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Behavioural and metabolic responses of *Unionida* mussels to stress

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Abstract

1. The aim of this study was to assess the extent to which the behavioural traits of freshwater mussels provide suitable indicators of stress in individuals, towards the advancement of non-invasive, remote monitoring techniques to examine population condition.
2. Variation in the expression of particular behavioural metrics was examined in accordance with measurements of oxygen consumption, across environmental stressors (aerial exposure and high concentrations of total suspended solids), and between two freshwater mussel species (*Margaritifera margaritifera* and *Anodonta anatina*).
3. Aerobic metabolic rate was quantified using intermittent respirometry, and behaviour was observed using time-lapse footage. Comparisons of metabolic response and the occurrence of behavioural traits, across the two stressors, focused on differences between the 24 h pre-exposure period (pre-exposure), the first 3 h of post-exposure (immediate post-exposure), and the time following the initial 3 h of post-exposure until the end of the experimental run (extended post-exposure).
4. The results of this study demonstrated a relationship between the frequency of occurrence of behavioural responses to stress exposure, associated with valve activity, and significant changes in the metabolic functioning of *A. anatina* and *M. margaritifera* mussels. The findings from the study also highlighted substantial intraspecific variation across species and stressors.
5. Data from this research could assist in the development of novel biosensors that track mussel valve activity remotely in their natural environment. When coupled with real-time data examining alterations in environmental metrics, this technology could assist in the monitoring of population condition and aid conservation management.

KEYWORDS

aerial exposure, emersion, remote sensing, stress response, sublethal endpoint, TSS, unionid

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1 | INTRODUCTION

Freshwater bivalve mussels of the order Unionida are one of the fastest diminishing taxa globally (Lydeard et al., 2004; Graf & Cummings, 2007; Geist, 2011). They are considered to be vital to the health of the wider freshwater ecosystems (Vaughn, 2018), functioning as biomonitors of adverse habitat conditions (Vaughn, 2010; Scheder et al., 2015; Lummer, Auerswald & Geist, 2016), enhancing nutrient cycling and trophic interactions in freshwater communities (Vaughn, 2010; Allen et al., 2012; Boeker et al., 2016), and contributing to habitat diversity in benthic environments (Spooner & Vaughn, 2008; Vaughn, Nichols & Spooner, 2008; Boeker et al., 2016). Despite this, conservation efforts have often been constrained by a limited understanding of their biology and insufficient identification of conservation units (Fraser & Bernatches, 2001; Ferreira-Rodríguez et al., 2019; Lopes-Lima et al., 2020).

One area of study that has witnessed increasing interest as a means of addressing these gaps in knowledge concerns the use of biomonitoring tools, or the tracking of specific biological processes and how these processes respond to alterations in the environment (Galloway & Depledge, 2001; Gagné et al., 2002; Blaise & Gagné, 2009; Farcy et al., 2013; Fritts et al., 2015). The study of biological responses may assist in detecting early warning signs before the occurrence of mortality (Handy & Depledge, 1999), provide a method to study the effects of sublethal stressors (Hartmann et al., 2016), and aid the evaluation of population condition in response to translocation and restoration efforts (Gray & Kreeger, 2014; Roznere et al., 2017; Salerno et al., 2018).

Behaviours reflect the response of an individual to a combination of environmental and physiological factors, and therefore have the capacity to provide sensitive, non-invasive indicators of stress in individuals (Robson et al., 2009; Hasenbein et al., 2015; Hartmann et al., 2016). Examples within the literature of non-lethal techniques for examining stress in freshwater and marine mussels often focus on two behavioural responses: movement (specifically, how a mussel may use its foot to move along the river bed or to burrow into the substrate—Johnson & Brown, 2000; Bartsch et al., 2010; Block, Gerald & Levine, 2013; French & Ackerman, 2014; Clements, 2015) and filtration (the active movement of water through the mantle, which facilitates respiratory and reproductive processes); previous studies suggest that both valve activity and clearance rates mirror individual responses to environmental change (Wilson, Reuter & Wahl, 2005; Nagai et al., 2006; Robson et al., 2012; Tuttle-Raycraft, Morris & Ackerman, 2017; Salerno et al., 2018). Both behaviours have the potential to provide an easy and cost-effective biomarker of stress (Kádár et al., 2001; Newton & Cope, 2006; Liao et al., 2009; Robson et al., 2009; Hartmann et al., 2016; Lummer, Auerswald & Geist, 2016), which could be scaled up to populations and species.

Despite interest regarding the use of behavioural traits as potential non-invasive indicators of stress in freshwater mussels,

few studies have researched the physiological mechanisms that may drive their expression during stress exposure (Farcy et al., 2013; Archambault, Cope & Kwak, 2014). Research examining oxygen consumption rates ($\dot{M}O_2$ —a measure of aerobic metabolic rate) in other aquatic species has received significant attention as a method for testing hypotheses that relate variation in physiological traits with intraspecific variation in behaviour and life history traits (Biro & Stamps, 2010; Burton et al., 2011; Rosewarne, Wilson & Svendsen, 2016), with recent research demonstrating the efficacy of these techniques to evaluate stress in unionid mussels (Gibson, 2019; Haney, Abdelrahman & Stoeckel, 2020). In the study reported here, stress responses in freshwater mussels were examined, through the analysis of their behavioural traits, in conjunction with aerobic metabolic rate.

Very few studies that link the expression of behavioural traits with physiological condition in freshwater mussel responses to stress have examined individual variability (Hartmann et al., 2016). During stress exposure, animals may prioritize specific physiological functions and behaviours (Killen et al., 2013); however, the expression of a trait may not be consistent across species and individuals (Dingemans et al., 2009; Biro & Stamps, 2010; Burton et al., 2011; Jolles et al., 2017). Specifically, the extent to which an individual prioritizes the expression of particular behavioural and physiological traits during stress exposure is thought to vary between conspecifics (Dingemans et al., 2009). Therefore, to assess the consistency of metabolic and behavioural responses to stress across unionid species, the responses of two unionid species were examined: the freshwater pearl mussel, *Margaritifera margaritifera*; and the duck mussel, *Anodonta anatina*. These species were chosen because they display similar distributional patterns and life-history traits yet appear to differ in their habitat requirements, which may assist in distinguishing species-specific thresholds in response.

Both *M. margaritifera* and *A. anatina* inhabit freshwater systems across Europe — ranging from the Iberian Peninsula in the south west, to Scandinavia in the north, and to Russia in the east (Zettler et al., 2006; Boon et al., 2019) — and have been known to exist in sympatry, using the same host, brown trout (*Salmo trutta*; Chowdhury et al., 2018). Within Europe, *A. anatina* is widespread, with a conservation status of Least Concern (IUCN, 2014); however, in some regions the species is listed as Near Threatened or Vulnerable (Lopes-Lima et al., 2016). The species appears adaptable to a wide range of habitat characteristics, inhabiting both lentic and lotic freshwater systems (Lopes-Lima et al., 2016). By contrast, the habitat requirements of *M. margaritifera* are more restrictive, with sustainable populations often associated with extremely oligotrophic conditions (Bauer, 1988). The species is classified as Critically Endangered in Europe (Moorkens, 2011), and populations are protected by the European Habitats Directive, which provides for safeguarding via designated Special Areas of Conservation (Council of the European Communities, 1992). Despite this, declines across *M. margaritifera* populations persist (Geist, 2010; Cosgrove et al., 2016; Lopes-Lima et al., 2016).

Recent work by Boon et al. (2019) underlined efforts to improve the monitoring of populations of *M. margaritifera* across the species' range to facilitate more effective conservation management. However, no known study has directly examined the response of *M. margaritifera* to isolated stressors in a laboratory setting to ascertain the thresholds at which environmental factors significantly reduce individual condition. Although such work has been undertaken with *A. anatina*, it has focused solely on identifying the response of behavioural traits to chemical pollutant stressors, such as road salts (Beggel & Geist, 2015; Hartmann et al., 2016), with little consideration towards other environmental stressors. The prevailing ecological conditions within an animal's habitat are known to accentuate the importance of particular traits; thus, different stressors may evoke different responses and, therefore, highlight otherwise subtle differences between populations and species (Cook, Wells & Herbert, 2011; Killen et al., 2013). To provide context to the expression of certain traits, individual responses should be observed across several environmental parameters, representative of common stressors a population experiences in the natural habitat, and presented at a magnitude necessary to evoke a response, thus determining whether the response is linear or has a threshold effect. Consequently, this study aimed to compare the response of the two species across two stressors.

There are two stressors to unionid mussels commonly identified in the literature that might be expected to induce stress: first, a reduced river discharge resulting in the emersion (i.e. exposure to air) of benthic living mussels (Bradley, Cadman & Milner, 2012; Environment Agency, 2013; Scottish Environment Protection Agency, 2014; Newton, Zigler & Gray, 2015; Lopes-Lima et al., 2016; Sousa et al., 2018); and second, heightened concentrations of total suspended solids (TSS), resulting from erosional and depositional processes (Naimo, Atchison & Holland-Bartels, 1992; Frank & Gerstmann, 2007; Addy, Cooksley & Sime, 2012; Tuttle-Raycraft, Morris & Ackerman, 2017).

The purpose of this study was to investigate mussel behaviour as a biomarker for stress in unionid mussel species towards the creation of new techniques to assist in their conservation. To do so, this study tested the following three hypotheses: (i) the physiology of mussels, measured as oxygen consumption, shows a quantitative response to stressors; (ii) the expression of certain behavioural traits, measured as frequency of occurrence, shows a quantitative response to stressors; and (iii) behaviour can be used as a non-invasive, non-destructive biomarker of underlying physiology in freshwater mussel species.

2 | METHODS

2.1 | Mussel collection

A sample of *A. anatina* was collected in December 2016 from Ryat Linn reservoir, East Renfrewshire, Scotland, during reservoir maintenance work when water depth was reduced, permitting access to deep, silty areas of the bed. A sample of *M. margaritifera* mussels was collected, under licence, in the summer of 2017 from a mill lade, hydrologically connected to the main channel of the South Esk River, Scotland. The substratum in the lade largely consisted of uniform beds containing fine silt and gravel, interspersed with larger boulders and wood. Adult mussels were removed by hand and stored in aerated cool boxes, lined with substrate and filled with water from the corresponding system. The individuals collected were held in two tanks (one for each species) at the Scottish Centre for Ecology and the Natural Environment. Each tank contained washed gravel (0.1–25 mm) to a depth of 100 mm and fed with water from Loch Lomond at ambient temperature ($14 \pm 4.2^\circ\text{C}$, annual mean temperature plus/minus SD) to a depth of 200 mm and a flow rate to mimic conditions of the respective habitats. Each individual mussel was marked for identification on the shell using typists' correction fluid, weighed, and measured for shell length, width, and height (Table 1).

2.2 | Experimental set-up

The experimental set-up (Figure 1) consisted of four metabolic chambers (1.25 L). Each chamber was placed within a separate holding tank (70 L) and submerged in water. Washed gravel (0.1–25 mm) was placed in each chamber to a depth of 50 mm. Air stones were placed within each holding tank to maintain maximum dissolved oxygen levels. Underwater digital cameras (GoPro Hero 8 Black; GoPro Inc., San Mateo, CA, USA) were positioned inside the holding tanks, facing the metabolic chamber and programmed to capture time-lapse footage (one image per minute). A wooden plank was placed across the length of the holding tank to accommodate individuals during the emersion exposure experiments, with camera mounts fastened to the plank in locations to enable continuous capture of behavioural responses. Adequate water circulation within the holding tanks was maintained using two Eheim pumps (1046 Universal; Eheim GmbH, Deizisau, Germany). The experimental apparatus was located within a constant-temperature unit at the Scottish Centre for Ecology and the Natural Environment, ensuring water temperatures were maintained at $15 \pm 0.5^\circ\text{C}$ throughout an experimental run.

TABLE 1 Summary of the morphological characteristics (mean \pm SD) found in the experimental sample populations of *Anodonta anatina* and *Margaritifera margaritifera*

Species	Length (mm)	Width (mm)	Height (mm)	Total weight (g)
<i>A. anatina</i>	90.45 \pm 3.03	49.14 \pm 2.16	27.75 \pm 2.20	81.98 \pm 7.35
<i>M. margaritifera</i>	93.33 \pm 8.18	41.70 \pm 3.57	25.41 \pm 2.34	62.80 \pm 13.74

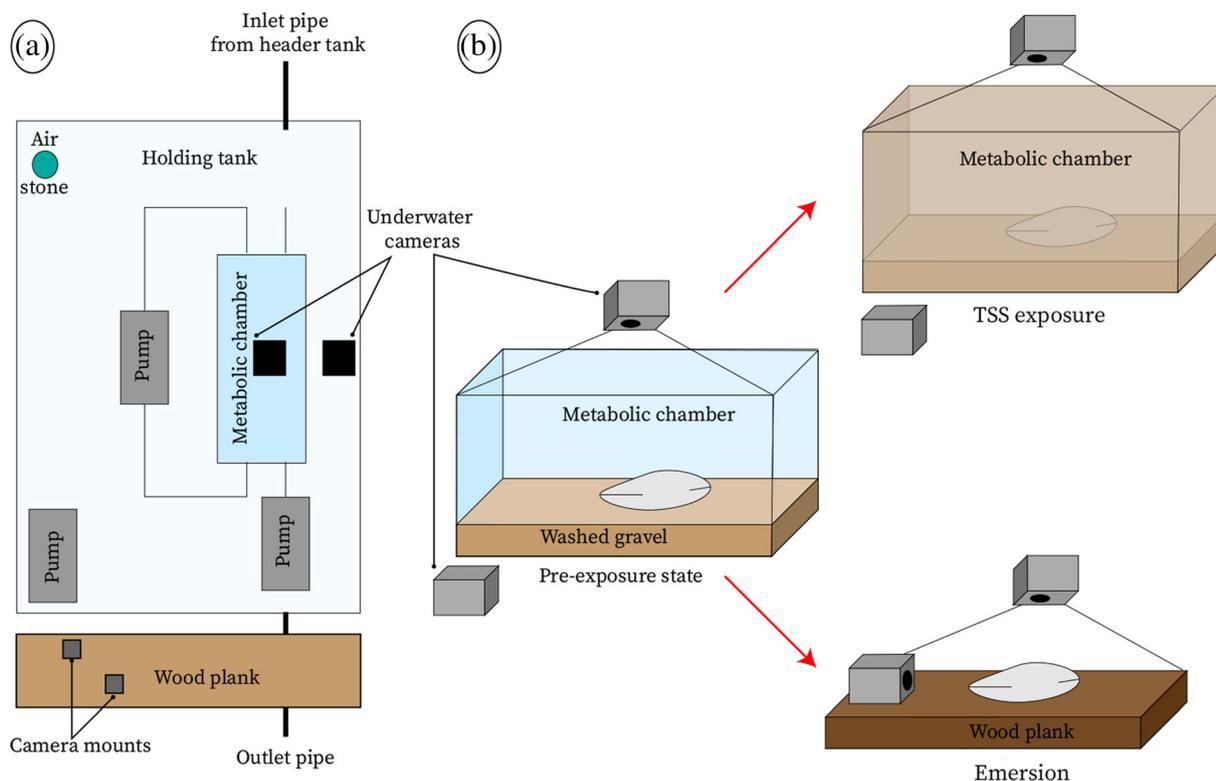


FIGURE 1 (a) Experimental set-up within each of the four holding tanks; (b) the conditions a mussel experienced during pre-exposure and two stressor exposures: emersion and increasing concentrations of total suspended solids (TSS)

TABLE 2 An overview of the stressors used to elicit a physiological and behavioural response in *Anodonta anatina* and *Margaritifera margaritifera* mussels

Stressor	Implementation of exposure	Control conditions	Low stress conditions	Medium stress conditions	High stress conditions
Emersion	Removal from metabolic chamber and placed in terrestrial conditions	Remain in metabolic chamber throughout experimental run	Placed in terrestrial environment for 1 h 30 min and then placed back into metabolic chamber	Placed in terrestrial environment for 3 h and then placed back into metabolic chamber	Placed in terrestrial environment for 4 h 30 min and then placed back into metabolic chamber
TSS	Addition of 'Polysperse 10' kaolin to the water of holding tank until a pre-set turbidity measurement (NTU) reached	0 NTU for 3 h 30 min	2 NTU (Polysperse 10: 1 mg L ⁻¹) for 3 h 30 min and then flushed out and replaced with 0 NTU water	20 NTU (Polysperse 10: 153 mg L ⁻¹) for 3 h 30 min and then flushed out and replaced with 0 NTU water	40 NTU (Polysperse 10: 320 mg L ⁻¹) for 3 h 30 min and then flushed out and replaced with 0 NTU water

Note: For the two stressors, the method for eliciting stress exposure and the variation in extent of stress exposure between the stressor magnitude groups is shown. For experiments examining heightened concentrations of total suspended solids (TSS) as a stressor, 'Polysperse 10' kaolin was added to the water of the holding tanks to achieve a desired nephelometric turbidity unit (NTU).

2.3 | Experimental overview

The experiment was designed to compare the physiological response (metabolic rate) and behavioural responses of the same individuals of two different mussel species to two different

stressors: air exposure and suspended sediment (Figure 1). A total of 40 mussels (20 from each species) were randomly selected for the experiment. Each individual was exposed to the two stressors or control conditions. There were four treatments for each of the emersion and TSS experiments, including one control condition

(see Table 2 for details of magnitude). Before, during, and after exposure to each stressor condition, the behaviour of each individual was quantified, with oxygen consumption rates recorded before and after exposure.

Each trial was conducted on four mussels simultaneously, each experiencing one of four treatment conditions (low, medium, and high stress magnitude and a control group), and consisted of six sequential steps: (i) a 2 day acclimation period; (ii) a 2 h background check; (iii) a 24 h pre-exposure period; (iv) a stress exposure (Table 2); (v) a minimum 18 h post-exposure period; and (vi) 2 h background check. An individual experienced two trials, one with each of the two stressors. A period of 6 weeks rest was given to each mussel between the two trials, with marginal differences in individual standard metabolic rate (SMR) suggesting this was adequate for recovery (Table 3).

Before the experimental stress exposure commenced, all mussels were acclimated to an experimental temperature, 15 ± 0.5 °C, for 2 days in a 30 L tank with untreated fresh water pumped from Loch Lomond and natural algal concentrations. Two background checks, undertaken in the absence of mussels, recorded oxygen reduction in the metabolic chambers for 2 h, before and after a trial, to obtain measures of microbial respiration (Svendsen, Bushnell & Steffensen, 2016). Oxygen concentration in the metabolic chambers during this period was regressed on time in both background check periods to quantify changes in background respiration over the course of a trial. Approximated background respiration was subsequently subtracted from measurements of mussel oxygen consumption. After the background check, individuals were placed in the corresponding metabolic chamber for a pre-exposure period. Here, mussels remained for 24 h undisturbed to record potential diurnal fluctuations in metabolic rate, and provide sufficient acclimation time (Gibson, 2019). Following this, mussels were exposed to the relevant stressor (Table 2). After stress exposure, mussels were left undisturbed in the metabolic chambers for a minimum of 18 h before removal from the experimental set-up and the final background check commenced.

Comparisons of metabolic responses across the two stressors focused on differences in oxygen consumption between the 24 h pre-exposure period (pre-exposure), the first 3 h of post-exposure (immediate post-exposure, and the time following the initial 3 h of post-exposure until the end of the experimental run (extended post-exposure).

TABLE 3 A summary of the calculations for standard metabolic rate (SMR; $\text{mg O}_2 \text{ hr}^{-1} \text{ kg}^{-1}$) in *Margaritifera margaritifera* and *Anodonta anatina* sample populations

Species	Mean (\pm SD)	Range	Average individual variation
<i>M. margaritifera</i>	0.013 \pm 0.004	0.006–0.024	6.13
<i>A. anatina</i>	176.290 \pm 55.360	89.52–262.16	2.93

Calculations of SMR in *A. anatina* used measurements of individual dry tissue weight, whereas calculation of SMR in *M. margaritifera* used individual wet tissue weights, confounding comparisons between species. The percentage difference between an individual's SMR readings for each of the two stressor experiments was calculated using $(\text{SMR emersion}/\text{SMR total suspended solids}) \times 100$, with average individual variation for each species shown.

2.4 | Oxygen consumption

Oxygen consumption ($\dot{\text{M}}\text{O}_2$; $\text{mg O}_2 \text{ h}^{-1}$) was measured using intermittent respirometry, using a computer-controlled set-up that recorded oxygen partial pressure and temperature (sampling rate, 10 s). Water oxygen content in the metabolic chambers was measured using optodes (FireSting 4-channel oxygen meters; Pyroscience, Aachen, Germany; www.pyro-science.com). Intermittent respirometry was conducted according to the technique described by Svendsen, Bushnell & Steffensen (2016). One complete measurement cycle ('loop') comprised a 5 min 'open-system flush period' (flush state) and a 30 min 'closed-system, metabolism determination cycle' (closed state). This loop repeated consecutively until the end of an experimental run. Individual $\dot{\text{M}}\text{O}_2$ was recorded during the 'closed state' by measuring oxygen reduction in the metabolic chamber, calculated using linear least-squares regression. The first 10 min and the final 2 min of the closed-state readings were excluded to ensure the linear component of oxygen reduction was captured in the absence of 'noise' (Svendsen, Bushnell & Steffensen, 2016) resulting from pump operation.

2.5 | Metabolic rate analysis

To determine an individual's metabolic rate as a proportion of metabolic tissue ($\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$) required metabolic tissue weights. To obtain *A. anatina* metabolic tissue weights, mussels were euthanized at the end of the experiment. Harvested tissues were dried at 70 °C for 2 days to provide final dry tissue weights. Individual *M. margaritifera* were not euthanized to obtain dry tissue weights owing to their endangered status. Instead, wet tissue weights were estimated. Empty shells were collected from the sampled population. To estimate live shell weight, the relationship between shell length, width, and height and dry weight was calculated using a linear regression constructed from dead shells. Estimated live shell weight was then estimated from shell linear dimensions and subtracted from the total wet weight of live individuals, to estimate wet tissue weight for live *M. margaritifera*. Mass independent metabolic rates (MIMRs) were calculated to standardize metabolic rates and reduce the intraspecific variation (up to threefold differences in SMR were observed between conspecifics), using residuals from a regression analysis between SMR and tissue weight ($P < 0.001$) (Auer et al., 2015). Body mass and metabolic rates were

\log_{10} -transformed before analyses to normalize and linearize the data. Individual SMRs ($\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$) were calculated using oxygen consumption measures in the final 10 h of pre-exposure. Readings taken during this period, within 1 SD of the mean, were averaged to generate a final estimated SMR for the corresponding individual (see Table 3 for summary of calculations).

2.6 | Behavioural analysis

Behavioural analyses were conducted only on mussels experiencing high and medium stress magnitude. Underwater digital cameras captured time-lapse footage from 2 h before stress exposure to 4 h after stress exposure. Behaviour was quantified from video film only during 30 min closed phases of intermittent respirometry cycles. Three behavioural metrics were quantified: (i) 'transition frequency', defined as the number of observations where the width of a mussel's shell aperture changed between successive images; (ii) 'avoidance behaviour', defined as the number of observations where the mussel's shell was closed; and (iii) 'foot extension', which recorded the number of observations where the foot of the mussel protruded from the shell and was clearly visible. This is distinctly different from the undisturbed resting behaviour of a mussel, during which the foot is anchored into the substrate and is no longer visible. Comparisons of observed behaviour comprised the mean time or frequency of each behaviour during each of three time periods (pre-exposure, stress exposure, and post-exposure).

2.7 | Statistical analysis

Data were investigated using mixed effects models in R version 3.5.3 (R Core Team, 2020).

2.7.1 | Physiological response

Three statistical approaches were used to analyse alterations in individual metabolic rate over time and in response to varied levels of stress magnitude. First, individual MIMR measures, recorded at each 30 min 'closed state', were used as the only response variable in a mixed-effects model, with 'time' (pre-exposure, immediate post-exposure, and extended post-exposure), 'stress magnitude' (high, medium, low, or control) as covariates and 'individual' as a random variable. Second, individual metabolic differential was calculated by taking the difference in mean MIMR between pre-exposure and immediate post-exposure, irrespective of the direction of change (i.e. positive or negative). The metabolic differential was entered as the primary response variable in a mixed-effects model, with stress magnitude as a covariate and individual as a random variable. Finally, metabolic variability was calculated using confidence intervals (CIs) of the mean MIMR for pre-exposure, immediate post-exposure, and extended post-exposure. The CI was entered into a mixed-effects

model as the primary response variable, with stress magnitude and time as covariates and individual as a random variable.

2.7.2 | Behavioural response

Analysis of each behavioural response (transition frequency, avoidance behaviour, and foot extension) was undertaken separately using two statistical approaches. The occurrence of the behavioural metric was analysed across an experimental run, using the average length of time the behavioural metric was observed during 30 min closed states as the primary response variable in a mixed-effects model, with stress magnitude (high or medium) and time (pre-exposure, stress exposure, and post-exposure) entered as covariates and individual as a random variable. A two-step approach was used to assess the relationship between the occurrence of a behaviour and an individual's metabolic rate, applying a mixed-effects model designed to handle zero-inflated data. The presence or absence of the behavioural metric during the closed state was entered as the primary response variable into an initial model, with time (pre-exposure, immediate post-exposure, and extended post-exposure), stress magnitude (high or medium), and MIMR entered as covariates and individual and time (minutes) as random variables. A second linear mixed-effects model was then created to determine which covariates affected the occurrence of the behavioural metric in the positive non-zero data, with individual and time (minutes) as random variables.

3 | RESULTS

3.1 | Metabolic rate

Analysis of MIMR revealed differences in metabolic response between the two species (Figure 2). There was a significant effect of stressor magnitude on MIMR in both species during emersion experiments. In *M. margaritifera*, a significant difference was observed between the control group and the high stress magnitude group ($P < 0.05$). For *A. anatina*, there was a significant difference between high-magnitude emersion and the control ($P < 0.05$) (Figure 2). For TSS exposure experiments, marginal differences in *M. margaritifera* MIMR between the control and low-magnitude grouping were found ($P = 0.08$), in addition to marginal differences between control and high-magnitude grouping ($P = 0.08$). No significant differences between the control group and the stressor magnitude groups were found in the TSS exposure experiments for *A. anatina*.

Analysis of MIMR over time during emersion experiments showed that MIMR was significantly lower during extended post-stress exposure ($P < 0.001$) than during pre-stress exposure in the high stress magnitude grouping for *M. margaritifera*. The same analysis for *A. anatina* found significantly lower MIMR during immediate post-exposure compared with pre-exposure ($P < 0.01$) in

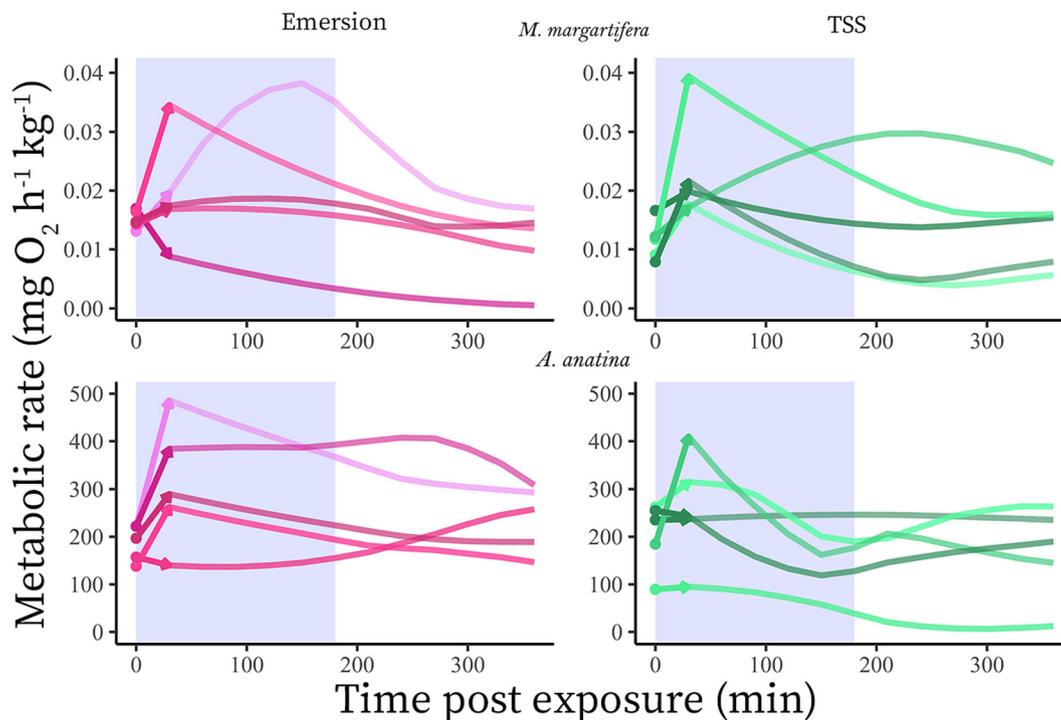


FIGURE 2 Variation in metabolic rate of individual mussels in response to high-magnitude stress exposure. A comparison of changes in metabolic rate of *Margaritifera margaritifera* and *Anodonta anatina* following emersion and total suspended solids (TSS) exposure. Each curve represents data for one individual and has undergone LOESS smoothing in R. Each line begins with a dot at the point of standard metabolic rate for the corresponding individual. An arrow from the point of standard metabolic rate to the first metabolic reading at 30 min displays the initial direction of change in metabolic rate. Shaded area represents immediate post-exposure period, with extended post-exposure present thereafter

the medium-magnitude group. By contrast, MIMR increased significantly during immediate post-exposure compared with pre-exposure in the high stress magnitude group for *A. anatina* ($P < 0.001$). No further significant differences in MIMR over time were identified for emersion or TSS exposure experiments in *M. margaritifera*. However, during TSS experiments in *A. anatina*, the MIMR increased significantly during immediate post-exposure when compared with pre-exposure ($P < 0.01$) across all stress-magnitude groups.

3.2 | Metabolic differential

Assessment of the metabolic differential revealed similarities between the two stressors and between *M. margaritifera* and *A. anatina* (Figure 3). A significant effect of stressor magnitude on the size of the metabolic differential during emersion experiments was shown in both species. For *M. margaritifera*, the differential was significantly higher in the low ($P < 0.05$) and high stressor magnitude ($P < 0.05$) groups than in the control group. Similarly, the metabolic differential was significantly higher in the low stressor magnitude groups than in the control ($P < 0.05$) in *A. anatina*. For TSS exposure experiments, no significant differences between stress magnitude groups and the control were found in *M. margaritifera* and *A. anatina*.

3.3 | Metabolic variability

Examination of metabolic variability (CI) for *M. margaritifera* and *A. anatina* revealed some similarities in responses (Figure 4). A significant effect of stress magnitude on metabolic variability in both TSS ($P < 0.05$) and emersion experiments ($P < 0.05$) was found in both species. Examination of emersion experiments showed a significant increase ($P < 0.05$) in CIs for all three stress magnitude groups when compared with the control, in both species, with no significant differences between stress magnitude groups shown in either species. Analysis of TSS exposure found a significant increase ($P < 0.05$) in CIs for all three stress magnitude groups when compared with the control in *M. margaritifera*. By contrast, this only applied to the medium- ($P < 0.05$) and high-magnitude ($P < 0.05$) groups for *A. anatina*.

Examination of CIs over time showed similarities between the two stressors and the two species. During emersion and TSS exposure experiments, *M. margaritifera* within each of the low ($P < 0.01$), medium ($P < 0.001$), and high stress magnitude ($P < 0.001$) groups displayed a significant increase in CIs during immediate post-exposure compared with pre-exposure. During emersion and TSS exposure experiments with *A. anatina*, the control group ($P < 0.05$), as well as each of the low ($P < 0.01$), medium ($P < 0.001$), and high stress magnitude ($P < 0.001$) groups, displayed a significant increase in CIs during immediate post-exposure compared with pre-exposure.

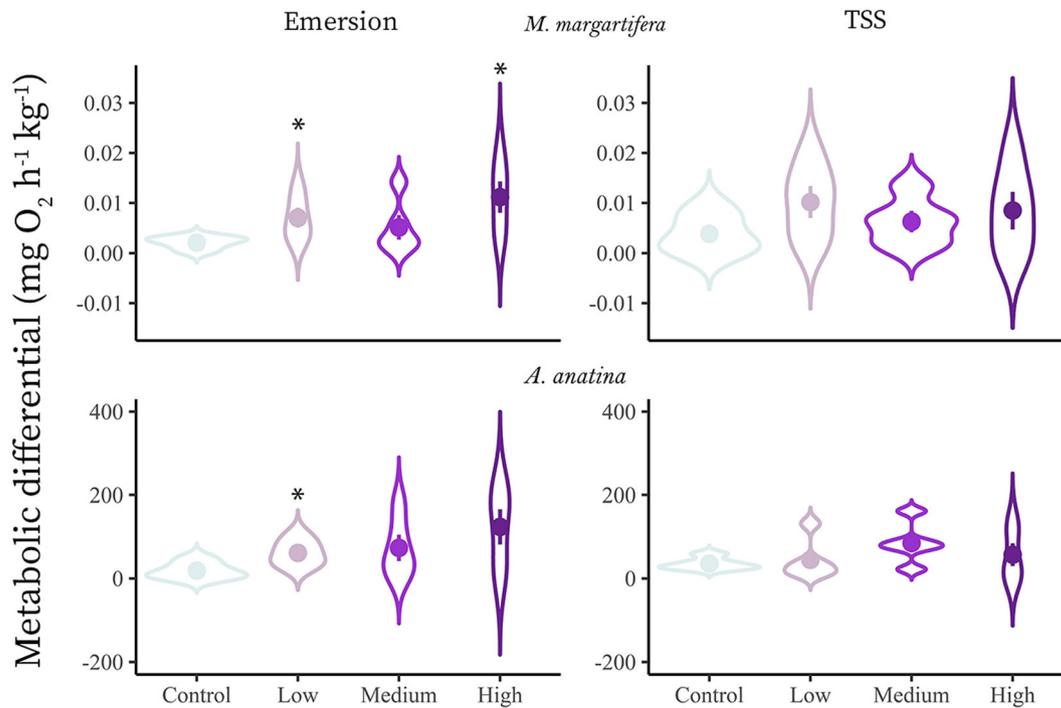


FIGURE 3 The effect of stress magnitude on the extent of change in metabolic rate in response to stress exposure. A comparison of metabolic differential in *Margaritifera margaritifera* and *Anodonta anatina* following emersion and TSS exposure. The violin plot shows the mean and SE for the metabolic differential of corresponding individuals across the stress magnitude groups. The significant differences highlighted in the output of Kruskal–Wallis tests, for comparisons of mean metabolic differential between stress magnitude groups and the control, are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

During emersion experiments, *M. margaritifera*'s extended post-exposure CIs remained significantly higher than pre-exposure in all three stress-magnitude groups ($P < 0.05$). For *A. anatina*, the extended post-exposure CIs remained significantly higher than the pre-exposure only in the medium stress magnitude group ($P < 0.05$). For TSS exposure experiments, no significant differences between pre-exposure and extended post-exposure CI readings were shown for either species.

3.4 | Transition frequency

Examination of the transition frequency (alterations to a mussel's shell aperture width) as a behavioural response revealed some similarities at the interspecific level (Figure 5). Stress magnitude did not significantly affect the occurrence of transition frequency during emersion experiments in *M. margaritifera*, but a significant effect was found for *A. anatina* ($P < 0.05$). During TSS experiments, stress magnitude was shown to have a significant effect on the occurrence of transition frequency in *M. margaritifera* ($P < 0.01$); by contrast, no significant effect was found in *A. anatina*. For *M. margaritifera* during emersion, time had a significant effect on the occurrence of transition frequency, with transition frequency shown to be significantly higher during stress exposure ($P < 0.001$) and post-exposure ($P < 0.001$) than with pre-exposure conditions. The same was true for *A. anatina*.

During TSS experiments, there was a significant effect of time on the occurrence of transition frequency in *M. margaritifera*; this was limited to a significant difference between stress exposure and pre-exposure ($P < 0.001$). A significant effect of time was also shown for *A. anatina* during TSS experiments, with both stress-exposure and post-exposure conditions shown to be significantly higher than pre-exposure ($P < 0.001$).

Mixed-effects models examining transition frequency with respect to metabolic activity found a significant association between the transition frequency and the metabolic rate (MIMR for *M. margaritifera*) in emersion experiments: higher levels of transition frequency were related to higher readings of metabolic rate ($P < 0.001$ for *M. margaritifera*; $P < 0.05$ for *A. anatina*) (Figure 6). Time was also a significant predictor of transition frequency in emersion experiments, with higher transition frequency during immediate post-exposure than during pre-exposure ($P < 0.01$) in both species. No significant predictors of transition frequency were found in TSS exposure experiments.

3.5 | Avoidance behaviour

Examination of avoidance (observations where the mussel's shell was closed) as a behavioural response showed some similarities at the interspecific level. There was no significant effect of stress magnitude

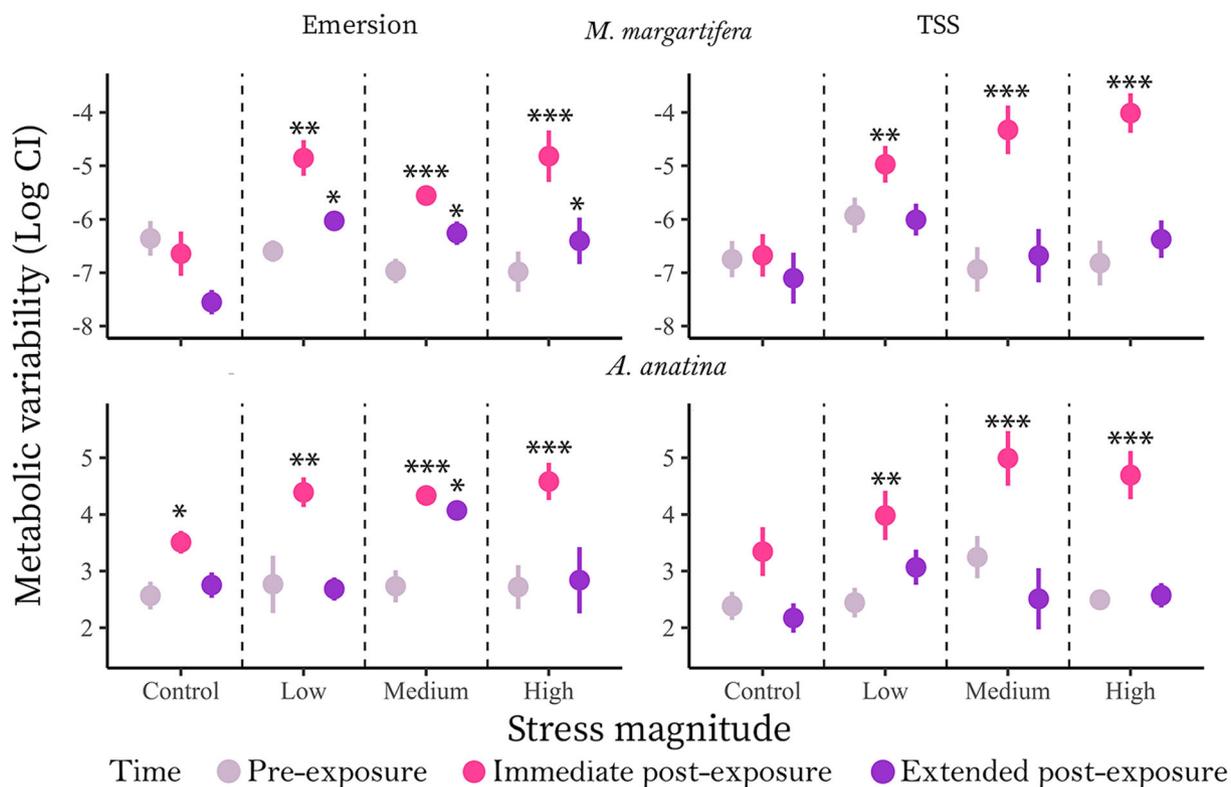


FIGURE 4 The effect of stress magnitude on variability in metabolic rate of individual mussels over time. A comparison of metabolic variability (confidence interval (CI) of mean metabolic rate of individual during corresponding time period) in *Margaritifera margaritifera* and *Anodonta anatina* following emersion and TSS exposure. Each point corresponds to the mean CI (\pm SE) for the relative stressor, stress magnitude, species, and time. The significant differences highlighted in the output of the Kruskal–Wallis tests, for comparisons of the mean confidence intervals within stress magnitude groups and between the pre-exposure time period and the two post-exposure time periods, are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

during emersion and TSS experiments. Time had a significant effect on the occurrence of avoidance behaviour for *M. margaritifera* in emersion experiments, with avoidance behaviour significantly higher during stress exposure ($P < 0.001$) and post-exposure ($P < 0.05$) than during pre-exposure conditions. There was a significant effect of time for *A. anatina* in emersion experiments, but this was limited to a significant difference between stress exposure and pre-exposure ($P < 0.001$). There was no significant effect of time on the occurrence of avoidance behaviour in either species during TSS experiments.

Mixed-effects models examining avoidance behaviour in *M. margaritifera* and *A. anatina* with respect to metabolic activity revealed no significant effects of the predictor variables on the presence of this behaviour across the two stressors.

3.6 | Foot extension

Assessment of foot extension as a behavioural response showed some differences between the two species. Stress magnitude significantly affected the occurrence of foot extension in *M. margaritifera* during emersion experiments ($P < 0.001$). No further significant effects of stress magnitude were found in either species

across both stressors. For *M. margaritifera* in emersion experiments, time had a significant effect on the occurrence of foot extension, which was significantly higher during stress exposure ($P < 0.05$) than during pre-exposure conditions. No further significant effects of time were found for the occurrence of foot extension in either species or across the two stressors. Mixed-effects models examining foot extension in *M. margaritifera* and *A. anatina* with respect to metabolic activity showed no significant effects of the predictor variables on the presence of this behaviour across the two stressors.

4 | DISCUSSION

The results of this study reaffirmed the notion that the behavioural response of unionid mussels to stress exposure provides a useful biomarker for examining the effects of environmental parameters on individual condition. Previous studies have established filtration and evasive behavioural strategies as biomonitoring tools to investigate tolerance to set concentrations of specific pollutants and between periods of rest and exposure (Tran et al., 2003; Liao et al., 2009; Hartmann et al., 2016; Haney, Abdelrahman & Stoeckel, 2020; Premalatha, Saravanan & Karuppanan, 2020). Nevertheless, this is

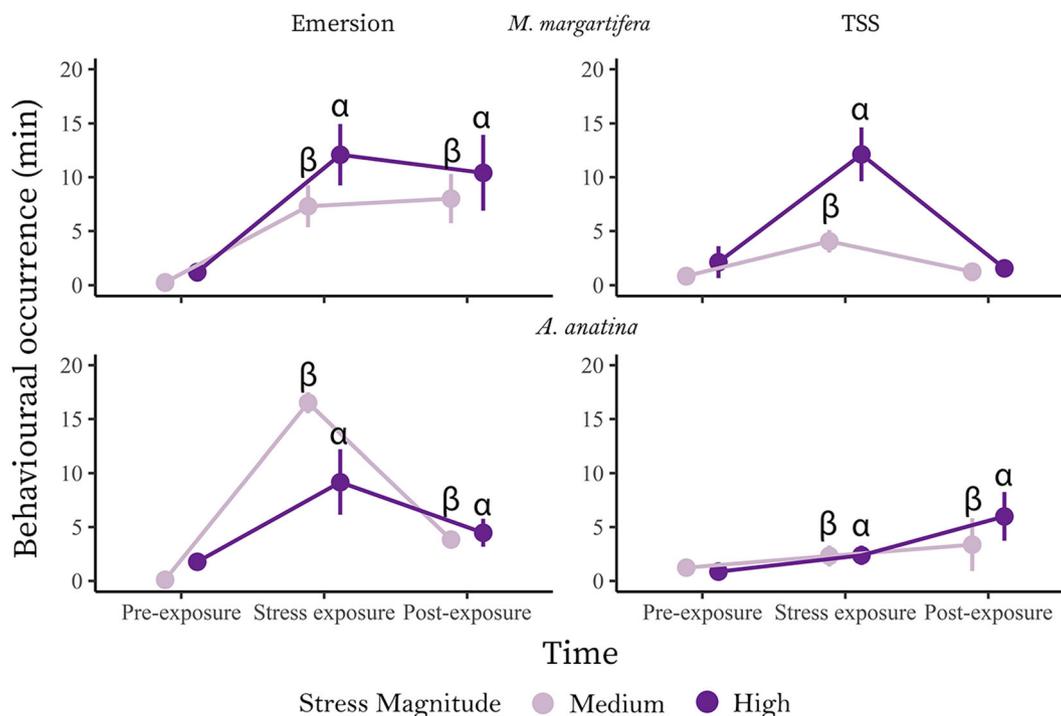


FIGURE 5 The effect of stress magnitude on the occurrence of transition frequency over time. A comparison of the mean (\pm SE) proportion of time transition frequency occurring in *Margaritifera margaritifera* and *Anodonta anatina* following emersion and total suspended solids (TSS) exposure, across the medium- and high-stress magnitude groups. Significant differences ($P < 0.05$) highlighted in the output of Kruskal-Wallis tests (for comparisons of the mean proportion of time mussels display the behaviour) between the pre-exposure time period and the corresponding post-exposure time period are marked with α and β respectively

the first known study that has attempted to identify common behavioural responses in freshwater mussel species across multiple environmental stressors and to associate these with measures of physiological stress. The results of this study provide evidence of behavioural responses to stress exposure that can be linked to physiological condition, specifically to metabolic rate, in *A. anatina* and *M. margaritifera* mussels. The study also revealed substantial intraspecific variation, highlighting the importance of individual variability when examining stress response across populations.

4.1 | Metabolic response

A key component of this study was to examine whether physiology, measured as oxygen consumption, displayed a quantitative response to stressors. Initial findings showed significant differences in individual metabolic functioning, across both species, with mussels found to exhibit idiosyncratic metabolic responses to stress exposure: some mussels appeared to heighten their metabolic rates, whereas others displayed a metabolic depression immediately after stress exposure. However, significant individual variation was already present before exposure to the stressors, with a threefold and fourfold difference between the maximum and minimum values for SMR in *A. anatina* and *M. margaritifera* respectively, which is a

common finding in many other aquatic species (Burton et al., 2011; Kristín & Gvoždík, 2012; Van Leeuwen, Rosenfeld & Richards, 2012; Metcalfe, Van Leeuwen & Killen, 2015). Therefore, quantification of physiological responses to stress required analysis that sought trends among the noise of individual variation.

Deviation from normal metabolic functioning for extended periods of time following stress exposure was found to be common across species and stressors, thus presenting metabolic variability as a potential method for quantifying response to stressors. This observed increase in metabolic variability and frequent failure to return to normal metabolic functioning within the experimental time limit following stress exposure is well documented in the literature: studies concerning metabolic response of bivalves to stress exposure provide evidence to suggest that individuals will sometimes require several days to return to pre-exposure levels (Newton & Cope, 2006; Robson et al., 2012; Ridgway et al., 2014; Lopes-Lima et al., 2016; Payton, Johnson & Jenny, 2016).

It is likely that both stressors used in this study would affect the physiological functioning of freshwater mussels. Emersion removes the appropriate medium for the mussel's specialized respiratory structures, consequently preventing filtration activity from fulfilling an individual's metabolic requirements. The subsequent establishment of an energy deficit may force a substantial reduction in energy dissipation to prevent fatal thermodynamic imbalance and cell death

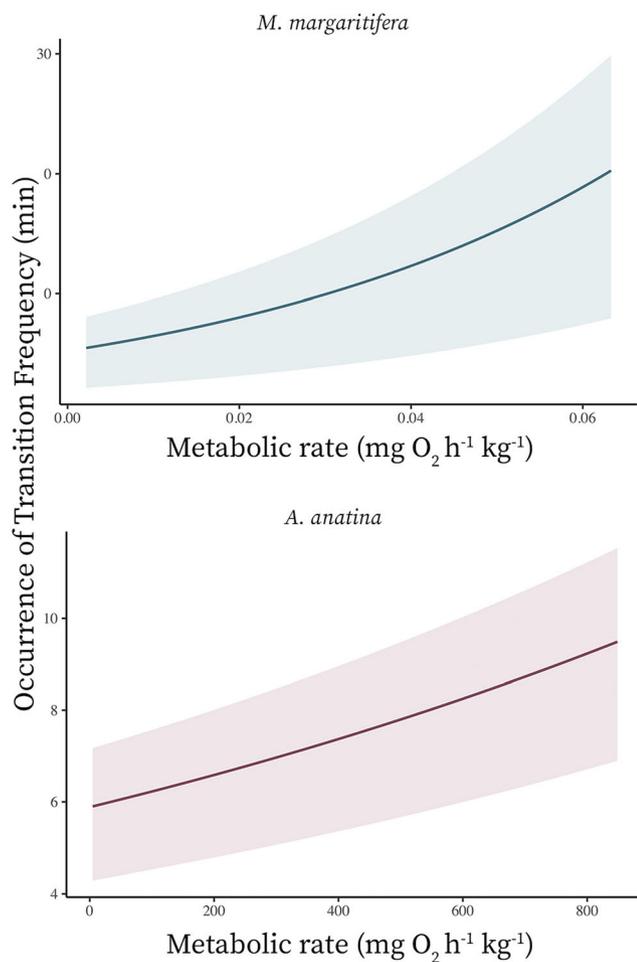


FIGURE 6 The average marginal predicted probability of transition frequency occurring during a 30 min 'closed state', in accordance with alterations in metabolic rate (MR). Results are shown for *Margaritifera margaritifera* and *Anodonta anatina* separately, owing to differences in the calculation of MR: calculations of MR in *A. anatina* used measurements of individual dry tissue weight, whereas calculation of MR in *M. margaritifera* used predicted wet tissue weights, confounding direct comparisons between species

(Widdows & Shick, 1985; Thomsen & Melzner, 2010). By contrast, high concentrations of inorganic suspended sediments are thought to increase the energetic demand of particle processing, with the active excretion of undesired compounds in pseudofaeces incurring an energetic cost to individuals (Vaughn, Nichols & Spooner, 2008; Lummer, Auerswald & Geist, 2016; Tuttle-Raycraft, 2018). For both stressors, the perceived deviation from the SMR following intense physiological activity is perhaps reflective of individuals' continued attempts to adjust their filtration rates to compensate for disturbance to osmoregulation (Hartmann et al., 2016), nutrient turnover (Lorenz & Pusch, 2013), and respiratory processes (Shick et al., 1986).

Previous studies, examining alterations to the clearance rates of freshwater mussels in response to TSS, have often alluded to a threshold in response at 8 mg L^{-1} , above which clearance rates are significantly diminished (Foster-Smith, 1975; Madon et al., 1998;

Gascho Landis, Haag & Stoeckel, 2013; Tuttle-Raycraft, Morris & Ackerman, 2017). In this study, attempts to define a similar threshold in the physiological response of mussels to increased stress from prolonged emersion or heightened concentrations of TSS found no substantial differences between stress magnitude groups, only between mussels that experienced stress and those that did not. Alexander, Thorp & Fell (1994) discovered a similar response in metabolic rate to increasing TSS with *Dreissena polymorpha*: acute exposure to suspended solids evoked a depressed metabolic rate; however, oxygen consumption did not cease or continue to decline at higher concentrations of TSS. Therefore, the results concerning physiological response to stress exposure suggest a binary response to the presence or absence of stress, contrary to a positive linear relationship between heightened response and greater levels of stress initially imagined.

4.2 | Behavioural response

In addition to examining individual physiology, this study also assessed whether behavioural responses to stress could be quantified. For both species and stressors, transition frequency increased in occurrence in response to stress exposure. Exposure to terrestrial conditions and suspended fine particulate matter are likely to have constrained the capacity of mussels to function as filter feeders (Widdows & Shick, 1985; Shick et al., 1986; Alexander, Thorp & Fell, 1994; Tuttle-Raycraft, Morris & Ackerman, 2017). To endure emersion, the adoption of brief periods of air breathing may have assisted in the removal of metabolic by-products through aerial diffusion, such as anaerobically produced CO_2 , thus permitting the conservation of energy stores and consequently preventing early fatigue (Shick et al., 1986). To cope with increased suspended fine particulate matter during TSS experiments, a consistent alteration of valve activity would assist in modulating an individual's exposure to suspended solids. Exposure may incur damage to the filter-feeding apparatus with inorganic solids overloading the gut and gills, interfering with filter-feeding functions and efficient gaseous exchange (Alexander, Thorp & Fell, 1994). Despite providing a potential coping mechanism, brief periods of aerobic respiration during exposure to either stressor are unlikely to entirely relinquish the negative physiological effects of stress exposure: mussels may remain reliant on anaerobic pathways (De Zwaan & Wijsman, 1976), with periods of closure interspersing phases of aerobic respiration to prevent physiological damage (Liao et al., 2009).

The implementation of anaerobic pathways to compensate for the energetic requirements of an individual during stress exposure would necessitate a recovery period after the removal of the stressor, dependent on aerobic metabolism (Richards, Heigenhauser & Wood, 2002; Burton et al., 2011; Robson et al., 2012; Haney, Abdelrahman & Stoeckel, 2020). To assist recovery, a constant movement of the shell aperture may have acted to facilitate an augmented filtration rate, by pumping the water over the gills, thus providing a pathway for reducing the oxygen deficit incurred and

removing potentially harmful substances (Widdows & Shick, 1985; Robson et al., 2012). It would appear, therefore, that the increased occurrence of transition frequency during and after stress exposure reflects a propensity of mussels to use behavioural traits to cope with stressors; however, this application appears to be specific to the stressor, the species, and the metabolic scope of an individual.

Individuals that display heightened transition frequency in response to stress exposure may be more likely to recover more quickly and, therefore, display a prompt return to normal activities after stress exposure (Marras et al., 2010). However, for transition frequency to occur, an individual is required to generate frequent shell movement, which necessitates the use of adductor muscles and is therefore likely to be energetically demanding (Shick et al., 1986). Individuals with a higher metabolic rate or aerobic scope are more likely to cope with the energetic requirements of transition frequency, and thus use this behavioural trait more often. Furthermore, individuals of the same species were collected from the same study site, suggesting that environmental conditions in the habitat were unlikely to shape the observable phenotypic variation in behavioural and physiological traits, provided that heritability of an individual's physiological profile is low (Burton et al., 2011).

Avoidance behaviour and foot extension were observed less frequently in mussels after stress exposure and varied between the two species, generating large zero-inflated data sets. Both avoidance behaviour and movement have been documented as responses to alterations in the environment (Allen & Vaughn, 2009; Gough, Gascho Landis & Stoeckel, 2012; Archambault, Cope & Kwak, 2013; Block, Gerald & Levine, 2013; Hartmann et al., 2016). The low frequency of occurrence of these two behaviours could result from the type of stressors used, with the expression of certain behavioural traits occurring more often in response to particular stressors. In addition, owing to limitations in the experimental design, oxygen consumption rates could not be obtained during stress exposure periods. It is during these periods that these behavioural metrics were often observed; thus, attempts to compare physiological change with avoidance behaviour may have suffered from a lack of data. Therefore, further tests, with an improved experimental approach that allows for continuous respiratory readings with larger mussel groupings and using different stressors, may be required to test the link between the presence of such behaviours and the physiological mechanisms underlying their occurrence.

4.3 | Variation in species and stressors

The results from this study suggest species-specific responses to the stressors, often perceived to reflect differences in physiology (Gough, Gascho Landis & Stoeckel, 2012; Ganser, Newton & Haro, 2013; Haney, Abdelrahman & Stoeckel, 2020). A key driver of these differences could also be the environmental conditions the populations experienced in their natural habitats. The sample populations used in this study were collected from ecosystems displaying very different habitat characteristics. The lentic system

from which *A. anatina* were collected was subjected to frequent water abstraction and displayed poor water quality, suggesting a potential tolerance to prolonged stress exposure and previous experience with both stressors. By contrast, *M. margaritifera* were taken from a mill lade, hydrologically connected to the main channel of the river, characterized by relatively consistent depth and flow conditions in addition to good water quality. This suggests there may have been differences in the sensitivity to the stressor (Hart, Miller & Randklev, 2019), with the *M. margaritifera* population less well adapted to the presence of the experimental stressors for extended durations (Lummer, Auerswald & Geist, 2016; Johnson et al., 2018). Owing to the significant differences in habitat where the species samples were taken, and the potential for this to be a significant driver of individual responses, this study was limited in its propensity to tease apart species differences.

In addition to interspecific differences in response, this study also highlighted differences in response to the two stressors, perhaps reflective of differences in the magnitude of stress caused by each stressor. However, there were differences in how these stressors were induced for this study: mussels were handled during the emersion experiments but were not handled during the TSS exposure study. Handling mussels might have heightened the extent of stress individuals experienced during the emersion experiments. Without handling the control mussels it is difficult to quantify the extent to which this evoked stress within mussels. Nevertheless, evidence from the literature regarding the impact of short-term handling on individuals suggests this may be negligible (Miller, Rach & Cope, 1995; Gray & Kreeger, 2014; Ohlman & Pegg, 2020).

It is likely that, for both species, suspended sediment presents a more commonly encountered environmental condition than emersion does. Furthermore, a suitable respiratory medium still exists in these circumstances. The lower magnitude of stress caused by exposure to suspended solids would probably have permitted a faster recovery, hence why both metabolic and behavioural metrics were often similar in the pre-exposure and post-exposure conditions during TSS experiments. Despite differences in the magnitude of stress caused, the data from this study would suggest that both stressors have a significant impact on the physiology of both species and cause stress to some degree. For both stressors, it is likely that prolonged exposure would cause significant loss of individual condition, eventually culminating in mortality.

4.4 | Implications for behaviour as a biomarker

The results from this study suggest that exposure to an environmental stressor can be detected by measuring transition frequency in unionid mussels. This study demonstrates a clear distinction in the presence of this behaviour between periods before stress exposure and following exposure, which can be linked to alterations in metabolic functioning. Measurements of transition frequency could, therefore, form the basis for a biomonitoring tool to detect the onset of stress in populations.

Recording the frequency of occurrence of this behaviour over time could assist practitioners in identifying when individuals are experiencing prolonged stress and requiring conservation intervention. This biomonitoring tool may also be deployed to aid relocation and restoration efforts towards the conservation of populations, with research concerning the use of bioindicators of unionid fitness already having demonstrated the applicability of such approaches (Gray & Kreeger, 2014; Roznere et al., 2017). For example, studies acting as prerequisites to a translocation scheme could deploy a small subset of a population into a habitat of interest and subsequently conduct monitoring of transition frequency to assist practitioners in gauging habitat suitability.

To quantify the extent of stress caused, research must account for individual variability. To do so, laboratory experiments could identify the most responsive individuals within a sample of the population to act as indicators for overall population condition. The thresholds for individual stress response could be identified by focusing on the presence of transition frequency across a variety of stressors in these indicator individuals, thereby accounting for population-specific variation in response.

This study focused exclusively on adult mussels, and hence did not account for variation in response across life stages. Research would suggest that juvenile mussels are perhaps more susceptible to environmental stressors such as heightened fine particulate matter (Geist & Auerswald, 2007; Geist, 2010; Tuttle-Raycraft, Morris & Ackerman, 2017), although, given their size, studies such as this may be difficult to replicate with a sample of juvenile mussels. Therefore, biomonitoring techniques reliant on the monitoring of transition frequency may be limited to adult mussels yet could be used as a proxy to infer juvenile population condition.

To identify the onset and frequency of occurrence of behavioural metrics, this study relied on direct observation using high-resolution camera technology. This provided a useful method of unrestricted categorization of behavioural traits but required extensive analysis of the image data, and this method would also be difficult to use in a field setting. The use of animal-attached remote-sensing technologies, such as Hall sensors, circumvents such issues and allows measurements of mussel valve movement (valvometry) to be acquired at high resolution and in real time (Nagai et al., 2006; Robson et al., 2012). Previous studies have provided evidence to suggest that both avoidance behaviour and transition frequency could be analysed using biosensor technology (Lorenz & Pusch, 2013; Hartmann et al., 2016; Lummer, Auerswald & Geist, 2016). However, this technology is currently limited to laboratory experiments and is yet to be tested in the field as a remote-sensing technique.

5 | CONCLUSION

To obtain information specific to certain species across variable, spatial, and temporal scales, in addition to being predictive, prescriptive, and scalable, ecologists must move away from 'long tail' scientific methods conducted by individual investigators over

limited spatial and temporal scales and reliant on funding models that provide limited scope for collaboration (Heidorn, 2008; Hampton et al., 2013). The adoption of a context-driven approach to ecology, which examines the physical attributes of the ecological landscape, in addition to how the animals respond to changes in their habitat, is likely to provide appropriate data for enacting successful conservation management. Using remote sensing to detect the occurrence of transition frequency in indicator individuals may assist such an approach. Data to suggest how a population is responding to alterations in environmental conditions before, during, and after conservation management (e.g. river restoration and reintroduction schemes) could assist the quantification of project success, providing population-specific thresholds to identify when particular environmental variables begin to impair the condition of individuals. This article highlights the potential of this approach for contributing to the conservation of endangered freshwater unionid mussels.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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