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Exposure to tritiated water at an elevated temperature: Genotoxic and transcriptomic effects in marine mussels (*M. galloprovincialis*)

Abstract

Temperature is an abiotic factor of particular concern for assessing the potential impacts of radionuclides on marine species. This is particularly true for tritium, which is discharged as tritiated water (HTO) in the process of cooling nuclear institutions. Additionally, with sea surface temperatures forecast to rise 0.5 - 3.5 °C in the next 30-100 years, determining the interaction of elevated temperature with radiological exposure has never been more relevant. We assessed the tissue-specific accumulation, transcriptional expression of key genes, and genotoxicity of tritiated water to marine mussels at either 15 or 25 °C, over a 7 day time course with sampling after 1 h, 12 h, 3 d and 7d. The activity concentration used (15 MBq L⁻¹) resulted in tritium accumulation that varied with both time and temperature, but consistently produced dose rates (calculated using the ERICA tool) of < 20 Gy h⁻¹, i.e. considerably below the recommended guidelines of the IAEA and EURATOM. Despite this, there was significant induction of DNA strand breaks (as measured by the comet assay), which also showed a temperature-dependent time shift. At 15 °C, DNA damage was only significantly elevated after 7 d, in contrast to 25 °C where a similar response was observed after only 3 d. The transcription profiles of two isoforms of *hsp70*, *hsp90*, *mt20*, *p53*

and *rad51* indicated potential mechanisms behind this temperature-induced acceleration of genotoxicity, which may be the result of compromised defence. Specifically, genes involved in protein folding, DNA double strand break repair and cell cycle checkpoint control were upregulated after 3 d HTO exposure at 15 °C, but significantly downregulated when the same exposure occurred at 25 °C. This study is the first to investigate temperature effects on radiation-induced genotoxicity in an ecologically relevant marine invertebrate, *Mytilus galloprovincialis*. From an ecological perspective, our study suggests that mussels (or similar marine species) exposed to increased temperature and HTO may have a compromised ability to defend against genotoxic stress.

Abbreviations: HTO, tritiated water; Fpg, formamidopyrimidine glycosylase; GoI, gene of interest; LSC, liquid scintillation counting; tDAC, tissue dry activity concentration; TFWT, tissue free water tritium; tTAC, tissue total activity concentration; woTAC, whole organism total activity concentration.

Keywords:

comet assay, gene expression, temperature, mussels, *Mytilus*, tritium

1. Introduction

Contaminants do not occur in the environment in isolation; organisms are exposed to fluctuations in biological (intrinsic), biotic and physico-chemical factors. These include competition between species, other contaminants, parasites, temperature, salinity and dissolved oxygen (Manti and D'Arco, 2010). Alterations in these parameters can influence spontaneous or contaminant-

7 induced damage (Dallas and Jha, 2015). As a result, assessment of the effects
8 of multiple stressors on biomarkers is a subject of increasing interest in both
9 ecotoxicology and radioecology (Altenburger et al., 2012; Dallas et al., 2012).
10 Despite this, the potential interactive effects of abiotic stressors when com-
11 bined with radiological contaminants have not been well characterised in
12 aquatic invertebrates (Vanhoudt et al., 2012).

13 Temperature is an abiotic factor of particular concern when it comes to as-
14 sessing the potential detrimental impacts of tritium (^3H) exposure in marine
15 species. This is particularly important as cooling water from nuclear installa-
16 tions is one of the major sources of ^3H to the aquatic environment. Thermal
17 discharge from nuclear facilities is considered to be one of the most impor-
18 tant environmental issues surrounding these establishments, second only to
19 the release of radionuclides (Kokaji, 1995). Discharged water is typically
20 8 - 12 °C above intake in temperate areas (up to a maximum of ~ 30 °C;
21 Bamber 1995), and thermal plumes (i.e. temperatures elevated by > 1 °C)
22 can extend up to 10 km (Tang et al., 2003). Consequently, animals close
23 to discharge pipes can be simultaneously exposed to radioactivity and heat.
24 This is especially significant for sessile aquatic invertebrates such as mussels.
25 Furthermore, climate change is one of the biggest issues facing environmental
26 protection today. As sea surface temperatures are forecast to rise by up to
27 3.5 °C in the next 100 years (IPCC, 2007), determining the interaction of el-
28 evated temperature with radionuclide exposure has never been more relevant
29 (Bamber, 1995; Madden et al., 2013; Kirillin et al., 2013).

30 The IAEA first described a ‘timely need’ for research into thermal dis-
31 charges from NPP/NFRPs in the 1970s (IAEA, 1974). Despite this, the

majority of studies examining the thermal effects of nuclear effluents do so without any radioactive contaminant (Hillman et al., 1977; Poornima et al., 2005; Teixeira et al., 2009). From the limited number of laboratory studies, it is generally agreed that higher temperatures increase radiosensitivity in fish via increased metabolic rates (e.g. Blaylock, 1974). The available data for aquatic invertebrates is consistent with this, however such studies have previously been restricted to only a few species, which are not necessarily representative of wider groups (e.g. *Artemia salina* Dallas et al., 2012).

To date, there is no literature on the effects of radiation and elevated temperature in mussels, a key group of model organisms, either at molecular or higher levels of biological organisation. There is, however, a growing body of work on these ecologically important animals exposed to elevated temperatures alone or in combination with chemical contaminants (e.g Bayne, 1976; Anestis et al., 2007; Mubiana and Blust, 2007; Baines and Fisher, 2008). In terms of chemical contaminants, the bioaccumulation of non-essential metals (Cd and Pb) in *M. edulis* increased at higher temperatures (Mubiana and Blust, 2007) and biokinetic modelling predicted increased accumulation of dietary Ag, Am and Zn in the same species at low temperatures (2 °C; Baines and Fisher 2008). Furthermore, the toxicity of Cu to developing *M. trossulus* embryos increased at temperatures > 15 °C (Yaroslavtseva and Sergeeva, 2007). Given these interactions with chemical contaminants and as mussels are poikilotherms, where metabolic rate is a direct consequence of external temperature, it is of particular interest to investigate the impact of co-exposure to radiation/heat (Buschini et al., 2003).

Molecular biomarkers of heat stress in *Mytilus* spp. are useful for eluci-

57 dating mechanisms behind temperature effects. Due to the highly conserved
 58 nature of many fundamental mechanisms, molecular approaches also pro-
 59 vide synergy between models of environmental and human health (Dixon
 60 et al., 2002). This type of approach has revealed complex effects of elevated
 61 temperature in mussels, such as increased expression of heat shock and met-
 62 allothionein genes (Núez-Acua et al., 2012; Franzellitti and Fabbri, 2005;
 63 Gourgou et al., 2010; Lockwood et al., 2010). Other genes of interest (GoI)
 64 for investigation in mussels under conditions of heat stress and radiation
 65 exposure include: *rad51*, indicative of DNA double strand breaks (Al-Amri
 66 et al., 2012); and *p53*, a tumour suppressor gene with multiple functions,
 67 including interactions with *rad51* (Greenblatt et al., 1994; Pantzartzi et al.,
 68 2010; Di et al., 2011).

69 Against the backdrop of the above information, this study was designed to
 70 fulfil the following aims and objectives: (a) to use tissue-specific accumulation
 71 of ^3H in mussels to determine the effects of elevated temperature on radiation
 72 dose; (b) to assess the impact of elevated temperature on the genotoxicity
 73 of HTO to mussel haemocytes, using the modified comet assay to determine
 74 oxidative DNA damage; and (c) to evaluate the transcription profile of key
 75 radiation and heat shock genes (two isoforms of *hsp70*, *hsp90*, *mt20*, *p53* and
 76 *rad51*) to elucidate potential mechanisms behind temperature-effects. De-
 77 tails of the proteins encoded by our GoI are given in Table 1. It was hypoth-
 78 esised that heat stress alongside tritium exposure would increase radiation
 79 dose and genotoxicity and that such enhanced effects would necessitate the
 80 upregulation of stress response genes.

Table 1: Summary of the major characteristics and functions of proteins encoded by genes targeted in this study.

Protein(s)	Characteristics	Functions
Metallothioneins	Low molecular weight, cysteine rich	Metal binding, radical capture
Heat shock proteins	Six highly conserved classes, based on molecular weight (HSP33, 60, 70, 90, 100 and the small HSPs)	Intra-cellular chaperones (assist with protein folding, prevent aggregation, aid in transport), antigen binding and presentation, vascular relaxation
p53	393 amino acids ^a , seven domains ^a , including transcription-activation, proline rich and DNA-binding	Cell cycle regulation (tumour suppression), DNA repair, initiation of apoptosis
RAD51	339 amino acids ^a , ATP-dependent DNA binding activity, DNA-dependent ATPase activity	DNA repair (homologous recombination)

^a in humans.

2. Experimental

2.1. Experimental design and mussel exposure conditions

Adult mussels (50.40 ± 0.36 mm) were collected from a previously used reference site, Trebarwith Strand (north Cornwall, UK), in April 2013, transported to the laboratory and depurated at 15 °C as described in Dallas et al. (2013a, 2016). Sea surface temperatures at nearby Bude (32 km away) are on average 10 °C in April according to NOAA data (min. 8.4 - max. 11.8 °C; Reynolds et al., 2007). As mussels were collected from rocks in the intertidal zone, air temperature is also relevant, and was 4.8 - 11.5 °C at Chivenor in that month (91 km away; Met Office, 2016). After depuration, mussels were transferred to glass beakers containing 2 L filtered seawater ($<10\mu\text{m}$) at a density of 4.5 mussels L^{-1} and allowed to acclimatise for 48 h (Dallas et al., 2013a). Beakers were randomly allocated to one of 5 treatment groups - a seawater control at 15 °C, a seawater control at 25 °C, 15 MBq L^{-1} HTO at 15 °C, 15 MBq L^{-1} HTO at 25 °C, and a positive control (40 $\mu\text{g L}^{-1}$ CuSO_4 ; D’Agata et al. 2014). The 15 MBq L^{-1} activity concentration was selected as it had shown genotoxic effects in previous experiments (data not shown).

Mussels were exposed to these conditions for 7 d and fed every 72 h (i.e. on day 0 and day 3) with live *Isochrysis galbana* (1.05×10^{-5} cells ml^{-1}) followed by a 100 % water change 2 h afterwards with complete replacement of the HTO, as described in Dallas et al. (2016). The 7 d exposure duration was based on previous work with mussels exposed to tritiated water (Jaeschke et al., 2011) or chemical genotoxins (methane methyl sulfonate and cyclophosphamide; Canty et al. 2009).

Water quality parameters during this experiment were measured daily

Table 2: Mean $\pm$ SD of measured temperatures ($^{\circ}\text{C}$) for mussels exposed to tritiated water (HTO) or Cu positive control at different nominal temperatures.

Treatment	Nominal temperature ($^{\circ}\text{C}$)	
	15 $^{\circ}\text{C}$	25 $^{\circ}\text{C}$
Control	15.34 ± 0.51	25.61 ± 0.43
15 MBq L^{-1} HTO	15.40 ± 0.37	25.58 ± 0.32
40 $\mu\text{g L}^{-1}$ Cu	15.37 ± 0.48	

(HQ40D, Hach-Lange, Dusseldorf, Germany) and were: salinity 34.27 ± 0.31 ; pH 8.33 ± 0.38 ; and dissolved oxygen 91.45 ± 3.74 %. Measured temperatures were close to nominal values (Table 2).

2.2. Sampling procedures

Sampling took place after 0, 1, 12, 72, and 168 h exposure. At the 12, 72 and 168 h time points, 9 mussels (i.e. one beaker) had their haemolymph extracted from the posterior adductor mussel using a 21 gauge needle, and were then dissected into their individual organs for liquid scintillation counting (LSC; gills, mantle, digestive gland, adductor muscle, foot, and ‘other’). Byssus was discarded due to the small weight making measurements inaccurate. Haemolymph samples (50 μl) were stored on ice in the dark until use in the enzyme-modified comet assay. During dissection of mussels, small ($\sim 5\text{mm}^2$) pieces of gill were also removed, weighed and flash frozen in liquid nitrogen. These gill samples were stored at -80°C for RNA extraction and gene expression analysis. Additionally, at the 0 and 1 h time points 9 mussels (i.e. one beaker) were sampled for gene expression only, in order to provide greater temporal resolution for the molecular analysis. Gill was

123 selected for measurement of gene expression as it has previously been shown
124 to exhibit the greatest induction of HSPs in response to heat stress in *M.*
125 *edulis* (Chapple et al., 1997).

126 *2.3. Liquid scintillation counting of water and mussel tissues and dosimetry*
127 *using the ERICA tool*

128 Water samples (100 μ L) were taken daily from each beaker. Both water
129 and tissue samples were processed for LSC as described in detail in Dallas
130 et al. (2016)(Method 4). This method produces measured values for tissue
131 free water tritium (TFWT), dry and total activity concentrations for each
132 tissue (tDAC and tTAC, respectively) and whole organism total activity
133 concentration (woTAC). tDACs are useful for comparison to previous studies,
134 whereas tTACs are summed to produce woTACs, which are then used for
135 whole organism dose calculation with the ERICA tool as described in Dallas
136 et al. (2016) and used by Devos et al. (2015) in oysters.

137 *2.4. Enzyme-modified comet assay to determine oxidative DNA damage*

138 Haemolymph was used for the enzyme-modified comet assay as in Dallas
139 et al. (2013a), except only two slides were produced per sample - one with
140 the buffer control, and one with formamidopyrimidine glycosylase (Fpg; to
141 detect oxidised purines). Slides were coded and randomised to ensure scoring
142 was unbiased.

143 *2.5. Determination of relative transcriptional expression of selected genes*

144 Extraction of total RNA and reverse transcription were performed us-
145 ing the GeneElute Mammalian Total RNA miniprep kit (Sigma-Aldrich, St

Table 3: Genes and primers used for qPCR on mussels exposed to tritiated water and elevated temperature.

Gene	GenBank Accession No.	Forward Primer	Reverse Primer
Alpha tubulin (<i>atub</i>) ^a	DQ174100	5'-TTGCAACCATCAAGACCAAG-3'	5'-TGCAGACGGGCTCTCTGT-3'
Elongation factor 1 (<i>ef1</i>) ^a	AF063420	5'-CACCAACGAGTCTCTCCAGA-3'	5'-GCTGTCAACACAGACCATTC-3'
Heat shock protein 90 (<i>hsp90</i>) ^c	AJ625655	5'-TCAGTGATGATCCTAGATTAGGCA-3'	5'-CGTTCTCTCTTTCCATCTGTAAAC-3'
Heat shock protein 70 sequence 1 (<i>hsp70-1</i>) ^b	AF172607	5'-GGGTGGTGGAACTTTTGATG-3'	5'-GCCGTTTGAAAAAGTCCTGAA-3'
Heat shock protein 70 sequence 2 (<i>hsp70-2</i>) ^d	AF172607	5'-CCCTTTCTTCAAGCACACAAGCA-3'	5'-AACTGGTTCATGGTTCTCTGTGAA-3'
Metallothionein 20 (<i>mt20</i>) ^d	AJ577131	5'-GACGCCCTGCAAAATGTGCAAGT-3'	5'-TCGGACCAAGTGCGGTCAACAT-3'
p53 anti-oncogene (<i>p53</i>) ^e	DQ158079	5'-CAAACTTGCTAAATTTGTTGAAGA-3'	5'-TTGGTCTCTCTACACATGAC-3'
rad51 (<i>rad51</i>) ^f	FJ518826	5'- TGGCATTGAGACTGGGTCAA-3'	5'- CCTTCACCTCCACCCATATC -3'

^a Ciocan et al. 2011; ^b Franzellitti and Fabbri 2005; ^c Banni et al. 2011; ^d Cefas (unpublished); ^e Ciacci et al. 2011; ^f Al-Amri et al. 2012

146 Louis, USA) and M-MLV reverse transcriptase as per the manufacturer's
 147 instructions and as described in Dallas et al. (2013a). Following cDNA syn-
 148 thesis, qPCR was performed on samples in duplicate. Each 15 μ L qPCR
 149 reaction contained 7.5 μ L SYBR Green Jumpstart Taq ReadyMix, 0.03 μ L
 150 of forward and reverse primers (100 μ M), 4.44 μ L of molecular grade wa-
 151 ter and 3 μ L of template cDNA. The qPCR reaction was carried out using
 152 an Applied Biosystems Step-One Plus real-time PCR system with StepOne
 153 Software (v2.2.2; Applied BioSystems). Thermocycling conditions were ini-
 154 tial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec
 155 and 60 °C for 1 min. A dissociation profile (melt curve) was added to verify
 156 the purity of PCR products.

157 Relative expression ratio (RER) of *hsp70-1*, *hsp70-2*, *hsp90*, *mt20*, *p53*
 158 and *rad51* was quantified using REST 2009 (v2.0.13; Qiagen Ltd) from PCR
 159 efficiency (measured using LinRegPCR v2015.3; Ramakers et al., 2003; Rui-
 160 jter et al., 2009) and threshold cycle (C_q), relative to the reference genes
 161 *atub* (alpha tubulin) and *ef1* (elongation factor 1) with control samples as
 162 calibrators (Pfaffl et al., 2002). Primer details are included in Table 3.

163 2.6. Statistics

164 Statistical differences between tDACs/tTACs were investigated using three-
 165 way ANOVAs with time, temperature and tissue as fixed factors. After visual
 166 inspection of residuals, tDACs were log transformed whereas raw tTAC data
 167 were used. Following H_0 rejection, Tukey's post hoc tests were used to de-
 168 termine specific differences. As whole organism total activity concentration,
 169 dose rate and total dose are mathematically related (i.e. by the ERICA tool
 170 algorithms and by a factor of time, respectively) significance is only reported

171 for total dose, but is equivalent between the three parameters. Median values
 172 for % tail DNA (comet assay) were calculated for each mussel and used in a
 173 two way ANOVA with time, treatment and buffer/fpg as fixed factors (Lovell
 174 and Omori, 2008; Dallas et al., 2013a). Spearman’s correlation analyses were
 175 performed to assess the relationship between gene expression and % tail
 176 DNA at all timepoints, and p -values were adjusted for multiple comparisons
 177 (Holm’s sequential Bonferroni adjustment). The gene expression parameter
 178 used for relationship analysis was C_q normalised for reference gene (i.e. ΔC_q
 179 $= C_{q[\text{GoI}]} - C_{q[\text{efl}]}$).

180 **3. Results & Discussion**

181 *3.1. Tritium accumulation and dose estimation*

182 Tritium activity concentrations in water showed good agreement with
 183 nominal values ($> 90\%$ of expected) at $14.3 \pm 0.6 \text{ MBq L}^{-1}$ (15°C) and
 184 $14.2 \pm 0.6 \text{ MBq L}^{-1}$ (25°C). Control water samples’ activity was below the
 185 LOD.

186 In general, tTACs were approximately 2-3 times tDACs (at both 15 and 25
 187 $^\circ\text{C}$; Fig. 1). The order in which tissues accumulated ^3H varied with time and
 188 temperature, but in general digestive gland, gill and foot showed higher con-
 189 centrations than mantle, muscle and other (Fig. 1). Both tDACs and tTACs
 190 showed significant effects of treatment and tissue (three-way ANOVAs, $p <$
 191 0.001) with significant interactions for treatment-timepoint, and treatment-
 192 timepoint-tissue (three-way ANOVAs, $p < 0.05$). However, when examining
 193 the results of post hoc tests for the three-way interaction (Tables 4 and 5)
 194 there were more significant differences among tTACs. For tDACs, specific dif-

Figure 1: Time-dependent tritium accumulation (between 0.5 and 7 days) in mussel tissues after exposure to 15 MBq L⁻¹ HTO at 15 or 25 °C: (a) and (b) are tDACs, i.e. tritium concentration in dry tissue after removal of water by freeze drying; (c) and (d) are tTACs, i.e. tDAC + TFWT, normalised for wet weight. Data are means $\pm$ one SE. Note that although the scales are the same, the y axis starts higher for tTACs.

ferences were most commonly between different tissues at the same timepoint and temperature (e.g. at 15 °C after 3 d, digestive gland was significantly higher than mantle, muscle and other). Whereas for tTACs, differences also occurred between the same tissue at different timepoints (e.g. at 15 °C after 12 h, gill was significantly different from 3 d and 7 d). Differences between temperatures were significant for foot tissue at 3 d (tDAC), all tissues at 12 h, gills, mantle, muscle and other at 3 d, and mantle after 7 d (tTACs). It is interesting to note that the majority of these temperature-related differences occurred within the first 3 d of the exposure period.

In this study, which exposed mussels to 15 MBq L⁻¹ HTO for 7d, woTACs were approximately 34-58% of the equivalent value from Dallas et al. (2016) where they were exposed to the same activity concentration for 14 d (3.90 ± <0.10 MBq kg⁻¹). This suggests that, in general, woTACs increase with duration of HTO exposure. When considering tissue-specific accumulation of tritium, tTACs also showed clear temporal trends, but these were highly temperature-dependent - decreasing in the first 12 h at 15 °C and increasing over the same period at 25 °C. The tTAC trends are reflected in the woTACs - highest at 12 h for the lower temperature and at 3 d for the 25 °C exposure. However, the biological cause of these peaks is less obvious. As ³H rapidly equilibrates with suspended sediment particles (Turner et al., 2009), one possible explanation is that ³H concentrations (particularly tDACs) are related to consumption of such material. This idea is supported by the highest tDAC levels in digestive gland, suggesting food intake is an important source of ³H. Previous authors have reported that *Mytilus* individuals acclimatised to 15 °C and then exposed to > 20 °C respond by reducing clearance rate (CR)

220 with only partial acclimatisation at 25 °C (e.g. Theede, 1963; Bayne, 1976).
221 Such a decrease could explain the lower values for the 12 h timepoint for both
222 tDAC and tTAC in the digestive gland in particular (Fig. 1 b, d). Quantifica-
223 tion of CR during combined heat shock and HTO exposure could potentially
224 address this question. Regardless of the biological cause, the variation with
225 time and temperature adds further weight to the idea that ^3H accumulation
226 in marine mussels is a dynamic and complex process with many contributing
227 factors.

228 There is a limited amount of literature with which to compare our ^3H
229 accumulation data. However, Cd exposure in the oyster, *Crassostrea gigas*,
230 resulted in linear accumulation increasing with temperature over 45 days
231 (0.10 , 0.53 and $0.56 \mu\text{g Cd g}^{-1}$ dry mass d^{-1} for 12 , 20 and 28 °C; Cherkasov
232 et al. 2007). Where temperature effects have been observed for metal accumu-
233 lation in mussels, they have been attributed to changes in solution chemistry
234 and physical kinetics, thereby increasing uptake with increased heat (Mu-
235 biana and Blust, 2007). These factors are thought to be less important with
236 tritium exposure, due to the chemical similarities of ^3H with H. However,
237 isotopic enrichment of ^3H in biopolymers, as a result of the preference of ^3H
238 for weak hydrogen bridges, has been described (Baumgartner and Kim, 2000;
239 Baumgrtner et al., 2001). Although, theoretically there is the potential for
240 increased energy (i.e. heat) to weaken hydrogen bridges (Khan, 2000) and
241 alter this enrichment, it seems unlikely that this would occur at 25 °C.

Table 4: Significant p values from Tukey's HSD post hoc test on the three-way interaction between temperature, time and tissue for mussel tDACs after exposure to 15 MBq $^{-1}$ HTO for 7 d. Individual tissues are digestive gland (Dg), foot (F), gill (G), mantle (Man), muscle (Mus), and other (O). For clarity, values ≤ 0.0005 are represented as 0.000. Non-significant values are not shown.

		Temp	15 °C						25 °C				
		Time	12 h	3 d	3 d	7 d	7 d	7 d	12 h	3 d	7 d	7 d	7 d
Temp	Time	Tissue	Dg	Dg	F	Dg	F	G	Dg	G	Dg	F	G
15 °C	12 h	Mus	0.0208										
	3 d	Man		0.011									
	3 d	Mus		0.000	0.004								
	3 d	O		0.001	0.018								
	7 d	Man				0.002	0.018						
	7 d	Mus				0.000	0.001	0.047					
25 °C	12 h	Man							0.007				
	3 d	F		0.013									
	3 d	Mus								0.004			
	7 d	Man									0.000		
	7 d	Mus									0.000	0.030	0.010
	7 d	O									0.005		

[illegible]

Table 6: Whole organism dose estimates for mussels exposed to tritiated water at either 15 or 25 °C, generated using the ERICA tool and whole organism total activity concentrations as per Dallas et al. (2016)(means $\pm$ SE, $n = 9$).

Temperature	Time (h)	woTAC (MBq kg ⁻¹)	Dose rate (μ Gy h ⁻¹)	Total dose (mGy)
15 °C	12	2.25 $\pm$ 0.02	18.49 $\pm$ 0.16	0.22 $\pm$ 0.01 *
	72	1.89 $\pm$ 0.02	15.58 $\pm$ 0.18	1.12 $\pm$ 0.01 * ^t
	168	1.84 $\pm$ 0.02	15.13 $\pm$ 0.19	2.54 $\pm$ 0.03 * ^t
25 °C	12	1.34 $\pm$ 0.01	10.94 $\pm$ 0.08	0.13 $\pm$ 0.01
	72	2.27 $\pm$ 0.01	18.72 $\pm$ 0.10	1.35 $\pm$ 0.01 ^t
	168	1.99 $\pm$ 0.02	16.35 $\pm$ 0.15	2.75 $\pm$ 0.03 ^t

* indicates significant differences from 25 °C ($p < 0.05$).

^t indicates significant differences from the previous timepoint at the same temperature ($p < 0.001$).

242 3.2. Dose calculations

243 As expected given the woTAC data, there was significant variation in total
244 dose across time and temperature and a significant interaction between both
245 these factors (two-way ANOVA, $p < 0.001$). Dose rates estimated using the
246 ERICA tool ranged from 10.94 to 18.72 μ Gy h⁻¹ giving total doses between
247 0.13 and 2.75 mGy. Interestingly, at 15 °C the highest dose rate was for the
248 12 h sampling point, whereas for 25 °C this was at 72 h (Table 6). At 12 h,
249 exposure to HTO at 15 °C gave a dose 1.7 times higher than that at 25 °C.
250 In contrast, at 72 and 168 h total dose was respectively 1.2 and 1.1 times
251 higher at 25 °C.

252 3.3. Genotoxicity

253 At 15 °C there was significant induction of DNA strand breaks at every
254 timepoint for the Cu positive controls. Although % tail DNA was elevated for
255 HTO exposed mussel haemocytes at each timepoint, it was only significantly

256 so after 168 h (Tukey HSD, $p < 0.05$; Fig. 2A) and did not show any
257 significant evidence of oxidative base damage (i.e. in Fpg-treated slides). At
258 25 °C there was significant induction of strand breakage at each time point
259 in the Fpg-treated HTO exposed samples (Tukey HSD, $p < 0.05$), but only
260 in comparison to the Fpg-treated controls, not the equivalent HTO buffer
261 treatments. At the higher temperature, significant induction of DNA damage
262 was observed faster in the buffer treated HTO-exposed mussel haemocytes
263 than at 15 °C - after 72 and 168 h (Fig. 2B).

264 It is interesting that our 25 °C control mussel haemocytes showed no
265 induction of genotoxicity, as *M. galloprovincialis* and *M. californius* haemo-
266 cytes have previously shown rapid (8 h) increases in DNA strand breaks (as
267 measured by comet assay) at higher temperatures (Yao and Somero, 2012).
268 It is, however, important to note that this was after exposure to 32 °C, higher
269 than that used here. In fact, mussels at 28 °C in the earlier study showed
270 much less induction of DNA strand breaks.

271 In terms of the interaction between elevated temperature and contam-
272 inants, haemocytes of a freshwater mussel (*Dreissena polymorpha*) showed
273 increased DNA damage after *in vitro* exposure to sodium hypochlorite and in-
274 creased temperature (peaking at ~27 % tail DNA after 1 h at 28 °C; Buschini
275 et al. 2003). The temperature-dependent effects reported here took longer
276 to appear, becoming evident only at 72 h. This may be due to the different
277 mechanisms of action of the different stressors used (chemical vs. radiologi-
278 cal). Differences between freshwater and marine mussel physiology, different
279 thermal histories of the animals or the thermal tolerances of these two species
280 may also cause variation in this response. Along this line, it would be in-

Figure 2: DNA strand breaks (as measured by the comet assay i.e. % tail DNA) in mussel haemocytes after exposure to tritiated water (15 MBq L^{-1}) for 7 days at (a) 15°C and (b) 25°C . Copper ($40 \mu\text{g L}^{-1}$) positive control is also shown on (a). Significant differences from the equivalent control (at the same timepoint) are indicated by * ($p < 0.05$). There were no significant differences from the equivalent buffer treatments.

281 teresting to assess the same endpoints in *M. edulis* exposed to tritium and
282 elevated temperature, as this closely related species is less thermally tolerant
283 (Bayne, 1973). Any species-specific differences would be especially interest-
284 ing as although the current mussels have been verified as *M. galloprovincialis*
285 (the Mediterranean blue mussel; Hilbish et al., 2002; Bignell et al., 2011) they
286 are from north Cornwall, where temperatures are significantly lower than the
287 Mediterranean Sea. Thus, the *Mytilus* species complex potentially represents
288 an excellent opportunity to look at both the mechanistic and evolutionary
289 basis of temperature effects on radiation toxicity, similar to recent studies on
290 chemical contaminants (Cheung et al., 2006; Dallas et al., 2013b).

291 This is the first description of a significant relationship between DNA
292 strand breaks and *rad51* expression in mussels, although a similar trend was
293 reported by AlAmri et al. (2012). Given the role of *rad51* in homologous
294 recombination and previous demonstration of its upregulation in irradiated
295 mussels, this association is unsurprising (Masson and West, 2001; Al-Amri
296 et al., 2012). It is tempting to use this correlation to suggest that most
297 of the strand breaks caused by HTO in this study were DSBs, however it
298 is important to note that there is considerable variation in the data, which
299 might be explained by single strand breaks (SSBs). Indeed, *p53* is known to
300 stimulate base excision repair (Zhou et al., 2001), consequently the observed
301 p53 upregulation at 72 h (for 15 °C HTO) may be in response to SSBs.
302 It is particularly necessary to fully characterise the nature of strand breaks
303 caused by HTO exposure in mussels as DSBs are specifically caused by high
304 LET radiation, i.e. α particles (Natarajan et al., 1993), whilst tritium is a β
305 emitter. Having said this, tritium's β particles are higher energy than most

306 other β emitters and have been predicted to cause DSBs (Chen, 2012). The
 307 data presented here for *rad51* expression support this idea. Nevertheless,
 308 investigation of genes associated with SSB repair processes, such as base-
 309 and nucleotide- excision repair, are highly recommended for future studies.
 310 Although the relationship between % tail DNA and *p53* is more complicated
 311 (a negative correlation at 72 h and a positive one at 168 h), it is still significant
 312 at both timepoints and is easily explained when looking at the data (at 72 h,
 313 *p53* is repressed where DNA damage is highest - i.e. 25 °C HTO). Together,
 314 these results add further weight to the idea that DNA damage may provide
 315 an indicator of other biological effects.

316 3.4. Alteration of transcriptional expression of key genes

317 PCR efficiencies were *atub* 1.499, *ef1* 1.863, *hsp70-1* 1.869, *hsp70-2* 1.756,
 318 *hsp90* 1.665, *mt20* 1.804, *p53* 1.760 and *rad51* 1.736. As the efficiency of
 319 *atub* was considerably lower than that of the other genes, it was discarded
 320 and *ef1* (C_q variability: 18.95 ± 0.80) was used as a single normalising gene.

321 For 15 °C exposure to both Cu and HTO, expression patterns were very
 322 similar between 1 and 12 h, before diverging at 72 h (Fig. 3 b, d). For
 323 example, both treatments showed a significant increase in the transcription
 324 of *hsp70-1* at 12 h ($p < 0.0001$). The 15 °C HTO treatment also induced
 325 significant upregulation of *hsp90* and *mt20* after 1 h ($p < 0.0001$), but this
 326 was gone by 12 h. After 72 h, expression of all genes (except *hsp70-1*) was
 327 significantly upregulated for the 15 °C HTO treatment compared to the con-
 328 trol, and for *hsp70*, *hsp90* and *mt20* in comparison to 1 h. Both Cu and HTO
 329 (15 °C) showed significant downregulation after 168 h, although this varied
 330 by gene. In contrast, there was much less variation in the transcriptional ex-

pression of the six target genes in the 25 °C HTO treatment. Downregulation of *rad51*, *hsp70-1*, *mt20*, and *hsp70-2* was significant at 1, 12, 72 and 168 h, respectively ($p < 0.05$). Statistical analysis also revealed significant decreases in expression compared to the 15 °C HTO treatment at 12 h (*hsp70-1*), 72 h (*hsp70-2*, *hsp90*, *mt20*, and *p53*) and 168 h (*hsp90* and *p53*; $p < 0.01$).

Spearman’s correlation analyses revealed significant relationships between DNA damage and the two genes associated with DNA repair (*p53* and *rad51*), although this varied with time for *p53* (Fig. 4 b,f). After 72 h, significant correlations were observed for % tail DNA with *hsp70-2*, *p53* (both negative) and *mt20* (positive). At 168 h, *hsp70-2* and *p53* were both still significantly correlated with % tail DNA, but now positively so, and *rad51* was now also positively correlated with the measure of genotoxicity.

There is limited information on the transcriptional responses of marine invertebrates to ionising radiation (Farcy et al., 2007, 2011; Devos et al., 2015), and even less data for *Mytilus* spp. (only Al-Amri et al. 2012). However, comparisons with the mammalian literature yield some interesting comparisons and support the general trends we have seen. For example, there is a well-known link between radiation exposure and increased expression of heat shock genes in mammalian *in vitro* systems (Nogami et al., 1993; Calini et al., 2003; Dote et al., 2006). Protective effects of metallothionein proteins in γ and UV-irradiated human cell culture systems have also been reported and are attributed to their oxygen radical scavenging ability (Cai et al., 1999, 2000). The current results show similar upregulation of metallothionein genes to that reported by Farcy et al. (2011), which suggests this defence mechanism may be generally important in radiation exposure. It is,

Figure 3: Relative expression ratios (RER) of six genes in gill tissue after exposure to control seawater at 25 °C (a); 15 MBq L⁻¹ HTO at 15 °C (b); 15 MBq L⁻¹ HTO at 25 °C (c) or 40 µg L⁻¹ Cu at 15 °C (d) over 7 d. Data are normalised for the reference gene (*ef1*) and the 15 °C control. Error bars indicate the 95% confidence intervals. The dashed grey line indicates no change in expression. Significant differences from the equivalent temperature control (*), 15 °C HTO treatment (§) and 1 h timepoint (#) are also illustrated (REST 2009 software, $p < 0.05$).

Figure 4: Spearman's correlations for DNA strand breakage with gene expression after exposure to control seawater and tritiated water (15 MBq L^{-1}) at 15 and 25°C . (A-C) % tail DNA and *hsp70*, *p53* or *rad51* expression at 72 h; (D-E) % tail DNA and *hsp70*, *p53* or *rad51* expression at 168 h. Data are median % tail DNA (of 100 cells) and ΔC_q values ($C_q[\text{GoI}] - C_q[\text{ef1}]$) for individual mussels across all treatments (control seawater, 15 MBq L^{-1} HTO and $40 \mu\text{g L}^{-1}$ Cu). All % tail DNA values are for haemocytes and are buffer-treated (not Fpg). Transcription of GoI is as ΔC_q , i.e. normalised to the housekeeping gene, *ef1*. The blue line indicates fitted values and dashed red lines represent 95 % confidence limits.

356 however, important to remember that comparisons of these two studies are
357 difficult, as the total doses varied approximately 2.5-fold (with concomitant
358 differences in dose rate) and different radionuclides were used. Interestingly,
359 it has been reported that temperature was inversely correlated with expres-
360 sion of genes such as *hsp70*, *hsp90* and *MT* in mussels sampled from the
361 French coast (Farcy et al., 2007), a trend only reflected in the current results
362 for HTO exposure (but not for temperature alone). The field-based work
363 of Al-Amri et al. (2012) found significant upregulation of *rad51* in mussels
364 exposed to dose rates as low as $0.61 \mu\text{Gy h}^{-1}$. In the current work, significant
365 upregulation of this gene after 72 h exposure to HTO (but not for Cu or the
366 25 °C control) supports the idea that this is radiation-induced.

367 Gourgou et al. (2010) report rapid induction of *hsp70* and *mt20* during
368 heat stress in *M. galloprovincialis* (30 °C for up to 8 h), which is at odds
369 with our 25 °C control treatment. It is, however, important to note that the
370 higher temperature (30 °C) caused 95 % mortality by 24 h, suggesting this
371 difference results in a considerably more stressful environment for mussels.
372 Despite the difference in outcome, use of selective inhibitors to potentially
373 link *hsp70* and *mt20* expression during HTO exposure with p38-MAPK or
374 JNKs (as in hyperthermic mussels; Gourgou et al. 2010), might be interesting
375 from a mechanistic point of view.

376 The data reported here indicate differential transcription of the two *hsp70*
377 sequences, suggesting that they belong to different isoforms of this gene.
378 Significant upregulation of *hsp70-1* was observed after only 1 h in two of
379 the treatment groups (HTO at 25 °C and Cu at 15 °C), whereas no such
380 change was observed for *hsp70-2*, which was upregulated only at 72 h. This

381 is consistent with previous data on differential expression of *hsp70* isoforms in
382 both proteins and genes of *M. galloprovincialis* (Franzellitti and Fabbri, 2005;
383 Tomanek and Zuzow, 2010), and suggests that the *hsp70-1* gene (sometimes
384 referred to as *hsc70*) is an initial response to acute stress, whereas *hsp70-2*
385 responds in a later phase (Franzellitti and Fabbri, 2005).

386 There are several splice-variants of p53-like genes, including ΔN isoforms
387 which have no ability to induce apoptosis and actually suppress functional
388 *p53*-like proteins, meaning they are oncogenic (Muttray et al., 2008). The
389 nomenclature surrounding which of these variants is present in *Mytilus* sp.
390 is often confusing (Muttray and Baldwin, 2007; Rotchell and Ciocan, 2007;
391 Štifani et al., 2009), but the ‘*p53*’ primers used herein are derived from a *M.*
392 *galloprovincialis* sequence of the *p63/73* family (see Table 3 for details) and
393 were designed to quantify total *p53*-like expression (Dondero et al., 2006b).
394 As a consequence it is possible that the increased *p53* expression observed
395 at 72 h is either anti-oncogenic (*p63/73*) or oncogenic ($\Delta Np63/73$). The
396 observed increase in genotoxicity at this timepoint at 25 °C (where *p53* is
397 downregulated in comparison to the cooler temperature) suggests that at 15
398 °C *p53* is either having a protective function or the protective effects of other
399 genes/proteins are compensatory (e.g. HSPs, MTs).

400 In general, the temperature-dependent difference between expression pro-
401 files for HTO-exposed mussel gill at 72 h suggests that downregulation of key
402 protective genes could be one explanation for the earlier genotoxicity of HTO
403 at 25 °C. Downregulation of these genes has been reported in conjunction with
404 DNA damage before (e.g. *p53* in mussels exposed to benzo(a)pyrene [Banni
405 et al. 2009a] and *hsp70* in γ -irradiated C3H 10T 1/2 cells [Calini et al. 2003]).

406 There is, of course, the potential that the lack of resolution in our sampling
407 schedule (i.e. a gap of 60 h) has obscured an earlier response by these genes.
408 For example, Tedengren et al. (1999) have reported that mussels pre-exposed
409 to elevated temperature showed increased resilience to Cd toxicity as a result
410 of more rapid synthesis of stress-induced cytoprotective proteins (e.g. HSPs).
411 However, our mussels had concurrent exposure to heat and HTO, with no
412 pre-treatment, so this effect is unlikely. The more rapid occurrence of DNA
413 strand breakage in the 25 °C HTO-exposed mussel haemocytes also suggests
414 a lack of protection, rather than a temporal shift. It is interesting that there
415 was no reduction in expression of *rad51* between the 15 and 25 °C HTO
416 treatments, as this gene is involved in double strand break repair (Al-Amri
417 et al., 2012; Di, 2012). Similarly, *rad51* was not upregulated before signifi-
418 cant strand breakage occurred (i.e. < 72 h) for HTO at 15 °C, suggesting
419 other DNA repair genes were involved in maintaining genomic integrity at
420 this stage. Yet again temperature altered this effect, with *rad51* upregulated
421 at only 1 h for 25 °C HTO exposure. Future studies on whether or not this
422 difference is due to different repair mechanisms or a temporal shift would
423 greatly enhance our understanding of the combined effect of radiation and
424 temperature on DNA.

425 The current study only analysed transcriptional expression in one tissue
426 - the gills. Though gills have been cited as showing the highest levels of
427 HSP70 and HSP72 proteins in *M. edulis* (Chapple et al., 1997) this does not
428 necessarily equal the highest mRNA expression, nor does it apply to other
429 genes/proteins. Expression of heat shock molecules is notably tissue-specific
430 in *Mytilus* spp. (Pantzartzi et al., 2010), as is expression of *p53* and met-

allothionein genes (*mt10* and *mt20*) in mussels exposed to benzo(a)pyrene and TiO₂ nanoparticles, respectively (Banni et al., 2009a; D’Agata et al., 2014). It is imperative that future studies consider this important variable, in order to fully characterise the response of these key genes to radiation and/or temperature stress.

4. Conclusions

This study is the first to investigate temperature effects on radiation-induced genotoxicity in an ecologically representative marine invertebrate, *M. galloprovincialis*. This represents an important step forward in radioecology in general, as to date there are temperature-dependent laboratory exposure data for only two other molluscs - *Physa* spp. (a freshwater snail; Ravera 1966; Cooley 1973) and *Crassostrea gigas* embryo-larvae (Nelson, 1971). Our study suggests that mussels (or similar marine species) exposed to increased temperature and HTO may have a compromised ability to defend against genotoxic insult at the molecular level. This is particularly pertinent in the context of rising sea temperatures and thermal pollution from nuclear institutions and suggests that there is still a pressing need to investigate the interactive effects of temperature and radiation exposure on aquatic organisms. Lastly, it is important to note that in addition to temperature there are many other physical factors which may interact with radiation exposure in aquatic animals (Dallas et al., 2012) and such interactions could also have implications for observed biological responses.

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