The environmental context of inducible HSP70 expression in Eastern Brook Trout

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Much research has focused on the population-level effects of climate change on Eastern Brook Trout (Salvelinus fontinalis). While some studies have considered here sub-lethal stress caused by warming waters, the role of multiple, interacting stressors remains largely unexplored. We used inducible heat shock protein 70 (HSP70) as a molecular biomarker to assess in situ response of Eastern Brook Trout in headwater streams to multiple potential stressors, including temperature. Over 7 sampling events during 2018 and 2019, we sampled 141 fish and found that HSP70 expression and 3-day mean water temperature exhibited a quadratic relationship ($R^2$-adj = 0.68). Further analyses showed that HSP70 expression was explained by temperature, relative water level and their interaction ($R^2$-adj = 0.75), while fish size and capture location were not factors. We observed a significant increase in HSP70 expression during periods of low relative water level with warm temperatures (∼18°C) and also during high relative water level with cold temperatures (∼8°C). Our results suggest that temperatures at the edges of the preferred range coupled with relative water level might act together to trigger the cellular stress response in Eastern Brook Trout and that there is greater variation in response at colder temperatures. These findings reinforce the need to consider complex, interactive stressors in influencing the health and persistence of Eastern Brook Trout populations into the future.

Keywords: Cellular stress response, climate change, heat shock proteins, multiple stressors, Salvelinus fontinalis

Introduction

Species distributions are shaped by a combination of both current and historic biotic and abiotic influences (Roubicek et al., 2010; Grenouillet et al., 2011; Araújo and Peterson, 2012; Bucklin et al., 2015). Land use and climate change can alter these influences (Araújo and Rahbek, 2006). For many species, such habitat changes may induce a stress response, which can ultimately result in a cascade of impacts at the organismal, population and even community levels (Petitjean et al., 2019). Cold-obligate, headwater stream fish species are among those most threatened by land use and climate change as they are highly dependent on historical temperature and flow regimes that have supported their persistence (Ficke et al., 2007; Nelson et al., 2009) and, in several cases, given them a competitive advantage for growth (Petty et al., 2014). Eastern Brook Trout (Salvelinus fontinalis; Mitchell, 1814) are threatened throughout their native range by anthropogenic activities, warming water temperatures, habitat fragmentation and outright loss of habitat (Flebbe et al., 2006; Hudy et al., 2019).
et al., 2008; Stranko et al., 2008; DeWeber and Wagner, 2015; Snyder et al., 2015). Although these threats are exacerbated in the southern portion of the Appalachian Mountains range, more central and northern populations are impacted as well (Hudy et al., 2008), where analyses have forecasted greater increases in mean annual air temperature and more frequent extreme precipitation events (Janowiak et al., 2018).

While large scale or persistent deviations from preferred or tolerable conditions may result in local extirpation, less severe and/or less frequent disturbances (e.g. intermittent heat stress and/or drought) could trigger sublethal responses, with two possible outcomes. The individual may attempt to modulate the stressor via a behavioural response (Wong and Candolin, 2015) or, failing that, they may instigate a stress response at the physiological and/or cellular level (Somero et al., 2017). Movement/dispersion is one of the common behavioural strategies to deal with environmental stressors, provided that the habitat is suitable to facilitate such response (Wong and Candolin, 2015). This is particularly relevant for cold-obligate, headwater fish such as trout due to their dependence on stream hydrologic conditions for mobility (Latterell et al., 2003; Cole et al., 2006; Warren et al., 2008; Breau et al., 2011). Under conditions not conducive to a behavioural response (e.g. low-surface water level, low flow and/or physical barriers), a stress response will be induced, which typically involves the endocrine-level response (e.g. elevation of plasma cortisol levels) and/or the cellular response.

One key component of the cellular stress response involves heat shock proteins (HSPs). The HSPs, particularly the HSP70 protein family, are among the first to respond to stressors (Smith et al., 2013; Somero et al., 2017). In non-stressful conditions, the constitutively expressed isoform, HSC70, assures proper folding of newly synthesized proteins. In contrast, the inducible isoform, HSP70, is rapidly expressed at high levels when protein denaturation is detected, as occurs under thermal stress and various other environmental influences (Lindquist and Craig, 1988; Wang et al., 2004; Gupta et al., 2010; Deane and Woo, 2011). The conditions at which HSP70 is expressed are often lower than the lethal thresholds for various stressors, but this response is not without energetic costs and physiological consequences (Feder et al., 1992; Krebs and Feder, 1997). For example, Chadwick & McCormick (2017) found that Brook Trout exposed to chronic or intermittent temperatures higher than 20°C initiated HSP70 expression and increased levels of plasma cortisol, which can lead to reduced food consumption, growth and reproduction, all of which may ultimately reduce the quality of a native Brook Trout fishery and threaten population persistence.

HSP70 expression may be useful as a molecular indicator of sub-lethal stress, as has been reported for several species (Iwama et al., 1999; Buckley et al., 2001; Tomanek, 2008; Chadwick et al., 2015). However, caution has been raised when using HSP70 in monitoring stress (Iwama et al., 2004), as the degree of expression is not only governed by the magnitude, duration and number of the stressors, but also by the history of past exposure, conspecific genetic differences, physiological status and developmental stage (Currie et al., 2000; Basu et al., 2002; Rendell et al., 2006; Fowler et al., 2009; Stitt et al., 2014). In response, efforts are underway to uncover the nature of cellular stress responses, including HSP70, in the context of multiple stressors (Gunderson et al., 2016; Petitjean et al., 2019).

For many salmonid species, an increase in HSP70 occurs upon thermal stress in all tissues examined (Currie et al., 2000; Lund et al., 2003; Rendell et al., 2006; Fowler et al., 2009; Deane and Woo, 2011). Branchial/gill tissue, in particular, is in direct contact with the environment and acts as a sensitive first responder to changes in the aquatic environment (Laurent and Dunel, 1980; Poleksic and Mitrovic-Tutundzic, 1994; Smith et al., 1999). In Eastern Brook Trout, Chadwick et al. (2015) found that HSP70 expression increased significantly in the gills of fish housed in laboratory tanks at 20.7°C, or from streams where the 7-day mean water temperatures were above ~21°C, both temperatures are well below Brook Trout’s lethal temperature threshold (25.3°C; Wehrly et al., 2007) but near the upper end of their 14°C–18°C preferred temperature range (Cherry et al., 1975; POWER, 1980). To that end, studying HSP70 in gill filaments has provided valuable insights into the cellular stress responses in fish upon exposure to multiple stressors under laboratory conditions (Alstad et al., 2005; Dorts et al., 2014; Eissa and Wang, 2016; Vargas-Chacoff et al., 2018).

Although a strong causative relationship between temperature and HSP70 expression in Brook Trout has been demonstrated in laboratory conditions, temperature is likely only one of several different factors influencing HSP70 expression in natural stream conditions (Deane and Woo, 2011; Eissa and Wang, 2016). As such, there have been recent calls to investigate potential synergistic and/or antagonistic interactions between temperature and other factors influencing the cellular stress response (Gunderson et al., 2016; Petitjean et al., 2019). For example, field studies have shown that temperature and high concentrations of pollutants could upregulate HSP70 expression in brown trout (Salmo trutta) and stone loach (Barbatula barbatula) (Köhler et al., 2001).

Fish are likely to experience multiple, cumulative stressors, yet there is a paucity of studies of the cellular stress response to multiple, environmentally realistic stressors. To address this gap, we (i) quantified HSP70 expression in Eastern Brook Trout gill tissues from the Beebe River watershed in central New Hampshire over time and naturally variable environmental conditions; (ii) characterized observed relationships between HSP70 expression and environmental conditions (temperature, conductivity and relative water level); (iii) identified temporal windows of these abiotic conditions that best explained HSP70; and (iv) attempted to explain observed variability in HSP70 response using multiple linear regression. We predicted that water temperature would be an important driver of HSP70 expression and that additional
environmental factors would contribute to alleviating or exacerbating the effects of temperature on Brook Trout stress responses. Our 2-year investigation raises important considerations that may guide future research of stress stimuli, how stress response may differ across geography and among individuals in a population and how future climate change and habitat alteration may influence stress responses.

Materials and methods
Study area
This study was conducted in a small headwater tributary (hereafter referred to as GR3) to the Beebe River in central New Hampshire (Campton/Sandwich, NH, USA; Fig. 1). The Beebe River watershed is a 12-digit hydrological unit sub-watershed to the Pemigewasset River that flows into the Merrimack River, ultimately draining into the Atlantic Ocean. The Beebe watershed is fed by high-elevation ponds, wetlands and groundwater springs and is bordered by the White Mountain National Forest and conserved land within the Squam Range. A privately maintained dirt road parallels the mainstem of the Beebe River, bisecting five south-facing, first-order tributaries. GR3 is the third stream along the upstream road corridor that flows into the Beebe River. The entirety of the GR3 catchment is forested, with the exception of an ~50 m reach, ~100 m upstream from its confluence with the mainstem. Where intact, the surrounding forest contains northern hardwoods (e.g. Sugar maple, Beech) and a mix of Balsam fir and Eastern hemlock. The ~50 m unforested reach is dominated by early-successional regrowth from road crossing disturbance and mowing that occurs on a 3–7-year rotation to maintain access to power transmission lines. Wild Brook Trout is by far the dominant fish species residing in GR3 and other headwater tributaries in this watershed, but we occasionally catch hatchery-stocked Brook Trout, Blacknose Dace (Rhynichthys atratulus) and Longnose Dace (Rhynichthys cataractae).

Environmental monitoring
Temperature, conductivity and water level were recorded using HOBO® sensors (Onset Computer Corporation, Bourne, MA, USA). Temperature sensors (U22-001) were deployed at multiple locations in GR3 to account for canopy-driven differences in water temperature (Table 2). Six temperature sensors were deployed in 2018 and five were deployed in 2019 (Table 2). One conductivity (U24-002) and one water level sensor (U20-001) were deployed 2 m upstream from the confluence with the mainstem and set to record data at 10-minute intervals (2018) and 15-minute intervals (2019). A second water level sensor was installed in a tree ~1.5 m off the ground to record barometric pressure and to compensate for barometric pressure in our water depth series. Water depth was calculated using the HOBO® Pro Barometric Compensation Assistant and relative water levels were calculated by subtracting the lowest recorded level of the season from the real-time water depth.

We calculated the average temperature as well as the maxima, minima, and average relative water level and conductivity for 7-, 5-, 3-, and 1-day prior to sampling events for subsequent analysis. Each fish captured was associated with the average of temperatures from the nearest upstream and downstream sensors. We divided the tributary into three sections based on the habitat type and temperature profile (Table 2).

Gill filament collection and protein extraction
Brook Trout were sampled via backpack electrofishing (Smith-Root LR-20) on seven dates in late June, July and early September 2018 and in late May, June, July and early September 2019 (2018: June 26, July 27, September 7; 2019: May 30, June 24, July 29, September 3) during monthly surveys associated with long-term population monitoring. We conducted a non-lethal gill biopsy from the first gill arch (per McCormick, 1993) of individuals >90 mm in total length and sampled individuals only once per year (New Hampshire Fish and Game Scientific Licenses F2018-94 and F2019-102). All sampled fish were uniquely identified with pit tags to minimize repeat sampling in a given field season. Only three Brook Trout were sampled twice in the same field season, and six were sampled in both seasons 2018 and 2019. Gill filaments were placed in SEID buffer (150 mM sucrose, 10 mM EDTA and 50 mM imidazole, pH 7.3, EDTA-free protease inhibitor and 0.1% deoxycholic acid) on ice until transport back to the laboratory. In the laboratory, gills were homogenized and centrifuged at 5000 g for 1 hour at 150 V. Bands were transferred onto a nitrocellulose membrane using iBlot®-2 Transfer System (Thermo Fisher Scientific, Waltham, MA, USA) and downstream sensors. We divided the tributary into three sections based on the habitat type and temperature profile (Table 2).

Western blotting and HSP70 quantification
Equal amounts (16 μg in 2018; 10 μg in 2019) of gill protein extract and a positive control of hepatic tissue from stressed S. fontinalis (from S. Currie, Acadia University, Nova Scotia) were loaded onto 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA). A total of 5 μl of Precision Plus protein standards (Bio-Rad) were run alongside samples as the molecular weight standard. Proteins were then separated in 1 x TGS buffer (Bio-Rad) for 1 hour at 150 V. Bands were transferred onto a nitrocellulose membrane using iBlot®-2 Transfer System (Thermo Fisher Scientific, Waltham, MA, USA). Total protein was visualized on the membrane by Ponceau-S to ensure equal sample loads.
(Romero-Calvo et al., 2010) and images were captured using ImageLab v4.0 (Bio-Rad). Pictures were transformed into 8-bit ImageJ (Schneider et al., 2012), and the total protein stain density was quantified by measuring the densitometry of all the bands per lane. The membrane was destined in 0.1 M NaOH, washed, equilibrated in PBS-Triton-X buffer (PBST: 0.0019 M NaH$_2$PO$_4$, 0.081 M Na$_2$HPO$_4$, 0.15 M NaCl, pH 7.3, 0.1% Triton-X-100) and blocked in PBS buffer with 5% BSA. We used a polyclonal antibody specific to the inducible form of salmonid HSP70 (AS05061; Agrisera, Vannas, Sweden) at a dilution of 1:2500 in PBS buffer with 5% BSA for 1 hour at room temperature. The membrane was rinsed in PBST, incubated in 1:5000 dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Portsmouth, NH) in PBS buffer with 5% BSA for 1 hour at room temperature, rinsed in PBST and developed in 1× DAB/peroxidase chromogenic solution (Thermo Fisher Scientific, Waltham, MA, USA). Bands were imaged using Image Lab v4.0 (Bio-Rad) and transformed into 8-bit pictures, and the adjusted band density was calculated in ImageJ by dividing the HSP70 band density by the total protein stain density. For interblot comparisons, fixed-point normalization was carried out by dividing the adjusted HSP70 band density to the control band density to obtain relative band density (Degasperi et al., 2014).

**Statistical analysis**

We report HSP70 expression as the relative band density for each gill sample. All statistical analyses were performed in Minitab 18 (State College, PA, USA) and we used $\alpha = 0.05$ to determine significance, unless otherwise noted. Relative HSP70 expression was found to be non-normally distributed (Anderson–Darling test, AD = 22.44, $P < 0.005$) and with unequal variances across samples (Levene's test, $P < 0.001$). To compare HSP70 values over time, we used Welch’s ANOVA for unequal variance and Bonferroni-adjusted Games-Howell non-parametric post hoc tests, an approach that reduces type I error in unbalanced data (i.e. unequal sample sizes across months) and tolerates heterogeneity of variance (Liu, 2015).

Linear and curvilinear regression analyses were conducted for HSP70 expression with respect to various time windows of temperature, conductivity and relative water level to better understand observed bivariate relationships. After comparing the distribution of the residuals, the highest adjusted $R^2$ and lowest multicollinearity (VIF factor) values were used to select the best fit relationships. We used multiple linear regressions to explore whether the variability in HSP70 levels could be explained by temperature, conductivity, water level, year, location of capture, total length and weight of fish. Total length and weight (as more informative than age) were integrated into our regression analyses, as HSP70 expression has been documented to decline over the lifespan of salmonids (e.g. Fowler et al. 2009). We also investigated the two-way interactions among temperature, relative water level and conductivity in explaining HSP70 variation. We applied a square transformation to 1-day relative water level and square root transformations to 3-day temperature and 7-day conductivity.
to linearize the residuals that existed from strong quadratic relationships with HSP70 levels. The transformed variables are referred hereafter as relative water level, temperature and conductivity, respectively. We used stepwise parameter selection, with an alpha-to-enter of 0.05 and an alpha-to-remove of 0.10 to identify the best fit models. All continuous variables were centered between −1 and +1 by their corresponding maximum and minimum values to reduce multicollinearity with interaction terms, to put independent variables on a comparable scales and to guide the interpretation when independent variables assumed zero values. The best fit model represents the optimal combination of high adjusted R², low P-value, low predicted residual error sum of squares statistic (PRESS) and low variance inflation factor (VIF) values.

### Results

#### Field sampling

Water temperatures in GR3 ranged from 8°C to 20°C over the two seasons of this study and did not exceed 20°C in either season. Hourly mean temperatures (±SD) over the period of late May to early September was 15.3 ± 2.6°C in 2018 and 14.1 ± 2.4°C in 2019 (Fig. 2a). Monthly mean conductivity from May to July was 13.0 ± 0.9 μS/cm in 2018 and 10.6 ± 1.3 μS/cm in 2019 (Fig. 2b). Mean relative water levels from May through July were 0.063 ± 0.030 m in 2018 and 0.048 ± 0.025 m in 2019 (Fig. 2c).

We assayed 43 and 98 fish for HSP70 expression in 2018 and 2019, respectively (Table 1). Forty gill samples produced insufficient total protein levels for use in western blotting. Approximately 77%–94% of fish captured per sampling event and assayed for HSP70 expression were captured in pool habitats (Table 1). The fish sampled in our study were predominantly age 1 individuals as determined by scale ageing (Hoekwater, 2020).

#### Variation in HSP70 expression over time

HSP70 expression was significantly different across individuals depending upon collection date (Welch’s ANOVA, F_{6,39.98} = 38.23, P < 0.0001; Fig. 3). The highest HSP70 expression was recorded in fish sampled in May 2019, followed by fish from September 2019 and September 2018, which were 2-fold and 5-fold lower, respectively (Games-Howell post hoc, α = 0.01, P < 0.005). There was no significant difference in HSP70 between September 2018 and September 2019 (Games-Howell post hoc, P = 0.013). Fish captured in June and July expressed lower HSP70 than in May or September in both years, producing a U-shaped relationship with the lowest values recorded in early to mid-summer both years (Fig. 3). Higher levels of HSP70 were recorded in June and July 2018 than that of June and July 2019 (Games-Howell post hoc, P < 0.005). Overall, HSP70 expression was significantly higher in fish sampled in May as compared to July (P < 0.005) and significantly higher in September than June or July in both years (P < 0.005). See Figure S1 for a representative Western blot.

#### Factors associated with HSP70 expression

Across all assayed fish, there was no significant difference in HSP70 expression as a function of weight (Welch’s ANOVA, F_{6,44.84} = 0.45, P = 0.843) or total length (Welch’s ANOVA, F_{6,44.23} = 1.00, P = 0.439). We found that HSP70 expression exhibited a quadratic relationship to temperature,

### Table 1: Summary of sampling effort

| Sampling event | Jun-'18 | Jul-'18 | Sep-'18 | May-'19 | Jun-'19 | Jul-'19 | Sep-'19 |
|----------------|---------|---------|---------|---------|---------|---------|---------|
| Fish > 90 mm   | 61      | 49      | 57      | 50      | 69      | 92      | 85      |
| # Gills sampled| 22      | 27      | 24      | 24      | 40      | 22      | 22      |
| # HSP70 samples| 14      | 19      | 10      | 23      | 40      | 22      | 13      |
| Total length   | 122.4 ± 10.5 | 129.1 ± 5.6 | 119.7 ± 11.1 | 129.5 ± 7.0 | 125.2 ± 6.3 | 123.6 ± 7.7 | 119.0 ± 13.3 |
| Weight         | 20.1 ± 4.3 | 22.6 ± 3.5 | 18.5 ± 6.8 | 23.2 ± 4.4 | 21.3 ± 3.9 | 19.7 ± 7.6 | 21.1 ± 5.2 |

- Report for HSP70 sampled fish in mm ± SE.
- Report for HSP70 sampled fish in g ± SE.
- Non-pool habitat includes cascade, riffle, glide and unknown.
Table 2: Summary of temperature, conductivity and relative water level prior to fish collection events

| Section (m) | Jun-'18 | Jul-'18 | Sep-'18 | May-'19 | Jun-'19 | Jul-'19 | Sep-'19 |
|-------------|---------|---------|---------|---------|---------|---------|---------|
| Temp (°C)   |         |         |         |         |         |         |         |
| 0–180°      | 12.8 ± 0.1 | 15.1 ± 0.3 | 17.6 ± 0.1 | 8.6 ± 0.1 | 12.5 ± 0.1 | 16.3 ± 0.1 | 13.6 ± 0.05 |
| 180–310°    |         |         |         |         |         |         |         |
| 310–650°    |         |         |         |         |         |         |         |
| Conductivity (µS/cm) | 13.7 ± 0.1 | 16.3 ± 0.1 | 16.4 ± 0.1 | 9.2 ± 0.05 | 11.0 ± 0.05 | 15.0 ± 0.05 | 15.0 ± 0.1 |

Sections lacking continuous data records are denoted with a dash, data are shown as mean ± SE.

*Reflects the average of sensors at 20 and 106 m (2018) and 20 and 100 m (2019).

Average of sensors at 180, 210, 270 and 310 m (2018) and a single sensor at 194 m (2019).

Average of sensors at 390 and 650 m (2019).

Table 3: Summary of multiple regression analysis (n = 141)

|                | Coded β + SE | Uncoded β | VIF | Contribution | F    | R^2-adj | PRESS |
|----------------|--------------|-----------|-----|--------------|------|---------|-------|
| Model 1        |              |           |     |              |      |         |       |
| Constant       | 0.32 ± 0.02*** | -0.21*** | -   |              | 129.31 | 0.65 | 8.42   |
| (Rel. WL)^2    | 0.48 ± 0.03*** | 85.09*** | 1.0 |              | 64.03% |     |        |
| Year           |              |           |     |              | 1.50% |       |        |
| 2018           | -0.06 ± 0.03* | -0.06*    | 1.0 |              | -    |       |        |
| 2019           | 0.06 ± 0.03*  | 0.06*     | -   |              | -    |       |        |
| Model 2        |              |           |     |              | 67.13 | 0.77 | 5.83   |
| Constant       | 0.24 ± 0.45  | -7.3      | -   |              | -    |       |        |
| (Rel. WL)^2    | 0.33 ± 0.68  | 549       | 716.0 | 12.52% | -    |       |        |
| Temp^1/2       | -0.22 ± 1.21 | 0.9       | 1807.9 | 56.72% | -    |       |        |
| Cond^1/2       | 0.12 ± 0.65  | 2.4       | 803.8 | 5.87% | -    |       |        |
| Temp^1/2 × Cond^1/2 | -0.12 ± 1.15 | -0.35    | 680.4 | 11.79% | -    |       |        |
| Cond^1/2 × (Rel. WL)^2 | -0.40 ± 0.69 | -139     | 372.8 | 0.06% | -    |       |        |
| Location       | 0.09 ± 0.04* | 0.003*    | 1.4 | 0.15% | -    |       |        |
| Year           |              |           |     |              | 1.09% |       |        |
| 2018           | -0.06 ± 0.14 | -0.06     | 59.7 | - | -    |       |        |
| 2019           | 0.06 ± 0.14  | 0.06      | -   | - | -    |       |        |
| Model 3        |              |           |     |              | 138.48 | 0.75 | 6.13   |
| Constant       | 0.21 ± 0.03*** | -1.31*** | -   |              | -    |       |        |
| (Rel. WL)^2    | 0.32 ± 0.04*** | 375***    | 2.7 | 18.37% | -    |       |        |
| Temp^1/2       | -0.15 ± 0.05* | 0.33*     | 2.5 | 46.72% | -    |       |        |
| Temp^1/2 × (Rel. WL)^2 | -0.33 ± 0.04*** | 89***   | 1.1 | 10.38% | -    |       |        |

Analyses were run using hierarchial stepwise regression procedure, except for Model 2. All continuous variables were centered as specified between −1 and +1 by their corresponding maximal and minimal values. Year was included as categorical variable and was coded to −1 and 1. Fish weight and length were included in the global models but were not significantly associated with expression. Temperature, conductivity and relative water level (Rel. WL) were calculated as specified earlier (Table 2).

\*P < 0.05, \**P < 0.001, \***P < 0.001.
Figure 2: Temperature, conductivity, and relative water level profiles in the GR3 tributary for 2018 (grey) and 2019 (black).

conductivity and relative water level (Fig. 4). In the case of temperature, we found this relationship using the average of 3-day temperature before sampling ($F_{2,138} = 144.20$, $P < 0.0001$, $R^2_{adj} = 0.676$; Fig. 4a). Other time windows (e.g., 1-, 5-, and 7-day(s)-before-sampling average temperature) were significant, but had lower $R^2$ values. We observed an increase in HSP70 expression at temperatures at $\sim 8^\circ C$ and also at $\sim 18^\circ C$ (Fig. 4a), with notable high expression when the 3-day temperature mean was $\sim 14^\circ C$. This seeming anomaly is discussed further in the context of stress interactions identified through multiple linear regression analysis. In addition, for fish sampled at temperatures less than $12^\circ C$, mean HSP70 expression was significantly higher (Welch’s ANOVA, $F_{2,138} = 73.38$, $P < 0.0001$, $R^2_{adj} = 0.515$; Fig. 4b). Although a third-order polynomial regression relationship between conductivity and HSP70 expression offered higher explanatory power, concern for overfitting and difficulty with biological interpretation prompted us to choose second-order regression. Finally, HSP70 expression and the 1-day average of relative water level before sampling also exhibited a quadratic relationship ($F_{2,138} = 189.81$, $P < 0.0001$, $R^2_{adj} = 0.733$, Fig. 4c). We observed an increase in HSP70 expression at relative water levels higher than 0.06 m above base flow (Fig. 4c).

Multiple linear regression analysis suggested that select measured parameters included in this study explained 74% to 77% of the observed variation in HSP70 expression (Table 3). The mean 1-day relative water level explained the largest proportion of observed variability in Model 1, whereas temperature, our *a priori* hypothesized stimulus, explained more of the variation in more complex models (Models 2 and 3). Location of capture contributed minimally to explaining HSP70 variation ($F_{1,133} = 3.93$, $t = -1.98$, $P = 0.050$; Model 2; Table 3), with a slight positive relationship with HSP70 expression (i.e., fish in more upstream locations had slightly higher expression). Similarly, year of collection contributed 1% to the overall $R^2$ value and was only a significant term in Model 1 ($F_{1,133} = 3.93$, $t(2018) = -1.98$, $t(2019) = 1.98$, $P = 0.050$; Model 2; Table 3). Total length, weight and capture location were not significant factors in any of the models.

Model 3 provided the best explanation of observed HSP70 variation in our study. As shown in Table 3, models ignoring interaction effects performed poorer (Model 1) than those that included interaction terms (Models 2 and 3). While
Figure 4: Quadratic relationships between the relative HSP70 expression and (a) average 3-day temperature, (b) average 7-day conductivity and (c) average 1-day relative water level.

Figure 5: Variation in HSP70 expression across temperature, with 98.3% confidence intervals of the standard deviations of 3 temperature windows, suggesting significant differences where intervals do not overlap (Levene’s with multiple comparison tests, P < 0.01).

Model 2 had the highest $R^2$-adj. value (0.77) and the lowest PRESS values (5.83), Model 3 is the most parsimonious model based on the relatively similar explanatory power ($R^2$-adj. = 0.75) and PRESS value (6.13), but substantially lower VIF value. Model 2, which included interaction effects between temperature and conductivity, exhibited severely high VIF values, which decreased the reliability of the coefficients by extending their confidence intervals (Table 3). Thus, we omitted conductivity and related interaction terms for Model 3.

In Model 3, temperature, relative water level and the interaction between temperature and relative water level all contributed to explaining variation in HSP70 expression. Both temperature ($F_{1,135} = 9.75, t = -3.13, P < 0.005$) and relative water level ($F_{1,135} = 55.51, t = 7.45, P < 0.001$) were positively related to HSP70 expression, while their interaction ($F_{1,135} = 57.15, t = -7.56, P < 0.001$) exhibited a negative relationship with the HSP70 level (uncoded $\beta$; Table 3). Temperature explained nearly 47% of observed variation in HSP70 ($\beta = -0.15, SE = 0.05$). Relative water level and the interaction of temperature and relative water level explained 18.37% and 10.38% of the variation in HSP70 expression.

We further explored the nature of the interaction between temperature and relative water level as contributors to HSP70 expression and uncovered a complex relationship. The coded coefficients (i.e. slope) of temperature and relative water level at low (−1, coded minimum), medium (0, coded midpoint between extreme values) and high (1, coded maximum) of the specified conditions were plotted. The HSP70 response to temperature differed with relative water level (Fig. 6a). At low relative water levels (e.g. > 0.02 m above base flow), HSP70 was positively related to temperature, while the inverse was found when the relative water levels were higher (Fig. 6a). In contrast, at low temperatures (~8°C), relative water level had a strong positive relationship with HSP70 (Fig. 6b). This relationship decreased substantially at moderate temperatures (13.2°C) and became slightly negative at high temperatures (17.9°C) (Fig. 6b).

Discussion

We measured HSP70 expression in Eastern Brook Trout from a cold, headwater stream in New Hampshire over two consecutive field seasons (May–August/September) to better understand the effect of temperature and other environmental factors on cellular stress response. Numerous published studies have investigated the sensitivity of streams to warming water temperatures (Mohseni et al., 1999; Caissie, 2006; Kelleher et al., 2012), others have documented the impacts of warming on trout at the population-level (Flebbe et al., 2006; Kanno et al., 2016) and few have narrowed focus to the cellular response of Eastern Brook Trout to warming waters (Chadwick et al., 2015; Chadwick and McCormick, 2017). Our results add to this growing body of literature by bringing to light important considerations that may guide future research on the cellular stress response in Eastern Brook Trout. We describe our main findings below and discuss their relevance in the context of understanding drivers of the cellular stress response and climate change implications for Eastern Brook Trout.
However, the branchial gills are a nexus point where HSP70 expression is instigated by a variety of different stimuli. The response is specific for protein denaturation/protein damage, but some studies have demonstrated the existence of a cross talk between the cellular and endocrine stress response axes (Basu et al., 2003; Deane and Woo, 2011). Moreover, we do not know if the measurable uptick in expression in the gills occurs in parallel with increases in other tissues or if plasma cortisol levels were also elevated in fish with increased HSP70 expression.

**Branchial gills and the cellular stress response**

We constrained our focus to measuring HSP70 in the branchial gills and used an antibody that specifically targets HSP70 (Rendell et al., 2006). Although the HSP70-based response is specific for protein denaturation/protein damage, HSP70 expression is instigated by a variety of different stimuli (Kültz, 2005). However, the branchial gills are a nexus point between the organism and its environment, and thus an increase in expression in this tissue likely reflects a real-time response of the individual to the environmental milieu. However, it is unclear whether or not an increase in HSP70 expression precedes or follows the endocrine stress response, but some studies have demonstrated the existence of a cross talk between the cellular and endocrine stress response axes (Basu et al., 2003; Deane and Woo, 2011). Moreover, we do not know if the measurable uptick in expression in the gills occurs in parallel with increases in other tissues or if plasma cortisol levels were also elevated in fish with increased HSP70 expression.

**Water temperature as a stressor**

Water temperature explained the largest portion of variation in HSP70 expression (46.72%), according to the best-fit multivariate regression. This aligns with numerous studies implicating temperature as the driver of the cellular stress response in Eastern Brook Trout and other salmonids, particularly at temperatures that exceed the upper ecological temperature threshold (20°C–21°C; Lund et al., 2003, Chadwick et al., 2015). Although temperature is consistently identified as the main stimulus for HSP70 response, several studies have documented geographic differences in the onset temperatures and/or magnitude of an HSP70 response (Fangue et al., 2006; Tomanek, 2008; Stitt et al., 2014; Cottin et al., 2015), suggesting northern populations, or those conditioned to cooler temperatures, may be more sensitive to warming waters. In agreement with these studies, we detected HSP70 in Eastern Brook Trout gills at temperatures nearly 3°C cooler (~18°C) than the onset temperature (20.7°C) documented in populations in western MA streams (Chadwick et al., 2015).

Surprisingly, but not unfounded, we also detected HSP70 expression in Eastern Brook Trout at ~8°C in May, well below the lower limit of their preferred thermal window (Cherry et al., 1975; Power, 1980; Ott and Maret, 2003). Cold temperatures, and coincident cold shock, elicit the stress response in both insects (Belén Arias et al., 2011) and fish (Donaldson et al., 2008; Parisi et al., 2020). Cold shock has been implicated in the expression of HSP70 in cultured rainbow trout and zebrafish cells (Yamashita et al., 1996; Airaksinen et al., 2003) and Atlantic salmon embryos exposed to 1°C laboratory conditions showed increased expression of HSP70 coincident with physical abnormalities (Takle et al., 2005). While it has long been documented that cold temperature results in changes in chromosomal number in Eastern Brook Trout (Lemoine Jr and Smith, 1980), we are the first, to our knowledge, to document HSP70 expression attributed to cold shock in Eastern Brook Trout. The implications of such bring us back full circle to the impacts of climate change, albeit not through the more common lens of warming streams. Increased rain on snow events have been documented throughout New Hampshire and New England. While these events cause rapid increases in stream flows during late winter and early spring, which are themselves problematic for headwater species like Eastern Brook Trout (Kanno et al., 2016; Blum et al., 2018), runoff waters tend to be substantially colder than the largely groundwater-fed streams themselves (Canjak and Power, 1986). The sudden introduction of cold waters may help explain the high levels of HSP70 expression we observed in May; however, an alternative explanation could be the interactive impact of high-water levels and other factors, as discussed below.

**Multiple stressors**

Although water temperature alone explained the largest portion of variation in HSP70 expression, relative water level and its interaction with temperature explained nearly as much (Model 2, Table 3). Collectively, temperature, relative water...
level and the interaction between these factors explained 75% of the observed variation in HSP70 expression during our 2-year study. The relationship of temperature and relative water level to HSP70 expression is complex, likely because the influence of these abiotic variables on HSP70 expression is context dependent.

As might be expected, although rarely tested, we found that HSP70 expression was positively related to temperature at low relative water levels (levels <0.02 m above the lowest seasonal flow; Fig. 6a). Under ideal habitat conditions, Eastern Brook Trout can avoid warmer waters by moving upstream, taking refuge in deep pools that are slower to warm or finding sites with groundwater upwelling (Baird and Krueger, 2003). However, low flow conditions (i.e. low relative water levels) can limit fish access to thermal refuge habitat or at least make access more stressful. Pools can become completely or functionally isolated in headwater streams during conditions of very low flow, trapping fish and/or greatly limiting their ability to move without stress. In these instances, we would expect that fish would have no recourse to mitigate temperature stress other than by increasing expression of HSP70.

High water levels may also present stressors to trout, particularly during cold conditions. We found that HSP70 expression was negatively related to temperature at high relative water levels (levels greater than 0.07 m above the lowest seasonal flow, Fig. 6a). In the early spring in northern New England, cold water conditions are often coupled with high water levels via episodic flooding from snowmelt and/or rain on snow events, which can present stressful conditions for overwintering fish living mainly on their storage energy (Cunjak, 1988). During the overwinter period, adult and juvenile Eastern Brook Trout tend to congregate in deep pools (often below ice) and groundwater seeps where groundwater is warmer than surface water (Cunjak and Power, 1986). We suspect that high relative water levels, which can pose movement challenges for trout, and temperatures colder than the preferred range can cause a significant increase in HSP70 expression, as we observed in May 2019. This stress response to high flows is not unfounded. Exceptional flow events have been linked with up-ticks in corticosteroid and glucose levels in three-spine sticklebacks in the UK (Pottinger et al., 2011), and this physiological stress response was greater when high flows were accompanied by exposure to wastewater effluents. Similarly, food deprivation has been linked to HSP70 expression in fish (Cara et al., 2005; Antonopoulou et al., 2013) and can exacerbate the HSP70 response in cases of thermal stress (Cara et al., 2005). The combination of energy depletion due to overwinter fasting, high relative water levels and cold temperatures may collectively instigate HSP70 expression. This raises a possible interactive impact of energy depletion and cold shock that warrants field and laboratory studies to better understand the nature of HSP70 expression under cold stress.

In central NH, low temperature tends to co-occur with high flows during the early season (May) and high temperature co-occurs with low flows (later season). In our study, we did not sample fish in low temperature and low flow conditions or in high temperatures and high flow conditions. Thus, we cannot unequivocally disentangle the relative impacts of temperature or water level in driving these patterns in HSP70 expression. However, this study highlights the importance of multiple stressors acting together on the response of Eastern Brook Trout to conditions that fall outside of optimal.

Other potential stressors considered, but not explicitly tested, include intraspecific competition for food refuge and spawning habitat that may result in energy depletion, evident in body condition. A reduction in the quantity and quality of critical habitat due to low flow conditions may trap fish in pools, prevent access to food-rich riffle habitats and increase competition within pools for macroinvertebrates. Hakala and Hartman (2004) found that during a severe drought year, Eastern Brook Trout confined to pool habitats in West Virginia headwater streams had lower body conditions related to reduced prey resources. Inequitable access to habitat and prey may help explain observed variability in HSP70 expression among sample trout. Although Hoekwater (2020) found no interannual variation in Fulton’s condition in a larger sample of this study population between 2018 and 2019, individual-level differences between May and September were not assessed, leaving the role of body condition in HSP70 expression unknown.

HSP70 expression may also be influenced by preparation for spawning. We documented elevated HSP70 levels in September 2019 (the pre-spawning period for this population), when 3-day mean temperature was well below expected onset temperatures. Currie et al. (2010) documented an increase in HSP70 expression in juvenile rainbow trout related to the establishment of dominance hierarchies. The fish sampled in our study were mostly age 1 or older (Morrill, 2019; Hoekwater, 2020) and constitute a mix of juvenile and adult fish. Therefore, it is possible that social interactions among fish occupying pools during the lead up to spawning may have contributed to the observed HSP70 expression.

**Implications**

The Northeast US has been and will likely continue to experience intermittent winter and early spring warm ups that produce rain-on-snow events and shorter snow pack durations into the spring. These changes, albeit subtle at times, occur during a critical period of fish development (eggs hatching into alevin) and metabolic vulnerability when prey is limited. In addition to warming, another expectation of climate change is the increased frequency of extreme weather events. Elevated water levels and increased water velocity, regardless of the source, may present important stressors for Eastern Brook Trout in the early season, which may have cascading negative impacts on growth, biomass and, potentially, population persistence. Indeed, Xu et al. (2010) found that the interactive effects of streamflow and temperature impacted...
growth of Eastern Brook Trout, also in a context-dependent manner. In addition, high flows in the early season due to snowmelt and rain on snow events combined with the shorter period of snow pack may actually increase the probability of low streamflow and drought conditions (Demaria et al., 2016). This sets the stage for potential stranding events for fish in the later spring/summer, during periods of warmer than preferred temperatures. During times like these (low flows and high temperature), fish may be forced to cope with warmer temperatures while isolated in warming pools. The interconnection of climate change on streamflow conditions with respect to both temperature and water levels brings to light an important set of stressors on Eastern Brook Trout that may be exacerbated in future years. Our study suggests that both high flows with low water levels and low flows with high temperatures may act in concert to induce the cellular response to stress in Eastern Brook Trout, creating, in some years, a period of intermittent stress from ice out through to spawning season.

Our findings raised several questions that will help to guide future research. What is the role of phenotypic variation in energy storage/body condition; prior exposure and magnitude of the HSP70 expression on shaping the response of Brook Trout to stressors? What is the connection between plasma cortisol and the HSP70 stress response, and how can access to refuge habitat, across seasons, influence this connection? Future efforts should undertake multiscale measurements of the stress response in fish exposed to environmentally realistic stressors. This data should be complemented by laboratory-controlled investigations of single and multiple stressors on individuals. There is a pressing need to clearly illuminate connections across biological scales, under environmentally realistic conditions, to better establish the nature of stress responses in wild populations, from molecular to population level impacts (Heidinger and Wada, 2019; Petitjean et al., 2019).

Conclusions
Climate change and other anthropogenic factors have led to the imperilment and extinction of many species, and Eastern Brook Trouts are no exception. Our study is one of few that examined the HSP70-based response of Eastern Brook Trout to multiple interacting stressors experienced in the wild, including temperature, relative water level and conductivity. This data provides a broad perspective of the cellular stress response in Eastern Brook Trout with two emergent components: (i) temperature and water level are important stressors; and (ii) water level plays an important role in modulating the effect of temperature, which helps to explain the seasonal shift in HSP70 expression. However, fish size and capture location were not significantly associated with variation in HSP70 in our study population.

The results from this study also suggest that Eastern Brook Trout populations in the northern portion of their range are responsive to temperatures lower than observed in even slightly more southern and/or lower elevation populations, but also at the colder end of the optimal temperature range. We also found that variability in HSP70 expression was much greater under cold conditions than warm, suggesting some inherent variability within the population and or differences in behavioural stress mitigation. The current study provides important insights into understanding the HSP70-based response to complex environmental conditions and may guide future studies of the cellular mechanisms of the stress response in the face of rapid climate change.

Acknowledgments
The authors extend a sincere thanks to The Conservation Fund as our research partner and for allowing us access to the Beebe River property. We would also like to thank Tyson Morrill for his efforts initiating the Beebe River Brook Trout study, Josh Hoekwater and Jared Lamy for continued management of the project, members of the Beebe River Brook Trout Field Crew (2016–2019), New Hampshire Fish and Game Department (particularly Ben Nugent) and members of the Pemigewasset Chapter (#726) of Trout Unlimited (particularly Dave Pushee) for tireless investment of energy into this work. We thank Roy Fruit for his earlier work on the HSP70 lab protocol (on stoneflies and mayflies). Critical assistance on the laboratory work was provided by the following: Chelsea Barnes performed immunoblotting in 2019, members of the Chabot laboratory at Plymouth State assisted with immunoblotting protocols/imaging, Suzie Currie (Acadia University, Nova Scotia) donated positive control tissue and Stephen McCormick (University of Massachusetts-Amherst) supplied expert advice on gill filament collection and protein extraction. Two anonymous reviewers provided critical feedback that led to an improved manuscript. This study was approved by the IACUC committee at Plymouth State University.

Funding
This work was supported by the New Hampshire Water Resources Research Center through the United States Geological Survey National Institutes for Water Resources Grants Program (section 104 g; grant number G16AP00070) and the National Fish and Wildlife Foundation ‘Bring Back the Natives’ program (2018). Support was also received through three sources at Plymouth State University: The Tourism, Environment and Sustainable Development Cluster, Student Research and Creativity Fund and The Center for the Environment Advancing Undergraduate Environmental Research program.

Conflict of Interest
The authors declare no conflicts of interest.
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