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Variation in genotoxic stress tolerance among frog populations exposed to UV and pollutant gradients

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ABSTRACT

Populations of widely distributed species can be subjected to unequal selection pressures, producing differences in rates of local adaptation. We report a laboratory experiment testing tolerance variation to UV-B and polycyclic aromatic hydrocarbons (PAHs) among common frog (*Rana temporaria*) populations according to their natural exposure level in the field. Studied populations were naturally distributed along two gradients, i.e. UV-B radiation with altitude and level of contamination by PAHs with the distance to emitting sources (road traffic). Tadpoles from eight populations were subjected to (1) no or high level of artificial UV-B; (2) four concentrations of benzo[a]pyrene (BaP) (0, 50, 250, 500 $\mu g\,L^{-1}$); (3) simultaneously to UV-B and BaP. Since both stressors are genotoxic, the number of micronucleated erythrocytes (MNE) in circulating red blood cells was used as a bioindicator of tadpole sensitivity. High-altitude populations appear to be locally adapted to better resist UV-B genotoxicity, as they showed the lowest MNE numbers. Conversely, no correlation was observed between levels of PAH contamination in the field and tadpole tolerance to BaP in the laboratory, indicating the absence of local adaptation for BaP tolerance in these populations. Nevertheless, the decrease of MNE formation due to BaP exposure with altitude suggests that high-altitude populations were intrinsically more resistant to BaP genotoxicity. We propose the hypothesis of a co-tolerance between UV-B and BaP in high-altitude common frog populations: local adaptation to prevent and/or repair DNA damage induced by UV-B could also protect these highland populations against DNA damage induced by BaP. The results of this study highlight the role of local adaptation along pollutant gradients leading to tolerance variation, which implies that is it necessary to take into account the history of exposure of each population and the existence of co-tolerance that can hide toxic effects of a new pollutant.

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1. Introduction

Spatial heterogeneity of the environment implies that populations of widely distributed species are unequally exposed to selection forces. Environmental gradients can induce plastic responses of traits leading to phenotypes optimized to local environmental conditions (Sultan and Spencer, 2002) but selection frequently favors local adaptation over plasticity when the gene flow is restricted among populations (Pigliucci, 2001; Sultan and Spencer, 2002). Besides low gene flow, strong selection and little variation in the local force of selection promote local adaptation (see Kawecki and Ebert, 2004, for a review). The number of anthropogenic chemicals likely to be found in the environment exceeds 100,000 ("White Paper on the Strategy for a Future Chemicals Policy," EU Commission, 2001). These substances, referred to as external stressors, can lead to internal stress states acting at various levels of ecological integration (Parker et al., 1999), such as species replacements, food web modifications, physiological (e.g. acclimation) and genetic (e.g. inherited tolerance) adaptations (Parker et al., 1999; Relyea and Hoverman, 2006). Stressors can affect evolutionary changes, such as a loss of genetic diversity before and after pollutant exposure (Belfiore and Anderson, 2001; Kerls and Weis, 1987; Van Straalen and Timmermans, 2002) through change of population size following acute mortality (review in Dixon et al., 2002), and local adaptations such as tolerance to heavy metals, air pollution, pesticides (Hoffman and Parsons, 1997; Hoffmann and Parsons, 1991; Linhart and Grant, 1996; review in Medina et al., 2007; Reznick and Ghalambor, 2001).

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Table 1
PAH and ΣPAH concentrations (mg/kg) of the sediments in aquatic sites housing common frog populations.

Compounds	Abbreviations	Fretterive	St. Laurent	Villard/Siard	Tines	Luitel	Creusates	Lautaret	Lac Rond
Naphtalene	Nph	–	–	–	0.101	–	–	–	–
Acetanaphtene	Ac	–	–	–	0.006	–	–	–	–
Fluorene	F	–	–	–	0.014	–	–	–	–
Phenanthrene	Ph	–	–	–	0.114	0.279	–	–	–
Anthracene	An	–	–	–	0.008	0.119	–	0.018	–
Fluoranthene	Fl	0.039	0.021	0.029	0.076	0.975	0.034	0.059	0.035
Pyrene	Py	0.036	0.006	–	0.183	0.745	0.023	0.051	0.027
Benzo(a)anthracene	BaA	0.093	–	–	0.007	0.440	0.007	2.041	–
Chrysene	Ch	–	0.012	–	0.013	0.327	–	0.029	–
Benzo(b)fluorenthene	BbF	–	0.022	0.013	0.039	0.302	–	0.042	–
Benzo(k)fluorenthene	BkF	–	0.007	–	0.009	0.005	–	–	–
Benzo(a)pyrene	BaP	0.084	0.008	–	0.017	0.277	0.032	0.072	–
Dibenzo(a,h)anthracene	DBahA	–	–	–	0.006	–	–	–	–
Benzo(g,h,i)perylene	BghiP	–	–	–	0.010	–	–	–	–
Indenopyrene	IP	–	–	–	0.008	0.106	–	–	–
ΣPAH		0.252	0.076	0.042	0.611	3.575	0.096	2.312	0.062

(–) non-detect.

Here, we report tolerance variation among frog populations exposed to two potential selective pressures. The focal species was the common frog *Rana temporaria*, a widespread amphibian in Europe (Gasc et al., 1997). The first studied gradient was the natural increase in UV radiation with altitude and the second gradient was the decrease in sediment contamination by PAH as the distance to emitting sources increases.

Spatial and temporal variation in UV levels on earth depends on various factors such as depletion of the ozone layer, latitude, and altitude (Cockell and Blaustein, 2001). An increase of 19% in UV-B per 1000 m is observed in the Alps (Blumthaler et al., 1997). UV radiation can cause formation of photoproducts inducing DNA damage and leading to cell death in amphibians (Cockell and Blaustein, 2001; Sancar and Tang, 1993). Thus, we predicted that frog populations would vary in their tolerance to UV radiation, with increased tolerance as altitude of population increases.

Polycyclic aromatic hydrocarbons (PAHs) are naturally produced by volcanic eruptions, forest fires, and organic matter decomposition in sediment (Blumer, 1976). However, human activities and, especially, urbanization are major sources of emissions (Nykolaou et al., 1984; Van Schooten et al., 1997). PAHs are transported through the atmosphere and the hydrosphere (Fernandez and Grimalt, 2003), and contamination increases with proximity of urban activity and road traffic (Eisler, 1987). PAHs are reported to be highly toxic to aquatic organisms (Bowling et al., 1983; Landrum et al., 1987), especially in amphibians species (Hatch and Burton, 1998; Mouchet et al., 2005). We thus tested tolerance to benzo[a]pyrene (BaP) among populations exposed to a wide PAH concentration range estimated by pollutant levels in sediments. We predicted that populations experiencing higher levels of PAHs would be more resistant to BaP than non-exposed populations. BaP was chosen in preference to other PAHs because of its persistence in the environment (due to its resistance to biological degradation) and because of its toxicity (Brondeau et al., 1997), in particular its capacity to induce genetic mutations and chromosomal aberrations in both *in vitro* and *in vivo* systems (Hollstein et al., 1979; Waters et al., 1991). Moreover, PAHs exposed to UV radiation are known to generate photoproducts that can be considerably more toxic than PAHs themselves (Bowling et al., 1983; Fernandez and L'haridon, 1992). Photo-induced toxicity of BaP is now well known (Fernandez and L'haridon, 1994). We thus also estimated the possible effects of simultaneous exposure to UV-B and BaP on frog populations along altitudinal and pollution gradients. The count of micronucleated erythrocytes (MNE) in circulating red blood cells of tadpoles was used to assess their genotoxic response to both stressors (Grinfeld et al., 1986; Jaylet et al., 1986).

2. Materials and methods

2.1. Study site

The present study was conducted in the French Alps (Department of Savoie). We selected eight populations, differing in their distance to roads: four were located close (less than 10 m) to roads with high traffic (1390 to >9000 vehicles/day). The other four populations were more than 1 km from the closest road. The altitude of these eight populations varied from 294 to 2450 m above sea level (Table 1).

2.2. PAH analysis

A sample of sediment (three replicates) was collected at the spawning place of each population for PAH content analysis. PAH extraction from sediments was carried out according to the protocol described by Verrhiest (2001) (details in Supplementary material S1). PAH concentration in the sediments is a good indicator of tadpole exposure, because tadpoles are benthic organisms feeding on dead organic matter mainly.

2.3. Embryos and larvae rearing conditions

Parts of 10 recently fertilized spawns per population were collected in the field. They were reared together in plastic tanks (length 40 cm; width 60 cm; height 30 cm, one tank per population), filled with 60 L of well water (pH 7.8; conductivity at 20 °C = 538 μS/cm; chloride ions = 1.1 mg L^{-1}; ammonium < 0.03 mg L^{-1}; nitrite ions < 0.03 mg L^{-1}; nitrate ions = 1 mg L^{-1}). After hatching, the jelly of the eggs was manually removed to prevent any degradation of the water quality. Water was changed every third day. Tadpoles were fed weekly with artificial dried food for aquarium fishes. These rearing conditions (density of about 15 tadpoles L^{-1}) continued until stage 36–38 (Gosner, 1960), when tadpoles were large enough to allow cardiac puncture. Rearing and experiments were conducted at constant (18 °C) temperature.

2.4. Experimental design

The experiments aim to test the effect of artificial UV (presence/absence), BaP exposure (four concentrations) and simultaneous UV × BaP exposure on tadpoles originated from eight different populations (described above) (Fig. 1). All experiments were conducted simultaneously. Three replicates (i.e. samples) per population were used for each experiment: each sample was made

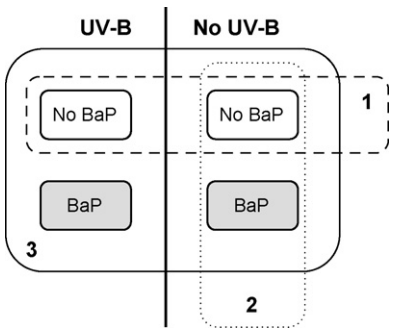

Fig. 1. Schematic description of the experimental design used for each of the eight studied populations of *Rana temporaria*. Experiment (1) tadpoles were exposed or not to UV-B without BaP, experiment (2) tadpoles were exposed or not to BaP without UV-B exposure, and experiment (3) tadpoles were exposed simultaneously to BaP and UV-B. Each condition is replicated three times with tadpoles from the same population. In experiments (2) and (3) tadpoles were exposed to four concentrations of BaP (0, 50, 250, 500 $\mu g\,L^{-1}$).

up with 10 tadpoles at stage 36–38 caught by netting them at random in each tank (i.e. each population), then put in a 500 ml glass beaker filled with well water for the duration of the following experiments:

(1) *Effect of UV exposure*. The tadpoles were exposed or protected from artificial UV with 3 replicates per lighting condition, resulting in 2 UV conditions × 3 replicates × 8 populations = 48 samples (no BaP exposure).
(2) *Effect of BaP exposure*. The tadpoles were exposed to four BaP concentrations (3 replicates per concentration), resulting in 4 concentrations × 3 replicates × 8 populations = 96 samples (no artificial UV exposure).
(3) *Synergistic effect of UV × BaP exposure*. The tadpoles were simultaneously exposed to BaP (4 concentrations) and artificial UV radiation, resulting in 4 concentrations × 1 UV condition × 3 replicates × 8 populations = 96 samples. Tadpoles exposed to artificial UV only in experiment 1 (24 samples) and tadpoles exposed to BaP only in experiment 2 (96 samples) were used as control for this third experiment.

Each experiment lasted 6 days; the rearing medium was changed after 3 days. Tadpoles were fed with artificial dried food for aquarium fishes at the beginning of the experiment and just after medium renewal. Dead tadpoles were counted daily, removed, and stored in 95% alcohol.

2.5. Choice of the artificial UV and BaP exposure

Pachard et al. (1999) provided values for UV spectral irradiance in the southern French Alps (latitude 44.90°N, longitude 6.65°E), at 1300 m a.s.l. from 5 to 11 July 1996 with highly variable weather during this week (cool and cloudy on the first days, then sunny and warm), which corresponds to usual conditions in this area. According to these results and other field measurements at several altitudes and regions of the Alps (Reiter et al., 1982; Blumthaler et al., 1997; Schmucki and Philipona, 2002), the total daily irradiance was estimated at 33 MJ/m^2, with UV-A = 1.72 MJ/m^2 and UV-B = 4.71 kJ/m^2 at 2500 m in the Alps (the highest altitude of the sampled populations). This irradiance was obtained with UV-A (Philips performance 100W) and UV-B (Philips TL 100W/01) tubes (Philips tubes Instructions for Use and D.3.M., Irigny, France, personal communication) with a daily lighting period of 8 h of UV-A (10:00 a.m. to 6:00 p.m.) and 6 h of UV-B (11:00 a.m. to 5:00 p.m.). Tadpoles were reared under a homogeneous UV dose produced by eight UV-B tubes and five UV-B tubes covering a surface of 5.7 m^2 at 90 cm above the level of water.

The control samples (i.e. samples away from artificial UV exposure) were reared in the same room, but protected from UV by a vertical black plastic screen. These samples were lit by aquarium tubes (Sylvania Standard F36W, white), which did not produce any UV radiation.

The BaP concentrations used in the experiments were chosen according to their non-acute toxicity (no direct mortality in the absence of UV exposure), and to their genotoxic potential (their capacity to induce a significant number of micronuclei in erythrocytes). Four BaP concentrations were used (0, 50, 250 and 500 $\mu g\,L^{-1}$), according to a previous study on the African Clawed frog *Xenopus laevis* (Zoll-Moreux, 1991). Because of its low solubility in water (from 0.2 to 6 $\mu g\,L^{-1}$), BaP was initially dissolved in dimethylsulphoxide (99%, Aldrich; DMSO) and, in all cases, final DMSO concentration was 0.05% (v/v) (AFNOR, 2000). This concentration did not affect egg and tadpole survival in *R. temporaria* (Marquis et al., 2006). Controls (i.e. samples in BaP free solutions) from each population received exactly the same concentration of DMSO. The concentration of dissolved BaP in the glass beakers was measured at the beginning of the experiment, after 1 and 3 days using the analytical method described above (PAH in sediments).

2.6. Micronucleus test

Among toxic effects, genotoxicity may durably affect the aquatic ecosystems. The interaction of genotoxic compounds with DNA initially may cause structural changes in the DNA molecule. Unrepaired damage can generate other cell lesions and thus lead to tumour formation (Vuillaume, 1987; Malins et al., 1990). In amphibian larvae, as in most eukaryotes, genome mutations may result in the formation of micronuclei, which are a consequence of chromosome fragmentation or malfunction of the mitotic apparatus. The first case takes place after chromosome breakage, while the second (chromosome loss) is due to an aneugenic event related to the spindle apparatus. Numerous mechanisms could destabilize chromosomes, including loss of mitotic checkpoint function, abnormal amplification of centrosome, defects in the kinetochore-microtubule attachement, and movement of chromosome relative to the pole, all potentially leading to micronucleus formation (Iarmarcovai et al., 2006). The sensitivity and reliability of the micronucleus test (MNT) to detect chromosomal and/or genomic mutations makes it a good method to analyze cytogenetic damages in erythrocytes of aquatic species, revealing interesting correlations with the exposure under laboratory and field conditions to a number of chemical and physical agents (Bolognesi et al., 2006; Udroiu, 2006). MNT has been first used in the late 1980s with *Ambystoma mexicanum* (Jaylet et al., 1986), and then with many amphibian species such as *Bufo bufo gargarizans* (Yin et al., 2009), *Bufo raddei* (Huang et al., 2007), *Pleurodeles waltl* (Mouchet et al., 2007), *Rana esculenta* (Barni et al., 2007), *Rana perzi* (Marques et al., 2009) and *X. laevis* (Mouchet et al., 2008). Since it reveals unrepaired damage, the main ecotoxicological relevance of MNT lies in its ability to lead to delayed adverse effects within impacted aquatic populations. In this way, the use of the MNT may provide an important tool for the prediction of the potential long-term effects on amphibians in the environment.

At the end of each experiment (UV, BaP and UV × BaP), five tadpoles were randomly chosen in each of the three replicates. The blood of each tadpole was extracted by intracardiac puncture and the five extracts were pooled and homogenized to ensure sufficient blood to perform a blood smear. The number of MNE was determined on 1000 erythrocytes observed using a microscope at 1000× magnification. Randomized slides were scored blind (details in Supplementary materials S2).

Table 2
Geographic characteristics and PAH analysis of sediments in aquatic sites housing common frog populations.

Site	Altitude (m)	Distance to the main road (m)	Mean annual road traffic (vehicles/days)[b]	ΣPAH (mg/kg)[c]	PAH isomer ratios and potential sources of PAHs[a]			
					Fl/(Fl+Py)	*An/(An+Ph)*	*BaA/(BaA+Chr)*	*IP/(IP+IP+BghiP)*
Fretterive	294	1200	25,822	0.252	0.519 Biomass/coal	–		–
St. Laurent	410	5	9,250	0.076	0.778 Biomass/coal	–	–	–
Villard/Siard	450	2670	1,083	0.042	1 Biomass/coal	–	–	–
Tines	800	1	1,900	0.611	0.294 Petroleum	0.066 Petroleum	0.347 Mixed	0.448 Biomass/coal
Luitel	1250	10	1,390	3.575	0.567 Biomass/coal	0.300 Biomass/coal	0.574 Biomass/coal	–
Creusates	1340	7500	939	0.096	0.596 Biomass/coal	–	–	–
Lautaret	2050	5	3,000	2.312	0.533 Biomass/coal	–	0.986 Biomass/coal	–
Lac Rond	2450	3350	1,500	0.062	0.565 Biomass/coal	–	–	–

[a] According to Yunker et al. (2002)'s classification. Petroleum = petroleum combustion; Biomass/coal = biomass or coal combustion; Mixed = combustion of both origin.
[b] *Source*: Road service of the Savoie Department.
[c] Concentrations (mg/kg) are expressed in dry mass of sediment.

2.7. Recorded variables and statistical analysis

We recorded two response variables, mortality (number of dead tadpoles, measured every 24 h) during the experiments and the number of micronuclei after 6 days of exposure. As no mortality was observed in samples exposed to only one stressor (UV or BaP), statistical analysis on mortality was performed only with samples subjected to simultaneous UV × BaP exposures. In this case, all tadpoles of a same container died at the same time interval (at 24 or 48 h), or tadpoles alive after 48 h also survived until the end of the experiment. It was thus not possible to calculate a mortality rate and we thus used the time to death (TTD = time of exposure when 100% of tadpoles are dead in a beaker), to compare mortality between populations. We distinguished three classes: (1) every tadpole died within the first 24 h of exposure; (2) every tadpole died between 24 and 48 h of exposure; and (3) all tadpoles survived until the end of the experiment.

We thus tested the influence of three factors, i.e. artificial UV (presence/absence), BaP (four concentrations), and population origin on the two response variables (mortality and number of micronuclei). Then, if we detected a significant effect of population origin, we evaluated whether among population differences were related to altitude of population or to PAH concentrations in sediments collected in breeding sites. This allowed us to individualize the factors that could have induced the differences among populations. We used multinomial regression to evaluate the effect of BaP and population of origin on the median of time to death (TTD). We used a generalized linear model (GLM) assuming a Poisson error distribution to evaluate the effect of UV, BaP, and population origin on number of MNE. Since population origin had a significant effect on the number of MNE (see results), Poisson regression was followed by Spearman's correlation to evaluate which features of the population origin were related to the differences in sensitivity. Per each population, data on MNE from the different containers were averaged and we evaluated whether average MNE number was related to altitude of populations or PAH concentrations in sediments collected in breeding sites. Multinomial regression was performed using the package nnet (Venables and Ripley, 2002). Significance in multinomial regression and GLM was assessed using a likelihood ratio test. All statistical analyses were performed under the R 2.1 environment (R Development Core Team, 2005).

3. Results

3.1. PAH analysis in sediments

Several PAHs were identified in the sediments studied according to their retention time and their absorption spectra. The distribution of 15 PAHs in the sediments is presented in Table 1. PAHs were present in the sediment of each site. The concentration of ΣPAH was not significantly correlated with the distance to the main road (Spearman's correlation, $r_s = 0.52$, $N = 8$, $P = 0.179$), the mean number of vehicles per day ($r_s = 0.21$, $N = 8$, $P = 0.61$), or the altitude ($r_s = 0.12$, $N = 8$, $P = 0.779$). Even the most isolated site, "Les Creusates," presented significant levels of pollutants. The highest ΣPAH concentrations were found in sediments adjacent to heavy traffic road (ΣPAH = 3.575 and 2.312 mg kg^{-1} in sites "Luitel" and "Lautaret," respectively), suggesting a petroleum combustion origin of PAH (Table 2). Four PAH isomer ratios were used to identify possible sources of PAH in the contaminated sediments and were compared to PAH isomer pair ratios compiled by Yunker et al. (2002). According to this classification, only "Tines" site was clearly identified by petroleum (gasoline diesel) contamination of the sediment, which was easily explained by the proximity (1 m) with a direct transfer of PAH by scrubbing of the road and/or by atmo-

spheric deposition of vehicular emissions. For the other sites, PAH isomer pair ratios were mainly derived from combustion source (biomass or coal combustion), even in sites "Luitel" and "Lautaret" closed to high traffic (Table 2). The amount of PAH recorded in the sediments of the breeding sites will thus be used to describe the pollutant gradient, instead of traffic or distance to main roads.

3.2. Tadpole survival

No mortality was observed in control groups that were not exposed to a stressor.

No mortality was observed in tadpoles that were exposed to artificial UV alone. Similarly, no mortality was observed in tadpoles that were exposed to BaP alone, whatever the concentration.

When tadpoles were exposed simultaneously to UV and BaP, mortality (expressed as time to death TTD), was observed at the three BaP concentrations. All tadpoles died within 24 h (six populations) or 48 h (two populations) in both the 250 and 500 $\mu g\,L^{-1}$ BaP exposures. At the lowest concentration (50 $\mu g\,L^{-1}$), mortality was complete in three populations within 48 h, while tadpoles in the other five populations were able to survive until the end of the exposure (6 days).

Both BaP concentration and population origin have a significant effect on mortality (multinomial regression: BaP: $\chi^2 = 130.97$ with 2 d.f., $P < 0.001$; Population: $\chi^2 = 94.62$ with 14 d.f., $P < 0.001$), whereas BaP × population interaction was not significant ($\chi^2 = 0.00$ with 14 d.f., $P > 0.999$). Populations from sites with lower total ΣPAH concentrations in the sediments tended to suffer higher mortality (Spearman's correlation: $r_s = 0.702$, $N = 8$, $P = 0.052$). Conversely, the median TTD value observed among a population was not related to altitude of the population (Spearman's correlation: $r_s = 0.472$, $N = 8$, $P = 0.237$).

The BaP concentration in water was measured after 1 and 3 days (method in supplementary material S1). Eighty to 90% of the BaP remain in the solution at day 1, and 30% at day 3, regardless of the initial BaP concentration. This BaP concentration decrease can be explained by degradation and/or accumulation in tadpole tissues and possible bio-transformation.

3.3. Micronuclei

Because most tadpoles died during experiment 3, the micronucleus test was performed in experiments 1 and 2 only. The mean number (per 1000) of MNE in non-exposed tadpoles (level of spontaneous MNE formation) was 0.67 ± 0.13 (range = 0.0–1.0). The level of spontaneous MNE formation varied between populations, decreasing with the altitude of populations ($r = -0.75$, $N = 8$, $P = 0.032$), but not with the concentrations of PAH in sediments of the breeding sites ($r = -0.43$, $N = 8$, $P = 0.29$).

The UV-exposure significantly increased the number of MNE of exposed tadpoles (all populations together, mean MNE ± S.E. = 3.5 ± 0.98, range = 0.33–7.5 in exposed tadpoles; difference between exposed and non-exposed tadpoles: GLM: $\chi^2_1 = 45.59$, $P < 0.001$; Fig. 2(a)). Moreover, there was a significant effect of population on the number of micronuclei ($\chi^2_7 = 43.10$, $P < 0.001$), but the interaction between UV-exposure and population was not significant ($\chi^2_7 = 7.16$, $P = 0.412$).

Tadpole exposure to BaP led to MNE formation (all populations and all concentrations together, mean MNE ± S.E. = 5.46 ± 3.64 in exposed tadpoles). The number of MNE was different in tadpoles exposed at different concentrations of BaP, and increased as the exposure concentration of BaP increased ($\chi^2_3 = 183.66$, $P < 0.001$; Fig. 2(b)). The number of MNE produced significantly varied between populations ($\chi^2_7 = 117.29$, $P < 0.001$; Fig. 2(b)). There was also a significant effect of the interaction between BaP exposure and population ($\chi^2_{21} = 40.98$, $P = 0.005$), suggesting that this factor has a different effect on the different populations.

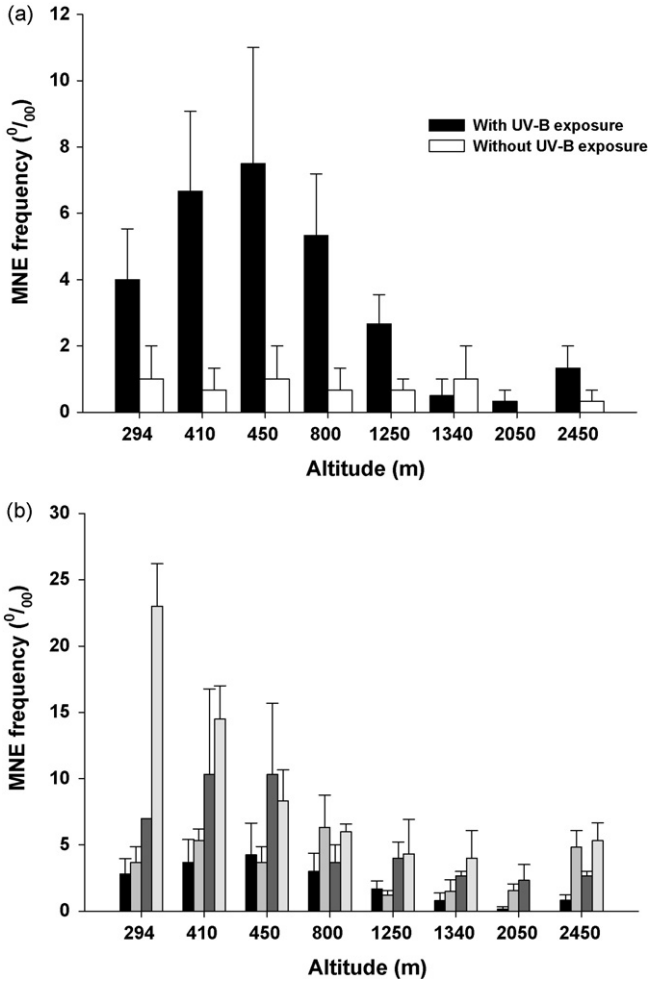

Fig. 2. Micronucleated erythrocytes (MNE) formation as stressor indicator of (a) UV exposure and (b) BaP exposure in tadpoles of the common frog according to their population of origin (altitude). *X*-axis: altitude of populations (m); *Y*-axis: mean number of MN (‰ ± S.E.). (a) Open bars: without UV exposure (control); closed bars: with UV exposure. (b) Closed to open bars = increasing concentrations of BaP (0, 50, 250 and 500 $\mu g\,L^{-1}$).

Since we detected population differences in the number of MNE, we evaluated the relationship of this number to population altitude (i.e. natural UV gradient) and to total PAH concentration recorded in the sediment (i.e. pollution gradient) of aquatic sites. The mean numbers of MNE induced by artificial UV-B significantly decreased as the altitude of populations increased ($r = -0.81$, $N = 8$, $P = 0.015$). These mean numbers of MNE were however not significantly related to the total PAH sediment concentrations ($r = -0.36$, $N = 8$, $P = 0.38$). The mean numbers of MNE induced by BaP exposures (using the average counts obtained from all the BaP concentrations) were not related to the total PAH sediment concentrations ($r = -0.47$, $N = 8$, $P = 0.24$). On the other hand, these mean numbers of MNE significantly decreased as the altitude of populations increased ($r = -0.77$, $N = 8$, $P = 0.024$).

Interpopulation variation in MNE count after stress exposures could result from variation in MNE formation baseline among populations. However, the net MNE numbers (number of MNE after stress exposure minus number of MNE without stress exposure) clearly decrease as the altitude of population increased (Fig. 2(a) and (b)), e.g. MNE number in the lowest altitude population increased from 2.8 to 23 when tadpoles were exposed to BaP while

this number increased from 0.83 to 5.33 in the highest altitude population. Exposed to non-lethal doses of artificial UV and BaP, tadpoles of different common frog populations expressed a variable phenotypic response, which clearly showed that high-altitude populations were more tolerant to both stressors.

4. Discussion

In the French Alps the common frog *R. temporaria* reproduces from May to July when UV-B radiation is at the annual maximum. Eggs and tadpoles are typically found close to the shoreline where solar radiation warms a shallow water layer and accelerates development. They are thus exposed to the full UV-B radiation impact, inducing protection mechanisms (Hofer and Mokri, 2000). Similarly, pollutants can be widespread in nature and common frog populations can be exposed to concentration gradients: PAHs are transported mainly in association with particles, and their distribution, including in high mountain regions, results from complex interactions between regional (Fernandez and Grimalt, 2003; Fernandez et al., 1999) and local factors (wind direction, stream speed, direction of stubbing waters, nature and characteristics of sediments) influencing dispersal and retention (Escartin and Porte, 1999; Pathirana et al., 1994). Our experiments show that tadpole tolerance to UV-B and PAHs vary among populations.

4.1. UV-B exposure effect and tolerance variation among populations

Tadpoles from the eight populations did not exhibited mortality when exposed to artificial UV-B that mimics high-altitude conditions, confirming that *R. temporaria* is very resistant to UV-B radiation (Cummins et al., 1999; Häkkinen et al., 2001; Hofer and Mokri, 2000; Langhelle et al., 1999; Marquis and Miaud, 2008; Pahkala et al., 2001, 2002a). However, the UV-B exposure acted as an external stressor that led to an internal stress state revealed by the micronucleus test. The MNE formation may be attributed to spontaneous events and depends on genetic and intraspecific factors such as age, sex, diet, health and reproductive status (Alsabti and Metcalfe, 1995). In our experiments, the mean number of spontaneous MNE per 1000 erythrocytes (i.e. without UV-B or BaP exposure) was 0.67 ± 0.13, and decreased as the altitude of populations increased (this relation was non-significant with the amount of PAH in the sediments), while tadpoles were reared in constant and similar conditions since hatching. This variation in baseline of MNE formation could have a genetic basis and remains to be studied among more populations and amphibian species.

The number of MNE in the circulating blood increased when tadpoles of the common frog were exposed to artificial UV-B but the extent of MNE frequency varied with the altitude of the exposed population. To our knowledge, no previous study has dealt with the use of the micronucleus assay to assess UV-B genotoxicity in amphibians along environmental gradients such as altitude. The higher induction of MNE in tadpoles from lowland than from highland populations may be related to photo-protection mechanisms. There are many ways to limit direct UV-B exposure in the field. Eggs that are laid close to the surface can be passively protected by the jelly surrounding the embryos (Crump et al., 1999; Häkkinen et al., 2001; Hofer and Mokri, 2000; Licht and Grant, 1997; Marquis et al., 2008; but see Rasanen et al., 2003; Smith et al., 2002) or by oviposition behavior (e.g. depositing eggs in deeper water, Palen et al. (2005)). Tadpoles are able to move under shelters, such as vegetation, or simply to deeper zones. However, especially in high-altitude populations, they often stay close to the shoreline where higher water temperatures allow faster development even if it makes them more exposed to UV-B radiations (Bancroft et al., 2008). It has been proposed that exposed tadpoles of the common frog can be protected by an accumulation of melanosomes in epidermal cells that provides a physical photo-protection; as well, UV-B absorbing substances increased in a dose-dependent manner after UV-B exposure in common frog tadpoles from a 1640 m above sea level population (Hofer and Mokri, 2000). However, the existence or variation of these two mechanisms remains to be tested among frog populations from different altitudes.

The observed decrease in MNE formation (induced by artificial UV exposure) as altitude of populations increased indicates that exposed tadpoles from lowland populations suffer un-repaired DNA damage to a larger extent than do those from highlands. Other traits such as life history, morphology, a behavior related to tolerance are usually directly linked to fitness (Van Straalen and Timmermans, 2002). Common frog eggs exposed to UV-B produce smaller tadpoles than do non-exposed eggs and this difference decreased as the altitude of populations (Marquis and Miaud, 2008) or latitude increased (Pahkala et al., 2000). This effect was attributed to the energetic cost of DNA repair systems in amphibians (Belden and Blaustein, 2002a; Belden et al., 2000). Variation of efficiency of repair systems or metabolic performance (e.g. quicker and/or more effective in highlands) remains to be analyzed among amphibian populations.

4.2. BaP exposure effect and tolerance variation among populations

The concentration of PAHs in surface and coastal waters is generally in the neighbourhood of $0.05\ \mu g\ L^{-1}$ and concentration above this point indicates contamination that can reach several $\mu g\ L^{-1}$ (review in Srogi, 2007; Zhang et al., 2007). In our study, the survival of tadpoles from eight populations was not affected by benzo[a]pyrene exposure alone, whatever the concentrations (i.e. up to $500\ \mu g\ L^{-1}$). This low toxicity has been observed in several terrestrial organisms from plants to invertebrates (Sverdrup et al., 2007). On the other hand, BaP has been shown to cause genotoxic effects in a board range of prokaryotic and mammalian cell essay system (US EPA, 1990) and the micronucleus test reveals that exposed tadpoles exhibited a dose-dependent response to BaP exposure and thus suffered DNA damage. It has previously been shown that BaP bioaccumulation in fish and amphibian larvae is mainly due to both trophic transfer and aqueous uptake *in situ* (Carlson et al., 2004; Garrigues et al., 2004; Wang and Wang, 2006). MNE formation with BaP exposure has previously been shown to be dose-dependent in several species of amphibians (*P. waltl*, *Ambystoma maculatum*, and *X. laevis* exposed to $0.01–0.06\ \mu g\ L^{-1}$, Fernandez, 1993; *P. waltl* exposed to $4–200\ \mu g\ L^{-1}$, Marty et al., 1998).

The variation of MNE number among populations was not correlated with variation in PAH concentrations in the sediment of breeding sites, suggesting that higher levels of exposure did not lead to local adaptation in pollutant tolerance among populations. High intensity of selection is one of the major prerequisites for local adaptation (Kawecki and Ebert, 2004), and the level and/or duration of the contamination of the aquatic sites could be too low to induce directional selection. Road traffic has occurred for one century in the study area, and a large increase in traffic (a twofold increase in 20 years) has been observed since the 1980s (data from the road service of the Savoie Department). PAH contamination was observed in all the breeding sites and is expected to increase and could lead to local adaptation in the near future.

4.3. Synergistic effects of UV-B and BaP exposure

Synergistic effects of UV-B with pH (Pahkala et al., 2002b), pathogenic fungi (Kiesecker et al., 2001), nitrates (Hatch and

Blaustein, 2000), and copper (Baud and Beck, 2005) that negatively affect tadpole survival have been reported in several amphibian species. In our experiments, tadpoles of all the eight common frog populations suffered complete mortality when exposed to 250 and 500 $\mu g\,L^{-1}$ BaP and artificial UV-B, indicating a strong synergistic effect. Due to their multiple aromatic ring system, PAHs can absorb sunlight in the visible and UV regions of the solar spectrum, causing structural modification (Toyooka et al., 2007). Hydroxyquinone, 9,10-phenanthrenequinone, and 1,6- and 3,6-benzo[a]pyrenequinone were identified as photomodified products of benzo[a]pyrene (Lampi et al., 2006), and photoproducts such as quinine have been confirmed as toxic (Cody et al., 1984; Sbrana et al., 1995). It has been previously shown that UV-B exposure can induce an important increase in acute toxicity of BaP in amphibian larvae (Fernandez and L'haridon, 1992; Hatch and Burton, 1998). The photoproducts of PAHs can disturb the membrane function by triggering lipid peroxidation, modifying the structure and/or the function of proteins, and can damage nucleic acids (Basaga, 1990; Chesis et al., 1984; O'Brien, 1991; Prinsze et al., 1990; Vaca et al., 1988). We thus hypothesized that the acute mortality observed in our experiments was caused by BaP photoproducts.

However, tadpoles exposed simultaneously to UV-B × BaP stress (at the lowest dose) tended to exhibit a lower mortality ($P = 0.052$) when originating from populations exposed in the field to the highest PAH concentrations in the sediment. This result remains only a tendency and is not able to bring any strong conclusion on a lower sensitivity to BaP × UV-B stress in populations from PAH contaminated areas. What can be highlighted is that even small amounts of pollutants reaching pristine areas with high UV exposure (e.g. mountains) may expose amphibians to increased mortality risk.

4.4. Two stressors, one effect: the co-tolerance hypothesis

In our laboratory experiments ("common garden" experimental design), the observed variation in tolerance to genotoxic stress among tadpoles originated from different populations argue for the role of genetic factors. No correlation was observed between tolerance to BaP exposure and level of PAH contamination in the field, but tolerance to UV-B increases as the altitudes of populations increases. This phenotypic variation can result from directional selection on populations, generating among populations tolerance divergence. Local adaptations, i.e. among population differences significantly attributed to genetic determinism, can appear in a few years (e.g. Hendry and Kinnison, 1999 for a review, Kinnison and Hendry, 2001; Reznick and Ghalambor, 2001) even in vertebrates (Hairston et al., 2005; Losos et al., 1997). Several adaptations along environmental gradients have been reported in amphibians; thermal preference among populations in the wood frog, *Rana sylvatica* (Skelly and Freidenburg, 2000); tolerance to nitrates in common frog populations from nitrate-contaminated/non-contaminated sites (Johansson et al., 2001); and tolerance to brackish water in the Naterjack toad, *Bufo calamita* (Gomez-Mestre and Tejedo, 2003). There has been only one attempt to demonstrate a variation of sensitivity to UV-B between populations along altitudinal gradient (Belden and Blaustein, 2002b) that showed that *Ambystoma macrodactylum* larvae from high-altitude populations had a higher survival than those from low-altitude population when exposed to UV-B. Results of the present study argue for the existence of local adaptation to genotoxic stressors among populations varying in altitude.

An unexpected result was that MNE formation caused by pollutant exposure decreased as the altitude of exposed populations increased, i.e. high-altitude *R. temporaria* populations would suffer fewer DNA damage induced by BaP than populations at lower altitudes. As BaP bioaccumulation in amphibian larvae was mainly due to trophic transfer and aqueous uptake (e.g. Wang and Wang, 2006), physical, e.g. epidermis would not be the main protection from this pollutant. These tadpoles must rather react *via* repairing systems as observed in other vertebrates exposed to PAHs (Binkova et al., 2007; Buet et al., 2006; Harrigan et al., 2004; Marty et al., 1989; Rocher et al., 2006; Vache et al., 2006). In mammals, the major DNA repair processes against UV-B- or PAH-induced damage are excision repair mechanisms, mismatch repair and recombination repair (Braithwaite et al., 1999; Moustacchi, 2000; Sancar and Tang, 1993). None of those mechanisms have yet been clearly demonstrated in amphibians (Hays et al., 1990), where the major DNA repair process following UV-B exposure is enzymatic photoreactivation (with CPD- and [6-4]-photolyases). Levels and activity of CPD-photolyase recorded in amphibian eggs vary among species (Blaustein et al., 1994, 2001; Hays et al., 1996), and were positively correlated with egg hatching success, and the rate of CPD induction and removal determines a UV-B toxicity threshold in *X. laevis* tadpoles (Pandelova et al., 2006). Species with different intrinsic repair capacities might show different toxicity thresholds under similar UV-B exposure (Pandelova et al., 2006), and testing this threshold effect hypothesis among populations widespread along environmental gradients (including pollutants) would be of particular interest.

High-altitude populations adapted to prevent or repair UV-B DNA damage could be also adapted to prevent or repair DNA damage due to BaP exposure. Multiple tolerances to two or more pollutants (e.g. metals) were explained by co-tolerance, namely, tolerance to one pollutant that confers tolerance to another (Cox and Hutchinson, 1981). The concept of co-tolerance defines the tolerance of a community to different compounds even if the community has not been previously exposed to some of these compounds (Forbes and Forbes, 1994; Vinebrooke et al., 2004). Numerous examples of multiple tolerance to heavy metals have been found in plants (Patra et al., 1994; Schat and Vooijs, 1997). Co-tolerance can appear to compounds with a similar chemical structure or similar toxicity mechanisms (Blanck et al., 1988). Both UV-B and BaP can lead to DNA damage having clastogenic and aneugenic effects (chromosome and genome alterations), which result in the formation of micronuclei (Gauthier, 1996; Udroiu, 2006). Similar DNA damage could thus necessitate similar DNA protection and/or repair mechanisms. High-altitude plants may have a simultaneous co-tolerance for several stress factors (Turunen and Latola, 2005); for example, a thicker epidermal layer and cutin, increased concentration of UV-B absorbing compounds in the epidermal cells, and higher levels of glutathione increase plant tolerance to other environmental stresses such as drought and low temperatures (Laakso et al., 2001; Turunen et al., 1999). In the common frog, tadpoles adapted to repair some DNA injuries caused by UV-B exposure (and potentially leading to micronuclei formation) may also be adapted to repair DNA injuries induced by BaP by using similar ways of DNA repair, showing an original case of co-tolerance in a vertebrate. In an evolutionary point of view, traits can evolve for a specific usage (or no function at all), and later be adapted to a different usage (so-called exaptation, Gould and Vrba (1982)). UV-B increase as altitude increases is a long-term situation allowing local adaptation selection (while PAHs are more recently widespread in the environment). Selection to UV-B tolerance observed in high-altitude populations, which confers PAH tolerance, could thus be an example of exaptation based on the co-tolerance to two genotoxic stressors. Likewise, Gomez-Mestre and Tejedo (2005) have hypothesized that early tolerance to salinity in aquatic stages could be linked to drought tolerance in the terrestrial juveniles and adult stages (via resistance to osmotic stress) of *B. calamita*. However, populations highly resistant to salinity were not more resistant to drought (Gomez-Mestre and Tejedo, 2005). Nevertheless this co-tolerance hypothesis remains to be tested by confirming the link

between PAH and UV-B DNA damage repairing systems and testing other genotoxic compounds with similar and different ways of toxicity.

Novel combinations of stressors may have particularly serious consequences for biodiversity and ecosystem functioning. This study highlights three important factors likely to alter relations between pollutant effect and organism response: (1) local adaptation along pollutant gradients can lead to pollutant tolerance variation, requiring studies to take into account the history of exposure of each studied population; (2) synergistic effects (e.g. photodecomposition) can strongly underestimate toxic effects; and (3) the existence of co-tolerance can hide toxic effects of a new pollutant that has a similar chemical structure or similar toxicity mechanisms to previously dispersed chemicals or environmental variables (such as salinity, UV, pH, metals).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.09.001.

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