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1 **The environmental cellular stress response: the intertidal as a multistressor model**

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35 **Abstract**

36 The wild poses a multifaceted challenge to the maintenance of cellular function. Therefore, a
37 multistressor approach is essential to predict the cellular mechanisms which promote homeostasis
38 and underpin whole-organism tolerance. The intertidal zone is particularly dynamic and thus its
39 inhabitants provide excellent models to assess mechanisms underpinning multistressor tolerance.
40 Here, we critically review our current understanding of the regulation of the cellular stress response
41 (CSR) under multiple abiotic stressors in intertidal organisms and consider to what extent a
42 multistressor approach brings us closer to understanding responses in the wild. The function of the
43 CSR has been well documented in laboratory and field exposures with a view to understanding
44 single-stressor thermal effects. Multistressor studies still remain relatively limited in comparison but
45 have applied three main approaches: (i) laboratory application of multiple stressors in isolation (ii)
46 multiple stressors applied in combination (iii) field-based correlation of multiple stressors against the
47 CSR. Application of multiple stressors in isolation has allowed identification of putative shared stress
48 pathways but overlooks non-additive stressor interactions on the CSR. Combined stressor studies are
49 relatively limited in number but already highlight variable effects on the CSR dependent upon
50 stressor type, timing and magnitude. Field studies have allowed identification of responsive
51 components of the CSR to various stressors *in situ* but are correlative, not causative. A combined
52 approach, involving both laboratory multistressor studies linking the CSR to whole organism
53 tolerance as well as field studies, are required if we are to understand the role of the CSR in the
54 natural environment.

55

56 **Keywords**

57 Multistressor, cellular stress response, heat shock proteins, cross-tolerance, ecophysiology,
58 integrative

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69 **Introduction**

70 In her seminal evaluation of the heat shock response, published in 1986, Susan Lindquist clearly laid
71 the foundations for the environmental stress response work carried out today (Lindquist, 1986). The
72 induction of a small number of highly conserved heat shock proteins in response to a range of
73 different stressors that denature the cellular protein pool was already well known. At the time, the
74 importance of these proteins in the CSR was underlined by the high level of conservation of heat
75 shock protein sequences between diverse species and the ubiquity of the response, even though
76 most studies focussed on model organisms (e.g. *Escherichia coli*, *Saccharomyces cerevisiae*,
77 *Drosophila melanogaster* and vertebrate cell lines) (Lindquist, 1986). It would be more than ten
78 years before the much wider role of heat shock proteins in non-model organisms responding to
79 stress in the natural environment would be promoted. In their highly influential review, Feder and
80 Hofmann, (1999) acknowledged the utility of heat shock proteins as environmental biomarkers
81 (particularly for toxicology), and also pointed out that in the aquatic environment, habitual exposure
82 to heat shock protein-inducing thermal stress was probably most commonly found in the intertidal
83 zone. Indeed, in the late 1990's and early 2000's research on intertidal species provided critical
84 information on the natural variability of the environmental heat shock response (HSR).

85

86 Much of this work concentrated on evaluating *HSP70* (heat shock protein 70kDa) genes and
87 proteins. This was largely because, in the early days of molecular biology, the high sequence
88 conservation of *HSP70* genes between species facilitated the, then complicated and lengthy, process
89 of candidate gene cloning (e.g. Yokoyama et al., 2006) and/or the identification of protein bands in
90 acrylamide gels, produced via the metabolic incorporation of ³⁵S-labelled methionine (e.g. Roberts et
91 al., 1997). This sequence conservation also enabled the use of heterologous hybridization of *HSP70*
92 antibodies raised in very distantly related model species (e.g. anti-*HSP70* human monoclonal
93 antibody applied to *Mytilus edulis* (Smerdon et al., 1995). Studies concentrated on well-known
94 intertidal species such as the bivalves *Mytilus sp.*, *Crassostrea gigas* and the marine gastropods
95 (*Lottia sp.* and *Tegula sp.*) (Dong et al., 2008; Hofmann and Somero, 1996; Tomanek and Somero,
96 1999; Yokoyama et al., 2006). Several of these species occur across wide latitudinal gradients and at
97 different tidal heights on the foreshore, providing natural model systems for dissecting the HSR
98 associated with the complex environmental stressors experienced in the intertidal (Tomanek, 2002).
99 At the time, work on model species was showing that heat shock proteins operated, not only as
100 molecular chaperones, but also had multiple functions and engaged in complex cellular interactions
101 (Lindquist, 1986). Similarly, it was becoming clear in environmental studies that these highly
102 conserved proteins were pivotal cellular thermostats (Feder and Hofmann, 1999). Nonetheless,

103 wider investigations were limited by the sequence technologies at the time and were largely
104 restricted to temperature effects on a limited number of candidate HSPs (heat shock proteins).

105

106 Since then, it could be argued that the HSR to multifactorial stressors has been well studied in wild
107 intertidal animals, albeit in certain taxa. For example, intertidal molluscs have often been
108 investigated using a combined laboratory and field approach (Dong et al., 2022). We would argue
109 that the field components in these studies have not been aimed at understanding the HSR in dealing
110 with multiple stressors *per se*, but have typically been temperature-orientated i.e. looking for
111 differences in the HSR in field organisms with different evolutionary thermal histories (e.g. along
112 vertical and latitudinal gradients) (Dong et al., 2022). Patterns between laboratory and the wild may
113 match in some cases (Dong et al., 2022) but even the earliest evaluations of the *HSP70* response
114 identified significant differences between field collected and lab acclimated individuals (Clark and
115 Peck, 2009). Although temperature is a major factor in initiating the HSR, the use of a few candidate
116 HSPs could not provide the detail on the contribution of secondary factors, such as nutrient
117 availability, salinity and predator status (Halpin et al., 2002; Sagarin and Somero, 2006). Certainly,
118 the majority of laboratory studies only subjected intertidal animals to a single stressor, and that was
119 usually temperature (Feder and Hofmann, 1999). However, the intertidal is not a single stressor
120 environment and these varied stressors have many different effects on physiology (Figure 1). Recent
121 advances in sequencing techniques and associated bioinformatics have provided opportunities to
122 investigate cellular adaptations to the intertidal more widely in a discovery-led approach (e.g. Clark
123 et al., 2017), which when linked with physiological and ecological studies, are revealing the true
124 complexity of the native intertidal CSR.

125

126 Hence, it is timely for a critical analysis of the responses of the CSR to multiple abiotic stressors with
127 a view to understanding its role in multistressor tolerance in the wild. We use organisms inhabiting
128 the intertidal zone (rocky shores, estuaries, shallow coastal polar and tropical organisms) as models.
129 We firstly review what is known about the HSR in terms of a focus on a single stressor -
130 environmental temperature. We then focus upon multistressor approaches conducted in the
131 laboratory and field. We conclude by discussing to what extent a multistressor approach brings us
132 closer to understanding the role of the CSR in the wild.

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135

136 **Patterns of HSP expression with a view to understanding responses to a single stressor:**
137 **temperature**

138 The intertidal environment represents an extreme environment, where harsh conditions prevail due
139 to the exposure to tides (Leeuwis and Gamperl, 2022). It is characterised by spatial and temporal
140 thermal heterogeneity, whereby temperature fluctuates markedly within hours and over short
141 distances. As a result, intertidal ectotherms experience rapid changes in body temperature over
142 short temporal and spatial scales, and therefore are excellent models to understand the mechanisms
143 underpinning temperature-sensitive physiological processes, both acute and chronic (Tomanek,
144 2010). Early experiments using the intertidal as a model system demonstrated the wide plasticity of
145 the HSR and its correlation with thermal tolerance and thermal history, thus contributing to our
146 understanding of how variation in the HSR contributes to biogeographic patterns (Dong et al., 2022).
147 Intertidal studies have revealed that, firstly, species living at the highest levels on the shore (and
148 therefore with the longest times for aerial exposure) have higher induction temperatures for the
149 HSR than those much closer to the water (Roberts et al., 1997; Tomanek and Somero, 1999;
150 Tomanek and Somero, 2000). Here, the low shore populations, which do not experience thermal
151 extremes as regularly as those on the high shore, need to resort to protective cellular mechanisms at
152 lower environmental temperatures to ensure the maintenance of homeostasis. A similar
153 phenomenon to that observed vertically in the intertidal is also apparent over broad biogeographic
154 scales, whereby populations on the cold edge of a species' distributional range, and therefore not
155 regularly experiencing thermal extremes, tend to have lower HSP70 induction temperatures
156 compared to those in the warmer edge (Hofmann and Somero, 1996). For example, the more
157 northern occurring *M. trossulus* was found to be less thermally tolerant and have lower induction
158 temperatures for *HSP70* compared with its more southern relative, *M. galloprovincialis* (Hofmann
159 and Somero, 1996). Secondly, thermal acclimation can modulate the HSR with changes in *HSP70*
160 induction temperatures, as well as maximum expression levels. However, there are limits to this
161 plasticity, as the temperature at which HSP synthesis (and protein synthesis more generally) ceases,
162 often remains unchanged following acclimation (Tomanek and Somero, 1999). In the field, seasonal
163 variation in production of HSP70 occurs in most species with more HSP70 produced constitutively in
164 the summer, as water and air temperatures naturally increase (Buckley et al., 2001; Roberts et al.,
165 1997). This can result in higher thresholds of induction of HSP70 (potentially by 6°C or more,
166 depending on the habitat) in summer-acclimated animals (Buckley et al., 2001; Hamdoun et al.,
167 2003; Hamer et al., 2004).

168

169 Early experiments also catalogued the varying time course and magnitude of the HSR in different
170 species, providing tantalising glimpses into the complexity of the response (Hofmann and Somero,
171 1996; Tomanek and Somero, 1999). Certainly, there was not the expected correlation with
172 latitudinal gradient, as the response to local site-specific factors and the effect of microhabitats
173 consistently overrode a more global temperature gradient (Halpin et al., 2002; Sagarin and Somero,
174 2006). Furthermore, it was becoming clear that such local conditions could influence the expression
175 of *HSP70* genes or proteins over longer timescales, rather than just for the duration of a tidal cycle.
176 Sessile species such as *Mytilus* and limpets showed temporal regulation of genes in response to
177 intertidal heat stress (Clark et al., 2008; Dong et al., 2008; Gracey et al., 2008; Lesser et al., 2010).
178 This observation led to the suggestion that elevated levels of HSP70 were always produced in these
179 populations, compared with subtidal populations and that these higher levels of HSP70 were
180 protective, acting as a “preparative defence strategy” against sudden stress (Dong et al., 2008). This
181 phenomenon was also called “constitutive frontloading” in some later studies (Barshis et al., 2013)
182 and appears to be restricted to sessile species, as no endogenous rhythms of *HSP70* expression were
183 detected in the tidal sculpin *Oligocottus maculosus* (Todgham et al., 2006). Expression of HSPs is
184 expensive at the cellular level and energetic trade-offs could be detected in some intertidal studies.
185 Elevated expenditure could also result in lower growth rates in high intertidal species (Tomanek and
186 Sanford, 2003) and their expression could vary with food availability (Lesser et al., 2010).

187

188 **Box 1. Temperature effects on the HSR - lessons from the intertidal** (see main text for references)

189

- 190 • **The stress response, including the expression of HSPs, may play a role in setting species’**
191 **biogeographical boundaries.** This is both in terms of their microhabitat (e.g. position on the
192 shore) and their latitudinal distribution.
- 193
- 194 • **HSP expression is highly plastic and varies with thermal history.** This is exemplified by two
195 phenomena that apply over different times scales: 1) evolutionary variation in the heat
196 shock response exists, so that organisms adapted to warmer environments have higher
197 induction temperatures than those in cold environments; and 2) threshold induction
198 temperatures are not fixed, but can be modulated as a result of thermal acclimation, over
199 relatively short timescales (e.g. seasonally).
- 200
- 201 • **The HSR, characterised by inducible HSP expression, varies with environmental stability.**
202 Organisms in highly variable thermal environments induce HSP expression regularly as a

203 cellular protection mechanism, whereas those in stable thermal environments do not often
204 resort to inducible HSP expression. In the former, this results in species with a larger
205 window of thermal tolerance, at least in the short-term.

206

- 207 • **Thermotolerance can increase upon both chronic exposure to thermal stress and repeated,**
208 **short term exposure to non-lethal extreme heat.** Exposure to elevated temperatures can
209 lead to elevated constitutive HSP levels. This ‘constitutive frontloading’ confers increased
210 cellular stress resistance, acting as a preparative defence strategy in thermally challenging
211 environments.

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213

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215 **Role of the CSR under multiple stressors**

216 The tidal cycle not only results in marked spatial and temporal heterogeneity in temperature, but
217 also in other abiotic variables, meaning coastal organisms have to cope with both synchronous and
218 asynchronous shifts in multiple abiotic factors with consequences from genes to the whole organism
219 (Leeuwis and Gamperl, 2022). Stressor effects on physiological function can be (i) additive - where
220 the combined stressor effect equals the sum of individual stressor effects, (ii) synergistic - where the
221 combined stressor effect is greater than sum of individual effects or (iii) antagonistic - where the
222 combined stressor effect is less than sum of individual effects (Todgham and Stillman, 2013). Studies
223 have tended to apply stressor combinations simultaneously and acutely resulting in an overemphasis
224 of detrimental synergistic effects in the scientific literature (Cote et al., 2016). However, the
225 temporal dynamics of stressors have recently begun to receive greater attention, i.e. the effects of
226 asynchronous stressors experienced in tidal habitats such as daytime high temperatures followed by
227 night-time hypoxia (Collins et al., 2021a; Gunderson et al., 2016). More recent studies have
228 considered whether pre-exposure to one stressor can either (i) reduce the ability of an organism to
229 tolerate a sequential, second stressor, termed “cross-susceptibility” (a type of synergistic effect) or
230 (ii) improve the ability to tolerate another, termed “cross-tolerance” (a type of antagonistic effect)
231 (Gunderson et al., 2016; Rodgers and Gomez Isaza, 2021). It should be noted that cross-tolerance
232 has multiple definitions within the scientific literature. If taking a specifically cellular view, “cross-
233 tolerance” is defined as the phenomenon by which exposure to one stressor improves the ability to
234 tolerate another as a result of shared protective mechanisms between stressors (Bueno et al., 2023;
235 Sinclair et al., 2013). In this review, we explore three main types of multistressor investigation of the
236 CSR in intertidal animals: (i) multiple stressors applied in isolation in the laboratory (ii) multiple

237 stressors applied in combination in the laboratory (iii) cellular stress responses measured directly in
238 the natural multifactorial environment. Recent studies have noted that the effect of multiple
239 stressors on the CSR still remains poorly studied compared to single stressors (Barrett et al., 2022;
240 Dong et al., 2022), so instead we draw upon selected multistressor case studies to illustrate the
241 advantages and disadvantages of each type of approach.

242
243

244 **Multiple stressors in the laboratory applied in isolation**

245 In terms of the CSR, studies have aimed to expose intertidal animals to a range of stressors to
246 identify common pathways of cellular response. Intertidal bivalves have often been the subject of
247 this approach (Gracey et al., 2008; Lockwood et al., 2015; Zhang et al., 2012). Microarray analysis of
248 mussels exposed to temperature and salinity stress separately revealed a shared CSR of 45 genes,
249 which were predominantly related to ion transport, but showed differential regulation in response
250 to these stressors (Lockwood and Somero, 2011; Lockwood et al., 2010). Such differential regulation
251 may reflect different challenges posed by temperature and salinity to membrane permeability
252 (Lockwood et al., 2015). One gene associated with the oxidative stress response was similarly
253 affected by both temperature and salinity, suggesting the importance of maintaining redox balance
254 during cellular stress (Lockwood et al., 2015). Another example comes from oysters where
255 transcriptome sequencing of organisms subjected to a wide variety of stressors was analysed
256 alongside the genome sequence (Zhang et al., 2012). Transcriptome data showed a shared cellular
257 stress response of 123 genes across four stressors (temperature, salinity, metal and air exposure)
258 but the identity of those genes was not discussed. This approach is a valuable first step in
259 determining the potential mechanisms used, but cannot necessarily determine what mechanisms
260 are actually realised when stressors are applied in combination as it neglects the potential for
261 unpredictable, non-additive effects on components of the CSR. Thus, there is a continued need to
262 investigate stressors in combination.

263

264 **Multiple stressors in the laboratory applied in combination**

265 Fully factorial designs involving two (or more) stressors allow identification of the effects of stressors
266 in both isolation and combination (Todgham and Stillman, 2013). In *M. edulis*, the CSR was
267 investigated via transcriptomics in response to different temperatures and salinities using a factorial
268 design (Barrett et al., 2022). At a control salinity, no functional enrichment of groups was observed
269 in response to temperature stress, but at low salinity temperature induced a more wide-ranging
270 response including elements related to the CSR such as the unfolded protein response (UPR) and cell

271 morphogenesis. This was associated with greater upregulation of CSR genes (*HSPs*, antioxidants and
272 MAP kinases) and increased mortality at low compared to high salinity. Therefore, with the addition
273 of just one simultaneous stressor, the cellular response of an organism to its environment is
274 markedly different. Similarly, there is evidence that sequential stressor exposures may also modify
275 the CSR. Although evidence documenting the role of HSPs in cross-tolerance for intertidal organisms
276 remain scarce using either targeted (Todgham et al., 2005) or global approaches (Collins et al.,
277 2021b; Collins et al., 2022). In our opinion, the study of Todgham et al., (2005) on the intertidal
278 sculpin is the most thorough assessment of HSP involvement in promoting tolerance of the
279 multistressor intertidal. The study investigated how pre-exposure to acute heat shock affected
280 mortality rates upon subsequent exposure to osmotic challenge. It was hypothesised that
281 temperature would induce HSP70 expression which would then confer protection to subsequent
282 stress. The priming of HSPs by one stressor is conceptually similar to the aforementioned
283 frontloading/preparative defence, which then would reduce future stress (albeit in response to a
284 stressor of a different a nature). Cross-tolerance was observed at the physiological level but was
285 dependent upon the magnitude of the initial heat shock and the recovery period between stressors,
286 requiring 8-48 h to develop. Interestingly, the key finding that emerged was that HSP70
287 induction/frontloading by the priming stressor was not clearly related to tolerance of a subsequent
288 stressor. Tolerance was only clearly related to the ability to acutely raise HSP70 levels in response to
289 the second stressor. Thus, the first stressor may prime some other mechanism within the cell that
290 then increases the ability to mount a HSP response to a second stressor. This finding for HSP70 was
291 interestingly mirrored by a recent transcriptomic study investigating cross-tolerance. Collins et al.,
292 (2021) explored whether thermal plasticity improves subsequent hypoxic performance in an
293 intertidal amphipod. Individuals acclimated to increased temperature out-performed cold-
294 acclimated individuals under hypoxia, which was associated with frontloading as the predominant
295 transcriptome-wide mechanism. Interestingly, frontloaded genes included those involved in immune
296 and cytoskeletal responses, but not specifically *HSPs*. *HSP* gene expression displayed a greater
297 response under hypoxia in warm acclimated individuals suggesting that, similar to Todgham et al.,
298 (2005), cross-tolerant phenotypes may be associated with the acute response of HSP to the second
299 stressor rather than their priming from a prior stressor. That said, the Collins et al., (2021)
300 experiment was conducted at the gene expression level which can be particularly dynamic (Dong et
301 al., 2022). There may be epigenetic regulation (Clark et al., 2018) and/or post-translational
302 modifications (Elowe and Tomanek, 2021), so future validation at the protein level is key. Overall,
303 our knowledge of molecular mechanisms underpinning two-stressor interactions is limited and, from
304 the studies here, reveal marked variation already between taxa (Fig. 2).

305

306 In the discussion above, we referred to two stressor studies applied simultaneously or sequentially,
307 but responses can be modified further by prior dual acclimation to two stressors before determining
308 responses to a subsequent stressor. Dual-acclimation for 7 days to temperature-pH combinations
309 (temp = 20 or 24 °C, pH = 8.1 or 7.8) results in markedly different *HSP70* responses to subsequent
310 acute thermal stress in intertidal limpets (Wang et al., 2018). Organisms dual acclimated to high
311 temperature and low pH demonstrated the greatest increase in *HSP70* gene expression, when
312 exposed to an acute warming event, compared to those acclimated to low temperature, high
313 temperature or low pH in isolation. Overall, studies of stressors in combination are essential to
314 understand the intricacies of the CSR, but given the variation observed with such a small number of
315 stressors, extrapolation to the wild will continue to be challenging.

316

317

318 **Disentangling the HSR in a multistressor context directly in the field**

319 Gene-network analyses can provide information about whole genomes and transcriptomes from
320 intertidal animals sampled *in situ*, i.e. directly in the natural multistressor environment. In an
321 impressive study, particularly at the time, Gracey et al., (2008) performed microarray analysis at high
322 temporal resolution on field-sampled *Mytilus* exposed to a tidal cycle. The study revealed distinct
323 phases of temporal gene expression over the tidal cycle corresponding with metabolism, cell division
324 and two phases of heat stress associated with *HSPs* (Gracey et al., 2008). Comparable studies using
325 RNA-Seq are few in number, likely due to sequencing costs, although costs are decreasing all the
326 time. Using RNA-Seq, Ruiz-Jones and Palumbi, (2017) provided an elegant study correlating
327 transcriptome profiles of tabletop coral *Acropora hyacinthus* with multiple stressors experienced in
328 the natural environment over 17 days. They identified groups of genes whose expression was
329 associated with various stressors such as temperature, pH and dissolved oxygen and previous day-
330 night variability in these factors. These environmentally-responsive genes were involved in
331 endoplasmic reticulum stress, the UPR (e.g. *HSP68*, *HSP70* and *HSP90*) and calcium ion binding. Thus,
332 a network approach may allow a formal statistical assessment of the nature of the CSR in response
333 to multiple abiotic stressors. The other benefit of network type analyses is that they facilitate an
334 integrative multilevel approach from genes to the whole organism. Groups of co-regulated genes
335 can be identified, and their expression correlated with whole animal physiological traits. For
336 example, coral reefs inhabiting back-reef pools can experience high thermal variability which may
337 result in bleaching (Barshis et al., 2013). At the molecular level, network analyses revealed bleaching
338 under heat stress may be associated with expression of genes whose function involves the

339 extracellular matrix and DNA-binding proteins (Rose et al., 2016). Thus, network analyses may
340 provide a clearer understanding of the mechanisms coordinating physiological responses to
341 environmental change directly in the intertidal zone. However, it could be argued that the weakness
342 of such a field-based approach is that it is correlative, and causative relationships cannot be drawn.
343 A combined laboratory and field approach may alleviate this difficulty. For example, Gracey et al.,
344 (2008) measured gene expression *in situ* but also compared it against gene expression profiles of
345 organisms exposed to a range of abiotic stressors in the laboratory. This hybrid approach may help
346 to better understand the environmental drivers of change observed in the wild.

347

348 **Insights into the CSR from genome analyses**

349 Whilst transcriptomic analyses provide a much more comprehensive overview of the CSR compared
350 to that of candidate genes, they are limited to those genes expressed under particular conditions.
351 This can lead to a lack of appreciation of the underlying genomic ability of the organism to respond
352 to stress. The best example of this to date is that of the oyster genome, which revealed intriguing
353 insights into life in the multifactorial intertidal. The oyster genome contains an over-representation
354 of genes that respond to environmental stress, with enrichment for genes encoding *HSP70* and
355 inhibitors of apoptosis (Zhang et al., 2012; Zhang et al., 2016). Whilst the expansion of *HSP* gene
356 family members had previously been documented in model species and correlated with thermal
357 tolerance (Bettencourt et al., 2008), this was the first time that such an expansion was demonstrated
358 in a non-model species. The evolution of the *HSP70* gene expansion in oyster is rather unusual, in
359 that the expansion has occurred in the atypical *HSPA12* family members, which in other species are
360 not associated with the stress response (e.g. Han et al., 2003; Hu et al., 2006). This gene family
361 expansion is bivalve-specific with further species-specific tandem duplications (Cheng et al., 2016),
362 resulting in circa 73 copies in *C. gigas*, 97 in the pearl oyster (*Pinctada fucata*), 55 in the invasive
363 golden mussel (*Limnoperna fortunei*), 57 in the scallop (*Patinopecten yessoensis*) and at least 43 in
364 the blue mussel (*M. edulis*) (Barrett et al., 2022; Cheng et al., 2016; Clark et al., 2021; Takeuchi et al.,
365 2016; Uliano-Silva et al., 2014; Zhang et al., 2012). The expansion of the *HSPA12* family in bivalves
366 raises the question of how other intertidal classes and families have modified their genomes and
367 adapted their stress signalling pathways. It is suggested that selection and maintenance of this
368 particular gene family was driven by adaptation to a sessile lifestyle in the dynamically changing
369 marine environment with its complex biotic and abiotic stresses (Cheng et al., 2016). Furthermore,
370 the association of specific sub-sets of these *HSPA12* genes with different stressors has led to the
371 suggestion that they act as complex intertidal stress regulators (Clark et al., 2021). This clearly needs
372 to be investigated further using gene network-type analyses (Ramsøe et al., 2020). Overall, these

373 studies show that intertidal organisms may have the potential for more robust multistressor
374 responses via genomic expansion of *HSP* families or shared mechanisms elicited by a suite of abiotic
375 stressors. There is also evidence for divergent mechanisms in terms of *HSPs* such as stressor-specific
376 *HSPA12* genes (Clark et al., 2021). Thus, there is a need to use cutting edge molecular tools
377 (including whole genome sequencing) alongside physiology experiments and ecological
378 observations/sampling to understand the multistressor CSR.

379

380 **Conclusions and future perspectives**

381 Understanding the CSR in the wild will continue to be challenging but multistressor approaches are
382 essential given the simple observation that even with just two stressors, one stressor can alter HSP
383 expression upon exposure to another. This similarly creates the problem of how we extrapolate
384 laboratory exposures to the natural environment where an estimated fifteen stressors can operate
385 in intertidal systems (Cote et al., 2016). Addition of stressors in the laboratory is logistically
386 challenging in terms of sample size. Field approaches avoid this pitfall but it is difficult to draw
387 causation (Spicer, 2014) and to assess how one stressor has altered the expression in response to
388 another. We argue that a combined laboratory-field approach provides the best ability to
389 disentangle complex patterns of the CSR and their role in understanding organism tolerance in
390 dynamic multistressor environments.

391

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399

400 **Competing Interests**

401 The authors declare no competing interests.

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577 **Figure legends**

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579 Figure 1: Schematic of the most common stressors in the intertidal region: A: periodic emersion with
580 potential for desiccation, high salt from sea spray, temperature stress from high temperatures in
581 summer to freezing in winter; B: heat and UV radiation; C: Wave action; D: isolated tide pools can be
582 affected by hypoxia, altered salinity (both high and low), temperature fluctuations with the potential
583 to freeze over in winter; E: rainfall will provide fresh water run-off over the intertidal region and
584 dilute salinity in tide pools. There may also be pollution run-off from the land including agriculture
585 (high nutrients and nitrates/nitrites from fertilizers), pollution from factories etc; F: predation. NB:
586 food supply is much more limited in the intertidal region compared with the subtidal.

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589 Figure 2: Conceptual mechanisms of multistressor effects on CSR depicting a two by two stressor
590 design: A: simultaneous exposure to two stressors - acutely both stressors, **S1** and **S2**, in isolation
591 induce the CSR. In combination, **S1+S2** cause increased stress and synergistic effects associated with
592 reduced organism tolerance e.g. Barrett et al. 2022 in mussels; B: sequential exposure to two
593 stressors acutely separated by a recovery period - **S1** is applied first and does not trigger the CSR
594 compared to control but may prime other mechanisms that allows for a greater CSR upon exposure
595 to subsequent stress (**S1+S2** exposed) than organisms exposed to just **S2**. This results in cross-
596 tolerance e.g. Todgham et al. 2005 in tidepool sculpins; C: sequential exposure to two stressors
597 involving chronic exposure to **S1** followed by acute **S2** exposure - chronic exposure to **S1** allows for
598 frontloading of cellular defences which reduces the need for an acute CSR upon exposure to another
599 acute subsequent stressor (**S1+S2**) than organisms exposed to just **S2**. This also may result in cross-
600 tolerance e.g. Collins et al. 2021 in amphipod crustaceans. Thus across phyla, there is wide diversity
601 in multistressor responses in terms of the CSR as cross-tolerance may be elicited with/without
602 frontloading of defences following **S1** or from increased/reduced CSR response to **S2**. Increased
603 reaction to **S2** could reflect either synergism or antagonism dependent on species, stressor timing
604 and duration.