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

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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

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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-

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

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

The IAEA first described a 'timely need' for research into thermal discharges 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-

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

Against the backdrop of the above information, this study was designed to fulfil the following aims and objectives: (a) to use tissue-specific accumulation of ^{3}H in mussels to determine the effects of elevated temperature on radiation dose; (b) to assess the impact of elevated temperature on the genotoxicity of HTO to mussel haemocytes, using the modified comet assay to determine oxidative DNA damage; and (c) to evaluate the transcription profile of key radiation and heat shock genes (two isoforms of *hsp70*, *hsp90*, *mt20*, *p53* and *rad51*) to elucidate potential mechanisms behind temperature-effects. Details of the proteins encoded by our GoI are given in Table 1. It was hypothesised that heat stress alongside tritium exposure would increase radiation dose and genotoxicity and that such enhanced effects would necessitate the 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 (<10μm) 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 μg 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 tritated 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 ± SD of measured temperatures (°C) for mussels exposed to tritiated water (HTO) or Cu positive control at different nominal temperatures.

Treatment	Nominal temperature (°C)	
	15 °C	25 °C
Control	15.34 ± 0.51	25.61 ± 0.43
15 MBq L^{-1} HTO	15.40 ± 0.37	25.58 ± 0.32
40 μg 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$5mm^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

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

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

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

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

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

2.5. Determination of relative transcriptional expression of selected genes

Extraction of total RNA and reverse transcription were performed using 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 (*atub*)[a]	DQ174100	5'-TTGCAACCATCAAGACCAAG-3'	5'-TGCAGACGGGCTCTCTGT-3'
Elongation factor 1 (*ef1*)[a]	AF063420	5'-CACCACGAGTCTCTCCCAGA-3'	5'-GCTGTCACCACAGACCATTCC-3'
Heat shock protein 90 (*hsp90*)[c]	AJ625655	5'-TCAGTGATGATCCTAGATTAGGCA-3'	5'-CGTTCCTCTCTTTCCATCTGTAAC-3'
Heat shock protein 70 sequence 1 (*hsp70-1*)[b]	AF172607	5'-GGGTGGTGGAACTTTTGATG-3'	5'-GCCGTTGAAAAAGTCCTGAA-3'
Heat shock protein 70 sequence 2 (*hsp70-2*)[d]	AF172607	5'-CCCTTTCTTCAAGCACACAAGCA-3'	5'-AACTGGTTCCATGGTTCCTCTGAA-3'
Metallothionein 20 (*mt20*)[d]	AJ577131	5'-GACGCCTGCAAATGTGCAAGT-3'	5'-TCGGACCAGTGCGGTCACAT-3'
p53 anti-oncogene (*p53*)[e]	DQ158079	5'-CAAACTTGCTAAATTTGTTGAAGA-3'	5'-TTGGTCCTCCTACACATGAC-3'
rad51 (*rad51*)[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

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

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

2.6. Statistics

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

for total dose, but is equivalent between the three parameters. Median values for % tail DNA (comet assay) were calculated for each mussel and used in a two way ANOVA with time, treatment and buffer/fpg as fixed factors (Lovell and Omori, 2008; Dallas et al., 2013a). Spearman's correlation analyses were performed to assess the relationship between gene expression and % tail DNA at all timepoints, and p-values were adjusted for multiple comparisons (Holm's sequential Bonferroni adjustment). The gene expression parameter used for relationship analysis was C_q normalised for reference gene (i.e. $\Delta C_q = C_{q[\mathrm{GoI}]} - C_{q[ef1]}$).

3. Results & Discussion

3.1. Tritium accumulation and dose estimation

Tritium activity concentrations in water showed good agreement with nominal values (> 90 % of expected) at 14.3 ± 0.6 MBq L^{-1} (15 °C) and 14.2 ± 0.6 MBq L^{-1} (25 °C). Control water samples' activity was below the LOD.

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

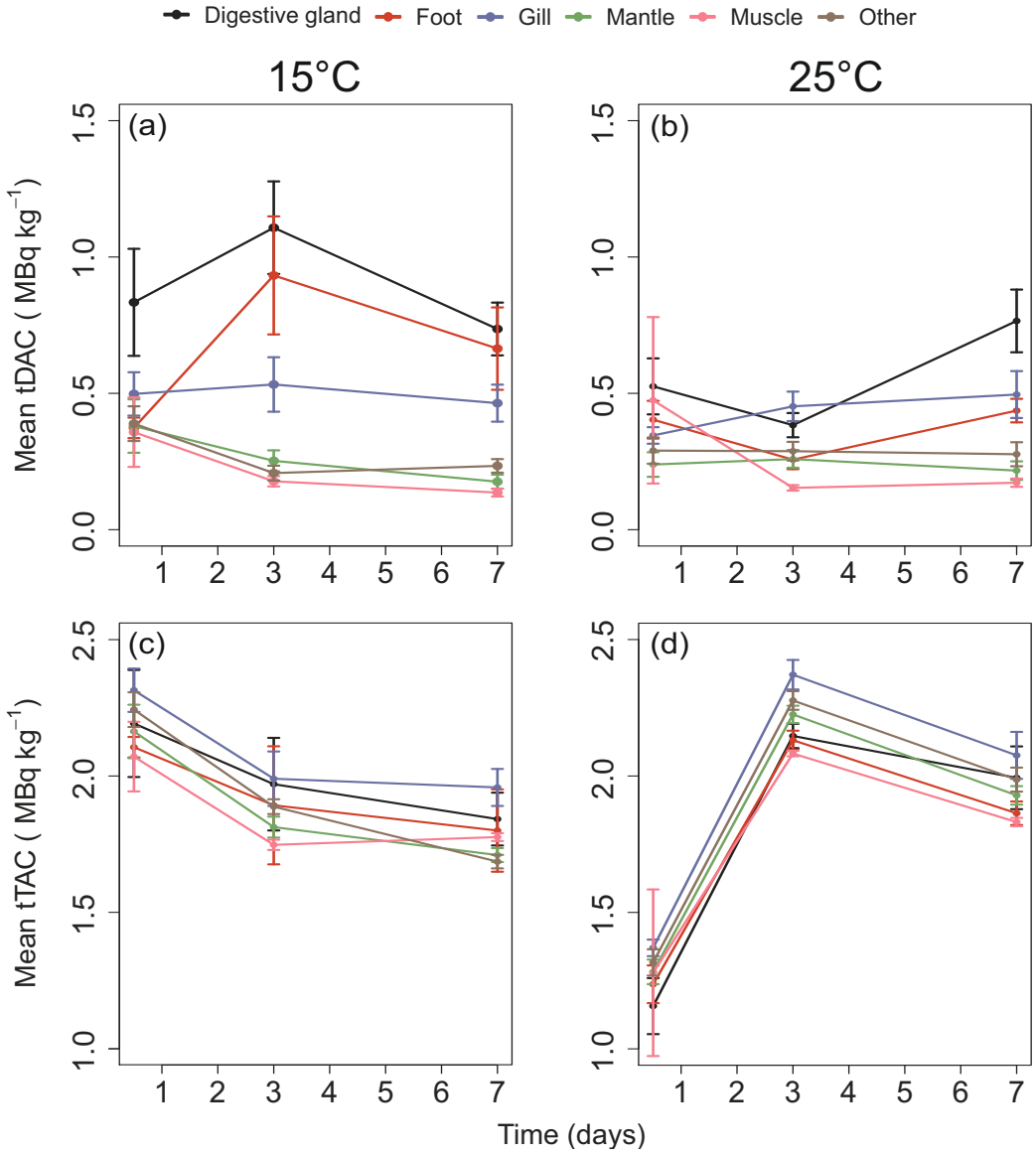

Figure 1: Time-dependent tritium accumulation (between 0.5 and 7 days) in mussel tissues after exposure to 15 MBq L^{-1} HTO at 15 or 25 °C: (a) and (b) are tDACs, i.e. tritium concentration in dry tissue after removal of water by freeze drying; (c) and (d) are tTACs, i.e. tDAC + TFWT, normalised for wet weight. Data are means ± 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 $\pm$ <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 ^{3}H rapidly equilibrates with suspended sediment particles (Turner et al., 2009), one possible explanation is that ^{3}H 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 ^{3}H. Previous authors have reported that *Mytilus* individuals acclimatised to 15 °C and then exposed to > 20 °C respond by reducing clearance rate (CR)

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

There is a limited amount of literature with which to compare our ^{3}H accumulation data. However, Cd exposure in the oyster, *Crassostrea gigas*, resulted in linear accumulation increasing with temperature over 45 days (0.10, 0.53 and 0.56 μg Cd g^{-1} dry mass d^{-1} for 12, 20 and 28 °C; Cherkasov et al. 2007). Where temperature effects have been observed for metal accumulation in mussels, they have been attributed to changes in solution chemistry and physical kinetics, thereby increasing uptake with increased heat (Mubiana and Blust, 2007). These factors are thought to be less important with tritium exposure, due to the chemical similarities of ^{3}H with H. However, isotopic enrichment of ^{3}H in biopolymers, as a result of the preference of ^{3}H for weak hydrogen bridges, has been described (Baumgartner and Kim, 2000; Baumgrtner et al., 2001). Although, theoretically there is the potential for increased energy (i.e. heat) to weaken hydrogen bridges (Khan, 2000) and 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 $\leq$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		

Table 5: Significant p values from Tukeys HSD post hoc test on the three-way interaction between temperature, time and tissue for mussel tTACs 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 $\leq$0.0005 are represented as 0.000. Non-significant values are not shown.

		Temp	15 °C																25 °C														
		Time	12 h	12 h	12 h	12 h	12 h	12 h	3 d	3 d	3 d	3 d	3 d	7 d	7 d	7 d	7 d	7 d	7 d	12 h	12 h	12 h	12 h	12 h	12 h	3 d	3 d	3 d	3 d	3 d	3 d	7 d	7 d	7 d	7 d	7 d	7 d
Temp	Time	Tissue	*Dg*	*G*	*F*	*Man*	*Mus*	*O*	*G*	*F*	*Man*	*Mus*	*O*	*Dg*	*G*	*F*	*Man*	*Mus*	*O*	*Dg*	*G*	*F*	*Man*	*Mus*	*O*	*Dg*	*G*	*F*	*Man*	*Mus*	*O*	*Dg*	*G*	*F*	*Man*	*Mus*	*O*
	12 h	Dg												0.000						0.000																	
	12 h	*G*			0.004		0.000		0.000						0.000						0.000																
	12 h	*F*		0.004												0.000						0.000														
	12 h	*Man*								0.000							0.000						0.000														
	12 h	*Mus*		0.000							0.000							0.000						0.000													
	12 h	*O*										0.000							0.000						0.000												
	3 d	*G*		0.000																							0.000									
	3 d	*F*																																			
15 °C	3 d	*Man*				0.000																							0.000								
	3 d	*Mus*					0.000																							0.000							
	3 d	*O*						0.000																							0.000						
	7 d	*Dg*	0.000																																		
	7 d	*G*		0.000													0.029		0.007																		
	7 d	*F*			0.000																																
	7 d	Man				0.000								0.029																					0.029		0.000
	7 d	*Mus*					0.000																														
	7 d	*O*						0.000						0.007																							
	12 h	Dg	0.000																					0.000					0.000								
	12 h	*G*		0.000																											0.000						
	12 h	*F*			0.000																																
	12 h	*Man*				0.000																							0.000					0.000			
	12 h	*Mus*					0.000																							0.000					0.000		
	12 h	*O*						0.000																							0.000						0.000
	3 d	*Dg*																		0.000						0.001										
	3 d	*G*							0.000															0.001	0.000	0.000			0.000							
	3 d	*F*								0.024																0.000							0.000				
25 °C	3 d	*Man*									0.000											0.000												0.000			
	3 d	Mus										0.000											0.000		0.000			0.014						0.000		
	3 d	*O*											0.000												0.000				0.014							0.000
	7 d	*Dg*																		0.000																
	7 d	*G*																			0.000						0.000						0.003		0.000	
	7 d	*F*																									0.000						0.003			
	7 d	*Man*															0.029					0.000						0.000								
	7 d	*Mus*																					0.000						0.000				0.000			
	7 d	*O*															0.000						0.000						0.000							

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 ± SE, $n = 9$).

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

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

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

3.2. Dose calculations

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

3.3. Genotoxicity

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

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

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

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

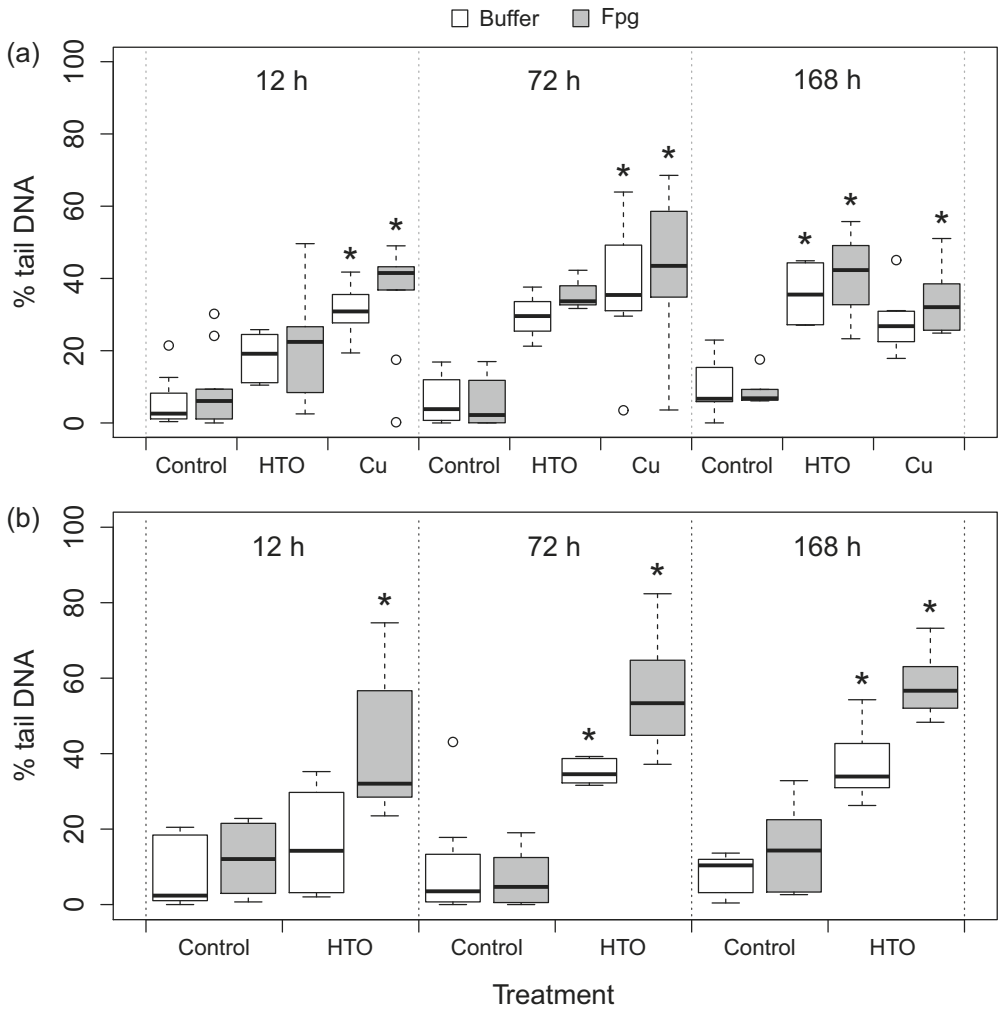

Figure 2: DNA strand breaks (as measured by the comet assay i.e. % tail DNA) in mussel haemocytes after exposure to tritiated water (15 MBq L^{-1}) for 7 days at (a) 15 °C and (b) 25 °C. Copper (40 μg L^{-1}) positive control is also shown on (a). Significant differences from the equivalent control (at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

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

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

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

3.4. Alteration of transcriptional expression of key genes

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

For 15 °C exposure to both Cu and HTO, expression patterns were very similar between 1 and 12 h, before diverging at 72 h (Fig. 3 b, d). For example, both treatments showed a significant increase in the transcription of *hsp70-1* at 12 h ($p < 0.0001$). The 15 °C HTO treatment also induced significant upregulation of *hsp90* and *mt20* after 1 h ($p < 0.0001$), but this was gone by 12 h. After 72 h, expression of all genes (except *hsp70-1*) was significantly upregulated for the 15 °C HTO treatment compared to the control, and for *hsp70*, *hsp90* and *mt20* in comparison to 1 h. Both Cu and HTO (15 °C) showed significant downregulation after 168 h, although this varied 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,

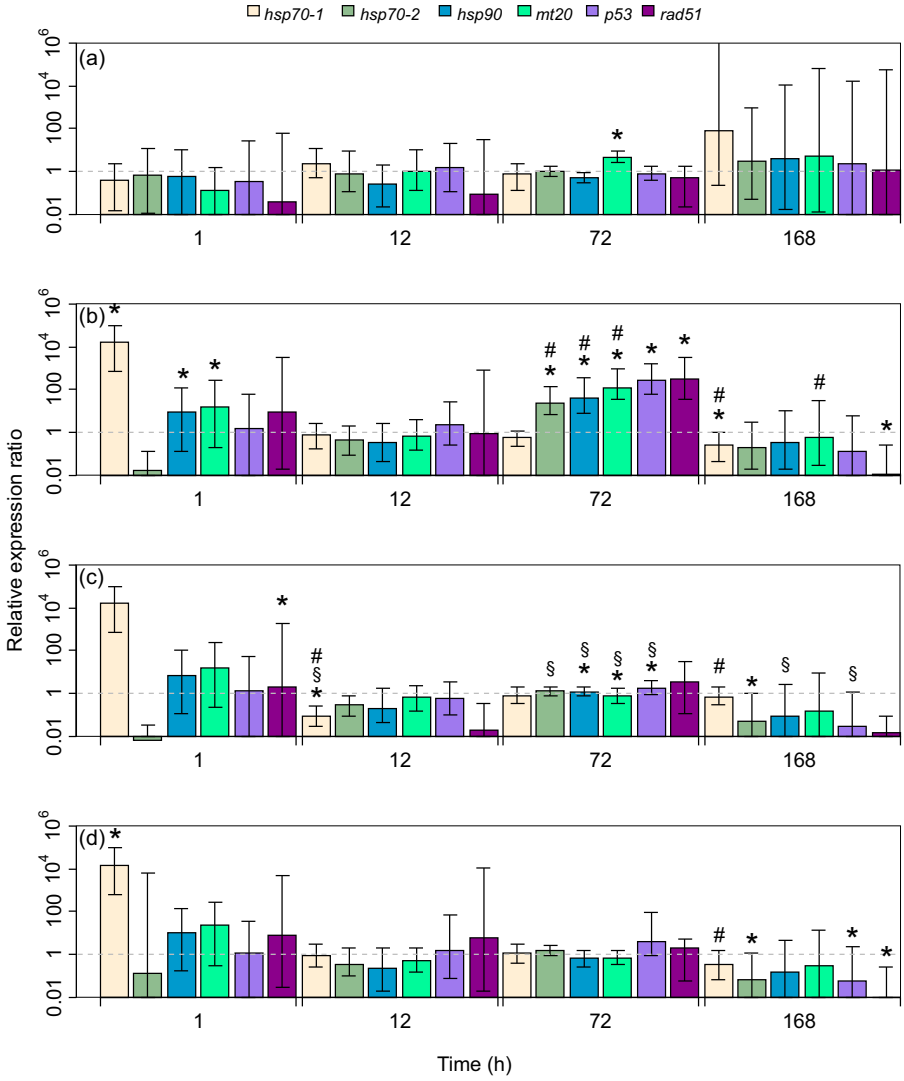

Figure 3: Relative expression ratios (RER) of six genes in gill tissue after exposure to control seawater at 25 °C (a); 15 MBq L^{-1} HTO at 15 °C (b); 15 MBq L^{-1} HTO at 25 °C (c) or 40 μg L^{-1} Cu at 15 °C (d) over 7 d. Data are normalised for the reference gene (*ef1*) and the 15 °C control. Error 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, $p < 0.05$).

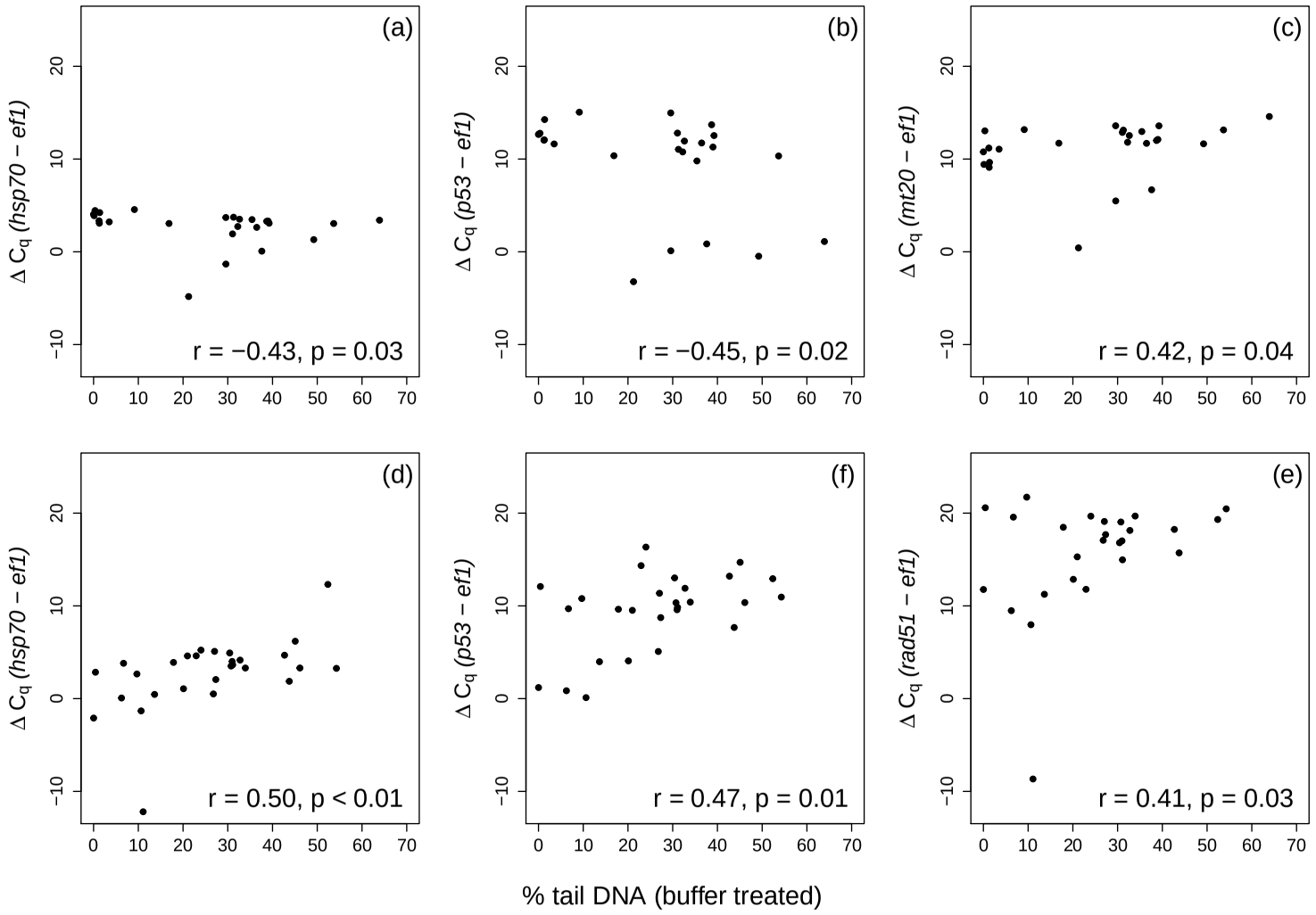

Figure 4: Spearman's correlations for DNA strand breakage with gene expression after exposure to control seawater and tritiated water (15 MBq L^{-1}) at 15 and 25 °C. (A-C) % tail DNA and *hsp70*,*p53* or *rad51* expression at 72 h; (D-E) % tail DNA and *hsp70*, *p53* or *rad51* expression at 168 h. Data are median % tail DNA (of 100 cells) and ΔC_q values ($C_{q[\text{GoI}]}$ - $C_{q[ef1]}$) for individual mussels across all treatments (control seawater, 15 MBq L^{-1} HTO and 40 μg 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.

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

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

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

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

There are several splice-variants of p53-like genes, including ΔN isoforms which have no ability to induce apoptosis and actually suppress functional *p53*-like proteins, meaning they are oncogenic (Muttray et al., 2008). The nomenclature surrounding which of these variants is present in *Mytilus* sp. is often confusing (Muttray and Baldwin, 2007; Rotchell and Ciocan, 2007; Štifani et al., 2009), but the '*p53*' primers used herein are derived from a *M. galloprovincialis* sequence of the *p63/73* family (see Table 3 for details) and were designed to quantify total *p53*-like expression (Dondero et al., 2006b). As a consequence it is possible that the increased *p53* expression observed at 72 h is either anti-oncogenic (*p63/73*) or oncogenic (*ΔNp63/73*). The observed increase in genotoxicity at this timepoint at 25 °C (where *p53* is downregulated in comparison to the cooler temperature) suggests that at 15 °C *p53* is either having a protective function or the protective effects of other genes/proteins are compensatory (e.g. HSPs, MTs).

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

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

The current study only analysed transcriptional expression in one tissue - the gills. Though gills have been cited as showing the highest levels of HSP70 and HSP72 proteins in *M. edulis* (Chapple et al., 1997) this does not necessarily equal the highest mRNA expression, nor does it apply to other genes/proteins. Expression of heat shock molecules is notably tissue-specific 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 TiO_2 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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