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Combined effect of temperature and ammonia on molecular response and survival of the freshwater crustacean *Gammarus pulex*

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ABSTRACT

Freshwater ecosystems are experiencing mounting pressures from agriculture, urbanization, and climate change, which could drastically impair aquatic biodiversity. As nutrient inputs increase and temperatures rise, ammonia (NH_3) concentration is likely to be associated with stressful temperatures. To investigate the interaction between NH_3 and temperature on aquatic invertebrate survival, we performed a factorial experiment on the survival and molecular response of *Gammarus pulex*, with temperature (10, 15, 20, and 25 °C) and NH_3 (0, 0.5, 1, 2, 3, and 4 mg NH_3/L) treatments. We observed an unexpected antagonistic interaction between temperature and NH_3 concentration, meaning survival in the 4 mg NH_3/L treatment was higher at 25 °C than at the control temperature of 10 °C. A toxicokinetic-toxicodynamic (TK-TD) model was built to describe this antagonistic interaction. While the No Effect Concentration showed no significant variation across temperatures, the 50% lethal concentration at the end of the experiment increased from 2.7 (2.1–3.6) at 10 °C to 5.5 (3.5- 23.4) mg NH_3/L at 25 °C. Based on qPCR data, we associated these survival patterns to variations in the expression of the *hsp70* gene, a generic biomarker of stress. However, though there was a 14-fold increase in *hsp70* mRNA expression for gammarids exposed to 25 °C compared to controls, NH_3 concentration had no effect on *hsp70* mRNA synthesis across temperatures. Our results demonstrate that the effects of combined environmental stressors, like temperature and NH_3, may strongly differ from simple additive effects, and that stress response to temperature can actually increase resilience to nutrient pollution in some circumstances.

1. Introduction

Consequences of global change are often considered independently as isolated drivers of biodiversity loss (Chapin et al., 2010; Loreau et al., 2001; Steudel et al., 2012). In natural ecosystems, multiple environmental forces interact, leading to multi-stress situations (Dehedin et al., 2013a; Travis, 2003). Despite the importance of considering these combined effects (Dehedin et al., 2013a, 2013b; Didham et al., 2007; Dukes and Mooney, 1999; Heino et al., 2009; Walther et al., 2002), synergisms and interactions between multiple stressors are difficult to conceptualize and quantify, and are often overlooked in ecological studies. Individual treatment of multi-dimensional stressors introduces uncertainty in predictive models for species distribution patterns (Chapin et al., 2000; Seneviratne et al., 2006).

Ammonia is a common anthropogenic pollutant in stream ecosystems (Alonso and Camargo, 2004; Piscart et al., 2009; Prenter et al., 2004). The most common sources of ammonia inputs include urban and agricultural runoff, industrial activity, and mismanaged waste water (Jeppesen et al., 2009; Maltby, 1995; Piscart et al., 2009; Wagner and Benndorf, 2007). While background concentration of ammonia is usually low in the environment, it may rise locally and periodically (Alonso and Camargo, 2015; Maltby, 1995) due to precipitation events or waste water runoff (Seager and Maltby, 1989). High water temperature can aggravate ammonia pollution because the decreased dissolved oxygen concentration associated with warmer water can impede nitrification and promote reduction of nitrate to ammonia by microorganisms, increasing ammonia concentration, particularly when nitrate concentration is high (Jensen et al., 1994; Navel et al., 2013).

Ammonium (NH_4^+), is typically inert in aquatic environments, whereas the un-ionized form, the ammonia (NH_3), is highly toxic (Alonso and Camargo, 2004). Ammonia induces severe stress on cells by disrupting respiratory metabolism and membrane Na^+/K^+-ATPase activity, impairing organism survival, activity and growth (Dehedin et al., 2013a; Li et al., 2014; Mummert et al., 2003; Naqvi et al., 2007; Prenter et al., 2004). Environmental factors such as water temperature and pH determine the equilibrium between ammonium and ammonia, with warm and alkaline water strongly favoring NH_3 (*e.g.* at neutral pH, an increase from 10 °C to 20 °C will approximately double the

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concentration of NH_3) (Emerson et al., 1975). While multiple consequences of ammonia on biodiversity have been described (Piscart et al., 2009; Williams et al., 1985), its physiological effects in combination with other environmental stressors have rarely been assessed (Maltby, 1995). In a context of global warming, exogenous and endogenous ammonia inputs as well as NH_4^+/NH_3 equilibrium shifts may become much more common, potentially enhancing deleterious environmental effects from ammonia in the future (Dehedin et al., 2013b; Handy, 1994).

The present experimental study was conducted on the species *Gammarus pulex* (L. 1758, Amphipoda: Gammaridae). This species plays a central role in leaf litter decomposition in streams (Piscart et al., 2009, 2011a, 2011b), and population crashes could trigger cascades through the whole trophic network. Gammarids are sensitive to both NH_3 concentration (Piscart et al., 2009) and temperature (Cottin et al., 2015; Foucreau et al., 2014) and are therefore good biological models to assess interactions between these parameters. Our study quantified gammarids survival under continuous exposure to temperature and ammonia, alone and in combination. Additionally, we measured expression of the *hsp70* gene to assess the molecular response to temperature and ammonia stressors. Several studies have demonstrated up-regulation of *hsp70* in response to a wide array of stressors, including thermal and NH_3 stress, in a variety of arthropod taxa (Feder and Hofmann, 1999; Sung et al., 2014). Therefore, we expected some response of *hsp70* transcript expression to isolated stressors and their combination.

The combination of multiple stressors can result in various interactions. Folt et al. (1999) and more recently Côté et al. (2016) defined these patterns in relation to the neutral additive interaction in which the effect of multiple stress is equal to the sum of each isolated stress. Therefore, any effect stronger than the one predicted using the additive hypothesis is as a synergism, while any lesser response is an antagonism. We predicted (i) an additive or a synergistic effect from high temperature and NH_3 concentrations on the survival of *G. pulex* and (ii) an up-regulation of the *hsp70* gene in response to both temperature and NH_3 stress, as well as a synergistic interaction between these stressors.

2. Material and methods

2.1. Organism sampling and rearing

Adult gammarids were manually harvested in a stream (47°32′27″N, 2°3′25″W, Sévérac, France) between February and March 2015. Stream water temperature at the end of the sampling campaign was 9 °C, pH was 6.8, and dissolved O_2 concentration 11.8 mg/L (was 100% saturation). The stream's surrounding was wooded and did not have intensive agricultural activity. Adult gammarids were stored 24 h in a climate chamber (Percival, CLF PlantClimatics, Germany) set at 15 °C, with a 12 h:12 h day/night cycle and with continuously oxygenated water collected from the stream. They were then transferred into plastic boxes containing synthetic freshwater (96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4$, 60 mg/L $MgSO_4$, and 4 mg/L KCl in deionized water) with pH buffered at 7 according to the US EPA method (Anon, 1991). Gammarids were left to acclimate for 5 days in this water at 15 °C with a 12 h:12 h day/night cycle and with *ad libitum* industrial food for shrimp (Novo Prawn, JBL, Neuhofen, Germany). We performed this acclimation process to standardize the abiotic factors before exposing gammarids to stressful conditions.

2.2. Combined exposure to NH_3 and temperature, and measures of survival

The experiment was performed in open glass petri dishes (Ø 15 cm) filled with 350 mL of synthetic water. Four temperatures were selected to be comparable with previous studies on gammarids: 10, 15, 20 and 25 °C (Cottin et al., 2012, 2015; Foucreau et al., 2014). This range includes optimal (10 °, 15 °C), mildly stressful (20 °C), and strongly stressful (25 °C) temperatures. These thermal conditions where crossed with six nominal NH_3 concentrations (0, 0.5, 1, 2, 3 and 4 mg NH_3/L) in a full factorial experimental design, leading to a total of 24 experimental conditions. The NH_3 concentrations were selected after several pretests, allowing us to adjust the treatments used by Dehedin et al. (2013b), to get a range of mortality going from 0 to at least 90% for the strongest dose at the end of the experiment. NH_3 concentration was increased by adding ammonium chloride (NH_4Cl), taking into account the influence of temperature and pH on the chemical equilibrium NH_3/NH_4^+ (Emerson et al., 1975). Each treatment was applied to 10 randomly selected adult gammarids, replicated three times and maintained 196 h under experimental conditions. Mortality was checked at least twice a day, dead individuals were counted and then removed. Water was renewed on a daily basis in order to limit any marginal decrease in NH_3 concentration due to oxidation or volatilization, therefore ensuring stable and continuous experimental conditions.

2.3. Combined exposure to NH_3 and temperature, and hsp70 expression measurement

For measurements of *hsp70* mRNA expression, gammarids were acclimated as previously described (15 °C, 5 d) and then exposed to four temperatures (10, 15, 20 and 25 °C) crossed with three NH_3 concentrations (0, 1 and 4 mg NH_3/L), resulting in a total of 12 experimental conditions. Gammarids were exposed to each experimental condition for 6 or 24 h. We looked at *hsp70* mRNA expression after 6 and 24 h to get an estimate of *hsp70* expression after short term exposure (mimicking pollutant spikes) and after longer exposure. We did not perform longer exposures to avoid sampling after the onset of mortality, which could bias the sampling in favor of tolerant individuals. Three replicates of three pooled gammarids from each experimental condition and each exposure duration were flash-frozen in liquid nitrogen and stored at −80 °C for subsequent rt-qPCR analyses.

2.4. RNA extraction and cDNA preparation

For each treatment combination, pools of three gammarids were ground in liquid nitrogen using a pestle. The RNA was extracted in 600 µL of extraction buffer (Nucleospin® kit, Macherey-Nagel, Düren, Germany) with 1% β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA) and then isolated on mini-spin columns (Macherey-Nagel) following the manufacturer instructions. We thus extracted three RNA replicates for each treatment combination. The quality of RNA was checked with NanoDrop® 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and by running 1 µL on 1% agarose gel. Samples were diluted in RNase-free water in order to standardize concentrations of purified RNA. Five hundred nanograms of poly(A)$^+$ total RNA were used in the reverse transcription to complementary DNA (cDNA) using Superscript III First-Strand Synthesis System for qRT-PCR (Invitrogen™, Carlsbad, CA, USA), according to manufacturer instructions. The cDNA was diluted 10 times in DEPC-treated water and stored at −20 °C until use.

2.5. Quantitative real-time PCR

We quantified *hsp70* transcripts with rt-qPCR for all the 24 combined treatments (12 conditions x 2 exposure durations). We investigated mRNA expression of an inducible gene, *hsp70* (form 1), as well as a housekeeping reference gene *Gapdh* for *G. pulex*, as described by Cottin et al. (2015). Primer sequences used for *hsp70* gene were CCGAAGCTTACCTTGGAGGCACTG for the forward strand and GTTCGCCCCCAGTTTTCTTGTCC for the reverse strand. Primer sequences used for *Gapdh* gene were CCGAAGCTTACCTTGGAG GCACTG for the forward strand and GTTCGCCCCCAGTTTTCTTGTCC for the reverse strand. Reactions were performed in a LightCycler® 480 system (Roche™, Boulogne-Billancourt, France) with a SybrGreen I mix (Roche™) according to Colinet et al. (2010). Two technical replicates