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**Effects of traffic noise on tree-frog stress levels, immunity, and color signalling**

MATHIEU TROÏANOWSKI<sup>1</sup>, NATHALIE MONDY<sup>1</sup>, ADELIN DUMET<sup>1</sup>, CAROLINE  
ARCANJO<sup>1</sup> and THIERRY LENGAGNE<sup>1\*</sup>

<sup>1</sup>, UMR 5023 Ecologie des Hydrosystèmes Naturels et Anthropisés, Université de Lyon,  
ENTPE, CNRS, Université Lyon 1, 6 Rue Raphaël Dubois Bât Darwin C 69622  
Villeurbanne, France , email [thierry.lengagne@univ-lyon1.fr](mailto:thierry.lengagne@univ-lyon1.fr)

\*Address correspondence to T. Lengagne.

Running head: Noise consequences on tree frogs

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## Abstract

Although it is known that noise exposure strongly influences human health, studies on the effects of noise on animals remain scarce. We experimentally exposed *Hyla arborea*, an anuran species, to traffic noise and altered the endocrine status of exposed animals to investigate physiological and phenotypic effects of noise pollution. Noise exposure significantly increased stress hormone levels (+63.5%,  $P < 0.01$ ). Moreover, the thickness of the swelling due to a phytohaemagglutinin injection showed a decrease of the frog immune response by 18% ( $P < 0.01$ ) Both traffic-noise exposure and stress-hormone application negatively affected *H. arborea* vocal-sac brightness (19.5% and 20% respectively,  $P < 0.01$ ). The best quality males with attractive vocal-sac coloration were the most affected by noise; thus, noise pollution may profoundly change sexual-selection processes. Our results suggest recent increases in anthropogenic noise worldwide may affect a broader range of animal species than previously thought because of alteration of visual signals and immunity. Examination of how our results may apply to other taxa is crucial for the conservation of biodiversity in an increasingly noisy world.

## **Introduction**

Modern human societies generate new patterns of noise that affect acoustic communication in many animal species (Barber et al. 2010). Noise from road traffic is one of the most sources of noise pollution (Forman 2000; Jamrah et al. 2006), and it may be a major factor in animal-population decline near roads (Reijnen et al. 1996). Traffic noise not only alters characteristics of sound- transmission channels, but it is also may threaten animal survival. Traffic noise affects human health negatively (e.g., myocardial infarction [Selander et al. 2009]; hearing loss [Barbosa & Cardoso 2005]), but little is known about traffic-noise impacts on wild animals (but see Kight & Swaddle 2011). There have been several comparisons of the responses of species in urban versus rural areas to traffic noise, but it is often difficult to confirm that observed physiological problems are specifically due to noise pollution (Partecke et al. 2006). Blickley et al. (2012) and Tennessen et al. (2014) found that experimental exposure to traffic noise leads to an elevation of corticosterone, the glucocorticoid hormone commonly used to measure a stress response. However, other researchers have identified either no such correlation (Potvin & MacDougall-Shackleton 2015) or a negative one (Crino et al. 2013). In the short term, an increase in corticosterone levels is adaptive because it has direct effects on behavior, metabolism, and energy allocation, which help organisms cope with perturbations (“emergency life history stage” [Wingfield 2003]) and enhance the probability of survival (Romero & Butler 2007). But, over the longer term (2-3 weeks), prolonged exposure to corticosterone due to chronic stress can induce a maladaptive response and affect fitness by inhibiting resource allocation to reproductive or immune activities, a condition known as allostatic overload (Mc Ewen & Wingfield 2003).

In addition to stress response, corticosterone is also involved in the regulation of immunity and color-signal expression. Corticosterone has direct effects on the immune

system: it inhibits the synthesis, release, and efficacy of cytokines; reduces activation and proliferation of T cells, B cells, and macrophages; and lowers the circulating level of lymphocytes or reduces the number of phagocytic cells at inflammation sites (Romero & Butler 2007; Martin 2009). Because traffic noise is widespread in space and time, animal populations might experience a chronic stress level and also face immunosuppression. A high level of corticosterone can also affect carotenoid-based colouration; stressed animals have been shown to become pale (Eraud et al 2007). Carotenoids are pigments responsible for orange-red ornamentations, but they have other functions such as immune stimulation or antioxidant effects (Blount et al. 2003). Vertebrates must acquire carotenoids through their food. Hence, in a carotenoid-limited environment, trade-offs between coloured ornamentation and immunity can exist. During an immune challenge, carotenoids may preferentially be used for immune function improvement rather than for colouration (Blount et al. 2003; Faivre et al. 2003). In the same way, stress factors may divert the use of carotenoids from colouration by means of allostasis (Landys et al. 2006).

We examined the impact of traffic noise on the European tree frog (*Hyla arborea*). In *H. arborea*, female discrimination of male quality is based on acoustic male-male interactions (Richardson et al. 2008) and on several acoustic parameters present in every emitted call (Richardson & Lengagne 2010; Richardson et al. 2010). Previous studies show that females use both the chroma and the brightness of a carotenoid-dependent signal, vocal sac colouration (Gomez et al. 2009, 2010), to select mates. The association of several signals may increase the reliability of the information (Candolin 2003), whereas the use of two modalities increases the discrimination accuracy (Gomez et al. 2011). Male *H. arborea* are unable to adjust their call structure and their vocal sacs pale in response to traffic noise (Troïanowski et al. 2015). To better understand this effect and its possible impact on population dynamics, we experimentally manipulated traffic noise exposure and stress levels of animals to investigate

physiological and phenotypic effects of noise pollution. We hypothesized that chronic exposure to traffic noise induces stress in *H. arborea*, traffic noise induces immunosuppression, and immunosuppression elicits a modification of vocal-sac coloration.

## **Methods**

### *Experimental Design*

Fifty-nine male tree frogs never exposed to road traffic noise were captured by hand when calling in the beginning of the breeding season (three consecutive days in the third week of April) near Lyon (France). After their capture males were immediately placed in individual terraria (25x17x15 cm) with a water-filled basin and artificial foliage and transported to EcoAquatron, a laboratory for keeping amphibians at the University of Lyon. The temperature was kept at 23.1 °C (1.5 SD). Each terrarium was placed under a fluorescent light (EXO TERRA Repti Glo 5.0 [Hagen, Montréal, Canada]) that mimicked natural light (30% UVA and 5% UVB). The lights were on timers set for a photoperiod approximating natural conditions. Throughout their captivity, males were fed ad libitum with domestic crickets (*Acheta domesticus*).

Frogs were captured on day 0 (D0). After 3 days of acclimation (D1, D2, and D3) (a sufficient period to observe natural calling behavior in males [Brepson et al. 2013]), subjects were assigned at random to treatment groups : control (C group, chorus noise during the night, no traffic noise, no hormonal supplementation,  $n = 19$ ); exposure to traffic noise (E group, chorus noise during the night, traffic noise 24h a day, no hormonal supplementation,  $n=20$ ); and corticosterone-supplemented treatment (S group, chorus noise during the night, no traffic noise, hormonal supplementation,  $n=20$ ). Starting on the evening of D3, the treatment lasted 10 days. Individuals in all 3 groups were housed in different rooms in the same

conditions except that subjects in E were housed in a separate room in which traffic noise was broadcast.

#### *Sound-track preparation and broadcast*

The chorus of 10 *H. arborea* males was recorded with Sony ECM-T6 microphones (Sony, Tokyo Japan) and Edirol R-44 recorders (Roland Tokyo Japan). Such a recorded chorus efficiently stimulates *H. arborea* calling activity (Brepson et al. 2013). The traffic-noise audio file was prepared from recordings at a busy road near Lyon, France (road N346), on which 40000 vehicles per month travel (average peak 3500 vehicles per hour in the early morning and in the evening). In the evening, a Sennheiser microphone ME62-K6 (Sennheiser, Hanover, Germany) was placed 10 m from the road at a height of 1 m and connected to a recorder. From a 1-hour recording, a 20-minute segment was selected and normalised to an averaged amplitude level (2 dB SD) with Avisoft SASLab software (Avisoft, Glienicke, Germany).

Both chorus and traffic noise were broadcast via an amplified loudspeaker (KH pas-100, Sennheiser) connected to a CD player directed toward the frog terrarium but situated on a table on the opposite side of the room in order to reduce seismic signals due to membrane loudspeaker vibrations.

For all 3 groups during the entire experiment (D1 to D13), we broadcast tree-frog chorus noises each night from 2030 to 0030 at 78 dB SPL (sound pressure level) (xx 1.4) (indicate what this 1.4 is, SD? SE?) (C weighted, 20 $\mu$  Pa) to reproduce the natural acoustic environment. The experimental groups (E and S) (?) were housed in a separate room wherein, in addition to chorus noise, traffic noise was broadcast from D4 to D13 24 hours/day at 76 dB

(xx 1.8). The pattern of traffic-noise level was constant during our experiment, which allowed us to compare our results with other published studies on the same topic. The noise level in our study matched values in the field at night (Bee & Swanson 2007; Pirrera et al. 2011).

#### *Hormonal treatments*

Amphibian skin and many of its secretions contain lipids (Schmid & Barden 1965; Wells 2007), and lipophilic molecules can easily cross skin. We applied a mixture of sesame oil and corticosterone daily to the skin of all S-treatment frogs to manipulate circulating levels of corticosterone. This non-invasive method has been used in lizards for corticosterone (Meylan et al. 2003) and in *H. arborea* for testosterone manipulation (Desprat et al., 2015). We diluted corticosterone (n° C2505 Sigma Aldrich [St Louis, Missouri, USA]) in sesame oil: 3 µg corticosterone /µL of sesame oil. We applied 4.5 µL of the corticosterone solution to the backs of S frogs at 1000 each day (D3 to D13). To frogs in the C and E treatments, we applied 4.5 µL of sesame oil on a daily basis (D3 to D13).

#### *Saliva sampling and corticosterone measurements*

Saliva samples of all 59 males were collected near the pond just after capture (D0) and at the end of the experiment (D13) to determine corticosterone levels. Within 3 minutes of capture, a dry cotton ball of known weight was inserted into the frog's mouth for 30 seconds to collect saliva. Each cotton ball was immediately weighed, put in a microtube equipped with a filter to retain the cotton fibre during centrifugation, and stored at -80 °C. This method for recording corticoid baselines has been used in toads and in mammals because stress responses due to manipulation of animals are not detectable in saliva until approximately 20–30 minutes after the onset of a stressor (Janin et al. 2012; Sheriff et al. 2011).

We measured corticosterone levels in saliva samples with an enzyme-linked immunoassay (EIA) validated for amphibians (Janin et al. 2012) and optimised for tree frogs.

We estimated the corticosterone concentration in 1 mg of saliva by dividing the amount of corticosterone in samples by the amount of collected saliva. Briefly, samples were reconstituted by adding 150  $\mu$ L of phosphate buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) to the cotton tubes, placing them in microtubes and centrifuging them at 8000 rpm for 5 minutes. Corticosterone analysis was carried out in duplicate with a colourimetric 96-well EIA assay kit (number 500651, Cayman Chemical Company, Ann Arbor, Michigan). This kit is based on the competition that occurs between corticosterone and a corticosterone-acetylcholinesterase conjugate for a limited number of corticosterone polyclonal antiserum binding sites. The colour reaction was developed using Ellman's reagent containing acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). The concentration of corticosterone in saliva samples was calculated using a standard curve run in duplicate on each plate.

#### *Immune-capacity measurement*

Josserand et al. (2015) used the following method to measure immune capacity in *H. arborea*. At the end of noise exposure (D13), we injected a small amount of phytohaemagglutinin (PHA) solution in each frog and measured the thickness of the resultant swelling to quantify the frog's ability to mount an immune response (Desprat et al., 2015; Josserand et al. 2015). One mg of PHA-P (no. L8754 [Sigma Aldrich]) was diluted with 20  $\mu$ L of sterile phosphate-buffered saline (PBS) (no. 231.791.2 [Sigma Aldrich]) to make an injectable solution. Using a 0.25 ml syringe, we injected 20  $\mu$ L of this solution intramuscularly into the right leg of each frog. We used a dial thickness gauge (Mitutoyo, ID-C112) to measure the thickness of the leg prior to injection and 16 hours after injection. After 3 seconds of contact with the leg muscle, three readings of thickness were taken in quick succession (gauge was removed between each reading) and averaged to give a mean

thickness value for the skin on each leg before and after injection. The same person (M.T.) performed all injections and measurements.

#### *Colouration measurement*

We measured coloration of vocal sac with a spectrometer (AvaSpec-3648SPU2 [Avantes, Leatherhead, Surrey]) and an Avantes AvaLight-DH-S deuterium-halogen light source lamp emitting in the range 200–1500 nm. The coaxial optic fibre (FCR-7UV200- 2-45 [Avantes]) had a terminal area (2 x 3 mm) cut at 45° to avoid specular reflectance and was protected by a silica window that guaranteed a constant distance between the detector and the measured patch. All spectra were taken relative to the dark and to a Spectralon white standard (Ocean Optics, Dunedin, Florida) and were then computed with software provided by Avantes. The vocal-sac colouration of each male was measured using reflectance spectrometry before and after the experiment (D1 and D13). To cover the entire vocal-sac surface, we get three reflectance spectra on the deflated vocal sac of each male. We averaged all the spectra taken for a given individual before analyses. We analysed vocal-sac reflectance spectra with AVICOL (version 5, available at <http://sites.google.com/site/avicolprogram/>). Hue characteristics did not show any substantial variation among individuals (Gomez et al. 2009). Consequently, we retained only chroma and brightness to characterise male colouration. Over the range 350–700 nm, we computed brightness (mean light intensity) as the mean reflectance over this range and chroma (spectral purity) as the difference between the maximal and minimal reflectance divided by the average reflectance. A more intense colouration is darker and more chromatic.

#### *Animal-Use Protocol*

The population from which the individuals were collected belongs to a metapopulation consisting of several thousand tree frogs. Hence, our sampling did not significantly affect reproduction of the population. No effect of transport on health or mortality was observed and housing conditions were approved by veterinary services (Préfecture du Rhône). After the behavioral experiment, all frogs were examined by the EcoAquatron staff. They were in good condition, showed no symptoms of disease, and were returned to their population. This study was conducted in accordance with French law on use of animal in science and was approved by of the ethical committee of Lyon 1 University (n°BH2011-6) according to decision 2012-086-0019 of the Préfecture de l'Isère specifying that animals have to be returned to their habitat after experiments) and permit 69266347 of the Direction des Services Vétérinaires. All frogs were released into the original pond the night after the end of the experiment.

### *Statistical analyses*

We analysed the temporal variation in colour, skin thickness, and corticosterone levels with linear mixed models in which the individual and date of capture were random effects and the treatments (C, E, and S) over time (D1, D13) were fixed effects. We used linear regression to test the potential impact of corticosterone on immune-capacity evolution (leg swelling) and to test a potential impact of immune capacity on vocal-sac colouration (brightness and chroma) changes. In the first case, we used corticosterone evolution (corticosterone D13 – corticosterone D0) as a fixed effect corrected by the mass of the males. In the second case, we used leg thickness as a fixed effect corrected by the mass of the males. All analyses were performed using R 2.15.1 statistical analysis software (R development Core Team 2013).

## **Results**

### *Impact of noise and hormone on animal corticosterone level*

Before the experiments started, corticosterone levels between groups were similar (all  $p > 0.726$ ). At the end of the experiment, the E group had higher stress levels (cover in Methods if not already there; I think it is there) than the C group (3.387 [SE 1.252],  $df = 54$ , Wald  $z$  test  $p = 0.009$ ) (Fig. 1). Corticosterone application did not induce change in corticosterone levels measured 24 hours after the last application (1.703 [1.229],  $df = 54$ , Wald  $z$  test  $p = 0.1715$ ) (Fig. 1). When we focused on hormone-level changes for each group, the corticosterone levels of frogs in E group increased over time (2.027 [0.89],  $df = 54$ , Wald  $z$  test  $p = 0.027$ ), whereas there was no significant change in the corticosterone levels of C and S groups (-1.36 [0.87],  $df = 54$ , Wald  $z$  test  $p = 0.1282$ ; 0.344 [0.858],  $df = 54$ , Wald  $z$  test  $p$  (remainder not marked) = 0.69, respectively) (Fig. 1).

### *Impact of noise and hormone on immune response*

Before PHA injection, the leg thickness of males from the different groups was similar (all  $P > 0.11$ ). A significant leg swelling was observed in the three groups (all  $P < 1.10^{-4}$ ) (Fig. 2). Nonetheless, both traffic-noise exposure and corticosterone supplementation decreased the immune-response intensity relative to the control group. Both E and S groups showed an immune responsiveness significantly lower than that of the C group (-0.034 [SE 0.012],  $df = 46$ , Wald  $z$  test  $P = 0.0067$ ; -0.033 [0.012],  $df = 46$ , Wald  $z$  test  $P = 0.0079$ , respectively) (Fig. 2). When testing for an impact of corticosterone change on leg swelling, we only found a tendency for a negative correlation between these two variables (-0.017 [0.009],  $df = 43$ ,  $P = 0.091$ ).

### *Impact of noise and hormone on vocal-sac colouration*

Before the start of the experiment, there was no significant difference in brightness (all  $P > 0.495$ ) or chroma (all  $P > 0.456$ ) of the vocal sac among groups. The brightness of the vocal sac of males exposed to traffic noise or supplemented with corticosterone increased relative to vocal sac of control males (0.855 [SE 0.28],  $df = 54$ , Wald  $z$  test  $P = 0.0035$ ; 0.842 [0.28],  $df = 54$ , Wald  $z$  test  $P = 0.004$ , respectively) (Fig. 3a). When we focused on brightness changes for each group, values in the C group did not change significantly between the beginning and the end of the experiment (0.032 [0.206],  $df = 54$ , Wald  $z$  test  $P = 0.877$ , fig. 3A), whereas the vocal sac of males of the E and S groups became significantly paler (0.887 [0.19],  $df = 54$ , Wald  $z$  test  $P < 1.10^{-4}$ ; 0.873 [0.19],  $df = 54$ , Wald  $z$  test:  $P < 1.10^{-4}$ , respectively) (Fig. 3a).

Conversely, chroma of the vocal sac did not change among groups during the experiment (all  $P > 0.293$ ) (Fig. 3b). Chroma did not change significantly within the C group (-0.062 [SE 0.081],  $df = 53$ , Wald  $z$  test:  $P = 0.443$ ), whereas there were significant decreases in this parameter within both the E and S groups (-0.175 [0.075],  $df = 53$ , Wald  $z$  test:  $P = 0.023$ ; -0.181 [0.076],  $df = 53$ , Wald  $z$  test  $P = 0.022$ , respectively) (Fig. 3b). Nonetheless, the more colourful males at the beginning of the experiment displayed greater changes in colour. That is to say, E individuals with the lowest brightness and highest chroma at D1 had the highest changes in vocal-sac colouration at the end of the experiment. Two significant correlations were found between the brightness or chroma of the vocal sac before the experiment and the changes in colouration (brightness:  $F = 15.8$ ,  $df = 18$ ,  $P < 0.001$ ; chroma:  $F = 37.9$ ,  $df = 18$ ,  $P < 0.001$ ). Neither rightness nor was significantly related to leg swelling (all  $P > 0.129$ ).

## Discussion

Corticosterone levels were significantly higher (63.5%) in tree frogs exposed to traffic noise compared with frogs in the control group, which supports the hypothesis that noise

pollution constitutes a chronic stressor. Because traffic noise was broadcast by night (period of frog activity) and day during (resting period of frogs), it seems probable that noise affects the well-being of animals independent of its effect on acoustic communication (Wright et al. 2007). Only two experimental studies have been conducted on the relationship between noise and stress in wild animals on *Lithobates sylvaticus* and *Centrocercus urophasianus* (Blickley et al. 2012; Tennessen et al. 2014) and their results were consistent with our study.

Surprisingly, the corticosterone levels of S frogs measured 24 hours after the end of the experiment were not higher than levels measured at the beginning of the experiment. Previous studies show that corticosterone implanted in *Physalaemus pustulosus* does not modify plasma corticosterone level, although there is a clear modification of calling behavior (Marler & Ryan 1996). In a preliminary experiment in which we used the hormonal-treatment method of the present study, 6 hours after supplementation the corticosterone levels of supplemented frogs were still four times higher than the corticosterone levels in control frogs (details in Supporting Information). Hence, although the stress levels of S frogs did not increase over the length of the experiment (no accumulation), they experienced a high stress level at least several hours per day. Although a relationship between calling activity and stress level has been described in several amphibians (Leary et al. 2008; de Assis et al. 2012), the effect of stress on calling may be too small to explain the corticosterone levels we found here because in a previous study (same noise level and exposure duration), we did not detect any effect of traffic noise on vocal activity (Troianowski et al 2015).

Increased corticosterone levels triggered by chronic stress has many known trade-offs. An important one is the effect of corticosterone on immunity (Butler et al. 2010; but see Crino et al. 2013). We measured immune response only once (at the end of the experiment) rather than twice (before and after experiment) because the second injection can lead to a greater swelling than the first due to the memory of the immune system (Brown et al. 2011).

Regardless treatment group, the PHA injection induced an immune response with substantial (use this term only if you mean a statistically significant swelling) leg swelling in each group. Nonetheless, there was a significant impact of treatment (traffic noise or corticosterone application) on swelling intensity. Leg swelling in the E and S groups was significantly lower than the swelling in C group (Fig. 3). This result, in addition to the relation between stress and immune response, suggests that traffic noise affects immune response through an immunosuppressive effect of corticosterone. Although the link between stress and immune response has been found previously (Saino et al. 2003), we found for the first time a significant relationship between stress induced by traffic noise and animal immune state. Because immunosuppression may be involved in amphibian decline (Carey et al. 1999), road-traffic noise, via its immunosuppressive effect, may be a large threat to amphibians. Next in wetlands near busy roads the adaptation process at the population level should be studied, although it may be difficult to ensure that all frogs studied in the wild have been exposed to the same noise level since metamorphosis. In some cases, common-garden or transplant experiments may overcome this problem (Partecke et al. 2006; Brady 2012).

Both traffic-noise exposure and corticosterone application negatively affected coloration of frog vocal sacs (your results). Frogs in these treatment groups became paler (high brightness value) and were less chromatic (low chroma value lowest) after treatment. In tree frogs, losing colour is bound to affect sexual selection processes because females tend to choose males that exhibit dark red vocal sacs (Gomez et al. 2010).

When male colouration decreases, two outcomes are possible. If all males lose attractiveness, the subjects with the darkest red sacs would be the most attractive for females. Alternatively, males with higher carotenoid levels in their vocal sacs have a larger colouration decrement and thus would have the same attractiveness of poor-quality males. Our results clearly support the second outcome because we found that males with initially

attractive vocal-sac colouration were the most affected by traffic noise. Loss of coloration may lead to an increase in the genetic contribution of poor-quality males in tree-frog population living in noisy areas. Evolution of a sexually selected trait after only several generations resulting from environmental changes has occurred in a bird (Yeh 2004). If sexual selection is weakened in some traits, this could strengthen selection of other traits under new environmental conditions (Candolin 2003). How large an effect anthropogenic disturbance has on the evolution of sexual selection and thus population viability is still debated (Candolin & Heuschele 2008).

Although traffic noise by itself affected male colouration, we do not know the precise mechanism behind the colouration loss. There was no direct relation between colouration loss and immunosuppression. In addition to their immunosuppressive effect, stress-related hormones are also involved in individual oxidative stress (Constantini et al. 2011). Moreover, exposure to white noise increases oxidative stress levels and has a negative impact on immune functions (Zheng & Ariizumi 2007). Hence, the colouration loss we observed may be due to an effect of traffic noise on both immune capacity and oxidative stress.

In many countries, there is growing concern about the ecological effects of unwanted environmental sounds such as roadway noise and aircraft noise, which are among the most pervasive sources of environmental noise. Many researchers have demonstrated effects of anthropogenic noise on animal communication in different taxa (frogs: Lengagne et al 2008; insects: Lampe et al. 2012; birds: Halfwerk et al. 2011; mammals: Siemers & Schaub 2011). We found a stress effect of traffic noise that may alter the metabolism of animals (i.e., immunosuppression) and thus affect the survival of animals living near roads and a coloration effect that could impact sexual selection. Hence, traffic noise may have a larger impact on wildlife than previously thought, and several countries have begun to implement widespread environmental mitigation for roads. Measures to reduce noise at-source are generally more

cost-effective than those designed to hamper its propagation. For instance, low-noise asphalt can reduce investments in noise abatement measures by up to 80% compared to noise barriers. Although the automobile industry is working to decrease noise production, a reduction in speed limits and education of car and truck drivers may be particularly efficient. For instance, an increase in car speed from 30 to 70 km/hour results in a 7 dB increase of noise level (Favre & Lamure 1987). We found that traffic noise had large effects on both physiology and behaviour of male tree frogs and suggest it is important to limit the impact of noise pollution on wildlife.

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**Fig. 1** Changes in corticosterone levels in European tree-frog (*Hyla arborea*) throughout the traffic-noise-exposure experiment (3 days of acclimation and 10 days of exposure) (C, control males; E, males exposed to traffic noise; S, males supplemented with corticosterone and not exposed to traffic noise; horizontal line, median value; box ends, upper and lower quartiles; whiskers, maximum and minimum values; different letters [a and b] above bars, significant difference between treatments; \*, significant change within a treatment [covered above]).

**Fig. 2** Impact of noise exposure and corticosterone supplementation on immune response in European tree-frog (*Hyla arborea*). (C, control males, no noise no corticosterone supplementation; E, males exposed to traffic noise during 10 days; S, males supplemented with corticosterone and not exposed to traffic noise). Measurements of leg swelling after an injection of phytohaemagglutinin allow us to quantify the immune response. Horizontal line, median value; box ends, upper and lower quartiles; whiskers, maximum and minimum values; different letters [a and b] above bars, significant difference between treatments; \*, significant change within a treatment; \*\*\*,  $p < 0.001$ )).

**Fig. 3** (a) Brightness and (b) chroma of the vocal sac of European tree-frog (*Hyla arborea*) pre- and post-exposure to traffic noise or corticosterone supplementation; (C, control males, no noise no corticosterone supplementation [diamond]; E, males exposed to traffic noise during 10 days [square]; S, males supplemented with corticosterone and not exposed to traffic noise [circle]; NS, no significant difference; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ). For brightness a high value indicates a paler vocal sac corresponding to a non-attractive male for females while for chroma a high value indicates a saturated vocal sac corresponding to an attractive male.