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Stress response of *Chironomus riparius* to changes in water temperature and oxygen concentration in a lowland stream

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ABSTRACT

The increasing impairment of lotic ecosystems has promoted a growing effort into assessing their ecological status by means of biological indicators. Community-based approaches are a valuable way of assessing ecosystem changes, but reflect local extirpation and long-term changes and might not be suitable for tracking and monitoring short-term and sub-lethal events. Responses to rapid changes in environmental conditions have been rarely studied under natural conditions. Here we used a field experiment to test how the synthesis of heat shock protein 70 (HSP70) and haemoglobin (Hb) in laboratory-reared larvae of *Chironomus riparius* (Diptera, Chironomidae) were influenced by short-term changes to water temperature and oxygen concentration in a lowland stream. Our aim was to determine whether HSP70 mRNA expression and Hb content could be used as an *in situ* "early warning system" for freshwater habitats undergoing environmental change. HSP70 exhibited a clear response to changes in temperature measured over a one-day period, confirming its suitability as an indicator of environmental stress. Hb concentration was related to oxygen concentration, but not to temperature. Our findings support the hypothesis that depletion in oxygen induces Hb synthesis in *C. riparius* larvae. Because tolerance to low oxygen is not only related to total Hb, but also to a more efficient uptake (binding to Hb, e.g. Bohr effect) and release of oxygen to the cell (Root effect), we cannot discern from our data whether increased efficiency played a role. We suggest that *C. riparius* is a suitable model organism for monitoring sub-lethal stress in the field and that the approach could be applied to other species as more genomic data are available for non-model organisms.

1. Introduction

Streams and rivers are among the most threatened ecosystems, having been modified globally by catchment land-use changes, water abstraction, channelization, pollution and invasion of alien species (Vörösmarty et al., 2010; Dudgeon et al., 2006). Additionally, climate change is expected to alter hydrology and temperature regimes with severe effects on organisms and ecosystem functions (Ormerod and Durance 2012; Li et al., 2012; Floury et al., 2013). This increasing impairment of lotic ecosystems has promoted a growing effort into assessing their ecological status by means of biological indicators and sentinel species (Friberg, 2014). The classification of the ecological status of rivers is officially based on the assemblage structure of key taxonomic groups (e.g., Hering et al., 2003; Traversetti et al., 2015). While assemblage-based approaches have been proven valuable in the assessment of ecosystem integrity (Bae et al., 2014), they tend to focus on the local extirpation of sensitive taxa and overall changes in community composition. This approach may not be suitable for identifying and monitoring the effects of short-term events such as low flows or other sub-lethal episodic events, whose frequency and magnitude is expected to increase in the near future (Ledger and Milner 2015). Biomarker assays (i.e. non-lethal responses of biological systems) are often used in eco-toxicological studies to assess the effects of pollutants, but their potential for tracking environmental change in the field has

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received little attention (Traversetti et al., 2017). Ideally, integrating indicators in a hierarchical fashion, from sub-organismal to organismal, population and community levels (Sures et al., 2015) should improve the assessment of ecosystem health over multiple spatio-temporal scales (Cajaraville et al., 2000; Lagadic et al., 2000; Colin et al., 2016).

A promising approach is to use multiple indicators of stress in organisms (Frank et al., 2013). Multiple biomarkers may produce the benefit of integrating biological responses at different time scales and levels of organisation (Den Besten, 1998; Lagadic et al., 2000; Scalici et al., 2015). Two potential biomarkers for measuring sub-lethal effects in stream macroinvertebrates are heat shock proteins (HSP) and haemoglobin (Hb). HSP70 is a set of chaperon proteins involved in ensuring the correct folding and unfolding of proteins, and its expression is rapidly regulated by changes in physical (i.e. temperature) and chemical conditions (Lencioni et al., 2009; Lee et al., 2006), but it is not affected by handling stress (Sanders, 1993). The expression of HSP70 is therefore considered a short-term "early warning" indicator of environmental changes (Yoshimi et al., 2009; Folgar et al., 2015). For example, Lencioni et al (2013) observed an increase in HSP70 expression after 1 h of heat stress at 26 °C in a cold-adapted non-biting midge (Diptera, Chironomidae) larvae.

Chironomidae larvae can be abundant in degraded freshwater habitats, and are thus considered indicators of poor water quality and early colonizers after large-scale disturbances (Serra et al. 2017). Resistance and resilience of chironomids is often attributed to the presence of hemoglobin (Hb), which allows them to tolerate low oxygen concentrations (Moller Pillot, 2009). In *Chironomus riparius,* Choi et al. (2001) observed a 151% increase in total Hb after 24 h of hypoxia. Chironomidae larvae have been reported to secrete up to 16 different Hb types (Choi and Ha, 2009; Green et al., 1998). Such diversity of Hbs with specific binding properties allows for a fine-tuned loading and unloading of O2 that regulates its delivery to specific tissues under variable environmental conditions (Choi and Ha, 2009; Ha and Choi, 2008; Weber and Vinogradov, 2001).

We used a field experiment to test how HSP70 expression and Hb production were influenced by short-term changes to temperature and oxygen concentration in a lowland stream. Laboratory-reared larvae of *Chironomus riparius* (Diptera, Chironomidae), a widespread species considered a model organism in aquatic toxicology (Lee et al., 2006; Lencioni et al., 2009; Morales et al., 2011; Marinkovic et al., 2011) were placed in a stream and sampled over a period of 1–8 days, while experiencing rapid variation in temperature and oxygen concentration.

2. Methods

The study was carried out in the lowland stream Groote Molenbeek in Limburg, Netherlands. In June 2010, two experimental reaches (upstream, downstream) were designated along the stream, each *ca.* 50 m in length and separated by *ca.* 200 m. In July 2010 the upstream and downstream reaches were separated by an artificial dam and a bypass was constructed (Fig. 1a and b). The aim was to simulate summer drought conditions in the downstream reach, e.g., reduced water flow, reduced oxygen concentration, and increased water temperature. Experiments were performed in the upstream reach in June, prior to dam construction, and in both upstream and downstream reaches in August, after dam construction. No experiment was conducted in the downstream reach in June because abiotic conditions were nearly identical to those in the upstream reach. In August, heavy rainfall caused large and rapid variations in oxygen and water temperature in both reaches. While this event disrupted the desired effect of the experimental drought, it provided the opportunity to quantify short-term responses to sub-lethal environmental change in all reaches. Therefore, we did not compare control and experimental reaches, but rather we measured the physiological responses of *C. riparius* to the environmental changes experienced *in situ*. The following environmental variables were measured each day at 08:00 throughout the sampling periods in June and August: Temperature (°C), dissolved oxygen (mg O_2 l^{-1}), conductivity (µS cm^{-1}), and pH (measured with a Multi 340i/SET immersion probe WTW, Weilheim, Germany), water depth (cm) and flow velocity (m s^{-1}) (measured using a 2030 flow-meter; General Oceanics, Miami, USA).

Chironomus riparius individuals were obtained from a permanent laboratory population at the Department of Aquatic Ecotoxicology in Frankfurt am Main, Germany. The single origin presumably minimized the genetic diversity among individuals (Nowak et al., 2012). Eggs were shipped to the IGB in Berlin, and after hatching, larvae were reared in aquaria for 4 months prior to the experiment according to the OECD (2004) guidelines. Laboratory aquaria were filled with fine quartz sand as substrate. Aquaria were constantly aerated and kept in a climate chamber in controlled conditions (20 °C, light:dark 16 h:8 h). Larvae were fed with commercial TetraMin® fish food (Tetrawerke, Melle, Germany). Mesh cages (16 × 12 × 12 cm; mesh: 0.2 mm; Fig. 2a) were designed ad-hoc from aquarium isolation chambers (Hagen Marina, Montreal, Canada) and used to transfer *C. riparius* larvae from the laboratory to the field and to introduce larvae into the experimental reaches. This procedure enabled rapid sample collection, thus minimising handling stress.

At the start of experiments, 25 mesh cages, with 100 larvae each (third and fourth instar), were placed on the stream bottom (Fig. 2b) in each reach (upstream in June, upstream and downstream in August). Fourth-instar larvae were used for HSP70 expression analysis (collected after 24, 96 and 192 h of exposure) and Hb analyses (24, 48, 96 and 192 h of exposure; sample sizes in Appendix 1). Larvae were removed from cages with forceps, placed in cryo vials (Eppendorf), immediately placed in liquid nitrogen and stored at −80 °C until analysis.

Total RNA was extracted from 7 individuals per time point and reach (n = 63; Appendix 1) using a Rneasy Mini kit (Qiagen, Hilden, Germany) with on-column DNase digestion (Trubiroha et al., 2009). RNA concentration was measured using a Nanodrop ND-1000 (Thermo Fisher Scientific, Darmstadt, Germany). Reverse transcription was carried out with Affinity Script transcriptase (Agilent/Stratagene, Waldbronn, Germany). Primers for HSP70 and ß-actin (Appendix 2) were designed using data from Park et al. (2010) and Morales et al. (2011) and specificity was confirmed by direct sequencing. QPCR was carried out with a Mx3005 (Agilent/Stratagene) using hot start polymerase (Phire Taq II, Life Technologies) and SYBR Green in a 20 µL reaction volume (2 µL diluted cDNA, 375 nM of each primer, 1x Taq buffer, 2 mM MgCl2, 0.5 mM each dNTP, 0.5 fold diluted SYBR-Green I solution, 1 U polymerase) under the following conditions: 98 °C initial denaturation for 4 min, followed by 40 cycles of 98 °C denaturation for 20 s, 62 °C primer annealing for 15 s, and 72 °C extension for 20 s. PCR efficiencies were determined in triplicate with a dilution series of pooled cDNA (ß actin 99.6%; HSP70 98.4%). All samples were determined in duplicate. Expression was determined by the comparative $\Delta\Delta$ C_T method (Pfaffl, 2001) with ß actin used as a baseline (housekeeping) gene considering a calibrator sample (pooled cDNA) and correction for efficiency. Specificity of amplification was monitored by melting curve analysis.

Total Hb was measured in nine individuals per time point and reach (n = 108; Appendix 1) using the cyanomethemoglobin method with a diagnostic haemoglobin reagent (DiaSys, International, Holzheim, Germany) as described by Wuertz et al. (2013). All samples were measured twice with an Infinite 200 microplate reader (Tecan, Mainz-Kastel, Germany) at 540 nm and concentration was calculated using a standard dilution series (120 mg/L haemoglobin standard, Diaglobal GmbH, Berlin, Germany). Total Hb was normalized to the total protein concentration determined by the Bradford (1976) method (RotiQuant Kit, Germany) as µg Hb/ µg total proteins.

Linear mixed-effect (LME) models were used to analyse variation in HSP70 expression and Hb concentration in relation to variation in environmental conditions. We used LME models to account for repeated sampling. After testing for collinearity using a Spearman test (also from