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# Impact of both desiccation and exposure to an emergent skin pathogen on transepidermal water exchange in the palmate newt *Lissotriton helveticus*

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**ABSTRACT:** Amphibians are the vertebrate group most affected by global change. Their highly permeable skin is involved in maintaining homeostasis (e.g. water and electrolyte equilibrium), which makes them particularly vulnerable to climate warming and skin pathogens. This study focused on the impacts of both desiccation (as a potential consequence of climate warming) and exposure to *Batrachochytrium dendrobatidis* (*Bd*), an emergent skin pathogen of amphibians. *Bd* causes chytridiomycosis, a lethal skin disease of amphibians, and is responsible for mass mortality events in several regions of the world. Because *Bd* colonizes the superficial layers of the epidermis, it is assumed to affect water transfer across the skin. We investigated the behavioural postures of the palmate newt *Lissotriton helveticus* expressed in response to desiccation and their influence on transepidermal water loss (TEWL) rate. We also investigated the effects of repeated 24 h exposure to *Bd* (i.e. every 4 d for 16 d) on the TEWL and ventral water absorption (VWA) rates of these newts. Our results suggest an efficient behavioural water-conserving mechanism, i.e. an 'S'-shaped posture associated with a restricted activity rate, not affected by repeated exposure to *Bd*. Similarly, TEWL was not significantly affected in exposed newts. VWA was significantly reduced after just 24 h exposure to *Bd* without modification until the end of the experiments. Our results suggest that *Bd* could rapidly inhibit rehydration of *L. helveticus* through fungal toxins and disrupt an essential function for survival.

**KEY WORDS:** *Batrachochytrium dendrobatidis* · Chytrid fungus · Emerging disease · Amphibian skin · Evaporative water loss · Water absorption · Osmotic balance · Water-conserving behaviour

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## INTRODUCTION

Climate warming and emerging infectious diseases (EIDs) are both major threats affecting biodiversity (Carey 2000, Harvell et al. 2002, Thomas et al. 2004). Climate warming is expected to lead to greater contrasts in rainfall distributions, with longer and more intense drought periods (Solomon et al. 2007). Such environmental changes may lead to detrimental ther-

mal and/or hydric conditions for organisms (e.g. shelter loss, intense drought periods with reduced water availability) that prevent them from maintaining homeostasis (e.g. water and electrolyte equilibrium) with direct physiological and functional consequences (Spotila & Berman 1976, Shoemaker et al. 1992, Spotila et al. 1992). EIDs can result from environmental changes in pathogen habitats, although they can also emerge in new geographical areas (novel patho-

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gen hypothesis; e.g. Laurance et al. 1996, Daszak et al. 1999, Rachowicz et al. 2005). The endemic pathogen hypothesis suggests that environmental changes (biotic and/or abiotic) can drive modifications in life history traits of native pathogens (virulence, survival, dispersal capacity) and/or host susceptibility (e.g. Kiesecker & Blaustein 1995, Schrag & Wiener 1995, Carey et al. 1999, Kiesecker et al. 2001, Rachowicz et al. 2005). EIDs can cause sub-lethal damage to hosts (e.g. developmental and physiological abnormalities) leading to the decline of host populations (Pounds et al. 2006, Garner et al. 2009, Blaustein et al. 2012). By disturbing the physiological functions of hosts, EIDs may also reduce the ability of individuals to maintain homeostasis and to respond to environmental change (Luquet et al. 2012). Consequently, climate warming can drive the emergence of infectious diseases, and EIDs can increase the detrimental impacts of climate warming (Garner et al. 2011, Blaustein et al. 2012, Fisher et al. 2012). It is crucial to study these 2 threats to biodiversity in concert in order to understand their detrimental consequences. In this context, our study focused on the impacts of both desiccation (as a potential consequence of climate warming) and exposure to an emergent pathogen in amphibians.

Amphibians are the vertebrate taxon most threatened by global change (Stuart et al. 2004). Their skin represents the key organ involved in maintaining homeostasis. As a consequence, these animals have a highly permeable and heavily vascularized epidermis allowing an efficient pathway for gas exchanges and for water and electrolyte absorption. However, these skin characteristics reduce the water retention ability and make amphibians highly vulnerable to transepidermal evaporative water loss (TEWL; Shoemaker et al. 1992, Spotila et al. 1992, Lillywhite 2006). A major challenge for amphibians in the terrestrial environment is therefore how to limit TEWL rate. TEWL is a passive diffusion process that depends primarily on physical conditions such as temperature and relative humidity of the atmosphere (Shoemaker et al. 1992, Spotila et al. 1992, Lillywhite 2006). Amphibians develop adaptations to limit TEWL rate and to replenish body water loss by evaporation. They show behavioural adaptations like water-conserving postures to reduce the skin area exposed to the atmosphere (e.g. Alvarado 1967, Gehlbach et al. 1969, Pough et al. 1983). A functional adaptation allows active water absorption in the ventral skin regions in contact with moistened substrates (called ventral water absorption, VWA). However, VWA efficiency depends on hydric environmental conditions (i.e. substrate water availability; Shoemaker et al. 1992, Spotila et al. 1992, Vi-

borg & Rosenkilde 2004). Consequently, climate warming, with frequent and longer drought events, can expose amphibians to pronounced water imbalance leading to high desiccation risks.

The physiological importance of the skin also makes amphibians extremely sensitive to chytridiomycosis, an emergent skin disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*). Cutaneous chytridiomycosis is a potentially lethal skin disease of amphibians known to contribute to population decline and mass-mortality events observed worldwide (Berger et al. 1998, Daszak et al. 1999, Bosch et al. 2001). As *Bd* is restricted to the superficial epidermis (Berger et al. 1998), this pathogen is suspected of affecting transepidermal water transfers. Two major, non-mutually exclusive, mechanisms have been proposed (Berger et al. 1998, 2005, Pessier et al. 1999): (1) epidermal hyperplasia and hyperkeratosis can physically block transepidermal water transfer, and (2) a fungal toxin, or other active compounds released by the pathogen, can interfere with the osmoregulatory functions of the epidermis. Understanding the mechanisms of pathogenesis is an important step to providing explanations on how exposure to or infection with *Bd* can cause such global amphibian declines (Daszak et al. 2003). Few studies have so far focused on the physiological effects of *Bd* on amphibians. Recent laboratory studies suggested impairments in normal skin functions in *Bd*-infected frogs, such as electrolyte depletion, osmotic imbalance (Voyles et al. 2007, 2009, 2012, Marcum et al. 2010) and disruption in the ability to rehydrate (Carver et al. 2010). However, physiological responses to *Bd* exposure/infection still remain unclear.

The objective of the study was to quantify the impact of both desiccation events and exposure to *Bd* on transepidermal water exchange (i.e. TEWL and VWA rates) in a urodele species, the palmate newt *Lissotriton helveticus*, during its terrestrial phase. We further investigated how newt behaviours (water-conserving postures) may influence the TEWL rate and how exposure to *Bd* affects these postures. Palmate newts are excellent models for such a study because this species is (1) active during the warmest months (terrestrial phase), (2) highly sensitive to dehydration due to a high body surface to volume ratio and (3) susceptible to *Bd* in the wild (Dejean et al. 2010). We performed laboratory experiments to test the following hypotheses: (1) palmate newts exposed to desiccating conditions should exhibit restricted activity and specific water-conserving postures, and (2) palmate newts exposed to *Bd* should exhibit disruption in TEWL and/or VWA rates.

## MATERIALS AND METHODS

### Animal collection

Adult male palmate newts ( $n = 64$ ; mean  $\pm$  SE body mass:  $0.627 \pm 0.016$  g, snout-vent length:  $2.97 \pm 0.02$  cm) were sampled in May 2010 during their aquatic phase in forest ruts near Mépieu, France ( $05^{\circ} 26' 28''$  E,  $45^{\circ} 43' 56''$  N). Both *Hyla arborea* (see Luquet et al. 2012) and *Bufo bufo* populations (E. Luquet, S. Plénet, J. P. Léna unpubl. data) have been widely sampled (ca. 30 ind. population<sup>-1</sup>) at the same sites as newts and at other sites throughout the Isère Département (France), and no *Bd* infection has been detected. As *Bd* is absent from the Isère Département, we are fairly certain that sampled palmate newts were not infected with *Bd* before the experiment began. All individuals from this population were housed in aquaria providing both aquatic and terrestrial environments allowing them to leave the water. When the newts began their terrestrial stage, they were housed individually in plastic boxes ( $19 \times 16.5 \times 9.5$  cm) lined with moistened cellulose paper, renewed weekly. The boxes were kept in climate-controlled rooms at  $20 \pm 1^{\circ}\text{C}$ ,  $60 \pm 10\%$  relative humidity and with a 16:8 light:dark regime. Newts were fed with small crickets ad libitum.

### *Bd* exposure

Individual newts at the terrestrial stage were randomly assigned to control ( $n = 30$ ) and *Bd* ( $n = 34$ ) treatments. We used a *Bd* IA2004 043 isolate generated from a dead *Alytes obstetricans* metamorph collected from a mass mortality event in Spain, and known to be highly virulent (Farrer et al. 2011). For the control and *Bd* treatments, newts were transferred to individual plastic Petri dishes with lids (height = 1 cm,  $\varnothing = 9$  cm) filled with 10 ml of tap water to ensure a fully hydrated body mass (i.e. the initial body mass,  $W_0$ ) of the animals. To ensure adequate ventilation, the Petri dish lids were pierced with holes. Depending on the treatment, newts were repeatedly exposed to either 5 ml culture, containing a total dose of 15 000 to 75 000 zoospores at each inoculation (exposed newts), or 5 ml of sterile culture medium (control newts). The newts were exposed for 24 h every 4 d for 16 d (4 exposures in total). Eight of the 30 control newts died during the second exposure because of an experimental error (i.e. these newts were transferred to unpierced Petri dishes), and 7 of the 34 exposed newts died in the interval between the 2 periods of measure-

ments. At the end of the experiment, we collected skin swab samples from 22 control newts (including the 8 dead individuals) and 22 exposed newts (including the 7 dead individuals). We were not able to perform infection detection on all individuals because of financial constraints. No further histological analyses were performed on the dead animals.

We performed real-time PCR to assess the skin swab samples for *Bd* DNA (Boyle et al. 2004). Extractions were diluted by 1/10 before real-time PCR amplification, performed in duplicate, and with *Bd* genomic equivalent standards of 100, 10, 1, and 0.1 zoospore genome equivalents. All PCRs were replicated twice, and we considered successful amplification in both reactions as a positive signal of *Bd* presence. In the event that only one replicate from any sample did not amplify, this sample was run a third time. If this third amplification attempt did not result in an amplification profile, the sample was scored as negative for infection. No amplification in both replicates was taken as a true negative signal. All exposed newts were ethically euthanized with an overdose of MS222 in accordance with the recommendations of the AVMA Panel on Euthanasia.

### Experimental design

Behavioural responses and transepidermal water exchange (TEWL and VWA rates) of all newts were investigated at 2 periods: after 24 h (Day 1;  $n = 30$  and 34 for control and exposed newts, respectively) and on Day 16 ( $n = 22$  and 27 for control and exposed newts, respectively) after the initial exposure. All measurements were carried out in a climate chamber ( $50 \times 60 \times 80$  cm), placed in a climate-controlled room at  $20 \pm 1^{\circ}\text{C}$ , allowing the relative humidity to be controlled (i.e.  $60 \pm 3\%$ ) using silica gel. In order to perform measurements without any modification of ambient humidity, 2 holes were made in the front wall of the chamber, each equipped with a flexible duct ending in a latex glove. It was thus possible to manipulate the newts without opening the chamber by slipping hands and arms into these ducts.

**Expt 1.** The purpose of this experiment was to study behavioural responses to desiccation (activity in moves  $\text{min}^{-1}$  and postural adjustment, i.e. adoption of 'S' or 'I' postures  $\text{min}^{-1}$ ) and how exposure to *Bd* affected these behaviours. Each newt was placed in a dry plastic Petri dish ( $h = 2.5$  cm,  $\varnothing = 9$  cm) laid on a microbalance (Scaltec sbc 31; 0.001 g sensitivity). We continuously monitored their behaviour using JWatcher (1.0 version) software for both treatments

during the desiccation period (see Expt 3 below). Newts with a posture characterized by a relatively straight body with no tail coil were considered to have the 'T'-shaped posture. When the body was huddled up with the tail coiling along it, newts were considered to have the 'S'-shaped posture (Fig. 1). We measured the time spent active relative to total dehydration time (moves  $\text{min}^{-1}$ ) and the time spent inactive with the 'T' or 'S' posture relative to total inactivity time (adoption of 'S' or 'T' posture  $\text{min}^{-1}$ ).

**Expt 2.** The purpose of this experiment was to evaluate the impact of postural adjustment ('T' versus 'S' shape) on the TEWL. For this purpose, we created agar models of palmate newts ( $n = 4$  for each postural adjustment; Fig. 1) using the method of Spotila & Berman (1976). To obtain replicas similar to our newts, agar models were cast from dead animals frozen in the desired posture and immersed in fluid alginate previously poured into a small plastic container. After hardening of the alginate, the dead newts were removed and moulds filled with a solution of 3% agar, 97% water. Each agar model was placed in a dry plastic Petri dish ( $h = 2.5$  cm,  $\varnothing = 9$  cm) laid on a microbalance (Scaltec sbc 31; 0.001 g sensitivity). We measured the body mass of agar models every 10 min until 10% loss of their  $W_0$  was reached.



Fig. 1. *Lissotriton helveticus*. Adult male palmate newt in the 'S'-shaped posture and its agar model. Photographs taken by T. Wardziak and T. Colin

**Expt 3.** The purpose of this experiment was, simultaneously with the behavioural experiment (see Expt 1 above), to quantify the impact of exposure to *Bd* on transepidermal water exchange (i.e. TEWL and VWA rates). We estimated the TEWL rate by measuring the body mass loss of the newts every 10 min until they reached a 10% loss of  $W_0$ . To avoid biases due to newts remaining in close contact with the sides of the dish, and thus artificially reducing the TEWL rate, the Petri dish rim was lined with wire mesh. We estimated the VWA rate by placing the newts in Petri dishes filled with 15 ml of tap water allowing them complete ventral absorption, immediately after the dehydration period. We measured body mass every 10 min, over a 60 min period, by moving newts to the microbalance after removing excess water with absorbent paper.

### Statistical analysis

The first model (Model 1) examined the effects of initial body mass ( $W_0$ ), treatment (control or exposed), day of measurement (Day 1 or 16) and their interactions on behavioural responses, i.e. the activity (moves  $\text{min}^{-1}$ ) and the postural adjustment (adoption of 'S' or 'T' posture  $\text{min}^{-1}$ ).  $W_0$ , treatment and day were considered as fixed effects. The second model (Model 2) investigated the effects of  $W_0$ , postural adjustment ('T' or 'S'), successive measurements made within the desiccation period (time) and their interactions on the TEWL rate of agar models.  $W_0$ , postural adjustment and time were considered as fixed effects. Both final models (Model 3 for the TEWL rate and Model 4 for VWA rate) examined the effects of  $W_0$ , treatment, day, time and their interactions on transepidermal water exchange (i.e. TEWL and VWA rates).  $W_0$ , treatment, day and time were considered as fixed effects.

All models took into account the correlated error between measurements made on the same individual as a random effect. For Models 3 and 4, another level of random effect was considered: the interaction individual  $\times$  day. We selected the covariance structure for each model using the Akaike Information Criterion (AIC). For Models 1 and 2, a compound symmetry covariance structure was used. For Model 3, a first-order autoregressive correlation between measurements was considered. For Model 4, because the re-absorption experiment lasted 60 min it was possible to use an unconstrained covariance structure to model correlation error between measurements made over the same period.

The significance of variance heterogeneity was examined using a likelihood ratio test (LRT), and the related covariance parameter was removed from subsequent analyses if not significant. Restricted maximum likelihood estimates were used to test the significance of the fixed effects. The significance of explanatory terms was examined using non-sequential  $F$ -tests based on the Kenward-Roger correction for degrees of freedom (SAS Institute, version 9.1.2 software). Non-significant interactions were then successively removed to obtain the final model. All statistical analyses were carried out using SAS (SAS Institute, version 9.1.2) software.

## RESULTS

### qPCR results and clinical chytridiomycosis

Real-time PCR revealed that the swabbed surviving control newts were negative for *Bd*. Furthermore, all dead control newts were also negative for the pathogen (Table 1). Repeated exposure to *Bd* infected 40% of the 15 surviving exposed newts, and all dead exposed newts were positive for *Bd* (Table 1). We can assume that the dead exposed newts died of chytridiomycosis. Indeed, all of these individuals were infected, and genome equivalents showed that the number of *Bd* zoospores was around 20 times greater than for surviving exposed newts (Table 1). In addition, all exposed newts, i.e. surviving as well as dead newts, showed a sloughing of the whole body (from the ventral abdomen, flanks, dorsum, legs and tail) versus only 2 control newts (pers. obs.).

Table 1. *Lissotriton helveticus*. Prevalence of chytrid infection and genome equivalents (GE) for all positive newts in this study. Prevalence was calculated as the proportion of individuals testing positive at 0.1 GE. GE are corrected for a 1/10 dilution factor

	n	% positive (95% CI)	GE ( $\pm$ SE)
<b>Control newts</b>			
Living	14	0 (0–27)	/
Dead	8	0 (0–48)	/
<b>Exposed newts</b>			
Living	15	40 (17–67)	1.13 (0.25)
Dead	7	100 (52–100)	20.35 (10.87)

Table 2. *Lissotriton helveticus*. Model 1 analyses for newt activity (moves  $\text{min}^{-1}$ ) and postural adjustment (adoption of 'I' posture  $\text{min}^{-1}$ ). The model included 3 fixed effects (initial body mass:  $W_0$ ; treatment: control or exposed; and day of measurement: Day 1 or 16) together with the interactions between these factors. Non-significant interactions were all excluded from the final model. The model took into account the correlated error between measurements made on the same individual as a random effect (see Table S1 in the Supplement)

	Moves $\text{min}^{-1}$			Adoption of 'I' posture $\text{min}^{-1}$		
	df	$F$	p	df	$F$	p
$W_0$	1, 63	0.03	0.8715	1, 68.1	1.22	0.2727
Day	1, 58.1	3.32	0.0735	1, 62.1	1.00	0.3211
Treatment	1, 54.2	0.12	0.7300	1, 59	0.06	0.8154

### Expt 1

In Model 1, we did not detect any significant variance heterogeneity between treatments either on activity ( $\text{LRT}_{2\text{df}} = 0.8$ , ns) or on postural adjustment ( $\text{LRT}_{2\text{df}} = 5.4$ ,  $p = 0.067$ ). Neither the  $W_0$  of the newts nor the treatment they underwent affected their activity (Table 2). However, animals tended to be less active on Day 16 (Day 1 versus Day 16 for control newts:  $0.12 \pm 0.02$  versus  $0.09 \pm 0.02$ ; for exposed newts:  $0.11 \pm 0.02$  versus  $0.08 \pm 0.02$ , moves  $\text{min}^{-1} \pm \text{SE}$ ; Table 2). Neither the  $W_0$  of animals, the treatment they underwent nor the day of measurement significantly affected the time spent in a given posture when inactive (Table 2). Newts did not modify their postural adjustment according to the treatment they experienced (control versus exposed newts on Day 1:  $0.19 \pm 0.03$  versus  $0.20 \pm 0.03$ ; on Day 16:  $0.16 \pm 0.03$  versus  $0.17 \pm 0.03$ ; adoption of 'I' posture  $\text{min}^{-1} \pm \text{SE}$ ; Table 2). Estimates of the random effects are available in Table S1 in the Supplement at [www.int-res.com/articles/suppl/d104p215\\_supp.pdf](http://www.int-res.com/articles/suppl/d104p215_supp.pdf).

### Expt 2

Model 2 indicated that the 'S' posture had a significantly lower TEWL rate than the 'I' posture (Table 3, Fig. 2). The model also showed that the TEWL rate of agar models reduced throughout the dehydration experiment (Table 3, Fig. 2), and lighter models had a lower TEWL rate than heavier ones (Table 3). Estimates of the random effects are available in Table S1.

### Expt 3

In Model 3, according to the AIC score, an autoregressive correlation was more supported than a con-

stant one to analyse the TEWL rate during dehydration experiments (AIC scores for autoregressive and constant correlations were  $-1232.9$  and  $-1223.5$ , respectively). TEWL rates were significantly more variable among the exposed newts than among controls ( $LRT_{4df} = 68.2$ ,  $p < 0.001$ ), and this variance heterogeneity was more salient for the second batch of measurements than for the first ( $LRT_{4df} = 72.5$ ,  $p < 0.001$ ). No effect of treatment on the TEWL rate depending on the day of measurement was detected (Table 4, Fig. 3a,b): contrast analyses showed no significant difference between both treatments on Day 1 ( $F_{1,86.7} = 0.22$ ;  $p = 0.64$ ) and Day 16 ( $F_{1,115} = 3.62$ ;  $p = 0.059$ ). Nevertheless, while the TEWL rate did not significantly vary between the 2 measurement days in control newts ( $F_{1,43.9} = 0.23$ ;  $p = 0.63$ ), we detected a significantly higher TEWL rate on Day 16 in the case of the exposed newts ( $F_{1,67.7} = 10.08$ ;  $p = 0.002$ ). However, the increase in TEWL rate in the exposed group, while statistically significant, was not biologically relevant, considering that the difference in water loss was about 0.9% of  $W_0$  over a 60 min period (Fig. 3a). The TEWL rate was constant over the duration of the desiccation experiments, regardless of the time period considered (Table 4, Fig. 3), but lighter animals had a lower TEWL rate than heavier ones (Table 4). Estimates of the random effects are available in Table S2 in the Supplement.

In Model 4, the unconstrained correlation structure was used to analyse the VWA rate during rehydration experiments (AIC scores for the unconstrained structure, the autoregressive and constant forms were 827.3, 863.6 and 857.5, respectively). We did not detect significant variance heterogeneity between treatments ( $LRT_{21df} = 31.5$ ,  $p = 0.0657$ ), but the variance heterogeneity was significant between time periods ( $LRT_{21df} = 45.1$ ,  $p = 0.0017$ ). The results of this analysis showed that exposed newts exhibited a significantly lower VWA rate than control newts, regardless of the measurement day (Table 4, Fig. 4). The difference was about 1.6% of  $W_0$  over a 60 min period for both measurement days (Fig. 4a). Moreover, the VWA rate declined as newts replenished their bodies with water (Table 4, Fig. 4), and the VWA rate was positively related to animal mass (Table 4). Estimates of the random effects are available in Table S3 in the Supplement.

Table 3. *Lissotriton helveticus*. Model 2 analysed for transepidermal water loss (TEWL) rate based on the posture of agar models. The model included 3 fixed effects (initial body mass:  $W_0$ ; posture: 'I' or 'S' shape; and successive measurements made within the desiccation period: time) together with the interactions between these factors. Non-significant interactions were all excluded from the final model. The model took into account the correlated error between measurements made on the same individual as a random effect (see Table S1 in the Supplement)

	TEWL rate		
	df	F	p
$W_0$	1,13.5	20.75	0.0005
Time	1,24.1	15.35	0.0006
Posture	1,19.9	27.77	<0.0001
Time $\times$ Posture	1,24.1	5.24	0.0311

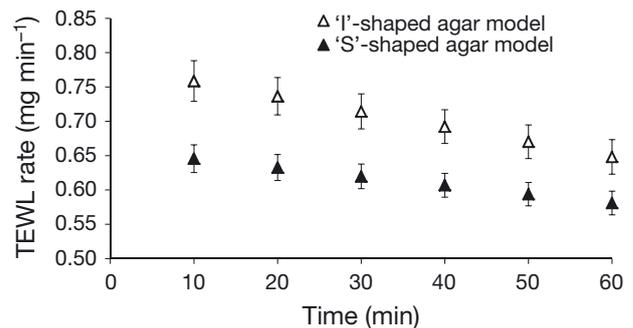


Fig. 2. *Lissotriton helveticus*. Transepidermal evaporative water loss rate (TEWL, mixed-models,  $\text{mg min}^{-1} \pm \text{SE}$ ) for a 60 min period for both the 'I'- and the 'S'-shaped agar models

Table 4. *Lissotriton helveticus*. Model 3 and Model 4 analyses for transepidermal water loss (TEWL) rate and ventral water absorption (VWA) rate, respectively. The models included 4 fixed effects (initial body mass:  $W_0$ ; treatment: control or exposed; day of measurement: Day 1 or 16; and successive measurements made within the desiccation period: time) together with the interactions between these factors. Non-significant interactions were all excluded from the final model. Two levels of random effect were considered. The models took into account the correlated error between measurements made on the same individual. Another level of random effect was considered: the interaction individual  $\times$  day (see Tables S2 & S3 in the Supplement)

	TEWL rate			VWA rate		
	df	F	p	df	F	p
Time	1,328	1.70	0.1931	1,101	236.03	<0.0001
$W_0$	1,73.5	4.92	0.0296	1,45.4	71.63	<0.0001
Day	1,56.2	6.60	0.0129	1,41.8	1.23	0.2745
Treatment	1,65.6	1.19	0.2800	1,37.6	25.22	<0.0001
Treatment $\times$ Day	1,53.6	3.70	0.0599			

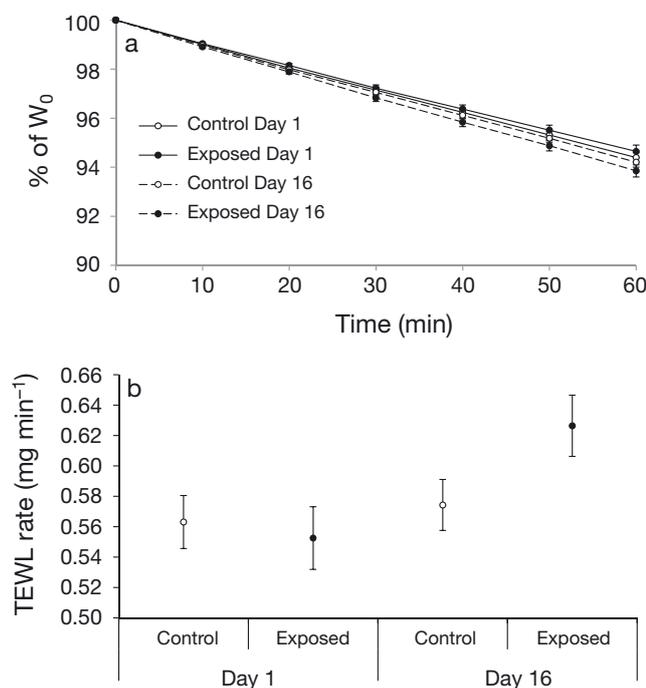


Fig. 3. *Lissotriton helveticus*. (a) Transepidermal evaporative water loss (TEWL, mean % of the initial body mass  $W_0 \pm \text{SE}$ ) for a 60 min period for both control and exposed newts 24 h after initial exposure to *Batrachochytrium dendrobatidis* (Day 1) and 16 d after repeated exposures. % of  $W_0$  denotes the percentage of the initial body mass. (b) TEWL rate (mixed-models,  $\text{mg min}^{-1} \pm \text{SE}$ ) for both treatments on Day 1 and Day 16

## DISCUSSION

Adult male palmate newts expressed a restricted activity rate when exposed to desiccation stress. As shown in other salamander species exposed to desiccating conditions, inactivity is associated with a postural adjustment that consists of tightly coiling the body and the tail (Alvarado 1967, Gehlbach et al. 1969) to form an 'S' shape. Alvarado (1967) reported that inactive terrestrial long-toed salamanders *Ambystoma macrodactylum* and tiger salamanders *A. tigrinum* with an 'S'-shaped posture experienced a lower water loss rate than active animals. Our results on agar models also showed that the 'S' posture improves water economy relative to the 'I' posture when inactive. The water economy of the 'S' posture certainly reflects a reduction in the surface exposed to evaporation. Thus, our results suggest that restricted activity associated with water-conserving postures can be used as an emergency response by newts unable to escape from desiccating conditions to increase their chance of survival (Pough et al. 1983).

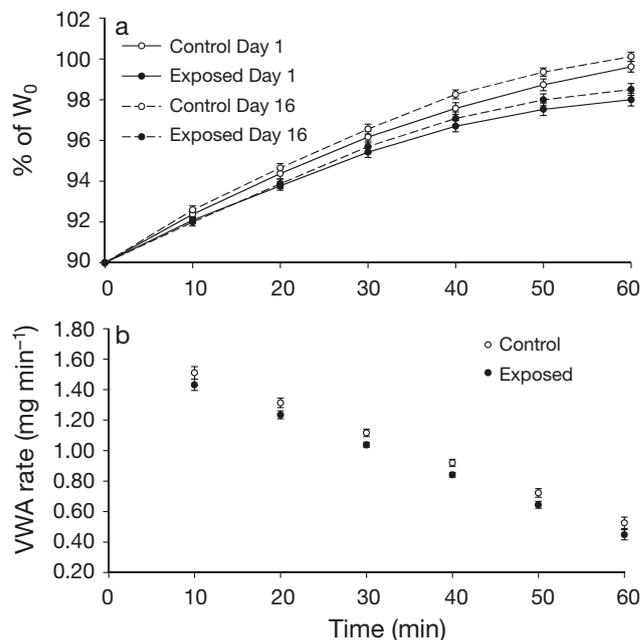


Fig. 4. *Lissotriton helveticus*. (a) Rehydration (mean % of the initial body mass  $W_0 \pm \text{SE}$ ) for both control and exposed newts 24 h after initial exposure to *Batrachochytrium dendrobatidis* (Day 1) and 16 d after repeated exposures. % of  $W_0$  denotes the percentage of initial body mass. (b) Deceleration of ventral water absorption rate (VWA, mixed-models,  $\text{mg min}^{-1} \pm \text{SE}$ ) for both control and exposed newts

Numerous studies have shown that exposure to *Bd* can lead to behavioural modifications in amphibians such as the expression of lethargic behaviours associated with abnormal and depressed postures (e.g. Bosch et al. 2001, Nichols et al. 2001, Carver et al. 2010). Our results do not show that exposure to *Bd* has a significant effect on activity and posture in the laboratory. In our experiment, the duration of exposure may have been insufficient to observe such lethargic and/or depressive behaviour in palmate newts. Furthermore, we found that the TEWL rate between treatments had not changed, although the difference in TEWL rate was significant in the exposed newts between the 2 measurement days. It therefore seems unlikely that *Bd* impedes the TEWL in newts, as suggested by Carver et al. (2010) in infected hylid frogs *Litoria raniformis*. In contrast, our results suggest that *Bd* has an impact on the ventral rehydration capacity of newts. *Bd* exposure slowed down the VWA rate, although the decrease was quite low, given that the difference in water uptake between groups was about 1.6% of  $W_0$  over a 60 min period. These results are consistent with the study of Carver et al. (2010) on infected hylid frogs, where a difference in water uptake of about 2 to 3% of  $W_0$  over a 60 min period was found 1 wk after inoculation with *Bd*.

Surprisingly, in our study, VWA was impeded 24 h after the first exposure to the pathogen without modifications until Day 16. Such an impact of *Bd* seems to reflect a disruption in the water uptake function of the ventral epidermis and suggests the action of a fungal toxin or other active compounds, rather than *Bd* itself. Indeed, *Bd* takes a few days to establish itself in the amphibian epidermis, its whole life cycle taking 4 to 5 d (Berger et al. 2005), and may produce lethal toxins either before or after infection (Blaustein et al. 2005). Furthermore, the study by Rosenblum et al. (2008) supports the role of a fungal toxin in the pathogenesis of *Bd*, which may be driven by zoospores and zoosporangial secretions. More interestingly, the recent *in vitro* study by Brutyn et al. (2012) reported rapid (less than 4 h) alterations in the structural integrity of the epidermis in *Xenopus laevis* after exposure to *Bd* zoospore secretions. As amphibian skin is physiologically active, disruption of epidermal structure through toxicity can compromise a number of critical skin functions including the ability to rehydrate or osmoregulate (Voyles et al. 2009, 2012, Carver et al. 2010, Marcum et al. 2010, Rosenblum et al. 2012). However, the exact mechanisms of VWA disruption in the palmate newt are still unknown. In White's tree frogs *Litoria caerulea* infected with *Bd*, a disruption in electrolyte ion transport was found (Voyles et al. 2009), suggesting an epidermal electrolyte channel disruption (Voyles et al. 2009) and a decrease in ion channel gene expression (Rosenblum et al. 2012). In amphibians, rapid VWA is dependent on water channels inserted into the epidermal layers (i.e. aquaporins; see Connolly et al. 1998, Hasegawa et al. 2003, Suzuki et al. 2007). We can assume that VWA is impeded by *Bd* through an epidermal aquaporin disruption. However, the distribution of aquaporins in the epidermis remains to be established in urodeles. Further experiments are needed to investigate whether a reduction in rehydration ability in amphibians is due to an inhibition of epidermal aquaporins.

Our results, together with those of Voyles et al. (2007, 2009, 2012), Carver et al. (2010), Marcum et al. (2010) and Rosenblum et al. (2012), support the epidermal dysfunction hypothesis, which suggests that *Bd* compromises the ability of amphibians to osmoregulate or rehydrate. Such disruption of a crucial physiological process, even when small, can at least alter the adaptability of amphibians during their terrestrial phase in the wild and even their survival, especially in the context of a warming climate and prolonged or intensified drought events. This refers to the climate-linked epidemic hypothesis (e.g.

Pounds & Crump 1994, Lampo et al. 2006, Pounds et al. 2006) and the drought-linked chytridiomycosis hypothesis (e.g. Burrowes et al. 2004, Lampo et al. 2006, Kriger 2009), which propose that abnormal climatic conditions can exacerbate chytridiomycosis outbreaks. However, these hypotheses remain highly controversial (Alford et al. 2007, Lips et al. 2008, Rohr et al. 2008, Kriger 2009). As stated by Kriger (2009), these hypotheses are inconsistent with our current knowledge of *Bd* physiology and ecology, and that dry conditions should reduce the severity of the pathogen. Nevertheless we have evidence that droughts actually increase outbreaks (Burrowes et al. 2004, Lampo et al. 2006). We can ask whether such impacts on transepidermal water exchanges by *Bd* in the palmate newt are sufficient to cause mortality in a climate change context. However, we are not in a position yet to answer this question, and further experiments are needed to conclude a link between climate change and the physiological effects of *Bd* on this species.

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