquito species to λ-cyhalothrin at 20 °C under static nutrition

The mortality in the negative control (NC) was 11.7 % for *Ae. albopictus* and 23.9 % for *Cx. pipiens*, thus the tests were valid if adopting the validity criteria in OECD guideline 219 for the non-biting midge *Chironomus riparius* (OECD 2004). Mortality in both mosquito species followed a clear concentration–response relationship (Fig. 2).

The lethal concentrations (LC_x) of λ-cyhalothrin with their respective 95 % confidence intervals (95 % CI) for *Ae. albopictus* were $LC_{10} = 39.7$ (30.1–52.5) ng L^{-1}, $LC_{50} = 96.4$ (86.0–108) ng L^{-1} and $LC_{90} = 234$ (193–284) ng L^{-1}, and for *Cx. pipiens* $LC_{10} = 21.1$ (9.99–44.6) ng L^{-1}, $LC_{50} = 92.2$ (69.4–122) ng L^{-1} and $LC_{90} = 403$ (240–674) ng λ-cyhalothrin L^{-1}. There was no significant difference between the LC_x values of the two species (extra sum-of-squares F test: $p = 0.71$; $F = 0.14$),
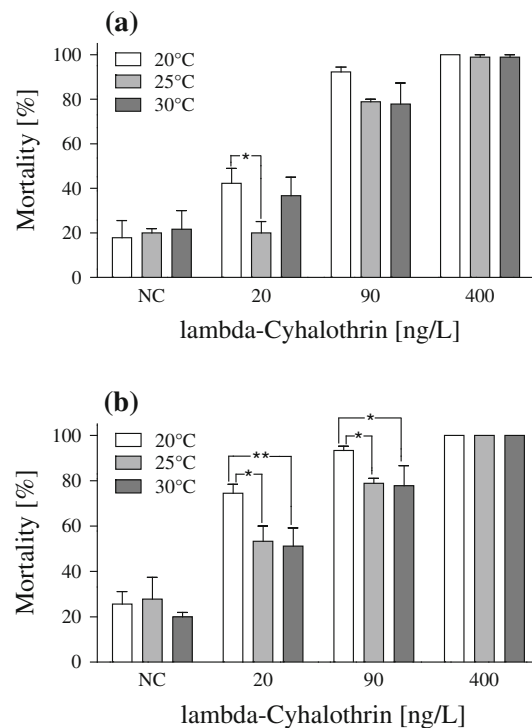

Fig. 3 Mortality [mean ± SEM] of *Culex pipiens* after a full-lifecycle exposure to λ-cyhalothrin at three temperatures at **a** static nutrition and **b** dynamic nutrition. 20 ng λ-cyhalothrin L^{-1} $\triangleq$ LC_{10}, 90 ng L^{-1} $\triangleq$ LC_{50}, 400 ng L^{-1} $\triangleq$ LC_{90}. *NC* negative control. Bonferroni's post hoc test: * $p < 0.05$; ** $p < 0.01$

but Hill factors (inclination at inflexion point) of the concentration–response-curves differed significantly, with 2.48 (1.89–3.07) for *Ae. albopictus* and 1.49 (0.9–2.07) for *Cx. pipiens* (Fisher's exact test: $P = 0.013$; $F = 6.51$).

Effects of λ-cyhalothrin on *Cx pipiens* at 20, 25 and 30 °C under static nutrition

The mortality in the NC did not differ significantly between the three temperatures (Fisher's exact test: $p \geq 0.52$) and was 21.7 % at maximum (valid test according to OECD 219). However, it has to be noted that one replicate of the NC had a mortality of 73.3 % and was detected as an outlier by Grubbs' test ($p \leq 0.05$; $Z = 1.15$) and therefore not included in further analyses. In nearly all cases, test concentrations of 20, 90 and 400 ng λ-cyhalothrin L^{-1} (LC_{10}, LC_{50} and LC_{90} at 20 °C, Fig. 3a) resulted in a higher mortality than observed in the NC; thus the factor insecticide is highly significant in the two-way-ANOVA (Table 1).

In the LC_{10} and LC_{50} treatments the mortality at 20 °C was always higher than at 25 or 30 °C. Although this difference was, in one case, significant in Bonferroni's post hoc test, the factor temperature was not in the ANOVA. The LC_{90} (400 ng λ-cyhalothrin L^{-1}) treatment resulted

Table 1 M/ANOVA results for the effects of insecticide exposure (LC_{50} of λ-cyhalothrin), temperature, and nutrition treatment on *Aedes albopictus* (a–b) and *Culex pipiens* (c–j)

Factor		Ae. albopictus		Cx. pipiens							
		a	b	c	d	e	f	g	h	i	j
Insecticide [ins]	p	<0.001	0.585	<0.001	<0.001	<0.001		0.568	0.255	0.413	0.229
	F	30.22	0.194	155	151.3	234.6		0.585	1.500	0.940	1.619
	df	1	1	3	3	3		2	2	2	2
Temperature [temp]	p	0.14	<0.001	0.23	0.003	0.007	<0.001	0.254	<0.001	0.631	0.037
	F	2.30	64.21	2.73	7.29	5.56	35.97	1.488	20.34	0.475	4.055
	df	2	2	2	2	2	2	2	2	2	2
Nutrition [nut]	p					<0.001	0.595				
	F					13.6	0.286				
	df					1	1				
Temp × ins	p	0.08	0.824	0.09	0.06	0.04		0.269	0.285	0.908	0.395
	F	3.15	0.30	1.47	2.39	2.40		1.422	1.389	0.246	1.089
	df	2	2	6	6	6		4	4	4	4
Nut × ins	p					<0.001					
	F					8.13					
	df					3					
Nut × temp	p					0.34	0.07				
	F					1.10	2.78				
	df					2	2				
Nut × temp × ins	p					0.83					
	F					0.46					
	df					6					
Residuals	df	12	73	23	24	47	62	17	15	14	16

[a] Mortality and [b] wing vein length of *Ae. albopictus*, [c] mortality of *Cx. pipiens* under static nutrition, [d] mortality of *Cx. pipiens* under dynamic nutrition, [e] mortality of *Cx. pipiens* under both nutrition protocols, and [f] wing vein length of *Cx. pipiens*, [g] egg floats per female of *Cx. pipiens*, [h] eggs per egg float of *Cx. pipiens*, [i] fertility of *Cx. pipiens*, and [j] larvae per female of *Cx. pipiens* under dynamic nutrition

almost always in a mortality of nearly 100 % which was independent from temperature.

Effects of λ-cyhalothrin on *Culex pipiens* at 20, 25 and 30 °C under dynamic nutrition

If used in combination with the dynamic nutrition protocol, the same test setup revealed an NC mortality that did not differ significantly between the three temperatures (Fisher's-Exact-Test: $p \geq 0.29$) and was 27.8 % at maximum (valid test according to OECD 219). Like in the test with static nutrition, exposure to λ-cyhalothrin significantly increased the mortality rate as shown in the two-way-ANOVA (Table 1). The mortality in the LC_{10} and LC_{50} treatments at 20 °C was always higher than at 25 or 30 °C and this difference was in most cases significant in Bonferroni's post hoc test (Fig. 3b). Additional investigated reproductive parameters were not always temperature dependent. Temperature had a high impact on the date of
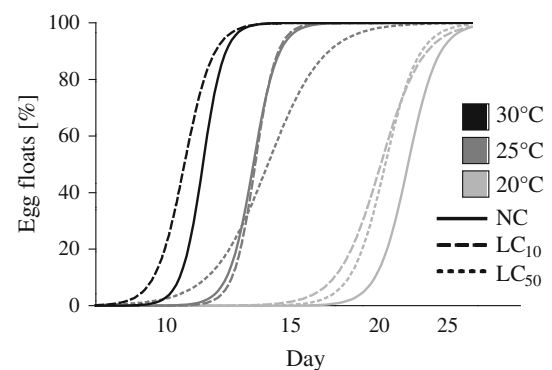

Fig. 4 Nonlinear regression for cumulative egg float production in *Culex pipiens* after a full-lifecycle exposure to λ-cyhalothrin at three temperatures. *NC* negative control. $LC_{10} \cong 20$ ng λ-cyhalothrin L^{-1}, $LC_{50} \cong 90$ ng L^{-1}. All $R^2 \geq 0.79$

oviposition (Fig. 4), but there were no significant differences in the number of egg floats per female (Fig. 5a) or the fertility of eggs (Fig. 5c). Nevertheless, higher temperature caused a highly significant decrease in the number

Fig. 5 Reproduction endpoints [mean ± SD] of *Culex pipiens* after a full-lifecycle exposure to λ-cyhalothrin at three temperatures. **a** Egg floats per female; **b** eggs per egg float; **c** fertility; **d** larvae per female. 20 ng λ-cyhalothrin $L^{-1} \triangleq$ LC_{10}, 90 ng $L^{-1} \triangleq LC_{50}$. Bonferroni's post hoc test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
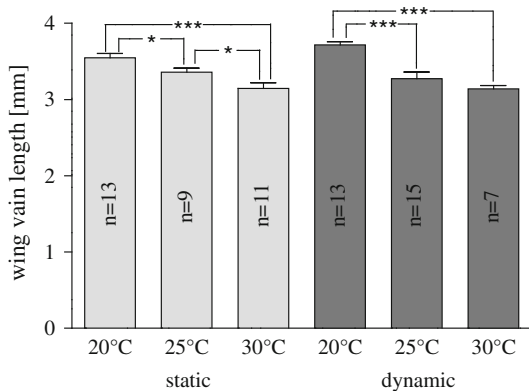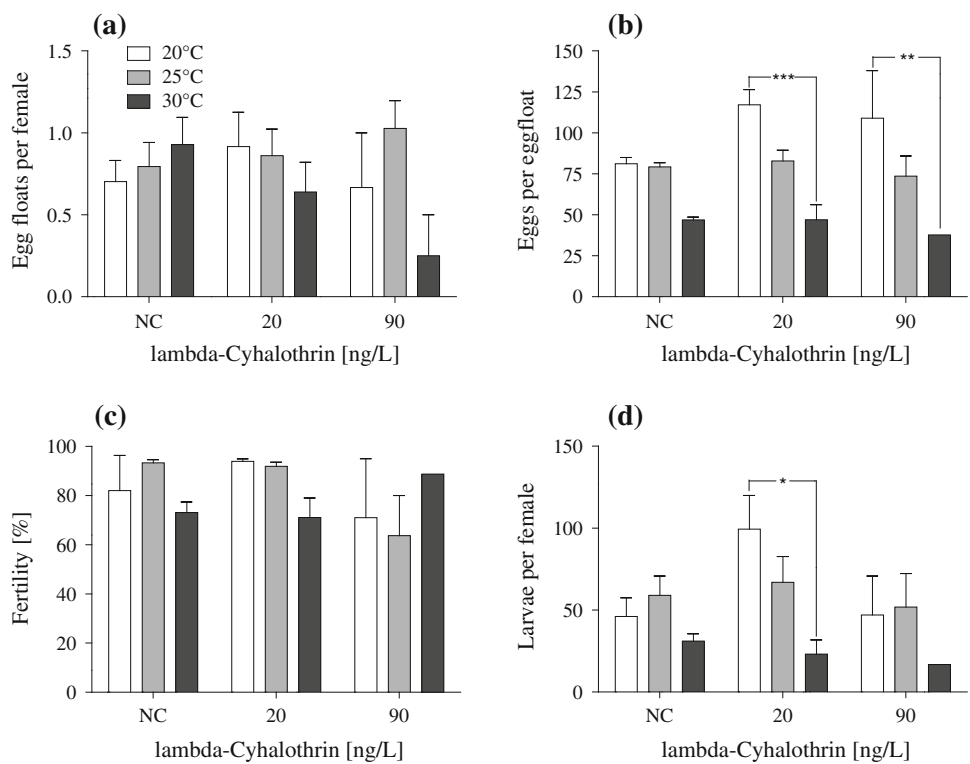

Fig. 6 Wing vein length [mean ± SD] of *Culex pipiens* exposed to three temperatures at static and dynamic nutrition

of eggs per egg float (Fig. 5b). This effect was so strong that it was still detectable in the number of larvae per female (Fig. 5d) and found to be significant in the ANOVA analysis (Table 1) despite the unclear response pattern to the interaction of the insecticide and the temperature in egg float numbers per female and fertility of eggs.

Comparison of the two nutrition treatments

There was no significant difference in the NC of both tests (Fisher's exact test: $p = 0.24$), but the mortality in the LC_{10} treatment under static nutrition was always lower at the respective temperature than in the LC_{10} treatment under

dynamic nutrition. The overall MANOVA (Table 1) revealed that the insecticide had the highest impact on variance, followed by nutrition, the interaction nutrition × insecticide, the single factor temperature and the interaction temperature × insecticide. Due to the high mortality in the groups exposed to λ-cyhalothrin, it was only feasible to analyze the length of the R_1 vein for the NC (Fig. 6). Temperature, but not nutrition, had a significant impact on wing length as calculated by Bonferroni's post hoc test and two-way-ANOVA (Table 1).

Effects of λ-cyhalothrin on *Ae. albopictus* under dynamic nutrition and 20, 25 and 30 °C

The mortality in the NC of *Ae. albopictus* (21.2–23.4 %) did not differ significantly between the three exposure temperatures (Fisher's exact test, $p \geq 0.86$, Fig. 7a), and the test is valid in terms of the OECD guideline 219 (OECD 2004).

Although mortality at 96 ng λ-cyhalothrin L^{-1} treatment (LC_{50} at 20 °C) differed by 31.6 % between 20 and 25 °C, and differed significantly by 40.4 % between 20 and 30 °C (Bonferroni's multiple comparison test: $p < 0.05$), temperature was not a significant factor for explaining differences in the variance as tested by a two-way-ANOVA (Table 1). In contrast, the length of the R_1 vein of females differed significantly between temperatures, but not between controls and the λ-cyhalothrin treatment groups
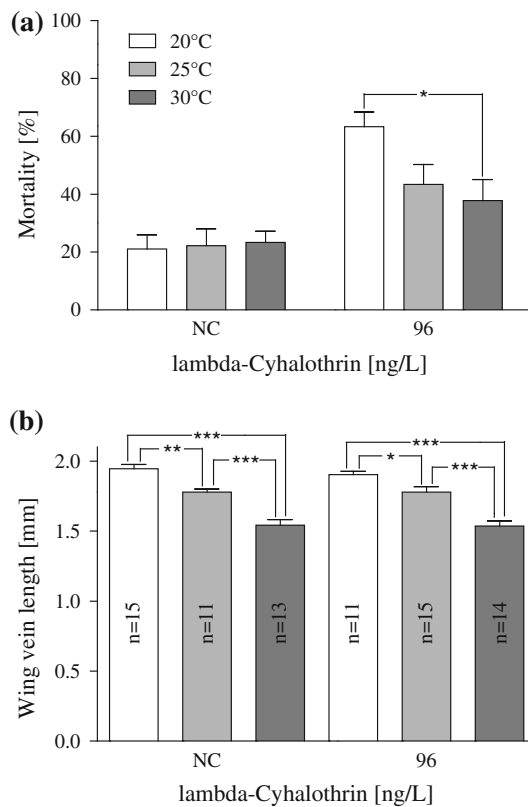
(a)

(b)

Fig. 7 **a** Mortality [mean ± SEM] and **b** wing vein length [mean ± SD] of *Aedes albopictus* after a full-lifecycle exposure to λ-cyhalothrin at three temperatures. 96 ng L^{-1} λ-cyhalothrin $\triangleq$ LC$_{50}$. *NC* negative control. Bonferroni's post hoc test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

(Fig. 7b, Table 1). For example, the NC differed significantly (by 0.402 mm) between 20 and 30 °C (20.7 % inhibition, Bonferroni's multiple comparison test: $p < 0.05$), but not between the NC and the respective λ-cyhalothrin treatment group.

Finite rate of increase

Unlike *Ae. albopictus*, *Cx. pipiens* produced a significantly lower number of eggs with increasing temperature, even though the λ' of both species increased in the negative controls (Table 2). The λ' was higher for *Cx. pipiens* than for *Ae. albopictus* at each respective temperature of the negative controls. All λ' had decreased under insecticide exposure due to mortality, but the impact was less for *Ae. albopictus* than for *Cx. pipiens* (mean reduction due to insecticide: 2.02 and 10.0 %, respectively). The strongest reduction of the finite rate of increase under insecticide exposure was detected for *Cx. pipiens* at 30 °C (−16.6 %). Thus, under insecticide treatment at high and low temperatures, the population growth of *Ae. albopictus* exceeded that of *Cx. pipiens*.

Table 2 Finite rate of increase (λ') of *Aedes albopictus* and *Culex pipiens* exposed to three temperatures and the LC$_{50\ (20\ °C)}$ of λ-cyhalothrin

Species	Treatment	Temperature [°C]		
		20	25	30
Ae. albopictus	NC	1.12	1.16	1.21
	LC$_{50}$	1.09	1.14	1.19
Cx. pipiens	NC	1.15	1.24	1.32
	LC$_{50}$	1.06	1.17	1.10

Discussion

A full-lifecycle biotest has been established to test the ecotoxicological responses of mosquitoes, in particular invasive species, under quarantine conditions. One advantage of the full-lifecycle biotest is that it enables researchers to carry out highly standardized, controlled investigations on invasive mosquitoes in areas where they are not (yet) established. Several life history parameters such as mortality, wing length, day of oviposition, number of eggs, fertility and mean finite rate of increase can be easily recorded. Using a low number of replicates the biotest generates reliable data with low variances (Fig. 2).

There was a difference in the ecotoxicological response of the invasive and the indigenous mosquito species to λ-cyhalothrin at 20 °C. Although the LC$_{50}$ was not significantly different (*Ae. albopictus*: 96.4 ng λ-cyhalothrin L^{-1}, *Cx. pipiens*: 92.2 ng λ-cyhalothrin L^{-1}), *Ae. albopictus* was more sensitive than *Cx. pipiens* at high insecticide concentrations and less sensitive at low concentrations (Fig. 2). The values are comparable to previous studies: Sulaiman et al. (1991) calculated an acute LC$_{99}$ of 153 ng λ-cyhalothrin L^{-1} for *Ae. albopictus* L4 larvae and Mohsen et al. (1995) calculated an acute LC$_{50}$ for larvae of the sister species *Cx. quinquefasciatus* with 66.7 ng λ-cyhalothrin L^{-1}. Moreover, the factor of decreasing mortality at increasing temperature was slightly higher for *Ae. albopictus* than for *Cx. pipiens* (1.7 and 1.2, respectively) (Figs. 3b and 7a). As *Ae. albopictus* is already a strong competitor for native container-breeding mosquitoes (Carrieri et al. 2003), vector control strategies aiming at the elimination of *Ae. albopictus* that include the use of λ-cyhalothrin as recommended by Whelan et al. (2009) or discussed by Lawler et al. (2007) must ensure concentrations at least above the LC$_{50}$ of both species and avoid hot weather not to increase the competitive capacity of *Ae. albopictus* in shared microhabitats.

In both species, there was a negative temperature–toxicity relationship for λ-cyhalothrin resulting in a loss of up to 70 % effectiveness of λ-cyhalothrin by a temperature increase of 10 °C (Figs. 3, 7a). Although this observation is

in contrast to those made with most other insecticides including the majority of pyrethroids (Mayer and Ellersieck 1986), λ-cyhalothrin as well as permethrin and bifenthrin also showed a negative temperature–toxicity relationship in other organisms, e.g., toxicity decreased by factor 1.9–2.3 per 5 °C increase in the amphipod *Hyalella azteca* (Weston et al. 2009). It also seems that the relationship depends on the organism itself as shown in a study with lepidopterans, where fenvalerate as well as deltametrin can show a negative temperature–toxicity relationship on *Trichoplusia ni* but a neutral or even positive relationship on *Spodoptera frugiperda* and *Heliothis virescens* (Sparks et al. 1982). Moreover, a negative temperature–toxicity relationship of λ-cyhalothrin and permethrin has been reported for the non-biting midge *Chironomus dilitus* with a factor of 3.1–3.2 at a 13–23 °C increase (Harwood et al. 2009). The mosquito *Ae. aegypti* showed the same relationship for six pyrethroids with an 1.33- to 3.63-fold increase in toxicity compared to a decrease of 10 °C (Cutkomp and Subramanyam 1986). Gammon et al. (1981) suggested that increased sensitivity to the pyrethroid allethrine at lower temperatures could be caused by an increase of the sensitivity of peripheral [sic] nerves. Salgado et al. (1989) showed that the steady-state resting potential of pyrethroid-exposed neurons was greater at lower temperature. In addition, the stability of the open-modified sodium channels is higher at low temperature, so that the duration of sodium influx is prolonged in affected invertebrates (Salgado et al. 1989). Certainly, temperature has to be taken into account when designing vector control programs. Especially in the face of global climate change, vector control measures should be adapted to better integrate the complex ecotoxicological responses of emerging insect vectors and native competitors in their rapidly changing environments.

An exposure to the 20 °C LC_{50} of λ-cyhalothrin reduced the finite rate of increase of both species compared to the NC (Table 2), even though all treatments had an $\lambda' \geq 1$ and thus a positive population growth rate. At all temperatures, this effect was stronger for *Cx. pipiens* than for *Ae. albopictus*. A rise in temperature by 5 °C compensated the effect of an exposure to the LC_{50} of λ-cyhalothrin on the finite rate of increase. According to the underlying data, this was mainly due to the greatly decreased development time trading off the reduction in egg mass. In a comparable study by Muturi et al. (2011) *Cx. restuans* (an important vector for West Nile virus and native in North America) and *Ae. albopictus* were exposed to the organochloride malathion as well in plastic containers, a similar density and temperatures like in this study. While revealing a positive temperature toxicity relationship for malathion they calculated comparable λ' for *Ae. albopictus* ($\lambda' = 1.15$ at 20 °C; 1.19 at 25 °C; 1.19 at 30 °C) and *Cx. restuans* ($\lambda' \approx 1.20$ at 20 °C; 1.26 at 25 °C; 1.24 at 30 °C) like we

did for *Cx. pipiens* (Table 2). Noteworthy is that even though there was a significant impact on survival of *Cx. restuans* by temperature as well as the exposure to malathion, the λ' of *Cx. restuans* was less sensitive to temperature than the λ' of *Cx. pipiens* and less sensitive to malathion than the λ' of *Ae. albopictus*. This finding shows a contrary pattern to our study where the λ' of the temperate *Culex* species is more sensitive to the impact of λ-cyhalothrin than the λ' of the tropic/subtopic invasive *Aedes* species. This shows the importance of considering all the different endpoints of the life-cycle in studies of multiple stressors to reveal the underlying mechanisms of the outcome of the λ' model for understanding different impacts and trade off strategies.

Our dynamic feeding protocol allows for an optimal development through all larval stages as already shown by Müller et al. (2013) for *Ae. albopictus* but conferred a higher sensitivity of *Cx. pipiens* at low concentrations of λ-cyhalothrin (Figs. 3, 5d). This effect might be caused by a higher accumulation of λ-cyhalothrin in a lower amount of nutrition in the first days because λ-cyhalothrin has a high log K_{ow} of 5.5 (PPDB Management Team 2014), and the first four feedings provided only 0.5 mg $larva^{-1}$ under the dynamic nutrition protocol and 1 mg $larva^{-1}$ under the static nutrition protocol. A different accumulation of the insecticide in the food could have resulted in a different biomagnification of λ-cyhalothrin in L1/L2 larvae. Biomagnification certainly took place in the mosquito as implied by the high bioconcentration factor (BCF = 4982; PPDB Management Team 2014). Moreover λ-cyhalothrin biomagnification was demonstrated in the midge *Chironomus dilitus* and stated to be the main mode of absorption (Harwood et al. 2009).

The higher sensitivity of *C. pipiens* at low λ-cyhalothrin concentrations under the dynamic nutrition protocol became disguised at higher concentrations of the insecticide (Fig. 3). Moreover, there was no impact of the different feeding protocols on wing length (Table 1, Fig. 6) and therefore body size (Lounibos et al. 2002). However, the comparison of the two feeding protocols shows that the ecotoxicological effect of λ-cyhalothrin and its temperature-dependent toxicity is stronger under the dynamic nutrition protocol recommended by Müller et al. (2013) compared to the static nutrition protocol according to OECD guideline 219 (OECD 2004). Besides, the dynamic nutrition protocol for mosquitoes like *Ae. albopictus* seems to give more clear results in biotests and is therefore strongly recommended for ecotoxicological studies. In conclusion, prior to vector control measures, the potentially large variety of trophic states of larval habitats targeted (e.g. newly emerged and oligotrophic receptacles or hypertrophic and anoxic ditches) has to be evaluated to take the impact of the state on the efficiency of an

insecticide into account (Roessink et al. 2005). Especially hydrophobic insecticides like λ-cyhalothrin are more likely to have a changed fate and mode of absorption and thus efficiency than hydrophilic substances in hypertrophic habitats.

It is conspicuous that several European non-target aquatic keystone species in temporary freshwaters such as the top predators *Chaoborus obscuripes*, *Notonecta glauca*, *Notonecta indica*, *Sigara striata*, *Corixa* sp., *Tropisternus lateralis* and *Sialis lutaria* are more sensitive to λ-cyhalothrin than the two mosquito species under investigation (Fig. 2, Maund et al. 1998; Dennett et al. 2003; Schroer et al. 2004). It is known, that some of these species are highly utilizing mosquito larvae and are effective biological control species as a top-down force. Especially invertebrates with a self distributing airborne life stage have the potential to reduce vectors in urban areas effectively (Kumar and Hwang 2006). But some of these species are up to 34.3 times more sensitive to λ-cyhalothrin than *Cx. pipiens* or *Ae. albopictus* (e.g., *Chaoborus obscuripes*: 48 h-EC_{50} = 2.8 ng L^{-1}) and the species sensitivity distribution model (SSD) predicts a high risk due to a field HC_{10} (hazardous concentration for 10 % of all species) of 5.1 ng L^{-1} (Maund et al. 1998, Schroer et al. 2004). Hence, the use of λ-cyhalothrin in or around freshwater habitats to control these mosquitoes as discussed by Lawler et al. (2007) and Whelan et al. (2009) should be considered as a last option only. As it is worthwhile to conserve biodiversity even in urban regions or in agricultural landscapes where it may be surprisingly great, the use of λ-cyhalothrin to control the larvae of these mosquitoes in, e.g., artificial receptacles ("anthrotelmata") such as trashed cans, old tires, blocked rain gutters as well as rice fields should be discouraged in favor of using more specific insecticides based on juvenile hormones or toxic proteins of *Bacillus thuringiensis*.

Conclusion

Studies with multiple stressors, including insecticides and rising temperatures add relevant knowledge for developing more specific and effective guidelines to combat invasive vectors like *Ae. albopictus* and support the protection of public health in due consideration of the protection of aquatic ecosystems as well. This study highlights the potential risk of vector control measures which, in the absence of detailed studies on the complex interactions between invasive and native species and environmental parameters, may increase the competitive advantage of the invasive target (*Ae. albopictus*) compared to a temperate competitor (*Cx. pipiens*). The observation that higher temperature can result in lower mosquito sensitivity to a

commonly used insecticide should also be taken into account when designing future vector control measures. The newly established full-lifecycle biotest provides support to further standardize experiments on *Ae. albopictus* and thereby answer important questions about its potential for invasion and establishment in new areas, its ecological niche, context-dependent vector competence, and mosquito control strategies.

Author contribution statement

AK and RM conceived and designed the study. AK conducted the experiments. AK, RM analyzed data. JO provided laboratory, rearing and infrastructural possibilities. All authors wrote, read and approved the manuscript.

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