Combined salinity and acidity stressors alter *Daphnia magna* population growth and structure under prolonged absence of photoperiod

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Running title: Combined physico-chemical stress affect *Daphnia* fitness

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Statement of contribution

All authors wrote the article. **MSK** conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, enriched and approved the final draft. **SAP** contributed to the extension of the concept of the experiment, performed the experiments, authored or reviewed drafts of the paper, approved the final draft. **RH** authored or reviewed drafts of the paper, approved the final draft.

Abstract

Daphnia represent a model keystone organism in the study of phenotypic plasticity and ecosystem health. Although natural and anthropogenic influences affect freshwater ecosystems globally at unprecedented levels, investigations of the effects of co-occurring physico-chemical stress on zooplankton phenotypic plasticity and population dynamics under disrupted photoperiod are surprisingly rare. The ability of *Daphnia* populations to reproduce, develop, and survive under these complex conditions remains largely unknown.

We exposed a laboratory-raised clonal population of *Daphnia magna* to different stress levels of acidity and/or salinity undergoing complete constant light or darkness over 30 days. Overall, in experiment 1, under 24h light, population size at day 10 day, considerably differed between specific stress conditions, with varying age structures. Nevertheless, all populations expanded compared to the starting population on day 1. On day 30, population size increased across treatments but showed significant differences between treatment groups in terms of population size and age structure. Surprisingly, *Daphnia* performed better under combined stress of salinity and acidity than under acidity alone as the extra salinity in the medium may have counterbalanced sodium loss caused by lower pH. Under complete darkness (experiment 2), significant mortality occurred by day 30 across all treatments, indicating a far more negative impact of constant light absence than presence on *D. magna* populations subject to chemical stress. Our findings reveal a considerable degree of differential reproductive and ontogenetic plasticity in response to combined light and environmental stressors that do not follow simple predictions about additive effects of individual stressors. Exposure to constant light led to increased population size, which may be a result of supercharged ion regulation that enables zooplankton to survive better under specific levels of chemical stress. Overall, the results of our work merit further molecular investigation of phenotypic plasticity of congeners across severe combined physical and chemical ecological stress conditions.

Introduction

Co-occurring chemical stressors

Natural and anthropogenic influences on aquatic ecosystems have increased the effects and occurrences of salinisation and acidification of freshwater ecosystems in a number of ways including seawater intrusions, mining, urbanisation and agricultural as well as industrial processes [1-5]. Daphnids are keystone cladoceran zooplankton and important models in the study of environmental stress and biodiversity in aquatic ecosystems [6]. Daphnids produce genetically identical daughters under temperate conditions through parthenogenesis. The genome of *Daphnia* may express different phenotypes in reaction to different environmental conditions; a phenomenon referred to as phenotypic plasticity [7]. Rapid responses to environmental stressors, such as ionic and osmotic regulation [8], which may be due to transgenerational epigenetic effects [9-10], are associated with high energetic costs [2,11], and may enable *Daphnia* to occupy a wider niche range compared to other fresh water inhabitants [12]. However, low pH levels have been shown to reduce zooplankton species richness and alter the structure of cladoceran populations [13-14], leading to diminished survival and growth of *D. magna* below pH 5 [15]. For example, *Daphnia* species are less abundant in acidified lakes compared to other arthropods (*e.g.*, calanoid copepods and insects), which may therefore dominate the ecosystem instead of *Daphnia* [2,16].

Like acidification, salinisation of aquatic habitats presents a key stressor of aquatic life. Some species, such as *Daphnia exilis* (Herrick) and *Daphnia pulex* (Leydig), can maintain a positive and constant osmolality difference with the environment at higher salinities via an osmoregulatory response [12]. In particular, *Daphnia magna* (Straus) is a generalist whose niche is defined by a much higher range of salinity tolerance than other aquatic *Daphnia* species. Populations of *D. magna* have been documented to inhabit both fresh and slightly salty waters [17], with strong evidence of their ability to adjust ion regulation (osmoregulation and osmoconformance) to cope with various levels of salinity [18]. This spans fresh to brackish waters, up to 10 gL^{-1}

salinity [19], with the upper limit of their tolerance depending on population life history and within species genetic variation [17,20, 21].

A further ecological challenge arises when changes in salinity may become coupled with water acidification [22]. For instance, osmoregulation in *Daphnia* is pH dependent as low pH is thought to inhibit sodium uptake and increase sodium loss much as it does in freshwater fish [21,22]. The negative effects of increased salinisation and acidification on freshwater zooplankton result from impairing the organism's osmoregulatory system; a compromised ion-regulation can lead to higher sensitivity to low pH, and the latter may alter tolerance to lethal and sub-lethal levels of salinity and *vice versa* [22]. Natural and anthropogenic causes lead to increasing levels of salinity and varying levels of acidity in many zooplankton habitats and thus increasingly create adverse conditions for these organisms [22]. This highlights the need to investigate the effects of co-occurring physical and chemical stressors on keystone species, such as *D. magna*, in freshwater ecosystems [23].

Lack of photoperiod as a novel environmental challenge

The duration and intensity of light exposure can have a strong influence on many organisms and research on zooplankton has studied effects on diapause [24,25]; egg hatching [26], circadian clock, diel vertical migration [27] and ontogenesis [28] in response to photoperiod and light quantity [29]. Somewhat surprisingly then, investigations of the effect of exposure to elevated light at night, constant light, or lack of light, on plastic responses of zooplankton in general, and *D. magna* in particular, has been overlooked for decades except for the area of diapause and sexual morph production [25], and alteration of their phototaxis [30]. The negative effects of too little or too much light extend beyond the direct effect on circadian rhythms. For instance, exposure to complete darkness for 7 days significantly reduced the survival of *Daphnia parvula* compared to exposure to 14:10 light/dark cycle by a mechanism that is not yet completely understood [31]. On the other end of the spectrum, testing zooplankton responses to constant light exposure remains unresolved.

Meyer & Sullivan (2013) demonstrated that ecological light pollution through increased night lighting modifies community composition, structure, and characteristics and thus ecosystem functioning via alteration of nutrient exchange and aquatic–terrestrial fluxes of invertebrates [32]. These altered fluxes may have cascading effects through riparian and aquatic food webs [33,34]. However, although a number of studies endeavoured to shed light on the effects of darkness and light at night on the physiology, behaviour, growth and development, and fitness of a range of aquatic organisms [34-36], the effect of lack of photoperiod, on daphnid phenotype, fitness and survival is surprisingly poorly studied. To date, very little is known about those effects on zooplankton [30,37] and their implications for ecosystems subject to light pollution which are simultaneously vulnerable to salinisation and acidification. This also includes waters trapped underground or in caves following storms, tsunamis, and floods [38-41, or in areas close to the earth's polar circles [36,42] that experience both extended periods of Siberian and Nordic nights or midnight sun (*e.g.,* [43]), while being subject to natural and man-made environmental changes.

These patterns and types of complex physico-chemical stress are predicted to lead to a reduction in biodiversity, especially when accompanied by upsetting the thermal regimes in aquatic and freshwater ecosystems because of climate change and environment modification (*e.g.,* arboreal and riparian vegetation alteration) by humans [34,44,45]. There is a significant gap in current knowledge about how the genotype of organisms in general, and zooplankton of economic importance in particular, would respond, in terms of reproductions and development, to the absence of photoperiod, let alone the combination of such physical stress with composite chemical stress (salinisation and acidification of water). In this work, we exposed a clone of *D. magna* to different stressors (salinity and/or acidity subject to constant exposure to light or dark conditions for 30 days in two separate experiments). We sought to answer the following specific questions: First, how does *Daphnia* reproductive success and age structure differ across varying levels of salinity and acidity,

121 and their combinations, under constant light exposure over time? Second, how do these traits vary
122 in response to the effects of salinity and/or acidity under continuous darkness?

123 Our findings show that exposure to physico-chemical stress (absence of photoperiod
124 combined with salinity and/or acidity) can differentially alter the reproductive success and age
125 structure of the he zooplankton population over time.

126 **Materials and Methods**

127 Model organism

128 The *Daphnia magna* (Straus) clonal population used in this experiment was established from a
129 single female sampled from a population purchased from Sciento©, Manchester UK. The resulting
130 clonal population was reared in a growth chamber, at the Faculty of Biology, Medicine and Health,
131 The University of Manchester, under a 16h:8h light/dark cycle at 23°C. Under these conditions,
132 daphnids reproduce asexually by parthenogenesis resulting in a female-only population. The ability
133 to endure a range of abiotic stressors or sensitivity to contaminants made daphnids a key model to
134 study tolerance [2,46], phenotypic plasticity [47], and toxicity in aquatic environments [48].

135 Feeding occurred every two days by injecting a diet mix into the medium. The diet consisted
136 of 1ml of 'Allinson dried active yeast' (*Saccharomyces cerevisiae*), purchased from a local store,
137 and 2ml of alga *Scenedesmus quadricauda* (sourced from Sciento ©, Manchester UK) in Artificial
138 *Daphnia* Medium (ADaM), *sensu* Ebert (2013) [49], at pH ~7 and 0.33 gL^{-1} salinity. One litre of
139 ADaM consists of 0.33 g sea salt, 2.3 ml $CaCl_2$ x $2H_2O$ (117.6 gL^{-1}), 2.2 ml $NaHCO_3$ (2.52 gL^{-1}),
140 and SeO_2 (0.007 gL^{-1}), all dissolved and homogenised in ultra-pure deionised Milli-Q water.

141

142 Experimental design

143 *Daphnia magna* population size (total number of daphnids in the beaker at each census, as a proxy
144 of reproductive success) and the proportion of juveniles relative to the total population in the beaker
145 per census (age structure, hereafter) were examined. The experimental conditions were based on

146 extensive pilot studies during which we established different levels of pH and salinity that were
147 lethal and sub-lethal to our clone of *D. magna* [47]. The pH treatment levels were 5, 5.5 and 6 and
148 the salinity treatment levels (plus the above-mentioned ADaM salinity) were 1.33, 3.33, and 6.33
149 gL^{-1}. The investigation of salinity and/or acidity stressors, as detailed below, was done in two
150 separate experiments of exposure to physical stress, *i.e.,* lack of photoperiod (constant light or
151 constant darkness). The negative control for all parts of the work was the standard ADaM with
152 optimal conditions (photoperiod of 16h light:8h darkness, salinity of 0.33 gL^{-1}, and pH = ~7).

153 We used Sigma Aldrich (USA) 'sea salts', and distilled acetic acid (Sarson's ©) procured
154 from a local supplier. To lower the pH in the treatment conditions the acetic acid was added
155 drop-wise to the medium in the beaker and measured continuously using a 'Mettler Toledo™ FE20
156 FiveEasy™ benchtop pH meter' until the desired pH was reached. Here, acetic acid provided a
157 practical way of acidifying the volume of water in question. Acetic acid can have extensive effects
158 on the metabolism of living organisms and it can be found in nature and also close to anthropogenic
159 activity such as bioethanol industry [50], pond preparation [51], as a product of anaerobic activity
160 associated with biological decay [52], waste decomposition and processing [53], biodegradation of
161 petroleum and hydrocarbon contaminants [54,55], ocean water, oilfield waters, and both cloud and
162 rain waters [56-60]. Furthermore, adjusting water pH using vinegar is applied in studies on the
163 effects of complex natural organic acids on larval stages of animals (*e.g.,* amphibians [61]).
164 Additionally, the application of vinegar is commonplace in fish farming to neutralise water
165 alkalinity or increase water acidity, owing to proton donation into the aqueous solution
166 ($CH3COOH_{(aq)} \rightleftharpoons CH3COO^{-}_{(aq)} + H^{+}$).

167 The salinity conditions were achieved by dissolving the correct weight of sea salts in the
168 beaker using a magnetic stirrer. All experimental beakers were kept in a dedicated growth chamber
169 at 23°C. Further, there were two light regimes: constant light (24h), and constant complete darkness
170 (24h).

171 In the first experiment, when photoperiod was lacking (constant light exposure), we
172 randomly selected 329 age-synchronised early nymphs from the stock clone reared in the
173 above-mentioned optimal conditions for 20 generations, and allocated from this population 7
174 nymphs to 9 different treatment groups (with 5 replicates each) plus a negative control (the standard
175 ADaM under 16h:8h light/dark cycle) and a positive control (the standard ADaM under 24h:0h
176 light/dark cycle). The treatment groups were as follows: Salinity1, Salinity2, Salinity3, Acidity1,
177 Acidity2, Acidity3, Salinity1 & Acidity1, Salinity2 & Acidity2, Salinity3 & Acidity3). Our pilot
178 study showed that survival was extremely low under severe experimental conditions as the exposed
179 *Daphnia* clone was not trained/acclimated prior to the exposure. This was done to imitate abrupt as
180 well as extreme environmental change. By Day 10 of the experiment, due to high mortality under
181 severe conditions, we ended up with 26 beakers as follows: *Salinity1* [1.33 gL^{-1} , 3 replicates],
182 *Salinity2* [3.33 gL^{-1} , 3 replicates], *Salinity3* [6.33 gL^{-1} , 2 replicates], *Acidity1* [pH 6 , 3 replicates],
183 *Acidity2* [pH 5.5 , 3 replicates], *Acidity3* [pH 5 , 2 replicates], *Salinity1 & Acidity1* [3 replicates],
184 *Salinity2 & Acidity2* [3 replicates], and *Salinity3 & Acidity3* [2 replicates]) plus the controls. The
185 beakers (12.5 cm x 10 cm x 10 cm) were cuboid and transparent, each filled with 600 ml of adjusted
186 ADaM. We randomised the positions of the beakers within the growth chamber upon every feeding
187 time.

188 Daylight LED bulbs (2800 K Warmwhite 230VAC [NorthLight] purchased from Clas
189 Ohlson ©, Manchester, UK) were used and the light in the growth chamber was surrounding all
190 sides except the base of each beaker, which was placed on a white base. The bulb is a daylight
191 mimic emitting warm soft yellowish light of more than 1100 lumens, which is equivalent to a
192 typical overcast midday in the UK and northern Europe. A 75% media change was conducted
193 weekly, in the light exposed *Daphnia* only, to refresh ADaM.

194 In the second experiment (complementary to experiment 1), when photoperiod was lacking
195 (constant absence of light), we repeated the above-mentioned 9 stress combinations plus a positive

control (the standard ADaM under 0h:24h light/dark cycle), with 7 early nymphs in the beaker, and 5 replicates per treatment combination. This resulted in 210 daphnids compartmentalised into the combinations under 24h darkness conditions. We also used the negative control defined above as reference frame. To ensure complete darkness, each beaker was completely double-wrapped with foil, with an obscured sealable opening for feeding with a pipette every other day. The media was not changed during the course of this part of the work.

Analysis of *Daphnia* traits

All statistical analyses were conducted in R [62] via RStudio [63]. The data were collected on Day 10 and Day 30 in the 24h light experiment and on day 30 for the 24h darkness experiment.

Experiment 1: Constant light (24h)

A generalised mixed effects model (GLMM), family 'Poisson', model optimiser 'bobyqa' [64], R packages 'lme4' [65], was applied to test *Daphnia* population size (as defined above) throughout the experiment duration (GLMM 1). There were two censuses. The census (count day) was randomised. This was followed by a posteriori test (Tukey), R package 'multcomp' [66], to examine multiple pairwise comparisons of the stressors in question. The explanatory variable (fixed effects) was stressor (salinity, acidity, salinity & acidity) under 24h light comprising the following levels: Baseline = positive control , *Salinity1* was 1.33 gL^{-1}, *Salinity2* was 3.33 gL^{-1}, and *Salinity3* was 6.33 gL^{-1}; *Acidity1* was pH 6, *Acidity2* was pH 5.5, and *Acidity3* was pH 5; *Salinity1 & Acidity1*, *Salinity2 & Acidity2*, and *Salinity3 & Acidity3*. Due to high mortality over time, several treatment combinations and/or replicates were omitted from the analysis. In other words, treatments/replicates without *Daphnia* on Day10 were excluded.

Another model (GLMM 2) was used to analyse age structure (the proportion of juveniles to total daphnid numbers in the beaker per census) with the same set up as above. Besides the model

summaries, the main effects of both models are respectively shown via analysis of deviance by using the Anova (Type II) command in R, package 'car' [67].

Experiment 2: Constant darkness (24h)

Data collection for this part of the experiment took place only at Day 30 because we could not count the *Daphnia* without exposing them to light and it was essential to eliminate all exposure to light during this part of the work. The same data were collected: *Daphnia* population size and age structure. As so few daphnids were able to survive the extreme conditions to the 30-day data collection, we provide descriptive statistics only.

Results and Discussion

Lack of photoperiod (constant light, 24h), acidity and/or salinity

Daphnia population size and age structure

Under constant light, 24h, *Daphnia* population size was significantly affected by overall physicochemical stress treatment (X^2 = 1117.3, Df = 9, P <0.0001), and specifically by each of the stress levels as follows: *Salinity1* (**P < 0.0001**), *Salinity2* (**P < 0.0001**), *Salinity3* (**P = 0.03**), *Acidity1* (**P < 0.0001**), *Acidity2* (**P < 0.0001**), Acidity3 (**P < 0.0001**), *Salinity1 & Acidity1* (**P < 0.0001**), *Salinity2 & Acidity2* (**P = 0.047**), *Salinity3 & Acidity*3 (**P < 0.0001**); (Fig 1); there were 35 daphnids on Day 10 in the negative control and 16 daphnids in the positive control, on Day 30 there were 119 daphnids in the negative control and 328 daphnids in the positive control, Supporting Information (Tables S1, S2, and S3) for multiple pairwise contrasts of the stress levels).

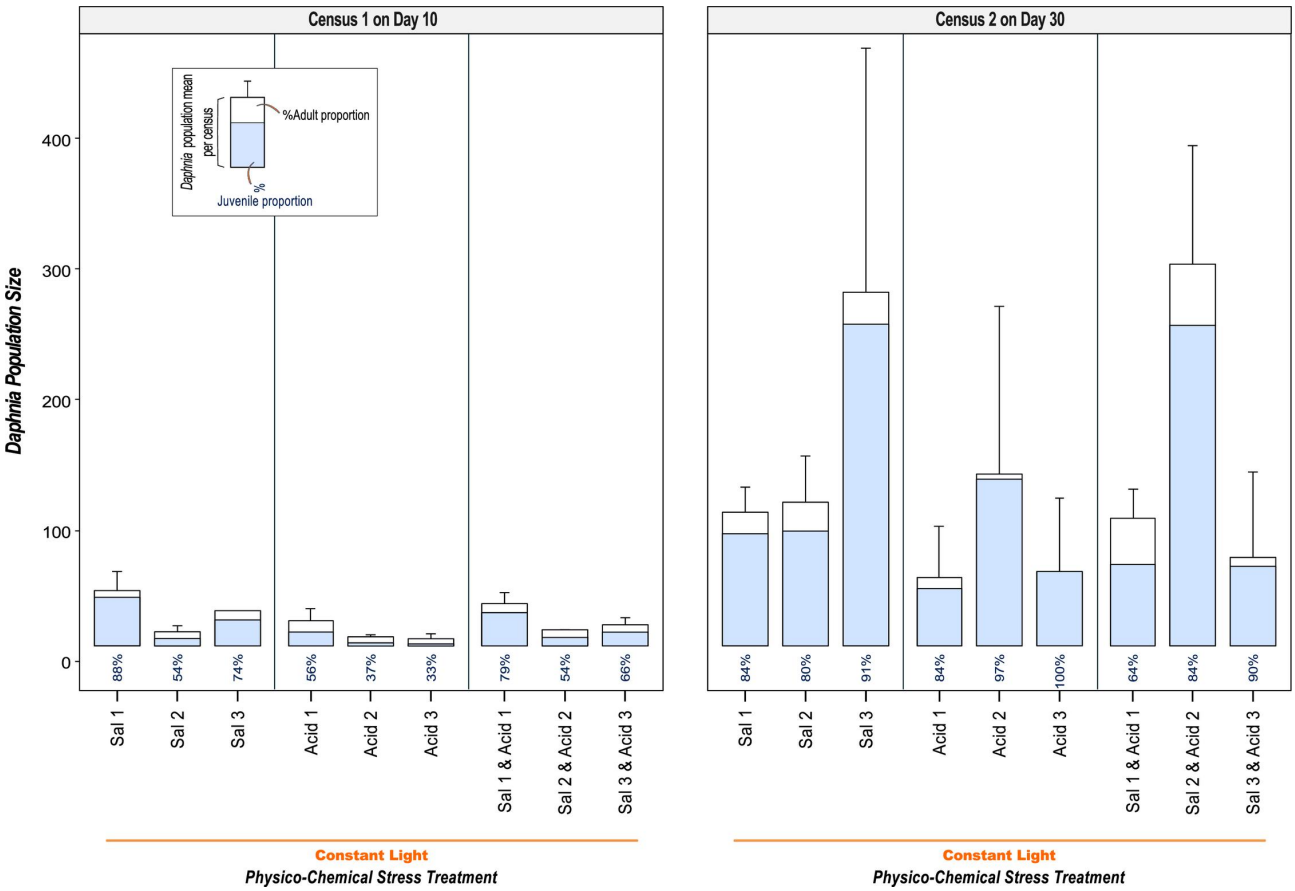

Fig 1. *Daphnia* population sizes and age structure under chemical stress in the absence of photoperiod (constant light). The bars illustrate the changes in *Daphnia* population (mean of total daphnid numbers in the beaker per treatment) ± SEM. The bar parts display the rounded proportions of juveniles against adults relative to the beaker population (*Daphnia* age structure). There were two counts on Day 10 and Day 30. The conditions were as follows: constant light (24h day:0h night), *Salinity1* [1.33 gL^{-1} , 3 replicates], *Salinity2* [3.33 gL^{-1} , 3 replicates], *Salinity3* [6.33 gL^{-1} , 2 replicates], *Acidity1* [pH 6 , 3 replicates], *Acidity2* [pH 5.5 , 3 replicates], *Acidity3* [pH 5 , 2 replicates], *Salinity1 & Acidity1* [3 replicates], *Salinity2 & Acidity2* [3 replicates], and *Salinity3 & Acidity3* [2 replicates]); Sal = Salinity, Acid = Acidity, Sal & Acid = Salinity & Acidity.

Compared to Day 1, overall population sizes were considerably larger on Day 10 under stress combinations; notable examples of average percentage of increase/decrease were as follows: *Salinity1* (495% larger population), *Salinity3* (279% larger); *Acidity1* (176% larger), *Acidity2* (5%

257 smaller); *Salinity1 & Acidity1* (362% larger); *Salinity2 & Acidity2* (67% larger), (Fig 1) and
258 Supporting Information (Table S4). Comparing population sizes on Day 30 to Day 10 reveals that *D.*
259 *magna* was able to dramatically increase population size over time. However, this differed in
260 magnitude contingent on the population ability to respond to different stress scenarios, with the
261 highest increases displayed under combined stress of *Salinity2 & Acidity2* (2400% larger), followed
262 by *Acidity2* (1865% larger), *Acidity3* (1030% larger) and *Salinity2* (965% larger), contrary to the
263 smallest increases reported under *Salinity1* (144% larger) and *Acidity1* (171% larger). The highest
264 level of combined stress, *Salinity3 & Acidity3*, corresponded with remarkable 352% larger
265 population size. Further, compared to the population at Day 1, the maximal population size, by Day
266 30, was reached in the combined stress of *Salinity2 & Acidity2* (4067% larger), and (3764% larger)
267 in *Salinity3*. By contrast, the minimal population increase, by Day 30, was observed under *Acidity1*
268 (648% larger) followed by combined stress of *Salinity3 & Acidity3* (857% larger), Supporting
269 Information (Table S4).

270 In the short run, acidity stress had a clear negative impact on *Daphnia* population size
271 markedly in *Acidity3* (Fig 1). The poor performance up to Day 10 is attributable to what is known
272 as pH shock [68] characterised by rapid harmful change in acidity from the culture's semi-neutral
273 pH to the severe experimental acidic conditions. The pH levels of the current study strongly limited
274 *Daphnia* reproductive success and resulted in delayed development to maturity (see age structure
275 below). But, *Daphnia* were able to acclimate to a degree to acidity as the population showed
276 variable resilience through time. By Day 30, the population emphatically recovered in *Acidity3*, but
277 the population was almost completely juvenile. *Daphnia* numbers burgeoned in *Acidity2* (mostly
278 juvenile population), whilst the margins of population growth and juvenility were comparatively
279 smaller in *Acidity1* (Fig 1) and Supporting Information (Table S4). This indicates that the surviving
280 daphnids were more reproductive at pH 5.5 (*Acidity2*) over time than at pH 6 (*Acidity1*) and pH 5
281 (*Acidity3*). Our findings are in line with the observations documented by Weber & Pirow (2009) in

282 *Daphnia pulex*, reporting that tolerance and acclimation at pH 5.5 can be high and plausibly be
283 linked to an induced compensatory active ion transport at this particular level of acidity, owing to
284 *Daphnia*'s ability to lower sodium efflux and increase its influx [2]. It has been shown that in the
285 long run, exposure to pH 5.5 may correspond with better survival and reproduction rates than
286 exposure to pH 5, as values less than 5 can lead to extinction of *Daphnia* [15].

287 Low pH levels are known to reduce survival and growth in *D. magna* due to sodium
288 deficiency [21,22], a sharp decline in respiratory rates, oxygen uptake depression, and impairment
289 of ion-regulation [15,69]. On the one hand, levels of free-form carbon dioxide (CO_2) in the
290 surroundings of *Daphnia* concomitantly increase in acidified freshwater, *e.g.*, due to acid spills and
291 acid rain akin to the pH levels tested in this work. At pH 6 the level of free-form CO_2 is usually
292 more than 60% and at pH 4 this rises to 100% [69]. As such, an external increase in water acidity
293 accompanied by abundant CO_2 will disturb the internal acid-base balance in *Daphnia;* a degree of
294 haemolymph acidosis that occurs interdependently with decreased CO_2 diffusion through the gills
295 leading to impeded oxygenation [2], in accordance with the Bohr effect [69]. In fact,
296 CO2-dependent freshwater acidification is a major new challenge for zooplankton; shown by Weiss
297 et al. (2018) to lead to impairment of *Daphnia* detection of predators with consequences for the
298 entire freshwater ecosystem [70]. Those impacts may worsen when 'acidified' saltwater intrudes the
299 land by rising sea levels [71,72].

300 On the other hand, sodium uptake and osmoregulation in *Daphnia* becomes rapidly
301 destabilised and compromised in acidified water [2,73,74]. The consequence of the above complex
302 interdependence for the functioning of the organism will be energetically costly due to
303 hyper-metabolism caused by increased stress [2]. Daphnids, therefore, may survive the acidity
304 stress dependent on their acidity sensitivity/tolerance [2,74,75]. In sum, daphnid acid-sensitivity and
305 shortage of ambient calcium in acidified water [76] with subsequent sodium deficit [2], oxidative
306 stress, increased maintenance under stress [8], and the resulting costly bioenergetics [2,11] were the

307 likely reasons behind the poor daphnid performance under the acidity levels of the current work.
308 These findings contribute to our understanding of the damaging effects of low pH levels on aquatic
309 systems that are presently vulnerable to constant change worldwide [5,45]. Like changes in ion
310 concentration in water [5,77], freshwater acidification is caused by multiple natural and
311 anthropogenic, direct and indirect, short- and long-term causes [78-80], *e.g.,* acid rain [81]
312 alongside with organic acids from land catchments and acid deposition on a local scale [78], pond
313 draining [82] and drainage waters particularly in areas with soils originated from granite or
314 weathering-resistant mineral aggregates [77,83], atmospheric and soil deposition of sulphur [78,80,
315 84], land use and management policies [80,83], afforestation [80] and mobilisation of acid anions,
316 via heavy rain, following forest fires and draughts [80,85-87], use of nitrogen-based fertilisers [88],
317 melt of snow rich in sulphuric and nitric compounds [81,83,84], and increased levels of carbon
318 dioxide and pCO_2 in water [70,89,90]. Indeed, water acidification are projected to lead to alarming
319 ecological consequences overtime [70-72,75,89-91].

320 For each level of comparison, the differences in population sizes between salinity and
321 acidity stressors were large but then decreased over time; for the contrast *Salinity2 vs Acidity2* the
322 margin of difference between Day 10 (55% larger in salinity) and Day 30 (16% smaller in salinity)
323 is nearly twofold, whilst the difference margin for the contrasts *Salinity1 vs Acidity1* or *Salinity3 vs*
324 *Acidity3* between Day10 and Day 30 is evidently smaller, Supporting Information (Table S5).
325 Overall, *Daphnia* population size was notably larger under each level of salinity stress compared to
326 acidity (Day 10: 116%, 55%, and 430%, respectively) and on Day 30 (94%, 16%, and 379%,
327 respectively). Thus, it is clear that salinity was far less damaging to the population than acidity. This
328 relates to halotolerance in *D. magna* [92] which may have contributed to resilience in the *Daphnia*
329 lineage to the severity of salinity over time in this experiment. However, as freshwater salinisation
330 significantly contributes to biodiversity loss and alteration of ecosystem functioning [93,94], acute
331 and elevated levels of salinity can be detrimental to *Daphnia* [92].

332 Both above and below ground freshwater resources are threatened around the globe by
333 elevated levels of salinity within and away from coastal regions due to primary (natural) and
334 secondary (anthropogenic) causes [95]. This includes runoff associated with road-treating salts with
335 consequent groundwater salinisation [93,96], hydrological alterations of waterways and changes in
336 water quality [4,97,98], mining and agriculture [4], and movement of saltwater inland following
337 storm surges, flash floods, rising sea level, and tsunamis [38-41]. For example, the consequences of
338 tsunamis may extend to include the salinisation of coastal/inland fresh surface water, groundwater,
339 and aquifers by means of intrusion, infiltration, and inundation [39,99,100]. It should be noted that
340 our knowledge of both ecosystem and organism responses to alterations of water ionic quality and
341 strength is not comprehensive [4,101] although increased salt toxicity is predicted to intensify
342 [4,102,103].

343 Under normal photoperiods, water salinisation negatively affects the performance and
344 survival of aquatic organisms due to osmotic stress and ion cytotoxicity [46]. Osmotic stress occurs
345 when the salt concentration of the environment is higher than the intracellular salt concentration and,
346 as a result, water moves out of the cells. By contrast, cytotoxicity refers to the disruption of the
347 hydrophobic and electrostatic forces that maintain the structure of proteins and can inhibit the
348 normal functioning of enzymes [46]. Our findings show that *Daphnia* was prolific under the highest
349 level of salinity (*Salinity3*) on Day 30 with a population size ~169% larger than the observation
350 under *Salinity*1, and ~161% larger than that under *Salinity2* (Fig 1) and Supporting Information
351 (Table S4). This can be attributed to *Daphnia*'s ability to acclimate to a range of salinity levels by
352 altering their osmotic and ionic regulation when water acidity and salinity levels are sub-lethal [8].

353 To our surprise, the effect of the chemical stressors under constant-light stress led to
354 unexpected results. *Daphnia magna* showed the same or greater population growth under combined
355 salinity and acidity. On Day10, there was greater growth in 1 (*vs Salinity2*) out of 3 scenarios

356 compared to salinity alone; and in 3 out of 3 compared to acidity alone. On Day 30, the population
357 size under combined salinity and acidity size was larger in 1 (*vs Salinity2*) out of 3 scenarios
358 compared to salinity, and in 2 out of 3 compared to acidity (Fig 1) and Supporting Information
359 (Table S6). In contrast to our prediction, across all treatment groups, population size was the largest
360 in the combined stress, *Salinity2 & Acidity2*. In this group, there were on average 291.66
361 individuals, generated from as little as 12 individuals on Day 10. The value of ~292 individuals on
362 Day 30 was 165% and 123%, respectively, larger than what was achieved by *D. magna* in *Salinity2*
363 and *Acidity2*. Further, the population size under combined stress, *Salinity2 & Acidity2*, was
364 respectively ~8%, ~187%, ~416%, ~457%, ~201%, and ~335% larger than what were achieved in
365 *Salinity3*, *Salinity1*, *Acidity3*, *Acidity1*, *Salinity1 & Acidity1*, and *Salinity3 & Acidity3*; see (Fig 1)
366 and Supporting Information (Table S4). As highlighted above, the uptake of sodium is reduced in
367 acidic conditions as sodium uptake takes place in exchange for hydrogen ions [73]. Moreover, the
368 cited authors found that the rate of sodium loss, in their *Daphnia magna* isolates, increased fourfold
369 between pH 7 and pH 3, and suggested that the increased loss and reduced uptake of sodium could
370 lead to death by sodium loss, especially when the concentrations of environmental sodium were low
371 [73]. This might explain why *D. magna* in *Salinity2 & Acidity2* obviously outperformed the
372 reproductive success in *Salinity1 & Acidity1* and *Salinity3 & Acidity3*. This could be associated
373 with the effects of the following factors: First, the ability of *D. magna* to regulate osmolarity in the
374 face of external ionic challenge is a result of the remarkable capacity of this organism to
375 counterbalance the effects of salinity beyond 5 gL^{-1} by switching from osmoregulation to
376 osmoconformance which enabled daphnids to cope with increased salinisation [12,47,104]. Our
377 results clearly demonstrate that the largest population sizes were generally under osmoregulation in
378 the short run; that was the case, as well, in the long run for combined stress *Salinity2 & Acidity2*;
379 but then there was a shift to osmoconformance in *Salinity3,* which increased survival and
380 reproduction in the longer term (Day 30). The second factor is tied to halotolerance as over time *D.*

381 *magna* may acclimate to the upper boundary of salinity, just below lethal levels contingent on the
382 acidity of the system. Such effects may occur within the 30-day period of our study. The third is the
383 capacity of the daphnid genotype to rapidly respond to ecological challenge and precondition the
384 phenotype of the offspring through adapting different strategies [9,10,105]. This ensures prioritising
385 energy allocation towards reproduction as opposed to growth and survival under stress [11,94]. The
386 fourth is an under-examined side effect of constant light exposure on the onset of increased
387 *Daphnia* reproduction under certain levels of salinity and/or acidity stress that are considered
388 otherwise unfavourable for exponential reproduction. Light pollution effects, in terms of artificial
389 light, sky glow and interference with daylight-night-time patterns, which increasingly affect aquatic
390 ecosystems and ecological interactions [34,106], are becoming a serious concern associated with
391 urbanisation and industrialised zones with increasing human population around the globe
392 [30,34,107-110]. The far-reaching effects of extended light exposure [34] may not only lead to
393 changes in vital phototactic behaviours with disruptive impacts on the aquatic system [30,34], but
394 may also extend to how zooplankton respond to a cocktail of stressors [77,111].

395 Further, the combined effect of salinity and acidity under constant light on population sizes
396 in our work was more severe than each of the individual stressors on Day 10. However, by Day 30,
397 the combined stress effect became more than additive for level 3 of salinity or acidity, considerably
398 less than additive for level 2, and almost equal to the total effect of salinity for level 1 (Fig 1) and
399 Supporting Information (Table S6). This is in line with the findings documented by Zalizniak et al.
400 (2009) who experimented on low pH effect on lethal and sub-lethal salinity tolerance in
401 macro-invertebrates [22]. They found that abundant ambient salinity may offset the negative effect
402 of acidity perhaps through exchanging the excess protons in the haemolymph with sodium. In our
403 study, however, the fact that population size at *Salinity3 & Acidity3* was larger than the population
404 size under *Acidity3*, at the first census by 11% then smaller by the same percentage at the second
405 census, but in both cases smaller than under salinity alone, is partly in accord with the common

406 view that increased acidity should aggravate salinity effects [22] and may be very well linked to the
407 physical-chemical process of response to combined stress associated with constant exposure to light
408 through a mechanism that is still nebulous. This becomes more intriguing given the fact that higher
409 sodium concentration in the ambient medium can aid sodium turnover [22]. Overall, our results
410 corroborate the view advocated by Altshuler et al. (2011) that a comprehensive understanding of the
411 combined effects of multiple stressors will require the specific assessment of the effects of these
412 stressors than only individually examining the effects of each stressor [23]. Further, a causative
413 relationship between physico-chemical stressors may exist as changes in one stressor can lead
414 changes in other stressors, *e.g.*, freshwater salinisation syndrome [4,77,112]. Hence, one could
415 argue that complex toxic effects of stressor interactions may underlie our results. To date, a
416 comprehensive understanding of the physiological responses to ionic changes (alone and
417 accompanied with pH changes) is still developing [4,103].

418 Under lack of photoperiod (constant light, 24h), *Daphnia* juvenile proportions were
419 significantly affected by overall physicochemical stress treatment (X^2 = 119.89, Df = 9, **P <0.0001**),
420 and specifically only by the following stress levels: *Acidity2* (**P = 0.035**) and *Acidity3* (**P < 0.0001**);
421 (Fig 1) and Supporting Information (Table S7) for details, and Table S8 for multiple pairwise
422 contrasts of the stress levels). There was an abrupt decrease in adult proportion from 67% adults on
423 Day 10 to 0% on Day 30 in *Acidity3*, and a sharp drop from 46% to 20% in *Salinity2*. Interestingly,
424 only in 2 out of all the 10 stress treatments were there more adults than juveniles on Day 10 with
425 respect to *Acidity2* (63% adults) and *Acidity3* (67% adults); this was markedly reversed on Day 30
426 as significantly more juveniles were recorded then under each of these stress scenarios, (Fig 1) and
427 Supporting Information (Table S9). Conversely, only two cases of the remainder (7 scenarios out of
428 10), *Salinity1* and combined stress *Salinity1 & Acidity1*, led to noticeable increases in adults on Day
429 30 compared to Day 10, (Fig 1) and Supporting Information (Table S9). Moreover, it is worth
430 pointing out that the proportions of juveniles (84%) and adults (16%), on Day 30 under constant

431 light for *Salinity1*, *Acidity1*, and combined stress *Salinity2 & Acidity2*, were identical despite the
432 very different stress conditions in these treatments, and the respective differences in average
433 population size at Day 30 (~102 individuals, ~52 individuals, and ~292 individuals, respectively).
434 However, overall, there were more adults on Day 10 in the acidity than in the salinity levels. In
435 contrast, the opposite was observed on Day 30 except for *Salinity1 vs Acidity1* where the adult
436 proportions were identical irrespective of the larger population size achieved under *Salinity1*, (Fig 1)
437 and Supporting Information (Tables S4 and S9).

438 *Daphnia* showed the greatest resilience under *Salinity2*, *Salinity3*, *Acidity2* (pH 5.5), and
439 combined stress *Salinity2 & Acidity2* as the population increased from means of (~10, ~27, ~7, and
440 ~12 individuals, respectively) on Day 10 to means of (110, 271, 131, and 292, individuals,
441 respectively) on Day 30, with large proportions of juveniles. Having a prolonged juvenile period
442 seemed to help *D. magna* in coping, as a form of resilience. Note that by Day 30 under the highest
443 level of water acidification in *Acidity3* the entire population consisted of juveniles. However, such
444 resilience was less apparent at Day 30 in *Acidity1* and combined stress, *Salinity1 & Acidity1*, as
445 more adults were found there in the population. At any rate, there were more juveniles with delayed
446 maturation when the photoperiod was lacking (constant light), but generally the population on Day
447 30 was more juvenile under acidity stress than salinity or the combination of both stressors. This
448 corresponds with the findings reported by De Coen & Janssen (2003) [113] that exposure to
449 sub-lethal chemical stressors might cause a decreased 'net energy budget' in *D. magna,* which
450 means that the physiological energetics of the stressed daphnids will be shifted more towards costly
451 compensatory metabolic mechanisms [11], such as respiration and exoskeleton formation [76]
452 rather than growth and reproduction. This view is supported by Glover & Wood (2005) who
453 suggested that *D. magna* sensitivity to acidic environments can be explained by a dependency of
454 sodium influx on sodium-proton exchange system and co-occurrent remineralisation of the
455 exoskeleton [74]. Acidic media (with low levels of sodium and calcium), accompanied by

456 exhausted respiration and ion-regulation, may slow metamorphosis, which occurs approximately
457 twice per week in optimal conditions. In turn, this may benefit *D. magna* in mitigating the flux
458 deficit of essential minerals with less allocation of minerals to re-mineralise the exoskeleton. Such a
459 survival strategy of more investment in longevity may be advantageous until conditions become
460 more hospitable [11]. Furthermore, the rates of sodium turnover in *Daphnia* are high, when
461 compared to those of other fresh water animals. However, the rate of sodium turnover is related to
462 their small size and greater surface area to volume ratio [73], which is even larger in juveniles than
463 adults [73]. Moreover, Na^+ uptake in *D. magna* is ATP-dependent (Na^+/K^+ pump), which is
464 different in juveniles compared to adults due to developmentally dependent biochemical differences
465 [114]. The affinity constant for sodium transport is much lower in adults, and juveniles have a lower
466 affinity for sodium, which is counterbalanced by a higher maximum capacity of sodium uptake
467 [114]. It can be argued that delayed maturation is a plastic trait governed by maternal
468 preconditioning of offspring phenotype [9,10,105], akin to diapause, *i.e.*, resting [28], which will
469 favour survival rather than reproduction in adverse environmental conditions. This may be
470 beneficial when accompanied by the ability of the preconditioned neonates to alter their ionic and
471 osmotic regulation [8]. In this respect, delay of reproduction for the advantage of investment in
472 energy accumulation to be available for sequent reproduction when the severity of conditions
473 lessens, has been shown as a survival tactic by *Daphnia* on deficient/limited resources [115,116].
474 However, these survival strategies may be context-dependent, clone-specific [17] and again affected
475 by transgenerational effects [9,10]. Overall, the excess ambient amount of Na^+ in combined stress
476 (*Salinity2 & Acidity2* and *Salinity3 & Acidity3*) could have played a vital role in mitigating the
477 negative impact of acidification on exhausting the haemolymph content of sodium [74]. This could
478 also explain the high mortality in acidity alone treatments in our study. Both weak and strong
479 acidification are known to directly and indirectly negatively affect the physiology, fitness, and

survival of freshwater biota [70,117] and thus leading to mortality and biodiversity loss [75,118], especially for taxa such as *Daphnia* that are sensitive to pH changes [74,75].

Lack of photoperiod (constant darkness, 24h), acidity and/or salinity

Exposure to the same salinity and acidity levels, but under 24-hour darkness, caused all but very few individuals to survive to the 30-Day census. The only treatment in the 24-hour dark phase where individuals in all three replicates survived was *Salinity1*; the average population increased by only 43% at Day 30 in comparison with Day 1. The negative effects of constant darkness were further pronounced in the treatments *Acidity1* and combined stress *Salinity1 & Acidity1*; only one replicate in each contained surviving daphnids on Day 30 with their population changes being a decrease of 95% (the population comprised only 0.33 individuals on average) and an increase of 48% (the population comprised only 10.33 individuals on average), respectively, Supporting Information (Table S10).

Under constant darkness, we made three major observations. First, the population under *Salinity1* with adult majority (56% of the population), was ~90% smaller than it was under constant light where juveniles made up the majority (84%), Supporting Information (Table S10). Second, the population under *Acidity1* was ~99% smaller than its counterpart treatment in constant light, and contained no adult individuals. And third, the population under combined stress *Salinity1 & Acidity1*, which had similar proportions of adults and juveniles, was ~89% smaller than its more juvenile counterpart treatment in constant light. This suggests that the lack of photoperiod may have a significant impact on the ability to tolerate and survive chemical stressors in *D. magna.* Survival in the darkness was associated with a specific single level of salinity, acidity, and their combination, hinting at certain thresholds beyond which *D. magna* seemed to fail to survive in the dark. Moreover, the effects of chemical stressors under constant light are clearly less negative than those under constant darkness. Our findings receive support from Connelly et al. (2016) asserting that

504 poor or abnormal light conditions can compromise *Daphnia* survival [31]. Furthermore, amid the
505 variety of adverse effects of freshwater acidification [70,75], disruption of olfactory and
506 chemosensory perception, which are crucial especially under low light conditions, has been shown
507 to result in maladaptive response to conspecific and heterospecific cues by aquatic organisms [5,81].
508 Notably, the ADaM medium in this experiment did not show any signs of foulness or turbidity in
509 any of the beakers. This suggests that there were no major negative effects of decaying organic
510 material behind the observed high mortality after the 30 day-spell of constant darkness. However,
511 the lack of water exchange, mimicking entrapment in water in cavities or underground, might have
512 led to poorer water quality that could explain the extremely low survival in the dark. Nevertheless,
513 our results imply that specific levels of chemical stress might have contributed to the ability of
514 small parts of the population to survive as all daphnids failed to survive onstant darkness that was
515 the only stressor applied. Finally, we observed no egg production under any conditions although
516 daphnids are known to produce diapausing eggs during periods of environmental stress induced by
517 natural and anthropogenic factors (*e.g.*, [104,119]). This further suggests that our results may be
518 reflective of plasticity found in *Daphnia magna* [47,120]; Supporting Information (*Note 1*) for
519 further information.

520 All individuals used in our experiments were genetically identical as they came from a
521 single mother and comprised the stock culture of the parthenogenetic clone. It is plausible, therefore,
522 that the variable population dynamics and age structure across treatments can be characterised by
523 within-clone ecological differentiation [17] arising from stress-induced phenotypic plasticity of the
524 particular *D. magna* genotype used here [7,20], but see evidence provided by Jose et al. (2009) who
525 found only small differences in the metabolic capacity across different clones of *Daphnia pulex* in
526 response to pH and thermal treatments [121]. We, nevertheless, speculate that different clones with
527 different life histories or communities of mixed clones, or colonies with sexual morphs, would

show differential responses as governed by the contexts they are embedded in, their tolerance, and their life histories [17,20,21].

Conclusion

The extent to which freshwater ecosystems are now exposed to environmental stress is unprecedented and whilst the effects of individual or pairs of stressors are well studied, few studies have explored the effects of abnormal photoperiods on the survival and population dynamics of aquatic organisms challenged by variable levels of salinisation, acidification and their combinations. This work is the first to demonstrate that exposure to physical stress (constant light) combined with chemical stresses (salinity and/or acidity) can differentially alter *Daphnia* population growth and age structure over time. Our findings, therefore, warrant further research on phenotypic plasticity (reproductive and ontogenetic) of important extremely plastic organisms in response to extreme conditions. Specific stress combinations counter-intuitively led to thriving populations, and delayed maturity was also widely observed. In the long run, *under constant light*, *D. magna may* considerably acclimate more to severe salinisation and acidification and their combination. By contrast, constant darkness had a much greater negative impact on population growth than constant light with differential effects on age structure. Our findings are a first step to a more comprehensive understanding of how aquatic organisms adapt to complex chemical conditions caused by anthropogenic activities, under prolonged light/night periods. Exploration of the metabolomic regulation of uptake and efflux of haemolymph ions needs to be the next step to test the organism's response and adaptability under combinations of multiple physical and chemical stressors.

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Data Availability: The data analysed in this work are available at the figshare data repository via the URL: https://figshare.com/s/34d8a98c279b2dc2cb81

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893 **Supporting information**

894 **Table S1. Effects of stress treatment levels on population size in chemical stress under**
895 **constant light (24h).**

896 **Table S2. Posteriori contrasts, *Daphnia* population size.**

897 **Table S3. Matrix of multiple pairwise comparisons, *Daphnia* population size.**

898 **Table S4. *Daphnia* population size differences.**

899 **Table S5. Meta-comparisons of *Daphnia* reproductive success under salinity and acidity in**
900 **constant light.**

901 **Table S6. Meta-comparisons of *Daphnia* reproductive success under salinity, acidity, and**
902 **their combination in constant light.**

903 **Table S7. Effects of stress treatment levels on age structure in chemical stress under constant**
904 **light (24h).**

905 **Table S8. Posteriori contrasts, *Daphnia* age structure.**

906 **Table S9. *Daphnia* age structure.**

907 **Table S10. *Daphnia population* size and age structure differences in salinity, acidity, and their**
908 **combination under constant darkness.**

909 *Note 1: Remarks on the relevance of the study's design and findings with regard to the multifaceted*
910 *environmental challenge of today's and tomorrow's worlds*