



Elevated temperatures interact with habitat quality to undermine survival of ectotherms in climatic refugia

Marcin Penk^{1,2*}, Ian Donohue^{1,2}, Vincent Récoules^{1,3} and Kenneth Irvine^{1,4}

¹Department of Zoology, School of Natural Sciences, Trinity College Dublin, Zoology Building, Dublin 2, Ireland, ²Trinity Centre for Biodiversity Research, Trinity College Dublin, Dublin 2, Ireland, ³École Nationale du Génie de L'eau et de l'Environnement de Strasbourg, 1 quai Koch, 67070 Strasbourg, France, ⁴UNESCO-IHE Institute for Water and Education, Westvest 7, 2611 AX Delft, The Netherlands

ABSTRACT

Aim Large-scale effects of climatic fluctuations can be masked by local micro-climatic variability, allowing species to retain fragments of their original ranges. However, downscaling predicted ecological effects of climate change to local scales and individual species comprises a key knowledge gap. As temperature modifies both abiotic and biotic processes, the fate of refugial populations may depend on interactions of temperature with susceptibility of organisms to other factors, rather than on the impact of temperature alone. To explore this, we examined whether temperature regulates the sensitivity to hypoxia of an aquatic glacial relict, *Mysis salemaai*, an opossum shrimp.

Location Loughs Oughter, Allen and Derg (field surveys) and Dublin (laboratory experiments), Ireland.

Methods We used laboratory experiments to determine thermal sensitivity of *M. salemaai* and quantify the effect of temperature on survival and regulation of aerobic metabolism in progressive hypoxia. The resulting temperature and oxygen sensitivity thresholds were then validated in the field.

Results Survival of *M. salemaai* in reduced oxygen conditions was significantly lower at higher temperatures, even though the tested temperature range did not cause mortality on its own. Results of respiration assays suggest that this was a consequence of impaired capacity at elevated temperatures for regulating oxygen uptake in hypoxic conditions.

Main conclusions Our findings demonstrate that biological effects of climate warming can depend more strongly on interactions of temperature with other factors than on the effects of elevated temperatures alone. Understanding species-specific responses to such interactions is essential to predict future distribution patterns, mitigate threats and prioritize conservation measures aimed at preserving global biodiversity.

Keywords

Climate change, climate refugia, hypoxia, lake, *Mysis salemaai*, nutrient enrichment.

*Correspondence: Marcin Penk, School of Natural Sciences, Trinity College Dublin, Zoology Building, College Green, Dublin 2, Ireland.
E-mail: penkm@tcd.ie

INTRODUCTION

Both the magnitude and rate of mean global temperature rise within the last century have been unprecedented within at least the last 1500 years and unusual for the entire Holocene (Marcott *et al.*, 2013). Such abrupt changes are expected to impact upon biotic assemblages significantly (McLaughlin *et al.*, 2002; Root *et al.*, 2003; Thomas *et al.*, 2004; La Sorte & Jetz, 2010). However, our understanding of such effects is

based primarily upon observational studies, generally at large spatial scales (Tingley *et al.*, 2012; Jueterbock *et al.*, 2013; Smith, 2013; Vittoz *et al.*, 2013). Such studies are unable to identify ecological mechanisms operating, particularly at local scales. Despite some notable experimental research (Moss, 2010; Kratina *et al.*, 2012; Woodward *et al.*, 2012; Brochier *et al.*, 2013; Ozen *et al.*, 2013), downscaling predicted broad-scale ecological effects of climate change to local scales, and to individual species, comprises a key knowledge gap

(Osmond *et al.*, 2004; Parry *et al.*, 2007; García-Molinos & Donohue, 2014).

Broad-scale effects of climatic fluctuations can be masked by local microclimatic variability (Pinsky *et al.*, 2013), allowing species to retain their ranges (Ackerly *et al.*, 2010; Stewart *et al.*, 2010; Davis *et al.*, 2013). Remnants of species distributions from the last glacial period are, for example, sheltered from summer temperature extremes in cooler habitat pockets (Zacharda, 1993; Sheldon *et al.*, 2008; Turlure *et al.*, 2009; Horsak *et al.*, 2010). This results in habitat fragmentation which not only precludes gradual change in distribution in response to further climate change, but makes species vulnerable to reduced habitat quality (Ilyashuk, 2002; Maitland *et al.*, 2007; Wezel, 2007). As temperature modifies both abiotic and biotic processes (Rao, 2010; Grigaltchik *et al.*, 2012; Hamilton *et al.*, 2012; Tavakoli & De Smedt, 2012), the fate of such refugial populations may depend on interactions of temperature with other factors rather than on the impact of temperature alone.

Species distributed within current climate envelopes may become restricted to refugial habitats by anticipated warming, similar to extant glacial relicts. The latter species therefore provide an opportunity to investigate the interactive effects of future climate scenarios with other globally important pressures and to elucidate ecological mechanisms in operation. Aquatic glacial relicts frequently find refugia in the relative thermal stability of the bottom zone of stratified lakes and are associated typically with low-nutrient and oxygen-rich waters (Kohn & Waterstraat, 1990; Sheldon *et al.*, 2008; Harrison *et al.*, 2012). Availability of such habitats has, however, been reduced considerably over the last century because of oxygen depletion caused by microbial decomposition of organic matter in nutrient-enriched waters (Mäkiläinen *et al.*, 2000; Horppila *et al.*, 2003). Further, this effect is magnified by increasing temperatures both accelerating microbial respiration and decreasing oxygen solubility in water (Moss *et al.*, 2011). Moreover, predicted warmer summers (Solomon *et al.*, 2007) are expected to drive stronger lake stratification, impeding oxygen mixing into the deeper waters and promoting nutrient remineralization from sediments (Moser *et al.*, 2002; Matzinger *et al.*, 2007). The latter, in combination with predicted higher nutrient run-off resulting from more flashy precipitation regimes, is expected to stimulate higher productivity (Jeppesen *et al.*, 2009) and exacerbate effects of eutrophication, including oxygen depletion, in aquatic environments (Jankowski *et al.*, 2006; Moss *et al.*, 2011; Foley *et al.*, 2012).

Biotic adaptations to oxygen deficits, such as reducing activity or increasing efficiency of oxygen uptake, are ubiquitous (Sutcliffe, 1984; Hagerman & Szaniawska, 1988; Richards *et al.*, 2009). Even under oxygen-saturated conditions, heat stress can depress aerobic scope, the capacity to match oxygen demand above basal metabolism (Portner & Knust, 2007; Rummer *et al.*, 2014). Thus, even below thermal mortality thresholds, elevated temperatures may reduce the ability of organisms to cope with hypoxic stress.

Using an extant aquatic glacial relict as a model species, we explored whether increased sublethal temperatures limit species' adaptability to hypoxia. We first determined thermal sensitivity using survival and thermal avoidance experiments. We then tested the hypothesis that survival in hypoxia deteriorates at higher, but sublethal, temperatures. We quantified shifts in regulation of aerobic metabolism in progressive hypoxia with temperature to elucidate possible mechanisms operating. The laboratory-derived temperature and oxygen sensitivity thresholds were validated in the field by examining vertical migration patterns in response to temperature and oxygen gradients.

The model species, an opossum shrimp, *Mysis salemaai* Audzijonyte & Vainola, 2005 (Crustacea; Mysidae), occurs at its southern distribution front in Ireland (Fig. 1). The species is considered highly vulnerable to both climate warming and eutrophication (Griffiths, 2007). *M. salemaai* shelters from visual predators in deep dark lake waters by day and redistributes to all depths by night, gaining access to the more productive surface waters (Southern & Gardiner, 1932). However, access to both of these habitats may be restricted by, respectively, reduced bottom oxygen concentrations and increased surface temperatures (Horppila *et al.*, 2003; Gal *et al.*, 2004; Boscarino *et al.*, 2007), the former being a key consequence of eutrophication (Diaz & Rosenberg, 2008).

METHODS

Experimental organisms

The organisms used in our experiments were collected from Lough Oughter, Ireland (54°01'05" N 7°29'20" W) at night. They were kept in lake water at 10 °C at a 12:12 h (dim : dark) light cycle and never exposed to strong light to avoid eye damage. They were fed with live zooplankton but fasted for at least 12 h before experiments. Animals were used within a week of capture, and there was good survival in storage tanks. Filtered (Whatman® (GE Healthcare, Little Chalfont, UK) GF/C, 1.2 µm) fresh Lough Oughter water was used in all experiments and was sterilized at 121 °C in an autoclave prior to respiration analysis to minimize microbial respiration. Body size or biomass variability among individuals was not confounded with the different treatments in any of our experiments. Animals were always introduced into the experimental set-up at 10 °C and full oxygen saturation water and gradually adjusted to treatment conditions over 15–30 min, depending on the set-up. This period is considered to be well within the rate of change encountered during vertical migration through the thermocline (as observed during our behavioural experiments) and facilitated gradual acclimation of experimental organisms.

Survival experiments

Lethal temperature limits and the effect of sublethal temperatures on survival in hypoxia were examined using two

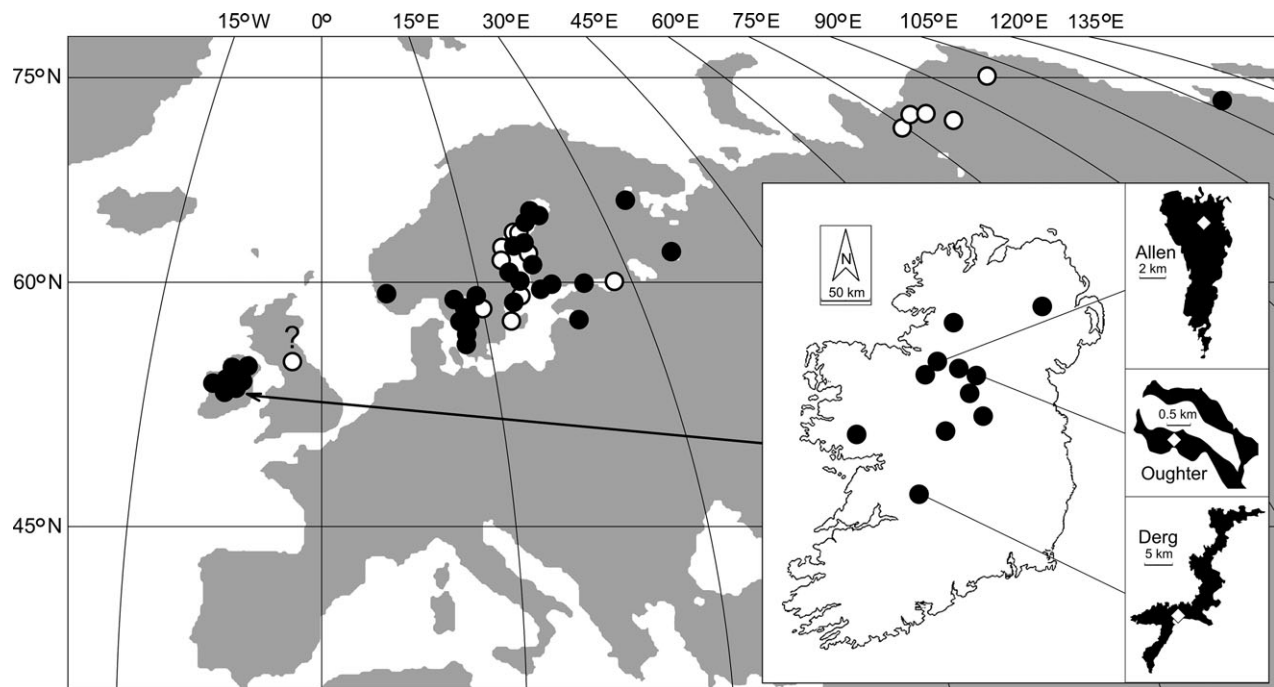


Figure 1 Global distribution of *Mysis salemaai*. Open circles indicate records > 50 years old, and the species is believed to be extinct from Great Britain (question mark). Insert shows location of the 11 lakes in Ireland from which *M. salemaai* is known, with our sampling sites (indicated with open diamonds) in Loughs Allen, Derg and Oughter.

separate sets of experiments. Lethal temperature experiments were done using 0.5-L rectangular bottles (base 7×7 cm, height 11 cm) placed in a temperature-controlled water bath. Temperature was held constant in each treatment and treatments ranged from 16 to 26 °C at 1 °C intervals. Whereas surface temperatures in Irish lakes during summer stratification typically fall within the lower half of this range, the upper limit was extended until near-full mortality was observed within the shortest recording time interval, to enable applicability of the results to future climate scenarios. In addition, a 10 °C 'control' reflecting summer bottom temperatures in stratified lakes in Ireland (Irvine *et al.*, 2001), and thermal refugia, was included to provide a measure of background mortality, against which effect of heat stress could be gauged. The resulting 12 treatments were run in randomized order in triplicate. Each replicate run comprised three bottles, each containing five *M. salemaai*, run simultaneously. Bottles were aerated gently during inspections to minimize risk of adverse effects of deoxygenation on experimental animals and oxygen concentration was always in excess of $7 \text{ mg O}_2 \text{ L}^{-1}$.

In the second set of experiments, the effect of temperature on survival in hypoxia was examined in a flow-through closed-circuit experimental system. Oxygen concentration was manipulated in a 5-L reservoir by bubbling either air or nitrogen gas through water, which was then circulated at 0.5 L min^{-1} using a submersible pump through a 1-L experimental bottle (base diameter 9 cm, height 16 cm; Fig. 2a). The entire unit was submerged in either a 10 or 15 °C water

bath. Oxygen was held constant in each treatment and treatments ranged between 1 and $5 \text{ mg O}_2 \text{ L}^{-1}$ at $1 \text{ mg O}_2 \text{ L}^{-1}$ intervals in addition to a saturated ($> 9 \text{ mg O}_2 \text{ L}^{-1}$) control. Ten *M. salemaai* were placed in each experimental bottle, and each of our 12 treatments was replicated four times.

Both survival experiments were initiated after 30-min acclimation performed within the experimental chambers. The diel rhythm of vertical migration determines the potential duration of the effect of heat and hypoxia on *M. salemaai*, but occasional wind-induced disruption of the thermocline may prolong exposure to heat stress. We therefore monitored survival of experimental animals at 1, 3, 6, 12 and 24 h in both sets of experiments, with an additional monitoring at 48 h in the temperature experiments only. Prolonging the experiments further would increase the likelihood of starvation affecting shrimp survival. At each time interval, surviving animals were counted and dead animals removed. Temperature and oxygen were monitored and maintained at each scheduled inspection, in addition to spontaneous checks, using a Hach® (Hach, Düsseldorf, Germany) HQd30 Flexi with a luminescent dissolved oxygen probe. Replicates during which any single oxygen concentration measurement deviated from target values by more $0.4 \text{ mg O}_2 \text{ L}^{-1}$ were repeated. The mean absolute deviation of individual readings from target concentrations (hereafter precision) was independent of the experimental treatment, but it increased with time from ± 0.05 to $\pm 0.17 \text{ mg O}_2 \text{ L}^{-1}$. Temperature stability was independent of both treatments and time (precision ± 0.2 °C).

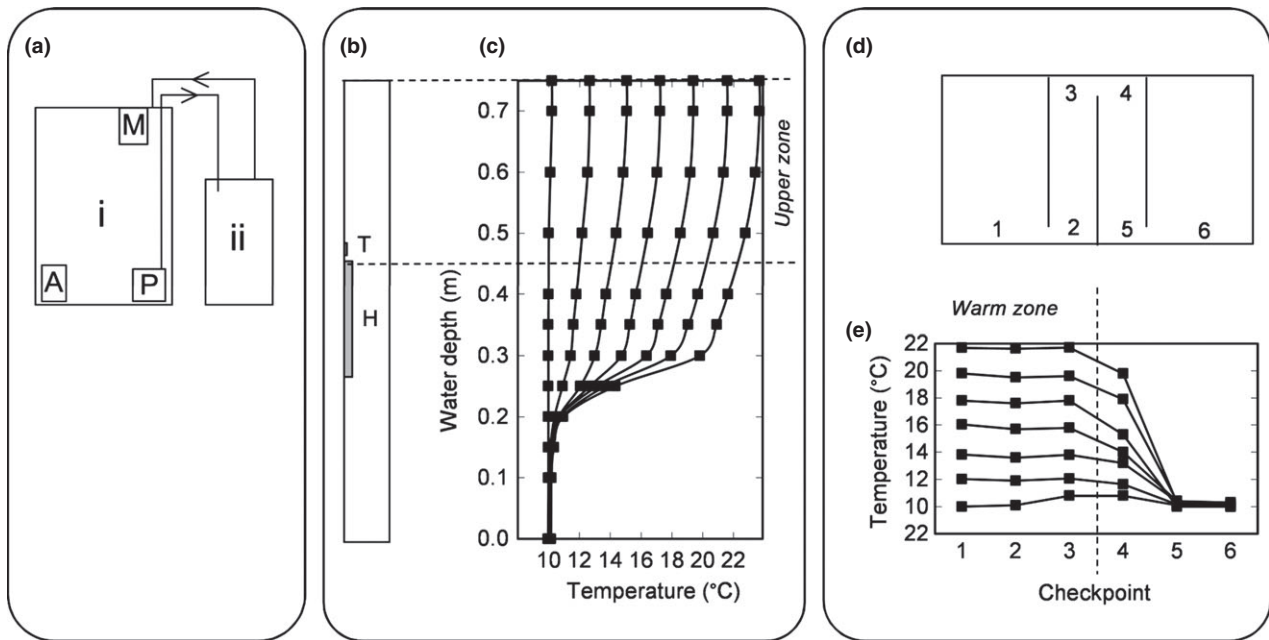


Figure 2 Experimental set-ups showing (a) the closed-circuit system used for examination of survival of *Mysis salemaai* in varying combinations of oxygen and temperature with adjustment reservoir (i) and experimental bottle (ii), indicating position of the submersible pump (P), air/nitrogen bubbling airstone (A) and temperature/oxygen monitoring probe (M), (b) the vertical migration experimental column in vertical cross section (height 75 cm, diameter 7 cm) indicating the position of the heating element and temperature sensor (H and T, respectively, to scale), (c) corresponding mean temperatures at pre-determined temperature treatments (10–22 °C, at 2 °C intervals), (d) the horizontal migration box in elevation view cross section (total length 33 cm, width 23 cm, height 20 cm, details to scale) indicating the position of temperature measurement checkpoints (1–6) and (e) corresponding mean temperatures at pre-determined temperature treatments (10–22 °C, at 2 °C intervals).

Regulation of aerobic metabolism experiments

The effect of temperature on regulation of aerobic metabolism in progressive hypoxia was examined by respiration analysis using a YSI® (YSI, Yellow Springs, Ohio, USA) 53 biological oxygen monitoring system. After 15-min temperature acclimation, a single *M. salemaai* individual was placed inside a cylindrical vial (diameter 20 mm) with 5 mL air-saturated water and a polarographic electrode (YSI® 5331) sealing the top of the vial. A magnetic stirrer was placed on the bottom of the vial to generate a gentle water current necessary for the probe to read accurately and was separated from the experimental organism by a 350-µm nylon mesh stage to prevent injury. The vial was submerged in a temperature-controlled water bath and shaded to minimize light stress. Five replicates, each containing a different individual organism, were run at each temperature treatment (i.e. 5–25 °C at 5 °C intervals; precision ± 0.2 °C). Each experiment commenced at $\geq 90\%$ O₂ saturation, and both time and saturation were recorded continuously until shrimp mortality occurred. There was usually an initial rapid decrease of the respiration rate, taken as an indication of handling stress, which typically stabilized within minutes. These initial measurements were excluded from analysis. Whereas longer acclimation times could affect aerobic metabolism, *M. salemaai* is typically exposed to frequent temperature fluctuations in lakes as a result of diel redistribution across the

thermocline, and thus, our protocol is consistent with field conditions.

Individual oxygen consumption rate ($\mu\text{g O}_2 \text{ h}^{-1}$) was calculated for each $\leq 10\%$ drop in oxygen saturation. After the experiment, each shrimp was blotted dry and weighed to the nearest 0.1 mg on a Mettler Toledo® (Mettler-Toledo, Greifensee, Switzerland) B154-S analytical balance. Individual oxygen consumption rates were divided by biomass to calculate mass-specific oxygen consumption rates (MO₂, in $\mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$). The MO₂ for each shrimp was modelled as a first-order function of the natural logarithm of oxygen saturation (SatO₂, in per cent; see Fig. S1 in Supporting Information). We then quantified critical oxygen saturation (SatO_{2 crit}) as the point at which the slope of the tangent to MO₂ equalled the slope of aerobic conformity (i.e. the straight line connecting MO₂ at 100% SatO₂ and the origin of the coordinate system) for each replicate. SatO_{2 crit} therefore corresponds to the point of inflection of the curve where the difference between MO₂ and aerobic conformity was maximal and marks the threshold where the organism's scope for regulation of aerobic metabolism decreases with decreasing oxygen saturation (see Fig. S1).

All elements of the respiration analysis set-up in contact with experimental water were cleaned and rinsed with Milli-Q® (EMD Millipore, Billerica, Massachusetts, USA) water before each run and sterilized daily to minimize microbial activity. The electrode was calibrated to 100% saturation

before each experiment in water-saturated air at the experimental temperature. To account for any potential shifts in measurement drift with increasing temperature, we ran two blank runs (same set-up, no shrimp) at each 2.5 °C interval over the entire experimental temperature range and used the resulting slope in this relationship ($0.027 \mu\text{g O}_2 \text{ h}^{-1}$, $t_{2,16} = 2.23$, $P < 0.05$) to correct the results. Drift of oxygen measurements was minor, ranging between 3 and 5% of our measurements, and unlikely to confound our findings. Three control runs, with open vial tops to allow replenishment of oxygen from air, were done at each temperature for a period exceeding the longest experimental run at a given temperature in order to examine whether factors other than oxygen depletion were resulting in mortality and, therefore, confounding hypoxic effects. Upon completion, shrimp survival was monitored for a further 24 h. All control animals survived at 5, 10, 15 and 20 °C and all died in <3 h at 25 °C, indicating that, while the set-up or handling did not lead to mortality, the highest temperature tested did.

Thermal avoidance experiments

Temperature avoidance thresholds of *M. salemaai* were examined in a stratified mesocosm representative of natural vertical arrangements of thermal gradients, where animals were introduced under cool, isothermal conditions. Experiments were done in 3.3-L perspex mesocosms (base diameter 7.5 cm, height 75 cm; Fig. 2b) filled with water and submerged in a 10 °C water bath to represent summer temperature in stratified Irish lakes. A thermostat-controlled 150 W heater lowered into half of the mesocosm column height allowed warming of the upper water while the lower section remained at 10 °C. Experimental temperatures in the upper part of the column were manipulated from 10 °C (isothermal) to 22 °C (stratified) at 2 °C intervals (Fig. 2c). Target temperatures were achieved at the depth of 45 cm and deviated up towards the surface by 0.7–1.7 °C over the experimental temperature range. Ten *M. salemaai* were introduced under isothermal conditions (10 °C throughout). After 30-min acclimation, during which stratification had established, the proportion of individuals above the target temperature was determined as a mean of ten counts done at 2-min intervals. Different individuals were used in each experimental run.

As the bottom temperature was kept constant, increasing surface temperature treatments resulted in steeper temperature gradients, possibly restricting upward migration and amplifying the observed thermal avoidance effect. For this reason, a second set of experiments was carried out in a horizontal arrangement, where equal numbers of *M. salemaai* were introduced to both cold and warm ends of a mesocosm, such that animals could reposition themselves across the thermal gradient to avoid unfavourable temperatures. If steepness of the temperature gradient inhibits migration, it should dampen thermal avoidance in the horizontal arrangement, in contrast to the vertical set-up, and the two experiments would be expected to produce different avoidance thresholds.

The horizontal thermal avoidance experiments were done in a 14-L insulated rectangular box (base 23×33 cm, height 18 cm) divided into two sections with three partition walls fitted in the centre. The partitions were vertically offset in relation to each other in alternating directions so as to facilitate passage below the two distal partitions and above the central one (Fig. 2d). This arrangement prohibited water in the two sides from mixing yet provided a migration corridor for experimental animals. Temperature-adjusted water was circulated independently through both sections of the box at 0.5 L min^{-1} . One section of the box was always kept at 10 °C (cold), while the temperature treatments in the other section were manipulated from 12 °C to 22 °C (warm) at 2 °C intervals, in addition to a 10 °C control treatment (precision ± 0.4 °C; Fig. 2e). Allocation of cold and warm temperature treatments to the sections was alternated between runs. After 15-min temperature acclimation, five animals were released from bottle enclosures into each side of the box and the proportion of animals in the warm side was determined as a mean of ten counts at 2-min intervals after 1.5 h. Both experiments were done in darkness and *M. salemaai* counted using an infrared camera. Each temperature treatment was replicated five times.

Field surveys

The vertical distribution of *M. salemaai* in field conditions was surveyed twice in the deepest points of three natural lakes {Loughs Allen [$54^{\circ}08'34'' \text{ N } 8^{\circ}03'09'' \text{ W}$; total phosphorus (TP) concentration $17 \mu\text{g L}^{-1}$], Derg [$52^{\circ}54'35'' \text{ N } 8^{\circ}23'03'' \text{ W}$; TP $24 \mu\text{g L}^{-1}$] and Oughter [$54^{\circ}01'05'' \text{ N } 7^{\circ}29'20'' \text{ W}$; TP $70 \mu\text{g L}^{-1}$]; Fig. 1}, representing a gradient of trophic conditions, in summer 2010. As severe water deoxygenation and concurrent mysid exclusion were recorded from Lough Oughter, a further three surveys took place in this lake in summer 2012.

At each sampling site, temperature and dissolved oxygen concentrations were determined *in situ* at 1-m intervals throughout the water column as the mean of two readings (during descent and ascent of the probe) with a polarographic oxygen probe (YSI® 52). Using a 500- μm zooplankton net with an opening diameter of 0.65 m and closing mechanism, samples of *M. salemaai* were collected, in triplicate, at each of between three and four discreet depth strata, corresponding to vertical changes in temperature and oxygen gradients in each lake (see Figs S2 and S3). After lowering to the desired depth, the net was rested for approximately 10 seconds to allow mysids to redistribute over the net opening and lifted at a low velocity (0.3 m s^{-1}) to minimize the pressure wave. All *M. salemaai* sampling took place by night to minimize the chance of suppression of its vertical distribution by natural light.

Data analyses

All survival analyses were done using R version 2.14.1 (R Development Core Team, 2011). The effect of temperature

(first experiment) and temperature and oxygen (second experiment) on survival of *M. salemaai* were modelled separately for each experiment as fixed effects using generalized linear mixed effect models (LMER, binomial errors) from the 'LME4' package (Bates, 2013), where repeated counts were nested within replicates (both as random effects). In the first experiment, effects of elevated temperatures were compared with 10 °C using orthogonal treatment contrasts. In the second experiment, effects of reduced oxygen concentrations were compared with oxygen-saturated conditions separately at 10 and 15 °C, and then temperature effects at different oxygen concentrations were tested using multiple comparisons with single-step *P*-adjustment using the general linear hypotheses function (GLHT) from the 'MULTCOMP' package (Hothorn *et al.*, 2008). Temperature and oxygen were aggregated into a single factor in these analyses to satisfy constraints of GLHT. To provide a measure of the effect of time-scale for each significant treatment in both experiments, a sigmoid curve was fitted to each replicate using generalized linear models (GLM, binomial errors) and time (\pm SE) to 50% mortality was estimated using the *dose.p* function from the 'MASS' package (Venables & Ripley, 2002).

We used first-order linear regression to test the effect of temperature on log-transformed SatO_2 crit as a measure of regulation of aerobic metabolism. At 25 °C, the slope of the tangent to modelled MO_2 was greater than that of aerobic conformity, indicating lack of regulation and precluding calculation of SatO_2 crit.

To test for thermal avoidance in the vertical experimental set-up (Fig. 2c), we used analysis of variance (ANOVA) to examine the effect of elevated temperatures on the log-transformed ($x + 0.01$) proportion of *M. salemaai* found in the upper part of the water column. Dunnett's *post hoc* test was then used to compare the effect of each stratified treatment to isothermal conditions. In the horizontal temperature avoidance experiments (Fig. 2e), we used individual *t*-tests to test the effect of each temperature treatment on the proportion of animals recorded in the manipulated box section against the theoretical neutral proportion of 0.5 (i.e. individuals evenly distributed between the sides) expected under a null scenario.

To validate laboratory-derived thermal and oxygen sensitivity thresholds in the field, we first analysed the relationship of mean volumetric density of *M. salemaai* in the surface and bottom water strata with, respectively, mean

temperature and oxygen concentration in the same strata using two separate linear regressions. To take account of potential differences in densities among lakes, mean densities in surface and bottom strata were expressed as a proportion of the all-depth average (i.e. relative density). To verify that this goal was achieved, lake was initially included in both models as a random factor, but never had a significant ($P < 0.05$) effect and was subsequently removed. We then looked for the temperature and oxygen concentration at which the corresponding regression lines (\pm 95% CI) intersected the neutral relative density of 1, as an indication of sensitivity thresholds. Surveys during which no *M. salemaai* were recorded or when isothermal conditions were encountered were not included in the analyses (respectively, Lough Oughter 28/07/2010 and Lough Derg 31/07/2010). The data were log-transformed ($x + 0.1$) prior to analyses.

RESULTS

Survival experiments

Survival of *M. salemaai* was reduced significantly at acute exposure to all temperatures ≥ 22 °C compared with 10 °C (LMER; *z*-value, respectively, -4.7 , -10.8 and -13.6 at 22 °C, 24 °C and 26 °C; $P < 0.001$ in all instances; Fig. 3). Estimated 50% mortality exposure time was 46 ± 1 h at 22 °C, 3.5 ± 0.6 h at 24 °C and 0.8 ± 0.1 h at 26 °C. Observed 100% mortality occurred between the 12–24 h intervals at 23 °C, 6–12 h at 24 °C and 1–3 h at ≥ 25 °C. Temperatures ≤ 21 °C had no significant (at $P \leq 0.05$) effect on survivorship compared with 10 °C within the 48-h exposure time (see Table S1).

Survival of *M. salemaai* was reduced significantly at oxygen concentrations ≤ 2 mg O_2 L^{-1} compared with > 9 mg O_2 L^{-1} at both 10 °C (GLHT; $z = -2.9$, $P < 0.05$ and $z = -9.5$, $P < 0.001$ at, respectively, 2 mg O_2 L^{-1} and 1 mg O_2 L^{-1}) and 15 °C (GLHT; $z = -6.5$, $P < 0.001$ and $z = -11.3$, $P < 0.001$ at, respectively, 2 mg O_2 L^{-1} and 1 mg O_2 L^{-1} ; Fig. 4). Further, survival at these concentrations was significantly lower at 15 °C compared with 10 °C (GLHT; $z = -7.7$, $P < 0.001$ and $z = -5.3$, $P < 0.001$ at, respectively, 2 mg O_2 L^{-1} and 1 mg O_2 L^{-1} ; Fig. 4). Estimated 50% mortality exposure times at 10 and 15 °C were, respectively, > 24 h and 14.3 ± 4.6 h at 2 mg O_2 L^{-1} and 3.2 ± 1.3 h and

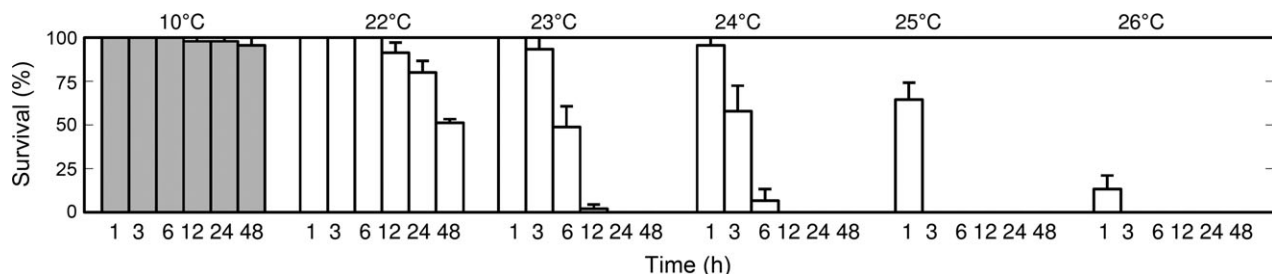


Figure 3 Survival (mean percentage, of 15 individuals, \pm SE, $n = 3$) of *Mysis salemaai* at elevated temperatures (white bars) at which it was significantly ($P < 0.05$) different from cool conditions (10 °C, grey bars) with increasing time.

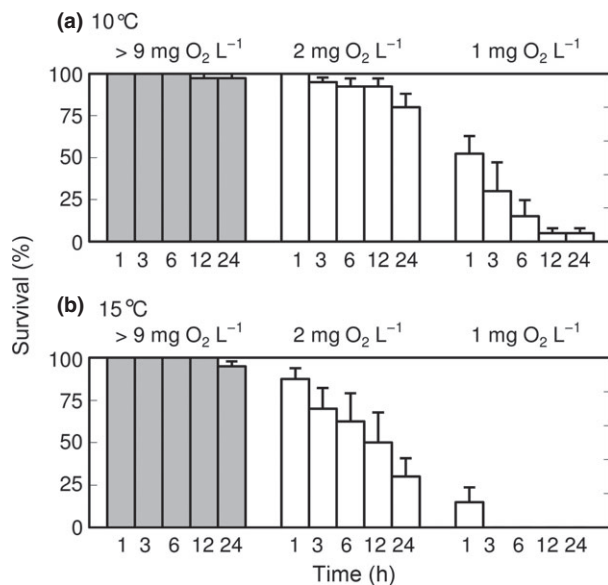


Figure 4 Survival (mean percentage, of 10 individuals, + SE, $n = 4$) of *Mysis salemaai* at (a) 10 °C and (b) 15 °C at reduced oxygen concentrations (white bars) at which it was significantly ($P < 0.05$) different from saturated conditions ($> 9 \text{ mg O}_2 \text{ L}^{-1}$, grey bars) over time. Survival at reduced oxygen concentrations was significantly ($P < 0.05$) lower at 15 °C compared with 10 °C.

$0.8 \pm 0.1 \text{ h}$ at $1 \text{ mg O}_2 \text{ L}^{-1}$. Observed 100% mortality occurred only in the combined 15 °C and $1 \text{ mg O}_2 \text{ L}^{-1}$ treatment in 1–3 h. Oxygen concentrations $\geq 3 \text{ mg O}_2 \text{ L}^{-1}$ had no significant (at $P \leq 0.05$) effect on survivorship compared with $> 9 \text{ mg O}_2 \text{ L}^{-1}$ within the 24-h exposure time (see Table S2).

Regulation of aerobic metabolism experiments

Critical oxygen saturation increased, and thus the ability to regulate aerobic metabolism in progressive hypoxia deteriorated, with temperature (linear regression; $t_{1,18} = 3.3$, $P < 0.01$; see Fig. S4). *M. salemaai* regulated aerobic metabolism in our experiment between 5 and 20 °C. At 25 °C, however, MO₂ was below conformity at all SatO₂ and all animals, including controls, died within 3 h indicating lethal heat stress, in line with the results of our survival experiments.

Thermal avoidance experiments

In the vertical migration experiment, approximately 50% of *M. salemaai* were swimming at any one time, regardless of the temperature treatment. However, the proportion of individuals swimming in the upper zone differed significantly among temperature treatments (ANOVA; $F_{6,28} = 7.0$, $P < 0.001$). Although the proportion swimming in the upper zone at 12 °C did not differ from that at isothermal conditions, the difference was approaching statistical significance at 14 °C and became significant at all

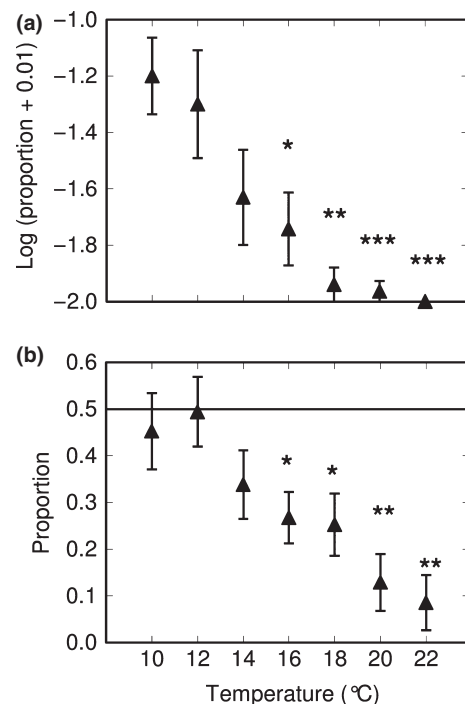


Figure 5 Relationships between temperature and proportion of *Mysis salemaai* recorded in the (a) upper zone of vertical column mesocosms (with 10 °C in the lower zone; mean \pm SE, $n = 5$) and (b) warm-temperature zone of the horizontal mesocosms (with 10 °C in the constant temperature zone; mean \pm SE, $n = 5$). Asterisks indicate statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) effects in relation to (a) isothermal conditions (10 °C) and (b) neutral proportion (horizontal line).

temperatures ≥ 16 °C (Fig. 5a). At 18 and 20 °C, *M. salemaai* was only recorded in the upper zone during one replicate run, and at 22 °C avoided it completely. Results of the horizontal migration experiment were entirely consistent with these effects (Fig. 5b), indicating that the steepness of the thermal gradient was not prohibitive. In accordance with these findings, there was no indication of reduced post-experimental survival in animals that breached steep gradients.

Field surveys

M. salemaai was never recorded from temperatures exceeding 18.5 °C in the field. Lake surface temperatures were associated negatively with its relative density in the top sampling stratum (linear regression; $t_{1,5} = -6.0$, $P < 0.001$, $R^2 = 0.88$; see Fig. S5a), and the corresponding estimated threshold of thermal avoidance was 17.2 °C (95%CI: 16.3 °C, 17.8 °C). This is consistent with our experimental results. Moreover, bottom oxygen concentration was associated positively with the relative density of *M. salemaai* in the lowest sampling stratum (linear regression; $t_{1,5} = 2.7$, $P < 0.05$, $R^2 = 0.59$; see Fig. S5b). The estimated threshold of hypoxic avoidance in the field was $4.1 \text{ mg O}_2 \text{ L}^{-1}$ (95% CI: $1.0 \text{ mg O}_2 \text{ L}^{-1}$, $> 5.9 \text{ mg O}_2 \text{ L}^{-1}$). Even though *M. salemaai* was found at

all oxygen concentrations recorded in the field, including down to $0.2 \text{ mg O}_2 \text{ L}^{-1}$, only single individuals were ever recorded below $4 \text{ mg O}_2 \text{ L}^{-1}$. This was in excess of our experimental mortality thresholds, suggesting active avoidance of sublethal conditions.

DISCUSSION

Temperature modifies some of the most important abiotic and biotic properties of the environment (Rao, 2010; Grigaltchik *et al.*, 2012; Hamilton *et al.*, 2012; Tavakoli & De Smedt, 2012). Results of our study demonstrate that warming can increase the vulnerability of aquatic organisms to reductions in habitat quality caused by deoxygenation. This highlights the importance of understanding biological responses to interacting multiple stressors (García-Molinos & Donohue, 2010; O'Connor & Donohue, 2013). We found that, in oxygen-deficient environments, the ability of *M. salemaai* to regulate oxygen-uptake deteriorates with increasing temperatures. However, even in oxygen-rich conditions, increased oxygen demand or impeded oxygen delivery driven by exposure to a wide range of environmental stressors, both in water and in air, can lead to cellular oxygen deficits (Zwart & Woutersen, 1988; Felten & Guerold, 2001; Lee *et al.*, 2009; Nesci *et al.*, 2011). Similar to the effect of environmental hypoxia, warming likely interacts with the effect of such stressors on ectotherms by increasing their oxygen demand and hence deepening deficits.

Our survival experiments show that elevated temperatures can increase sensitivity of invertebrates to hypoxic stress, even where temperature does not have a detectable effect on its own. Whereas this has been established for fish (e.g. Schurmann & Steffensen, 1997), our results indicate that it is likely a more general characteristic of ectotherms. This suggests that, at local scales, the effects of climate warming can depend more strongly on interactions of temperature with other factors, rather than on the impact of temperature alone. Results of our field surveys were consistent with our laboratory experiments and indicate that both temperature and oxygen conditions are already restricting distribution of *M. salemaai* at the most southerly and westerly points of its range. Restricted access to productive surface waters at high temperature limits food supply and, as found in the closely related *M. diluviana*, may lead to retarded growth, prolonged generation time and lower fecundity (Morgan, 1980; Chess & Stanford, 1998). Reduced oxygen concentrations may, on the other hand, restrict access to dark refugia, thus accentuating the risk of being eaten by visual-hunting predators in the bottom waters (as found for *M. relicta*; Horppila *et al.*, 2003). This is likely to result in reduced overall abundances in affected areas, concurrent with the significant restriction of horizontal distribution to marginal habitats, as found in the stratified eutrophic Lough Oughter, where severe hypoxia is frequent (Penk, 2014). Periodic deoxygenation below observed mortality thresholds is affecting the wider refugial range of *M. salemaai* (see Table S3), and increasing temperatures are likely exacerbating these effects. The Irish

populations of *M. salemaai* studied here mark both the most westerly and southerly distribution of this species, which is likely extinct from Great Britain, where it has not been recorded since 1952 (Bratton, 1991). Extinction from Ireland would, thus, reduce its distribution range significantly. Our results indicate clearly that the interactive effect of temperature with other stressors is likely to be of particular importance in refugial habitats, where small-scale climatic heterogeneity may offer protection against acute heat stress, but the interaction of warming with other habitat-specific or locally important stressors may be driving and accelerating retreat of the distribution front. Factoring such interactions among stressors, including hypoxia, into species distribution models could improve greatly their predictive power.

Thermal stress limits reported here are much higher than those of another closely related relict *M. diluviana*, which lives at similar latitudes (Smith, 1970; Chipps, 1998; Rudstam *et al.*, 1999; Boscarino *et al.*, 2007). This may suggest selection pressure towards higher heat tolerance as a result of exposure to higher temperatures, compared with the more consistently stratified habitats of *M. diluviana*. However, unlike temperature extremes, which are moderated by the high specific heat capacity of water, oxygen depletion can be quite severe and rapid as, once stratification has established, deoxygenation will progress until the water is mixed again. Therefore, genetic adaptation keeping pace with such an extreme oxygen depletion as was observed in this study is unlikely.

Owing to disjoint distributions in refugial ranges, local extinctions tend to lead to significant jumps of the biogeographic front, rather than gradual migration. Similar to glacial relicts, ranges of many species that once thrived in particular areas are likely becoming reduced to isolated habitat pockets by rising temperatures. Our findings highlight that survival in these pockets, and ultimately survival in a given geographical range, is likely to depend strongly on simultaneous and interactive effects of temperature with other stressors, both abiotic and biotic. Understanding species-specific responses to interactions among these pressures is essential to predict future distribution patterns, mitigate threats and prioritize conservation measures.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 The effect of elevated temperatures on survival of *Mysis salemaai* and time to 50% mortality.

Table S2 The effect of reduced oxygen concentrations on survival of *Mysis salemaai* compared with saturated conditions at 10 °C and 15 °C, effect of temperature on *M. salemaai* survivorship at different oxygen concentrations and time to 50% mortality.

Table S3 Offshore bottom oxygen concentrations exceeding mortality thresholds recorded in Irish lakes containing *Mysis salemaai*.

Figure S1 Oxygen consumption rate by *Mysis salemaai* in response to oxygen saturation.

Figure S2 Night-time vertical distribution of *Mysis salemaai* in relation to water temperature and dissolved oxygen concentration during two surveys in Loughs Allen, Derg and Oughter in summer 2010.

Figure S3 Night-time vertical distribution of *Mysis salemaai* in relation to water temperature and dissolved oxygen concentration in Lough Oughter in summer 2012.

Figure S4 Relationship between temperature and critical oxygen saturation below which *Mysis salemaai* loses scope for regulation of aerobic metabolism.

Figure S5 Relationship between mean relative density of *Mysis salemaai* and mean temperatures in the uppermost water stratum and mean oxygen concentrations in the lowest stratum.

BIOSKETCH

Marcin Penk is a freshwater ecologist interested in how interactions among organisms and their environment shape ecosystem functions, which contribute to biosphere integrity. His primary focus is on environmental change and multiple stressors, including climate change and introduced species.

Author contributions: M.P., I.D. and K.I. conceived the ideas; M.P. and V.R. collected the data; M.P. analysed the data and led the writing; all authors contributed to the writing of the manuscript.

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