1 **Is altered behavior linked to cellular energy regulation in a freshwater mussel (*Elliptio***
2 ***complanata*) exposed to triclosan?**

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ABSTRACT

Environmental stress may alter the bioenergetic balance of organisms by resulting in greater energy investment into detoxification processes, which diverts energy from other biological functions. Here, we examine responses to triclosan (TCS) exposure in a freshwater mussel across multiple biological levels: behavioral (*e.g.*, burrowing and movement activity), organismal (*e.g.*, metabolic rate and heart rate), and subcellular (*e.g.*, gene expression and protein abundance/activity). At the subcellular level, we employed both energetic (*i.e.*, AMP-activated protein kinase (AMPK)) and traditional (*i.e.*, heat shock protein (HSP70), superoxide dismutase (SOD), glutathione-S-transferase (GST)) biomarkers. We found a significant reduction in burrowing and movement behaviors, a 1.8-fold increase in total-AMPK protein abundance, and a 2.8-fold increase in AMPK activity after 21 d. GST activity increased after 4 d, but not after 21 d. Our findings suggest TCS exposure results in an energetic tradeoff between detoxification at the cellular level and whole-animal activity.

Keywords: triclosan, bivalves, AMPK, bioenergetics, biomarkers, behavior, energy allocation

1. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) have been flagged as high priority pollutants because they pose potential ecological hazards at low concentrations (Harada *et al.*, 2008; Murray *et al.*, 2010; Mearns *et al.*, 2013). Triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy) phenol) is a broad-spectrum antimicrobial agent found in numerous PPCPs at concentrations typically ranging between 0.1% to 0.3% active ingredient by weight (Sabaliunas *et al.*, 2003). Of the estimated 300 tons of TCS used annually in the USA, 85% is contained within PPCPs, which are typically disposed of through residential drains (Bedoux *et al.*, 2012). Consequently, TCS is commonly detected in municipal wastewater treatment facility influent as well as effluent (McAvoy *et al.*, 2002; Antoniou *et al.*, 2009; Bell *et al.*, 2011; Bedoux *et al.*, 2012 and references therein). Global environmental inputs of TCS exceed 600,000 kg yr⁻¹ and could be as high as 10,000,000 kg yr⁻¹ (TSCA, 2003). Environmental concentrations of TCS vary temporally and among water bodies, with surface water TCS concentrations ranging from <1.0 - 2,300 ng L⁻¹ (see reviews by Barber *et al.*, 2000; Kolpin *et al.*, 2002; Bedoux *et al.*, 2012; Dhillon *et al.*, 2015). The continuous introduction of TCS into aquatic environments makes it a pseudo-persistent pollutant that is bioavailable and potentially harmful to aquatic organisms (Coogan and La Point, 2008; Gatidou *et al.*, 2010; Kookana *et al.*, 2013).

Several studies have demonstrated that exposure to TCS can alter biochemical processes in bivalves. For instance, acute TCS exposure increases activities of detoxification enzymes (Binelli *et al.* 2009a,b,2011; Matozzo *et al.*, 2012a), like glutathione-S-transferase (GST) and superoxide dismutase (SOD). SOD and GST are phase I and phase II detoxification enzymes, respectively, and are typically used to evaluate cellular oxidative stress. Phase I enzymes oxygenate xenobiotics, producing either polar metabolites that are directly excreted, or substrates for phase II enzymes. Phase II enzymes transform xenobiotics into compounds that may be incorporated by an organism (Gregus, 2013). TCS can also up-regulate heat shock proteins, which act as protective scaffolding proteins (Riva *et al.* 2012). Additionally, recent *in vitro*

experiments with mammalian cell lines suggest TCS acts as a mitochondrial uncoupler and reduces ATP production (Weatherly *et al.*, *In press*), though this mode of action has not been investigated in bivalves. Although these and other studies (see Canesi *et al.*, 2007; Cortez *et al.*, 2012; Matozzo *et al.*, 2012b; Falfushynska *et al.*, 2014) provide strong support for TCS causing disruption of cellular and biochemical processes, it is unclear whether cellular responses are relevant at higher levels of organization (*e.g.*, organismal or population level; Maltby, 1999; Forbes *et al.*, 2006). Indeed, recently there has been greater effort to elucidate adverse outcome pathways by establishing linkages across levels of biological organization (Ankley *et al.* 2010). For this reason, we examined energetic effects of TCS exposure in mussels at multiple levels of biological organization

Energy is a common currency that spans all levels of biological organization (Brown *et al.*, 2004), thus a bioenergetics approach to evaluating toxicity may help link subcellular effects to effects at higher levels of biological organization. According to the dynamic energy budget hypothesis (reviewed in Kooijman *et al.*, 2009), organisms are typically energy-limited by resources in their environment and metabolic capacity, and must allocate available resources between various biological functions (*i.e.*, reproduction, activity, growth, maintenance). Environmental stress can alter the allocation of resources due to increased energy requirements for repair costs or reduced energy (*i.e.*, ATP) producing potential (Selye, 1950; McEwen and Wingfield, 2003; Romero *et al.*, 2009). As a result of cellular energy depletion, metabolically costly processes, like movement and reproduction, may be impaired (Sokolova *et al.*, 2012). Therefore, biomarkers of cellular energy status may serve as informative predictors of effects at higher levels of biological organization (De Coen and Janssen, 2003).

Energy homeostasis at the cellular level is regulated in part by the heterotrimeric protein AMP-activated protein kinase (AMPK), which is ultrasensitive to changes in ATP availability (Hardie *et al.*, 2006). During an energetic challenge, AMPK acts to maintain ATP concentrations by promoting ATP-producing pathways (*e.g.*, glucose uptake, glycolysis, fatty acid beta-

oxidation) and, in tandem, inhibiting ATP-consuming pathways (*e.g.*, protein synthesis, fatty acid synthesis, glycolysis synthesis; Hardie *et al.*, 2006). The regulatory function of AMPK is essential to maintaining energy homeostasis because the ΔG from the hydrolysis of ATP to ADP is dependent on cellular ATP:ADP ratios. For this reason, organisms can be energetically stressed without observable changes in ATP concentration. In mammals, AMPK is comprised of a catalytic subunit (AMPK α) and two regulatory subunits (AMPK β , AMPK γ ; Carling *et al.*, 1989). Although the structure of AMPK has not been validated in mussels, AMPK is highly conserved between different eukaryotes (Hardie, 2007), and we have therefore assumed this kinase has a heterotrimeric structure in mussels as well (Guevelou *et al.*, 2013; Jost *et al.*, 2015). Recently, we found evidence for AMPK as a sensitive energetic biomarker to municipal effluent exposure *in situ* (Goodchild *et al.*, 2015), but it is unknown whether this is associated with effects at higher levels of biological organization.

The present study evaluated energetic effects caused by subchronic TCS exposure in the freshwater mussel *Elliptio complanata* (Bivalvia: Unionoida; hereafter referred to simply as mussels) across multiple biological levels: behavioral (*e.g.*, burrowing and movement activity), organismal (*e.g.*, metabolic rate, heart rate), and subcellular (*e.g.*, protein abundance/activity and gene expression). At the subcellular level, we examined AMPK as a biomarker of cellular energy status, heat shock protein 70 (HSP70) as an indicator of general stress, and antioxidant enzymes (*i.e.*, SOD, GST) as indicators of oxidative stress. We hypothesized that the energetic cost of detoxification disrupts cellular energy homeostasis, resulting in increased AMPK levels and an energetic tradeoff with other biological processes.

2. METHODS

2.1 Animal Collection and Maintenance

Mussels (60 mm $\pm$ 5) were collected in August 2013 from the Sheepscot River ME, USA (44.180695, -69.626566), which has been annually monitored from 1998-2012 and has received a high water-quality rating, determined by macroinvertebrate diversity and water chemistry (see

Tsomides *et al.*, 2013). Collected mussels were immediately transported to the University of New England's Marine Science Center in plastic bags of river water. Mussels were allowed to acclimate for 3 weeks in a 500 L tank with continuously aerated dechlorinated tap water and 90% water renewals occurring every other day. During acclimation, mussels were fed PhytoPlex Phytoplankton (Kent Marine, Walnut Creek, CA, USA) *ad libitum*. Throughout acclimation and TCS exposure, water conductivity (100 ± 20 mS), temperature ($18 \pm 2^\circ$ C), dissolved oxygen (9.27 ± 0.41 mg/L), and pH (7.14 ± 0.37) were monitored, and photoperiod was maintained at 12:12 D:L.

2.2 Experimental Design

TCS exposures occurred in 80 L glass aquaria (3 L per mussel) at nominal concentrations of 300, 600, or 900 ng L⁻¹ (Sigma-Aldrich, CA, USA). Exposure concentrations were maintained with 100% water renewals every 48 h. We did not measure TCS concentrations analytically; nonetheless, our methods are consistent with previously conducted studies that employed similar protocols (*e.g.*, Binelli *et al.*, 2009b; Gatidou *et al.*, 2010; Riva *et al.*, 2012; Matozzo *et al.*, 2012a). Additionally, Kookana *et al.* (2013) investigated TCS bioconcentration in a marine mussel (*Mytilus galloprovincialis*; TCS concentration: 100 ng L⁻¹; density: 4 L per mussel) and found only a 20% reduction in TCS after 72 h. Thus, although some caution should be used when interpreting our results, there is strong evidence to suggest a 48 h renewal period is sufficient to maintain TCS concentrations. A stock solution of 1 g TCS L⁻¹ dimethyl sulfoxide (DMSO) was further diluted in dechlorinated tap water for a final concentration of 5 mg TCS L⁻¹. DMSO was used as a carrier molecule to deliver TCS to tissue. The control group was exposed to DMSO and in all treatments DMSO concentrations were <0.0001% v/v DMSO by water. Mussels were fed 2 h prior to each water renewal during TCS exposure. Our experimental design allowed us to investigate responses at multiple levels of organization, but in doing so, precluded us from measuring all responses for each individual. Consequently, behavior, metabolic rate, heart rate, and subcellular effects were measured independent of each other (*i.e.*, tissue of mussels from the

behavior assay was not analyzed for subcellular endpoints). All protein and mRNA effects were assessed for the same individual. Responses were measured after 4 and 21 d except behavior, which was only measured after 21 d.

2.3 Behavior

Burrowing and movement assays were conducted according to Waller *et al.* (1999) with slight modifications. Briefly, prior to TCS exposure, identification buoys were attached to the posterior slope of the left valve with fishing line, dental wax, and super glue, allowing identification of completely burrowed individuals. After TCS exposure, mussels (n = 10 per treatment) from all treatment groups were transferred to a single fiberglass tank (1.83 m long, 0.91 m wide, and 0.41 m deep) with a 0.15 m-deep layer of sand and TCS-free dechlorinated tap water. Mussels were placed on the substrate and location, given by the umbo, was recorded by dropping a weighted line through an overlaid plastic grid (cell size = 1.5 cm). Burrowing and movement behaviors were recorded at 1, 2, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h, and mussels were described as inactive, burrowed, or relocated (*i.e.*, movement event). In nearly all instances, righting occurred with burrowing or relocation events and was not differentiated in this study. In mussels, activity is an ordered process, and relocation is preceded by righting and burrowing (Waller *et al.* 1999). For this reason, slight movements (<7.5 cm) occurring prior to burrowing were disregarded, as such movements are an artifact of the burrowing process and not a true relocation event.

2.4 Metabolic rate

Basal metabolic rate was determined by closed vessel respirometry. Mussels (n = 7 per treatment) were placed in sealed respirometry jars, and oxygen concentrations were taken in triplicate at 5 min intervals using an OxySense® system and an oxygen sensitive OxyDot®. To avoid stress from hypoxic conditions, mussels were removed from respirometry jars before oxygen concentration dropped below 85% of initial concentration.

2.5 Heart rate

Heart rate ($n = 7$ per treatment) was measured by impedance pneumography (see Braby and Somero, 2006). Briefly, two 1.8 mm holes were made on the postero-dorsal edge of the right valve next to the pericardial cavity using a Dremel tool. Silver electrodes (40 AWG wire, with 3 mm of tip exposed) were inserted into each hole and held in place with superglue and dental wax. Heart rate was monitored for 3-5 min by converting an analog impedance signal to a voltage signal (UFI, Impedance Converter model 2991, Morro Bay, CA, USA) and recorded using a data acquisition system (ADInstruments, PowerLab, Colorado Springs, CO, USA).

2.6 Protein abundance and activity

Protein abundance and activity were measured using previously established protocols (see Frederich *et al.*, 2009; Jost *et al.*, 2012). Hepatopancreatic tissue (~1g) was isolated from mussels ($n = 5$ per treatment), snap frozen in liquid nitrogen, and stored at -80°C . To prevent dephosphorylation of enzymes, tissue homogenization occurred in a phosphate buffer containing a cocktail of protease inhibitors (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride at 104 mM, aprotinin at 80 μM , bestatin at 4 mM, E-64 at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM; Sigma-Aldrich, St Louis, MO, USA). Homogenized tissues were centrifuged at $1700 \times g$ for 10 min; the supernatant was collected and centrifuged again at $21000 \times g$ for 40 min. The Bradford method was used to determine total protein concentration for each sample (Bradford *et al.*, 1976).

Relative quantities of total-AMPK, phosphorylated-AMPK (*i.e.*, AMPK-activity), and total HSP70 were determined by western blotting. Proteins from homogenate (50 μg) were separated on a 10% polyacrylamide/SDS gel at 180 V for 30 min and transferred to a nitrocellulose membrane at 70 V for 2 h. The membrane was then blocked with 3% non-fat dry milk. The proteins were visualized with primary mouse anti-HSP70 (Sigma, St Louis, MO, USA; product no.: H5147), rabbit anti-AMPK (Millipore, Billerica, MA, USA; product no.: 07-681) and rabbit anti-pT172 (AmpliCruz, Santa Clara, CA, USA; product no.: sc-33524) antibodies, and quantified on a LiCor Odyssey Imaging system with secondary donkey anti-rabbit antibodies and

goat anti-mouse antibodies (Sigma-Aldrich). Mouse anti-actin antibodies (Sigma-Aldrich) were used as a loading control. Protein abundance was determined by densitometric analysis using ImageJ software (<http://imagej.nih.gov/>; see Perez and Pascue, 2013) relative to controls (Heidebrecht *et al.*, 2009).

Enzyme activities of SOD and GST were measured in duplicate according to McCord and Fridovich (1969) and Habig *et al.* (1974), respectively. Reactions were carried out in a phosphate buffer (Sigma-Aldrich) using standard cuvettes and a Beckman Coulter 700 Series UV spectrophotometer. SOD competes with cytochrome C for superoxide radicals produced enzymatically by hypoxanthine and xanthine oxidase. SOD activity was measured as inhibition of cytochrome C reduction at 550 nm for 5 min. GST activity was determined by the conjugation of glutathione and dinitrobenzene facilitated by GST and monitored at 330 nm for 5 min.

2.7 mRNA Expression

Relative mRNA concentration of AMPK γ , HSP70, SOD, and GST mRNA were quantified by semi-quantitative real-time PCR according to Frederich *et al.* (2009). Briefly, hepatopancreatic tissue (~1 g) was isolated from the same individuals used for protein analysis. Tissue was placed in RNeasy[®] Solution (Life Technologies, Carlsbad, CA, USA), and stored at 4° C until analysis. Total RNA was purified using the Total RNA Isolation System (Promega, Madison, WI, USA). Tissue was homogenized in RNA denaturing solution using a Bullet Blender and RNAase-free zirconium oxide beads (Next Advance, Averill Park, NY, USA). Homogenate was centrifuged at 1700 x g for 10 min. The supernatant was collected, acidified with sodium acetate, extracted with phenol-chloroform, and centrifuged at 21000 x g for 40 min. RNA was precipitated overnight in isopropanol at -20° C. After centrifugation, the pellet was washed, resuspended in RNAase-free water, and stored at -80° C until analysis. 1.8 μ g of total RNA (260/280 absorbance ratio: 2.0 ± 0.1) were reverse transcribed using the Super-Script III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Alien RNA (Agilent, La Jolla, CA, USA) was added as an external control for alerting reverse transcription inhibition and

normalizing PCR efficiency (Huggett *et al.*, 2005). Gene targets were amplified with primers developed by Goodchild *et al.* (2015; Table 1) and an Agilent Brilliant SYBR Green qPCR Kit (Agilent, La Jolla, CA, USA) on a Stratagene MX3005s instrument. After 40 cycles with an annealing temperature of 53° C for AMPK γ and HSP70, or 55° C for SOD and GST, a melting curve analysis confirmed that only one DNA product was amplified per gene target.

2.8 Statistical Analysis

To analyze behavioral data, two statistical analyses were employed to describe (1) time-dependent intensity of whole-animal performance and (2) distribution of behavioral responses within treatment groups. Use of the Cox proportional hazard model (Cox, 1972) is increasingly common in behavioral ecology studies in general (e.g., Cole and Quinn, 2014) and has been used multiple times to specifically assess mussel burrowing performance (e.g., Waller *et al.*, 1999; Hazelton *et al.*, 2014). This modeling approach generates a proportional hazard also described as intensity of an event given a condition. In our case, we used this modeling approach to describe intensity of burrowing and movement following TCS exposure. This approach to analyzing mussel behavioral data is appropriate because some individuals did not conduct behaviors during the study duration and therefore the data are considered right-censored, which the proportional hazard model accommodates (see Waller *et al.*, 1999; Hazelton *et al.*, 2014); the alternative approach is to remove non-responding individuals from the dataset and use a group means statistical test (e.g., Flynn *et al.*, 2013). The parameter estimates are generated as a proportional likelihood of an event occurring relative to the control group. In a second analysis, we evaluated the overall effect of TCS on mussel activity, regardless of when events occurred. To do so, we used an ordered logit model to test if TCS affects mussel vigor, with the assumption that increasing activity (inactivity < burrowing < relocation) indicated greater vigor (Waller *et al.*, 1999).

A repeated-measures analysis of variance was used to analyze heart rate data (standard assumptions were tested and Mauchly's test confirmed that the model did not violate the assumption of sphericity ($p = 0.89$)). Metabolic rate and GST and SOD activity were analyzed

using two-way analysis of variance. Western blot and qPCR data were quantified relative to the controls of the respective sampling day (*i.e.*, 4 d or 21 d), therefore mRNA concentrations and protein abundance of AMPK and HSP70 were analyzed *via* one-way analysis of variance within sampling time-points. All statistical tests were done with R Version 3.0.2 (R Core Team, 2013) using RStudio© Version 0.97.551 interface (RStudio Team, 2015).

3. RESULTS

3.1 Behavior

TCS exposure impaired burrowing and movement behaviors (Fig. 1). The Cox proportional hazard model revealed that mussels exposed to 900 ng L⁻¹ TCS concentration had a lower likelihood of burrowing ($p = 0.03$) and relocating ($p = 0.02$) relative to the control group (Table 2). Activity decreased in mussels exposed to TCS (Fig. 1b), and the time-independent ordered logit model provided further evidence that TCS exposure decreases mussel vigor ($p = 0.01$).

3.2 Protein abundance and activity

After 21 d of exposure to TCS there was a 1.8-fold increase in total-AMPK α ($p = 0.04$; Fig. 2a), and mussels in 900 ng L⁻¹ group had a 2.8-fold increase in AMPK-activity ($p = 0.02$; Fig. 2b). There was no difference in HSP70 concentrations at either sampling point (Fig. 2c). GST activity was significantly increased after 4 d in the 300 ng L⁻¹ ($p = 0.02$) and 900 ng L⁻¹ ($p = 0.03$) groups, but not in the 600 ng L⁻¹ group (Fig. 3a). However, there was no difference in GST or SOD activity after 21 d (Fig. 3).

3.3 mRNA Expression

There was no change in AMPK mRNA expression after 4 d. However, after 21 d, the 900 ng L⁻¹ group had elevated AMPK mRNA expression relative to the control, but this difference was not significant ($p = 0.06$; Fig. 4a). GST mRNA expression trended upward after 4 d, though this trend did not achieve statistical significance ($p = 0.06$) and there were no differences after 21 d (Fig. 4d). mRNA expression of HSP70 (Fig. 4b) and SOD (Fig. 4c) were unchanged.

3.4 Heart rate and oxygen consumption

At the organismal level, there were no significant differences ($p > 0.05$) in resting metabolic rate (Fig. S1) or heart rate (Fig. S2) after 4 and 21 d of TCS exposure. Heart rate remained unchanged at 8.9 ± 0.6 beats per minute, and oxygen consumption stayed constant at $0.27 \pm 0.07 \mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$.

4. DISCUSSION

Environmental contaminants can have a wide range of physiological effects that vary depending on level of biological organization and exposure duration. In this study, we examined effects across multiple scales and after acute and subchronic exposure durations. Our data suggest TCS exposure reduces burrowing and relocation, which are energetically costly behaviors essential to mussel ecology. During high river discharge or other disturbances, mussels can become dislodged and must reorient themselves by righting, burrowing, and relocating (Waller *et al.*, 1999). An inability to perform these activities may increase predation risk (Bowers *et al.*, 2005), diminish reproductive success (Amyot and Downing, 1997, 1998; McMahon and Bogan, 2001), and decreases drought tolerance (Gough *et al.*, 2012). Moreover, reduced mussel activity may impact the broader benthic community because disoriented mussels are unable to conduct important ecological services such as water column filtration (Caraco *et al.*, 2006; Loayza-Muro and Elias-Letts, 2007; Allen and Vaughn, 2009), aeration of benthic sediments through bioturbation (Loher *et al.*, 2004), and biodeposition of nutrient dense material (Vaughn and Hakenkamp, 2001; Spooner and Vaughn, 2012). Because burrowing and movement behaviors are vital to mussels' reproductive strategies and their ability to perform ecological services, energy stress may have proximate (*i.e.*, individual fitness) and ultimate (*i.e.*, population health and community stability) effects (Calow, 1989).

In a variety of taxa, energetically demanding activities are associated with cellular energy stores (*e.g.*, Carmi *et al.*, 1992; Milligan, 1996; Richards *et al.*, 2002); therefore a reduction in burrowing and movement may indicate that TCS exposure causes a reallocation or depletion of cellular energy (Sokolova *et al.*, 2012). However, we did not detect any differences in resting

oxygen consumption or heart rate at the organismal level, suggesting the energetic cost of TCS exposure is not compensated for by an increase in resting metabolic rate. Palenske *et al.* (2010) report increased heart and metabolic rates in frog larvae exposed to TCS, but at concentrations much higher (47-500 $\mu\text{g L}^{-1}$) than those used in this study or even typically observed in the environment. Increased metabolic and heart rates have been observed in bivalves exposed to pollutants (Bakhmet *et al.*, 2009; Manachini *et al.*, 2013), though some pollutants known to be highly toxic, such as cadmium, did not alter heart rate or metabolic rate (Lannig *et al.*, 2008). Due to experimental limitations, it was not possible to measure behavior and resting metabolic rate simultaneously for the same individual, as doing so would likely disrupt the animals' behavior. Thus we were not able to directly compare these endpoints within the same individual. Additionally, we did not examine whether TCS exposure reduced metabolic scope (*i.e.*, the difference between maximum metabolic rate and resting metabolic rate; Clarke and Pörtner, 2010). A reduction in metabolic scope may be impeding burrowing, though further experimentation is needed to explicitly test this hypothesis.

Although we did not detect differences in resting metabolic rate, previous studies have demonstrated that environmental stressors do not necessary alter all components of an organism's energy budget, but rather result in complex energetic trade-offs (DuRant *et al.*, 2007). At the cellular level, protein abundance and mRNA expression data revealed induction of total-AMPK at 900 ng L^{-1} TCS and increased AMPK-activity after 21 d exposure, indicating that TCS inhibits ATP production or facilitates ATP depletion. Protective and repair mechanisms are energetically demanding (Sokolova *et al.*, 2012), thus increased AMPK activity is in accord with the many studies describing oxidative stress, DNA damage, lysosomal membrane instability, and endocrine disruption in mussels exposed to TCS (Binelli *et al.*, 2009a,b,2011; Riva *et al.*, 2012; Falfushynska *et al.*, 2014). Additionally, TCS may target mitochondria and impede ATP production (Weatherly *et al.*, *In press*), which would also cause AMPK activation. Taken as a

whole, our data suggests that TCS alters energy homeostasis at the cellular level, which may result in an energetic trade-off between activity (*i.e.*, burrowing and movement) and repair.

We found marginal evidence for detoxification costs. GST enzyme activity increased, and a similar trend was mirrored in the GST mRNA data, though is not significant ($p = 0.06$). Increased GST activity in response to TCS has been reported previously by Binelli *et al.* (2009a, 2011) at concentrations similar to those used in this study. Importantly, the greatest increase in enzyme activity reported in other studies occurred within the initial 48 h of exposure, and GST activity returned to baseline by 96 h. In our study, we observed increases in GST levels after 4 d, but no effect on GST levels after 21 d. We did not observe any difference in SOD levels, but as with GST, prior studies observed increased SOD activity during the initial 72 h and a return to baseline by 96 h (Binelli *et al.*, 2011). Since prior studies saw a return to baseline after 4 d of exposure, it is plausible that we only captured the GST response as it returned to baseline, and we potentially missed any effect on SOD. By comparing results from acute exposure reported elsewhere (Binelli *et al.*, 2009b, 2011) and our subchronic exposure reported here, it appears that SOD and GST are important in the initial response to TCS exposure, but are not part of protective strategies under subchronic conditions (Binelli *et al.*, 2009b; Hinthner *et al.*, 2011).

Evaluating toxicity from an energetics perspective may be useful for establishing adverse outcome pathways (Ankley *et al.*, 2010) for toxicants that potentially target mitochondria; however, several different methods exist for examining cellular energy stress and it is unclear which methods are most sensitive to mitochondrial disruption. For instance, some studies have focused on measuring ATP concentrations or ATP:ADP ratios (*e.g.*, Dickinson *et al.*, 2011), but as we discussed elsewhere, such data might not capture energetic stress. Alternatively, De Coen and Janssen (2003) proposed measuring concentrations of cellular macromolecules to determine “cellular energy allocation”, which indirectly assesses ATP consumption by assuming energetically stressed animals will catabolize macromolecules to satisfy their energy needs. In our case, we used AMPK because it has been mechanistically identified as an upstream regulator of

macromolecule metabolism and is directly activated by slight decreases in ATP concentrations (Hardie *et al.*, 2006). Therefore, we propose that AMPK may be a valuable energetic biomarker for use in ecotoxicological studies because it is sensitive to low concentrations of pollutants in both controlled and field scenarios (see Goodchild *et al.*, 2015), though further comparative studies are needed to evaluate which methods are most informative.

Ultimately, adverse outcome pathways aim to make links between effects at multiple levels of organization (Groh *et al.*, 2015), thus in order for AMPK to be an informative component of adverse outcome pathways, more evidence is needed that links AMPK activity with effects at higher levels of organization. Previous studies have demonstrated linkages between running endurance and AMPK activity in marine crustaceans following various types of environmental stress, such as elevated water temperatures, low salinity conditions, and hypoxia (Frederich *et al.*, 2009; Jost *et al.*, 2012). AMPK is also known to have a functional role of accelerating ATP producing pathways in skeletal muscle during exercise (Hardie and Sakamoto, 2006). Nonetheless, further study examining the relationship between AMPK in muscle tissue and mussel activity will strengthen the energetic link between cellular and whole-animal effects.

5. CONCLUSION

Exposure to environmentally relevant concentrations of TCS may result in a reallocation of available energy. Behaviorally, mussels exposed to TCS were less likely to burrow and move, which might reduce fitness *in situ*. Additionally, TCS exposure resulted in increased total-AMPK protein and mRNA levels, along with an increase in AMPK-activity. Elevated AMPK levels suggest that detoxification of TCS results in an energetically taxing situation. By pairing our molecular and behavioral data, we speculate that TCS exposure may result in an energetic tradeoff between detoxification and movement behaviors.

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 615

Tables:

Table 1. Specific primers for *Elliptio complanata* for RT-PCR analysis of relative mRNA concentrations of respective gene targets.

Target	Primer	Nucleotide Sequence
AMPK γ	F	5'- GAA GTG GAG AGA GGA ATT GAA GG- 3'
	R	5'- TGT GGA CAT GGT GTT GGA TTA G- 3'
HSP70	F	5'- GGT GTG TTG ATC CAG GTC TAT G -3'
	R	5'- GCT GGT GGA ATT CCC TGT AA -3'
SOD	F	5'- CAA CCC TAG CAA GCA GGA A -3'
	R	5'- CAT GTG CCA CTC CAT CAT CT -3'
GST	F	5'- GCT GAG GTA CTT GGG AAG AAA G -3'
	R	5'- AGC AAG TCG ATA GTC CTC CA -3'

Table 2. Cox proportional hazard model describing the likelihood of burrowing and movement behaviors following 21 d exposure to triclosan. Model outputs are odds ratios relative to the control group. Values < 1 indicate diminished likelihood of a behavior occurring. Bolded values indicate $p < 0.05$.

	Burrowing	Moving
300 ng/L	0.68 (p = 0.50)	0.60 (p = 0.36)
600 ng/L	0.47 (p = 0.19)	0.51 (p = 0.23)
900 ng/L	0.28 (p = 0.03)	0.23 (p = 0.02)

Figure Legends:

Fig. 1. Burrowing and movement performance of *Elliptio complanata* after 21 d exposure to nominal concentrations of triclosan. (A) Time until burrowing. (B) Number of mussels that were inactive, burrowed only, or relocated within treatment groups (n =10 per treatment); none of the mussels in the control or 300 ng L⁻¹ group remained inactive.

Fig. 2. (A) Total-AMPK, (B) AMPK-activity, and (C) HSP70 protein abundance in *Elliptio complanta* after 4 (gray bars) and 21 (white bars) d exposures to nominal concentrations of triclosan. Representative western blot panels are displayed above respective proteins. Data are normalized to set the data for control group to 1. * denotes statistical significance (p < 0.05) compared to relative control. Mean ± S.E., n=5 per treatment.

Fig. 3. GST (A) and SOD (B) enzyme activity in *Elliptio complanta* after 4 (gray bars) and 21 (white bars) d exposure to nominal concentrations of triclosan. * denotes statistical significance (p < 0.05) compared to relative control. Mean ± S.E., n=5 per treatment.

Fig. 4. mRNA expression for AMPK γ (A), HSP70 (B), SOD (C), and GST (D) in *Elliptio complanata* after 4 (gray bars) and 21 (white bars) d exposures to nominal concentrations of triclosan. Data are normalized to set the data for control group to 1. No treatments achieved statistical significance (p < 0.05) compared to relative control; however, # denotes groups that approached significance (p < 0.10). Mean ± S.E., n=5 per treatment.

653 Supplementary Figure Legends:

654 Fig. S1. Resting metabolic rate measured by oxygen consumption in *Elliptio complanata* after 4
655 and 21 d of exposure to nominal concentrations of triclosan (mean $\pm$ S.E., n= 6 per treatment).

656

657 Fig. S2. Heart rate in *Elliptio complanata* after 4 and 21 d exposure to nominal concentrations of
658 triclosan (mean $\pm$ S.E., n= 7 per treatment).

659

Figure 1

Figure 2

Figure 3

Figure 4