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The microbiome mediates the interaction between predation and heavy metals

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Abstract

Gut microbiota communities are fundamental ecological components in the aquatic food web. Their potential to mediate how organisms respond to multiple environmental stressors remains understudied. Here we explored how manipulations of the gut microbiome of *Daphnia pulex*, a keystone species in aquatic communities, influenced life history (size at maturity, age at maturity, somatic growth rate and clutch size), morphology (induced defence) and body condition (lipid status deposits) responses to combined anthropogenic (copper) and natural (predation risk) stress. Data from a factorial experiment revealed that the effect of predation risk on traits was often mediated by copper (predation risk and copper interact). These patterns align with theory linking predation risk and copper contamination via digestive physiology. We also found that each stressor, and their combination, was associated with the same community composition of the *D. pulex* microbiome. However, antibiotic manipulation of the microbiome reversed 7/12 the trait responses across life history, morphology and body condition. This was associated with dramatically different communities to control conditions, with clear and unique patterns of microbiome community composition for each stressor and their combination. Our study revealed that microbiome community composition is highly correlated with the response of organisms to multiple, simultaneous stressors.

INTRODUCTION

In freshwater ecosystems, organisms are exposed simultaneously to a wide range of biotic and abiotic stressors including predation, bacterioplankton, metals, nutrition, pH and temperature (Coors and De Meester, 2008; Coors et al., 2004; Hecky and Kilham, 1988; Hunter and Pyle, 2004; Jackson et al., 2016; Long et al., 2004; Martins et al., 2017). Evaluating how multiple simultaneous stressors impact on organisms currently focuses on understanding how life history, morphological and behaviour responses reflect additive or interactive effects among stressors (Jackson et al., 2016; Orr et al., 2020). Multi-stressor research has grown enormously in the past decades as our understanding of modes of action of stressors have allowed hypotheses and experiments to address whether stressors combine additively or interactively (Altshuler et al., 2011; Folt et al., 1999; Jansen et al., 2011; Loureiro et al., 2010; Orr et al., 2020).

Understanding how such effects arise requires a more thorough understanding of organism physiology. An emerging approach to achieve this understanding centres on examining how the microbiome of target species might mediate responses to different stressors (Banerjee et al., 2018; Mushegian et al., 2019; Sison-Mangus et al., 2015). The gut microbiome is a potential 'interface' to mediating the response to stressors in freshwater which enter organisms via contact or ingestion. Here we focus on the role of the gut microbiome of *Daphnia pulex* in mediating its response to the heavy metal Copper and the biotic stress of predation risk.

These are common and co-occuring stressors in many aquatic communities around the world and each form of stress is represented by a large body of historical empirical research that together provides the platform for predicting how variation in the identity and relative

61 abundance of taxa in the gut microbiome might mediate physiology, life history and
62 morphology under threat from both stressors.
63
64 *How Copper affects life history*
65 Exposure to anthropogenic sources of heavy metals such as Copper generate major responses
66 in life history and behaviour leading often to dramatically reduced fitness (De Schamphelaere
67 et al., 2007; Martins et al., 2017; Sadeq and Beckerman, 2019a; Shuhaimi-Othman et al., 2010).
68 Theory and empirical data indicate that sub-lethal concentrations of Copper influence foraging
69 and assimilation of nutritional resources which can lead to as increased metabolic cost and
70 reduced energy acquisition. Copper's impact on digestive physiology is strongly predicted by
71 classic life-history theory where reduced energy intake translates into delayed maturity at a
72 smaller size, reduced reproduction and slower somatic growth rates (Barata and Baird, 2000;
73 Barata et al., 2000; Bui et al., 2016; De Schamphelaere et al., 2007; Sadeq and Beckerman,
74 2019b).
75
76 *How Predation Risk affects life history and morphology*
77 Several decades of research on predation risk reveal a wide array of responses in Daphnia spp.
78 Much of predation risk research, like that on sub-lethal concentrations of metals, is framed
79 around how risk of predation alters foraging, habitat use and life history and on the allocation
80 of energy to growth versus reproduction (Beckerman et al., 2007; Benard, 2004; Noonburg and
81 Nisbet, 2005; Stoks and McPeek, 2003; Taylor and Gabriel, 1992). Predation risk is well
82 known to alter foraging behaviour (Balseiro et al., 2007; Noonburg and Nisbet, 2005) with very
83 clear changes life history (Beckerman et al., 2010; Black and Dodson, 1990; Campero et al.,
84 2007; Pestana et al., 2009; Pestana et al., 2010; Rose et al., 2002; Rose et al., 2001; Schulz and

Dabrowski, 2001) and morphology (Carter et al., 2017; Hammill et al., 2008; Tollrian, 1995; Tollrian and Dodson, 1999) driven by the size-selectivity of the predator.

Under small size selective predation, the conditions of predation risk favour somatic growth over reproduction and typically leads to later age and larger size at maturity, often along with induced morphological defence. In contrast, under large size selection by predators, prey favour reproduction over somatic growth, typically leading to early maturity at a small size (Beckerman et al., 2010; Beckerman et al., 2007).

A null model for Copper-Predation Risk interactions

Thus, sub-lethal concentration of Copper drive changes in life history that are typically associated with starvation. In contrast, predation risk drives changes in life history that are not directly aligned with theory about resource limitation and are instead driven by the size-selectivity of the predator. Such 'univariate' patterns provide a template and null expectation for how the two forms of stress might combine additively. The central role of predator size and resource allocation to life history and morphological defences suggests that any abiotic (e.g. Copper) stress that interferes with digestive physiology may alter the response to predation risk (and vice-versa). Table 1 provides an overview of aligned and contrasting effects of metals and predation that underpin the expectation of their joint effects.

The relationship between stress responses and microbiota: a case study with Daphnia pulex

In addition to providing a template for additive effects between the stressors, the null predictions also provide a reference point for evaluating how the gut microbiota mediates responses to these stressors.

TABLE 1 HERE

Based on this history of research centred around digestive physiology and the allocation of energy to growth and reproduction, we propose that the gut microbiome may mediate interactions between Copper and predation risk. This mechanistic hypothesis stems from assuming that digestive physiology is a shared 'mode-of-action' for the response to metals and to predation risk (*sensu* ecotoxicology). Here we evaluate this hypothesis in *Daphnia pulex* facing stress from copper and from predation risk by manipulating their microbiome with antibiotics.

The *Daphnia* gut is colonized by a wide range of bacteria. They obtain these microbes via the transmission from host parents to offspring or acquisition from sediments and food in ponds and lakes that enter organism's gut via filter feeding (Gillis et al., 2005; Grossart et al., 2009; Mushegian et al., 2019). As a result, the *Daphnia* gut offers a niche for selective microbes that may provide benefits and services to their hosts (Sison-Mangus et al., 2015). Gut microbiota offer a variety of functions and physiological processes to their hosts associated with metabolism, development, fecundity immunity, and behaviour (Dattagupta et al. 2009, Nicholson et al. 2012, Sommer et al. 2013, Gorokhova et al. 2015, McKenney and Pamer 2015, Sampson and Mazmanian 2015, Sison-Mangus et al. 2015). Bacterial communities may thus influence the potential interaction between metals and predation (Gorokhova et al. 2015) at the nexus of energy acquisition, assimilation and allocation to growth, defence and reproduction.

To evaluate whether the gut microbiome mediates response to multiple stressors and offer insight into how this might happen, we manipulated the microbiome of *Daphnia pulex* using antibiotics as part of a fully factorial experiment evaluating how body condition, life history and morphological defences respond to exposure to predation risk and copper. Our experimental design is motivated by asking the following two questions: First, does the effect

of predation risk on body condition, life history, and induced defences vary by the presence of Copper? This question centres around evaluating the predictions shown in Table 1 and are a formal test for synergistic or antagonistic effects of the two stressors. Second, does antibiotic treatment which will alter directly the gut microbiota, disrupt the interaction(s) between copper and predation risk leading potentially to augmentation or reversals of the patterns, defined in Table 1. These questions are motivated by the shared importance of digestive physiology on the response to predation risk and metals.

We first report on whether the effects of predation risk vary by copper in the absence of antibiotic exposure (e.g. Table 1). We then document the reversal of numerous interactions between predation risk and copper under antibiotic treatment suggesting a central role of the digestive physiology and the gut microbiota in mediating response to copper and predation risk. We then associate these responses, and the experimental treatments causing them, with clear changes in taxonomic and functional diversity of the microbiota. This final step formalises emergent hypotheses about functional groups of bacteria that appear to mediate the response to combined metal (anthropogenic) and predation risk (natural) stressors.

MATERIAL AND METHODS

Daphnia culturing

The clone *D. pulex* (LD33) was collected from field populations (in 2010) in the UK and maintained in long-term stock culture in the Department of Animal and Plant Sciences, University of Sheffield. Stock cultures were acclimated in ASTM hard water under controlled conditions at a temperature of 20±2 ºC, photoperiod 16h light: 8h dark and light intensity 26 $\mu E\ M^{-2}s^{-1}$. Prior to experiments, animals were acclimated to test media over three weeks as recommended in the OECD guideline. The cultures were maintained in 2L tanks with

approximately 25 individuals and fed every day with the green algae *C. vulgaris* fo. *Viridis* (strain number: CAAP 211/12). The algal cultures were grown in Ebert medium (Ebert group, Zoologisches Institut Evolutionsbiologie, Switzerland) and kept on a table shaker in controlled room at 20±2 ºC under an 8 h dark16 h light photoperiod with 40 µE $M^{-2}s^{-1}$.

Experimental media

Daphnids were exposed in a factorial experiment to copper, predator cues (*Chaoborus flavicans* extract) and antibiotics. The control medium and other treatment groups consisted of autoclaved ASTM water, 300µl Marinure (nutritional seaweed extract), and the green algae *C. vulgaris* at 2 x 10^5 cells/ml.

We used a concentration of 5µg/l aqueous copper (II) chloride dihydrate (Fisher Scientific UK C/7920/48) for the copper (Cu) treatment (see Sadeq and Beckerman, 2019a). For predation exposure, concentrated chemical cues were extracted using the procedure of (see Beckerman et al., 2010; Carter et al., 2017; Dennis et al., 2011; Hammill et al., 2008; Lind et al., 2015; Tollrian, 1995) and added to treatment media at a concentration of 1µl / ml. Nominal and realised concentrations of Cu were strongly correlated (ICP-MS; $r2 = 0.99$, $F = 1.91$, $p < 0.002$; performed in a separate specialist chemistry laboratory at the University of Sheffield; see Sadeq and Beckerman, 2019b).

The antibiotic treatment was made using ampicillin (Sigma-Aldrich Company Ltd A9393-25G) and kanamycin sulphate (Sigma Aldrich Company Ltd 60615-5G) delivered together at 9.5µg and 4µg per 150ml (sensu Sison-Mangus et al., 2015). Antibiotic treatments were delivered only on Day 1 of the experiment to 'clear' the microbiome; this allowed re-colonisation of the gut microbiota, in-situ, for the remainder of the experiments (see below).

185

186 *Experiment set up*

187 We performed two factorial experiments to acquire data. First, we performed a classic life
188 table experiment with n=15 replicates per treatment to collect data on the life history and
189 morphology of daphnia exposed to predation risk and copper, with and without antibiotic
190 exposure. Second, we repeated this experiment, but collected n=30 individuals at maturity for
191 microbiome analysis. The two experiments were required because analysis of the microbiome
192 requires destructive sampling before the life table assays are completed.

193

194 Both experimental groups were exposed to the same conditions and following treatments:
195 Control, Cu, Predation, Cu-Predation, Antibiotics (AB), AB-Cu, AB-Predation and AB-Cu-
196 Predation.

197

198 *1. Life History Experiment*

199 Experiments were initiated by transferring <24-hour old neonates individually into six-well
200 plates (10ml). The daphnids were fed daily, their media changed and each individual
201 photographed every day using a Cannon camera (EOS 350D DSLR) placed onto a Leica MZ6
202 modular stereomicroscope (Leica Microsystems GmbH, Wetzler, Germany).

203

204 Using these photos and observations, we captured data on six response variables: size at
205 maturity, age at maturity, clutch size, induction of morphological defences, somatic growth
206 rate and body condition represented by lipid status. Age and size at maturity were estimated as
207 size and age of adults on the day neonates first appear in their brood pouch (max 12 days). Size
208 was estimated with image analysis (linear measurement from head to the base of the carapace
209 spine) using ImageJ(Rasband, 1997-2018). Clutch size was recorded by counting the number

of eggs in the first clutch. Somatic growth rate was calculated as ln(size at maturity/initial size) / (age at maturity (days)). Lipids were counted daily and calculated as the sum of droplets/exposure period (Gilbert, 2004; Wacker and Martin-Creuzburg, 2007). The induction score was calculated based on a composite of pedestal size and spike number (Carter et al., 2017; Dennis et al., 2011; Hammill et al., 2008; Lind et al., 2015).

2. Microbiome Methods (Bacterial communities' identification)

As in the life history experiment, all treatments were initiated on embryos/neonates and guts were collected from adults who had just released their first brood (e.g. Age at Maturity above). As the antibiotic treatment was on day 1 only, the guts were expected to have a microbiome acquired from living and feeding naturally after antibiotic exposure but under the control or experimental treatments.

The guts were dissected under a stereomicroscope with sterilised needles and transferred into phosphate buffered saline buffer (PBS) and then to micro-centrifuge tubes (1.5ml). The guts from each treatment were pooled to ensure sufficient material for microbiome sequencing and as such represent an average microbiome community among 30 individuals in each treatment. Samples were frozen in liquid nitrogen and then stored in the freezer at -20 ºC for bacteria abundance and diversity analysis.

Samples of *D. pulex* guts were analysed using 16S rDNA sequencing (RTL Genomics, Texas, USA). This technique is a well-established method for identifying taxonomy and phylogeny of bacteria. The analysis yielded numerous OTU (operational taxonomic unit) data (Woo et al. 2008). OTU were taxonomically classified by identifying sequences to the highest similarity among bacterial taxa using the SINA method (Pruesse et al., 2012).

Statistical analysis – microbiome data

Genus level OTU data for the microbiome data were analysed using the phyloseq package for R (McMurdie and Holmes, 2013). We subset the data to exclude counts of less than 200 and assessed the changes in bacterial diversity and community composition among treatments using hierarchical clustering and non-metric multidimensional scaling (NMDS). Results were qualitatively similar with exclusion criteria of 1000 counts.

Statistical analysis – phenotype data

All phenotype data were analysed using R 4.0.2 (R Core Team, 2020). We first analysed the non-antibiotic treatment data using MANOVA to evaluate the baseline question: does the effect of predation risk on body condition, life history and induced defences vary by copper exposure. We then analysed the full trait data with MANOVA to evaluate whether the effect of predation risk on all phenotypic traits varied by copper and then whether this interaction (or not) varied by the antibiotic treatment manipulating the gut microbiome. In both cases, MANOVA was followed by univariate ANOVA for each individual trait. In each analysis, we used Type II sums of squares implemented in the Anova() function of the car package (Fox and Weisberg, 2019) for R to assess significance in the MANOVA and ANOVA models.

Data availability

All data and analysis scripts are available at www.github.com/andbeck/microbiome_lifehistory

RESULTS

We first report on whether and how the microbiome was affected by the two stressors under control and antibiotic conditions. These data provide two fundamental results: whether the

microbial community changed – which is necessary to understand the effects reported from the life table experiment – and how the microbial community changed – which is necessary to generate our functional hypotheses linked to the association between microbiome and stress response. Against these microbiome data, we then report on the effects of stressors and antibiotic treatment on life history, morphology and condition (lipids). Specifically, we report on a set of interactive and additive effects of Cu and predation that were often reversed by the antibiotic treatment.

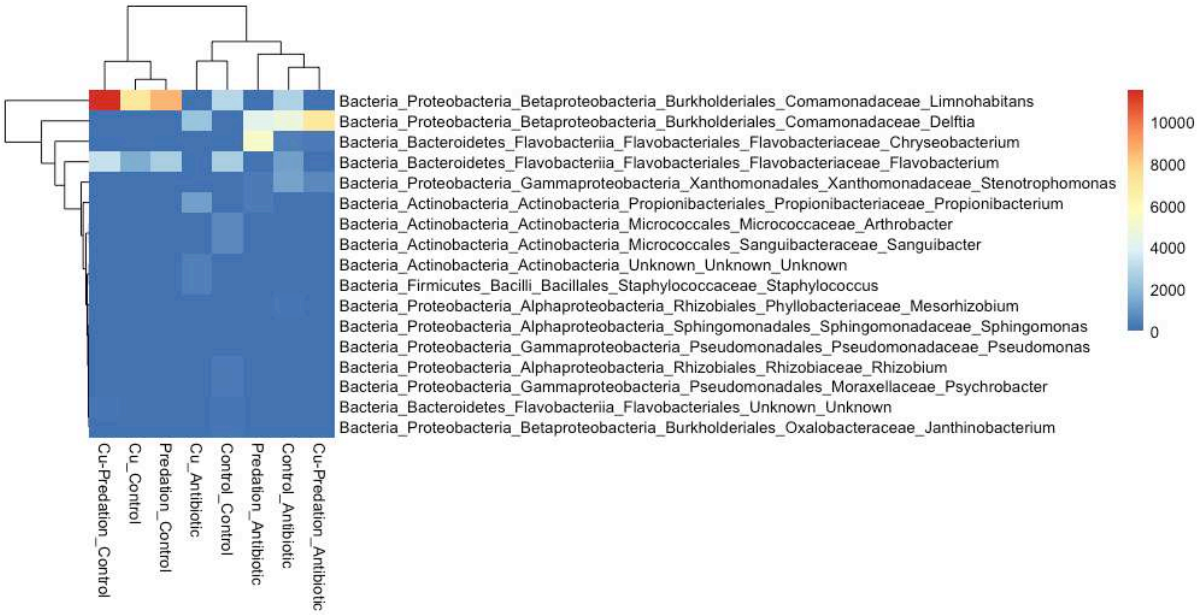
1. Microbiome

1.1. The diversity and composition of the bacterial communities in the digestive tract in response to stressors

We detected ~10,000 unique OTUs among our samples. Specifically, we detected OTUs in Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes phyla, Betaproteobacteria, Flavobacteriia, Gammaproteobacteria, Actinobacteria, Bacilli and Alphaproteobacteria classes, Burkholderiales, Flavobacteriales, Xanthomonadales, Propionibacteriales, Micrococcales, Bacillales, Rhizobiales, Sphingomonadales and Pseudomonadales orders, Comamonadaceae, Flavobacteriaceae, Xanthomonadaceae, Propionibacteriaceae, Micrococcaceae, Sanguibacteraceae, Staphylococcaceae, Phyllobacteriaceae, Sphingomonadaceae, Pseudomonadaceae, Rhizobiaceae, Moraxellaceae and Oxalobacteraceae families and *Limnohabitans, Delftia, Chryseobacterium, Flavobacterium, Stenotrophomonas, Propionibacterium, Arthrobacter, Sanguibacter, Staphylococcus, Mesorhizobium, Sphingomonas, Pseudomonas, Rhizobium, Psychrobacter and Janthinobacterium* genera.

284 Hierarchical clustering of bacterial abundances (Fig. 2) revealed substantive changes caused
285 by antibiotic, Cu and predation treatments in the diversity of the bacterial community in the
286 gut of *D. pulex*. Across treatments, the dominant classes were Betaproteobacteria,
287 Gammaproteobacteria,

Figure 1. Heatmap based on hierarchical clustering of the bacterial community composition in different taxa (row labels; phylum, class, order, family and genus) associated with the *Daphnia pulex* gut in six treatments (column names; chronic exposure to different stress(ors). OTUs are classified using 16S rDNA gene sequences and are plotted for values >200.



288

289 Alpaproteobacteria and Flavobacteria. The data suggest that antibiotics and our stressors alter
290 the relative abundance of four key genera: *Limnohabitans, Delftia, Chryseobacterium* and
291 *Flavobacterium.* Specifically, *Limnohabitans* and *Flavobacterium* dominated the control, no-

antibiotic conditions, but these were replaced by *Delftia, Chryseobacterium* and *Stenotrophomonas* under antibiotic treatments (Fig 1).

We gained further insight into the microbiome community changes linked to predation, copper and antibiotic treatments via nonmetric-multidimensional scaling analysis (Fig. 3). First, under conditions with no antibiotics, the copper treatment, the predation treatment and their combination each shifted the microbiome to the same community structure (Fig 2, point A). Second, the addition of antibiotics shifted the control microbiome (Fig 2, point B). Finally, under antibiotic treatment, each stressor also shifts the community, but now uniquely (Fig 2, points C 1,2,3). In contrast to their effects under no-antibiotic conditions, each stressor is here associated with a microbiome community with a distinct relative abundance

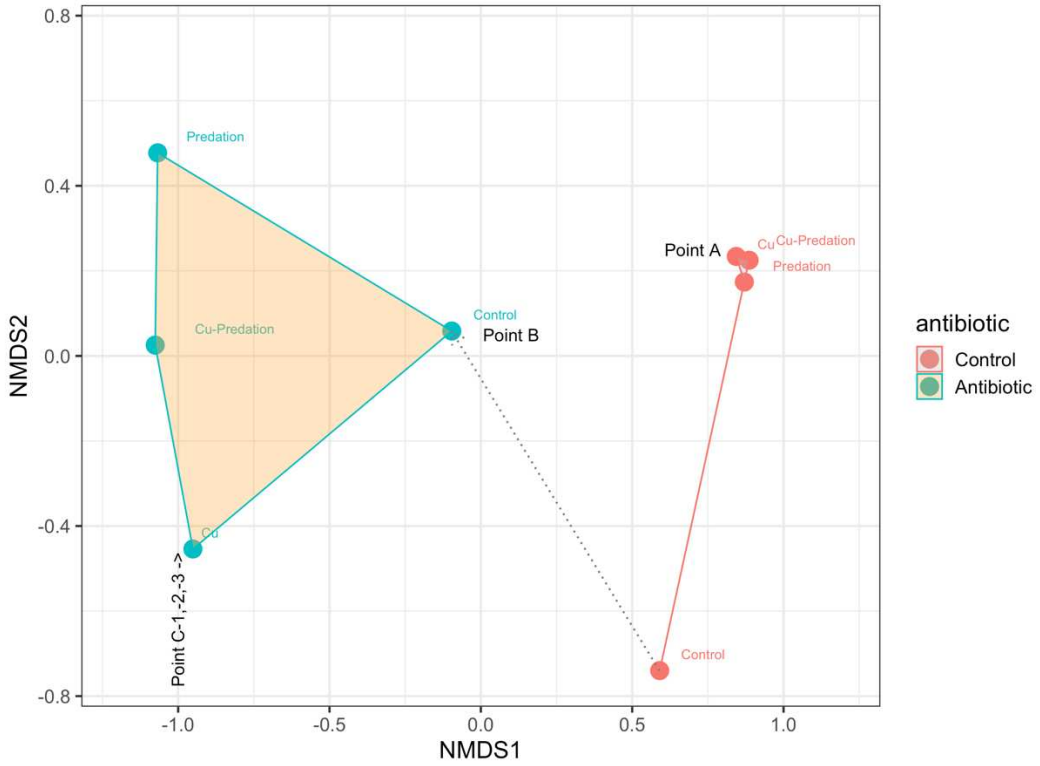


Figure 2. The bacterial diversity in *D. pulex* gut in response to two stressors, Cu and predation and to antibiotic treatment. The data are analysed via NMDS using bray-curtis dissimilarity. The red dots represent the control treatments (without antibiotics), while the blue dots are the treatments under antibiotics. Point A designates the community under copper, predation or combined stress, but in the absence of antibiotics. Point B represents the shift in the control community in the absence of antibiotics to their presence (grey dotted arrow). Points C-1, -2, and -3 indicate the community under copper, predation or mixed stressors, but after the antibiotic treatments.

under antibiotic treatments. These data show that our antibiotic treatments had substantial effects on the gut microbiome and that the response of the microbiome to stressors also varies by antibiotic treatments.

2.1 Life Table Experiment – Copper–predation interactions (No antibiotics)

The left-hand block of each Fig. 4 panel provides substantial insight into whether and how the natural predation risk stress and the anthropogenic copper metal stress interact among six traits. Overall, the effect of predation on the phenotype under control conditions varied by copper (Table 1A; MANOVA, Pillai's Trace = 0.568, approximate F = 7.9, p <0.001). Underpinning

this multivariate response are several significant univariate responses (Table 2A; all patterns described below are associated with p.values < 0.05): Predation risk and Cu both reduced size at maturity, but their effects were additive; The effect of Cu on Age at Maturity varied by predation rsk - Cu increased age at maturity in the absence of predation, but because age was later under predation risk without copper, there was no Cu effect under predation risk. The effect of Cu on somatic growth rate varied by predation risk - Cu reduced somatic growth from a much higher point in the absence of predation risk; Cu reduced reproductive output (clutch size), but there was no effect of predation. The effects of Cu and predation risk on mprhological defences were additive - Cu reduced the morphological defence induced by predation risk; Predation risk reduced body condition, but there was no effect of Cu.

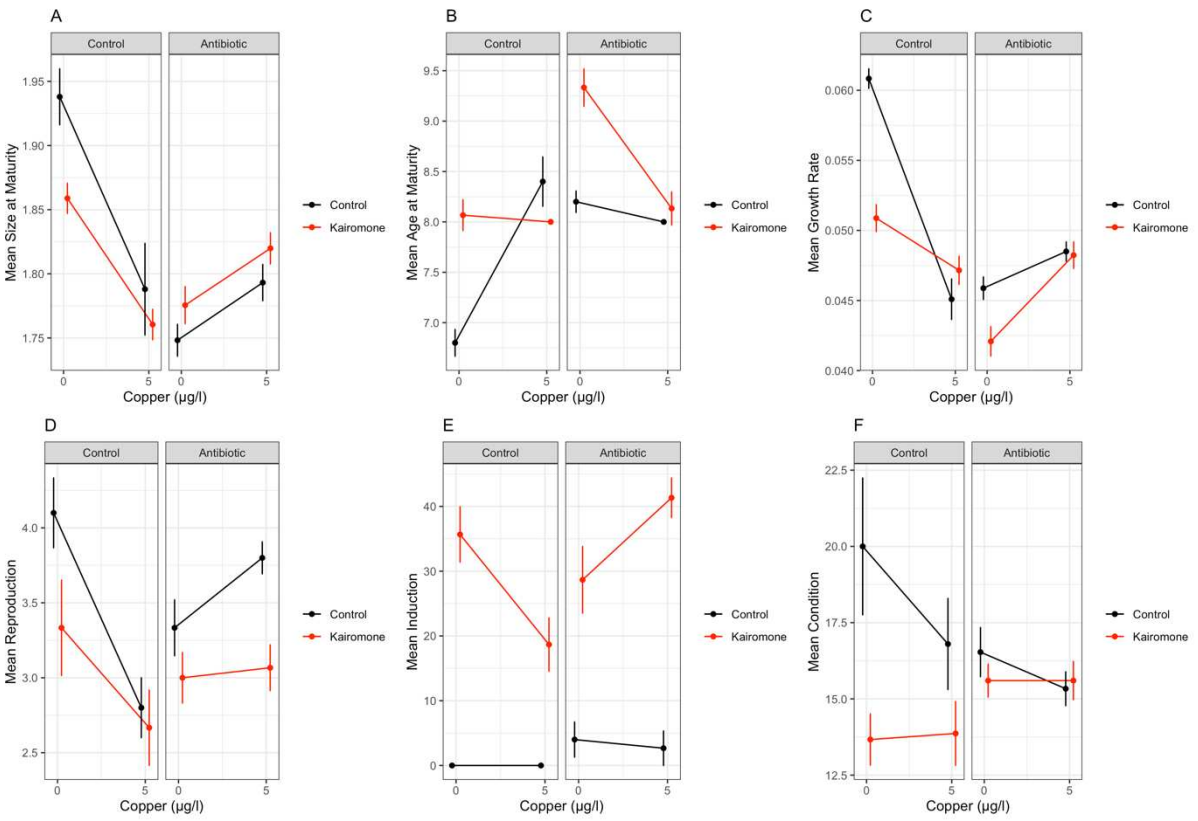


Figure 3. Interaction plot of the effect of copper and predation risk (multi-stressors) on life history, morphological defence to predation and body condition, under control and antibiotic treatment conditions (3-way ANOVA): size at maturity (A), age at maturity (B), somatic growth rate (C), clutch size (D), induced defence (E) and body condition (F). All values are mean ± SE, n=15.

2.2. Whole phenotype (MANOVA)

Here, and in the next section, we first report first whether the three-way interaction among stressors (predation risk, Cu and antibiotics) was significant. If this was not significant, we then report on significant two-way interactions (all p.value < 0.05).

The effect of predation risk on the phenotype comprised of the six traits we measured varied by the presence of copper, and this interaction varied by the antibiotic treatment (Fig 3; Table 3A; 3-way interaction; Pillai's trace = 0.156, df = 6,92, approximate F = 2.846, p = 0.0137). Against the background that the microbiome community shifted dramatically with treatments (Fig 1,2), this result suggests a strong association between how organisms respond to multiple stressors and their gut bacteria community.

2.2 Univariate Trait Responses

Table 2 documents the 7/12 reversals of treatment effects caused by the exposure to antibiotics at Day 1 (neonates) of the experiment. These reversals underpin the statistical interaction among copper, predation and antibiotic treatments revealed in the following univariate ANOVAs of each trait (Table 4A; see text below).

TABLE 2 HERE

2.2.1 Size at maturity

There was no evidence of a three-way interaction (Cu: Predation:Antibiotics interaction; F = 1.4, df = 1,97, p = 0.25; Fig. 4a; Table 4A). We found that the effect of Predation on size at

maturity varied by antibiotics (Predation:Antibiotics interaction; $F = 14.3$, $df = 1,97$, $p<0.0002$) where Predation reduced body size for the bacteria-free daphnids, but increased body size under antibiotics. Furthermore, Cu had significant effect on body size which varied by antibiotic treatments (Cu:Antibiotics interaction; $F = 56$, $df = 1,97$, $p<0.0003$). Cu reduced body size in control treatment, but increased the size under antibiotic treatments.

2.2.2. Age at maturity

We found no evidence of a three-way interaction (Cu:Predation:Antibiotics interaction; $F = 2.7$, $df = 1,97$, $p = 0.1$; Fig. 4b; Table 4A). Cu had significant effect on age and this varied by antibiotics (Cu:Antibiotics interaction; $F = 49$, $df = 1,97$, $p<0.0003$) where maturation happened later as Cu increased under control, but earlier under antibiotic conditions. The effect of Cu on age varied by predation (Cu:Predation interaction; $F = 40$, $df = 1,97$, $p<0.0008$) where maturation happened later as Cu increased under control, but earlier under predation treatments.

2.2.3. Growth

The results indicated that the impact of predation on somatic growth rate did vary by Cu and this interaction varied by antibiotic exposures (Cu:Predation:Antibiotics interaction; $F = 8.5$, $df = 1,97$, $p<0.004$; Fig. 4c; Table 4A). Cu reduced somatic growth rate under control, but increased growth under antibiotics. The reduction without antibiotics was much weaker under predator conditions and the increase under antibiotics was much stronger under predator conditions.

2.2.4. Clutch size

There was no evidence of a three-way interaction (Cu: Predation:Antibiotics interaction; $F = 2.4$, $df = 1,97$, $p = 0.12$; Fig. 4d; Table 4A). However, we found that the effect Cu on clutch size varied by antibiotics (Cu:Antibiotics interaction; $F = 12.2$, $df = 1,97$, $p<0.0007$) where Cu reduced number of eggs in the absence of antibiotics and increased them in their presence.

2.2.5. Induced defence

We found that the effect of predation on neck-teeth production varied by Cu and this interaction varied by antibiotics (Cu: Predation:Antibiotics interaction; $F = 7.4$, $df = 1,97$, $p<0.008$; Fig. 4e; Table 4A). Cu reduced neck-teeth production under control, but increased spike production under antibiotics.

2.2.6. Body Condition

There was no evidence for a three-way interaction (Cu: Predation:Antibiotics interaction; $F = 0.6$, $df = 1,97$, $p = 0.5$; Fig. 4f; Table 4A). We found that the effect of predation on lipid droplets varied by antibiotic exposures (Predation:Antibiotics interaction; $F = 9.2$, $df = 1,97$, $p<0.003$). Predation risk reduced the number of lipid droplets under control conditions, but had no effect under antibiotics where condition was on average lower than control-control conditions.

DISCUSSION

Freshwater communities including lakes, ponds and streams harbour valuable biodiversity and provide important ecosystems services like freshwater and recreation. However, the organisms in them experience multiple simultaneous threats which impacts on their capacity to deliver these services. Here we evaluate the response of a keystone species in freshwater lakes and ponds – daphnia – to the combined effects of an anthropogenic stress – the heavy metal copper – and a natural stress – predation risk.

As detailed at the end of the introduction, we aimed to answer two major questions. The first was whether the two stressors combine to affect life history, induced morphological defences and body condition of daphnia in an additive versus interactive manner. The second was whether the gut microbiome mediated these responses. Our experiments were motivated by theory and empirical data suggesting the responses of *D. pulex* to predation and copper are both mediated, in part, by digestive physiology. We found strong evidence of interactions among the stressors, strong associations between gut microbiota composition and experimental treatment responses and detected the antibiotic treatment driven reversal of 7/12 interactions between Cu and predation risk. The manipulation of the gut microbiota is associated with major changes in life history responses to multiple stressors.

In the following sections, we first review the array of responses to copper and predation risk under no-antibiotic conditions (no manipulation of the microbiome) against research on this topic from other laboratories. Then, we discuss in more detail how these patterns changed and were often reversed under manipulation of the gut microbiome. Finally, we discuss potential functional links between the substantial change in the abundance of the four genera of bacteria, feeding/digestive physiology and our life history, morphology and condition traits.

Phenotype Results - No Antibiotics

Our results demonstrate that the effects of predation risk on several life history traits varies by the presence of copper (Fig 3, all left panels; Table 1A, 2A). This analysis is framed by ecological and ecotoxicological theory associated with resource limitation and predation risk by small size-selective predators experienced by *D. pulex*. (Table 1) and is one of the most comprehensive analyses of these two stressors on multiple phenotypic traits. The theory that

our results align under is defined by resource limitation associated with Cu where life history theory predicts later maturity, decreased somatic growth rates, smaller size at maturity and smaller clutches and theory about size selective predation risk which predicts that under threats from small size-selective predators, individuals mature later, but at the same or larger sizes, experience faster juvenile somatic growth and have the same or larger clutches (Beckerman et al., 2010).

Some of our 'no antiobiotic' results are in line with the findings of several other groups. For example, Pyle and colleagues (DeMille et al., 2016; Hunter and Pyle, 2004; Mirza and Pyle, 2009) have investigated the interaction between predation risk and Cu in several *Daphnia* species and for several traits. In their 2004 work, they found that concentrations of Cu and Ni had inhibitory impacts on neck tooth induction in *D. pulex* (more clearly in Cu). In their 2009 work, they showed that Cu and predation had morphological changes in *D. pulex* neonates leading to fewer and shorter neckteeth compared to kairomone treatments alone. DeMille et al. (2016) further revealed that clones of *D. pulicaria* collected from lakes representing a gradient of Cu concentrations exhibited different responses to *Chaoborus* kairomones in the presence of Cu. This work suggests that acclimation or adaptation to local metal concentrations is linked to changing capacity to respond to predation risk.

Our results, however, are more comprehensive than these bodies of research which are typically at the scale of a single trait response and do not evaluate how metals (or salts for example) might interfere with predator induced morphological and life history (but see the work of the Pyle group). We, and Pyle et al., suggest that because Cu interferes with digestive physiology, it may impact on induced defences by altering the pathways for allocating energy to defences.

We extended this conjecture to invoke the microbiome. We now review in more detail the results from our manipulation of the microbiome.

Microbiome mediation

Our experimental manipulation of the gut microbiome with antibiotics generated four distinct patterns in the bacterial microbiome community (Fig 2, Point A-C) and clear changes in the relative abundance of bacteria taxa among the treatments (Fig 1). Our data show that *Limnohabitans* and *Flavobacterium* dominated the control conditions, but these were replaced by *Delftia, Chryseobacterium* and *Stenotrophomonas* under antibiotic treatments. Given our experimental design, the new gut communities under antibiotic treatment are acquired throughout development via feeding from the existing bacterial community in the water column. The most striking pattern in the data are that under no antibiotic conditions, where parents and offspring experience the same bacterial community, exposure to either treatment or their combination leads to the same microbiome community composition (Fig 2, Point A). This suggests, that against the background, natural microbial community, these natural and anthropogenic stressors act generically to alter gut biota in a similar way.

Our data on taxanomic diversity and relative abundance complement evidence focused on life history and the role of specific microbiome taxa but in the absence of other stressors. Our data suggest a functional relationship between *Limnohabitans sp* (Betaproteobacteria), who's relative abundance was dramatically altered in our experiments and multiple traits (body condition, life history and induced morphological defences) that respond to Copper and predation risk. Qi et al. (2009) foundthat re-infection of aposymbiotic *D. pulex* to *Limnohabitans sp*., the dominant bacterial species in *D. pulex,* led to elevated reproduction. Peerakietkhajorn et al. (2016) found that *Limnohabitans* sp. play an essential role in conferring

fecundity by showing that bacteria-free *Daphnia* recovers fecundity when inoculated with *Limnohabitans* sp. However, host-microbiota interactions within *D. magna* are known to be highly specific, e.g., only certain strains of the genus *Limnohabitans* are able to recover the fitness of germ-free individuals after re-inoculation (Peerakietkhajorn et al., 2015). Indeed, the work of Akbar et al (2020) on associations between the microbiome, diet quality, and life history revealed substantial variation in the genus Comamonadaceae, the genus in which Limnohabitans is classified.

We are, however, unable to assess whether these changes are caused by stress induced alteration of the gut microbiome, stress induced limited uptake of the bacteria from the community or stress induced change in the external bacterial community. The latter is unlikely, however, as under antibiotic treatment, the subsequent adults contain a vastly different community depending on the stressors. This suggests that stressors either alter the gut community or the uptake, but only when the community starting point is first altered by antibiotics in the first place. Distinguishing among these mechanisms requires observation and experiments that evaluate and manipulate environmental and host filtering of the microbiome through ontogeny or among environmental conditions (e.g. Skelton et al., 2017).

A striking result of our antibiotic treatment and associated change in gut bacterial community are the 7/12 instances where the pattern under no antibiotics is reversed under antibiotic treatments (Fig 3; Table 2). For example, not only did antibiotics increase age at maturity but the effect of copper on age shifted from positive to flat under no-predator conditions and from flat to negative under predation conditions (Fig. 4). Antibiotic treatment also shifted the negative effect of copper on somatic growth and reproduction to a positive one. Finally, the negative effect of copper on induced morphological defences under predation risk with un-

497 manipulated microbiota was reversed when the gut microbiome had been manipulated. In the
498 absence of information on the chemical, functional and nutritional properties of all of bacteria
499 genera that comprise the change in microbiota community structure, we are unable to ascribe
500 causal inference to these patterns. However, it is clear that the community composition of
501 daphnia guts is deeply connected to their life history, to morphological defences and to body
502 condition.
503
504 Overall, our data, albeit on a single clone, support continued work to test the compelling
505 hypothesis that digestive physiology, mediated by the microbiome, has a substantial influence
506 on *D. pulex* response to multiple simultaneous stressors. This study is the first to combine a
507 study of interactions between natural and anthropogenic stress with manipulation of the gut
508 microbiota. Our data highlight stressor interactions, stress mediated changes in microbiota and
509 microbiota linked reversals of stressor interactions.
510

Table 1. Copper and Predation both affect the energy budget of organisms leading to various aligned and contrasting predicted responses in life history, inducible defences and body condition. Theory and empirical data about copper is linked strongly to life history theory about starvation where reduced energy leads to later maturity at a smaller size, reduced growth rates and reduced reproduction (de Shelampeare 2007). This also parallels theory on non-size selective predation (Abrams and Rowe 1996). In contrast, theory about small size selective predation, as implemented in this study, predicts investment into growth over reproduction, a decoupling of growth and development leading to increase in size and age at maturity, induced morphological defences (Beckerman et al 2010) but equivocal data on reproduction. Copper does not generate induced defences, hence the null symbol.

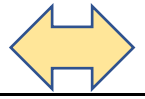
Trait	Copper	Predation Risk
Size at Maturity		
Age at Maturity		
Growth Rate		
Reproduction		
Induced Defence		
Body Condition		

Table 2. Summary of the impact of antibiotics treatment on life history, induced defence and body condition. Five of six trait responses to copper are reversed by the antibiotic treatment and two of six trait responses to copper are reversed. These underpin the statistical interactions detected (see Fig. 1) from the 2 x 2 x 2 factorial phenotype assay. The terms in brackets represent the direction of effect of the stress (Copper or Predation) under control (normal text) or antibiotic (italics). For example, copper and predation Decreased size at maturity average under control conditions. However, copper and predation *Increased* size at maturity under antibiotics (see Fig 3a).

Trait	Copper	Predation Risk
Size at Maturity	Reversal (Decrease - *Increase)*	Reversal (Decrease - *Increase)*
Age at Maturity	Reversal (Increase - *Decrease)*	Consistent (Increase - *Increase)*
Growth Rate	Reversal (Decrease - *Increase)*	Consistent (Decrease - *Decrease)*
Reproduction	Reversal (Decrease - *Increase)*	Consistent (Decrease - *Decrease)*
Induced Defence	Reversal (Decrease - *Increase)*	Consistent (Increase - *Increase)*
Body Condition	Consistent (Decrease - *Decrease)*	Reversal (Decrease - *No Change)*

Appendix

Table 1A. MANOVA Results – No Antibiotics. This table reports multivariate Pillia's trace, approximate F-values, numerator and denominator degrees of freedom and p-values testing whether the effect of Cu on six traits varies by predation risk (Cu:Predation).

	Df	Pillai's Trace	approx F	n_Df	d_Df	p.value	
Cu	1	0.768	19.895	6	36	4.29E-10	***
Predation	1	0.684	12.994	6	36	9.15E-08	***
Cu:Predation	1	0.568	7.9003	6	36	1.80E-05	***

Table 2A. Univariate ANOVA Results – No Antibiotics. This table reports Sums of Squares, F-values, degrees of freedom and P-values testing whether the effect of Cu on each of the six traits varies by predation risk (Cu:Predation). 'size' = size at maturity; 'age' = age at maturity; 'growth' = somatic growth rate; 'repro' = reproduction/clutch size; 'morph' = induced morphological defence; 'lipid' = body condition.

model	term	SS	df	F-values	p.value	
size	Cu	0.141	1	45.2	4.05E-08	***
size	Predation	0.0343	1	11	0.00195	**
size	Cu:Predation	0.00614	1	1.96	0.169	
age	Cu	2.16	1	11.4	0.0016	**
age	Predation	3.82	1	20.2	5.54E-05	***
age	Cu:Predation	6.41	1	34	7.56E-07	***
growth	Cu	5.97E-04	1	49	1.63E-08	***
growth	Predation	2.78E-04	1	22.8	2.31E-05	***
growth	Cu:Predation	3.33E-04	1	27.4	5.31E-06	***
repro	Cu	8.04	1	8.17	0.00667	**
repro	Predation	2.67	1	2.71	0.107	
repro	Cu:Predation	0.926	1	0.94	0.338	
morph	Cu	1500	1	8.3	0.00629	**
morph	Predation	8270	1	45.7	3.57E-08	***
morph	Cu:Predation	667	1	3.69	0.0618	
lipid	Cu	7.76	1	0.361	0.551	
lipid	Predation	246	1	11.5	0.00157	**
lipid	Cu:Predation	26.7	1	1.24	0.271	

Table 3A MANOVA Results – Full Model with Antibiotics. This table reports multivariate Pillia's trace, approximate F-values, numerator and denominator degrees of freedom and P-values testing whether the effect of Cu on six traits varies by predation risk and whether these interactions vary by Antibiotic treatment (Cu:Predation:Antibiotic).

	Df	Pillai's Trace	approx F	n_Df	d_Df	p.value	
Cu	1	0.140	2.507	6	92	0.0271754	*
Predation	1	0.652	28.746	6	92	< 2.2e-16	***
Antibiotic	1	0.517	16.470	6	92	8.38E-13	***
Cu:Predation	1	0.330	7.571	6	92	1.29E-06	***
Cu:Antibiotic	1	0.634	26.667	6	92	< 2.2e-16	***
Predation:Antibiotic	1	0.233	4.663	6	92	0.0003499	***
Cu:Predation:Antibiotic	1	0.156	2.846	6	92	0.0137695	*

Table 4A – Univariate ANOVA Results – Full Model with Antibiotics. This table reports Sums of Squares, F-values, degrees of freedom and P-values testing whether the effect of Cu on six traits varies by predation risk and whether these interactions vary by Antibiotic treatment (Cu:Predation:Antibiotic). 'size' = size at maturity; 'age' = age at maturity; 'growth' = somatic growth rate; 'repro' = reproduction/clutch size; 'morph' = induced morphological defence; 'lipid' = body condition.

model	term	sumsq	df	f-statistic	p.value	sig
size	Cu	0.0125	1	4.35	0.0397	*
size	Predation	0.0012	1	0.418	0.519	
size	Antibiotic	0.0682	1	23.7	4.38E-06	***
size	Cu:Predation	0.00225	1	0.783	0.378	
size	Predation:Antibiotic	0.16	1	55.7	3.69E-11	***
size	Cu:Antibiotic	0.0413	1	14.3	2.65E-04	***
size	Cu:Predation:Antibiotic	0.00389	1	1.35	0.248	
age	Cu	1.24	1	5.2	0.0248	*
age	Predation	9.83	1	41.1	5.23E-09	***
age	Antibiotic	12.2	1	51.1	1.62E-10	***
age	Cu:Predation	9.53	1	39.8	8.31E-09	***
age	Predation:Antibiotic	11.7	1	48.7	3.64E-10	***
age	Cu:Antibiotic	0.135	1	0.566	0.454	
age	Cu:Predation:Antibiotic	0.635	1	2.65	0.106	
growth	Cu	8.31E-06	1	0.691	0.408	
growth	Predation	2.75E-04	1	22.8	6.25E-06	***
growth	Antibiotic	7.34E-04	1	61.1	6.65E-12	***
growth	Cu:Predation	2.77E-04	1	23.1	5.73E-06	***
growth	Predation:Antibiotic	0.00104	1	86.6	4.28E-15	***
growth	Cu:Antibiotic	3.72E-05	1	3.09	0.0819	
growth	Cu:Predation:Antibiotic	1.03E-04	1	8.55	0.00431	**
repro	Cu	1.1	1	1.75	0.189	
repro	Predation	6.93	1	11	0.00127	**
repro	Antibiotic	0.00495	1	0.00787	0.93	
repro	Cu:Predation	2.44E-04	1	3.88E-04	0.984	
repro	Predation:Antibiotic	7.69	1	12.2	7.12E-04	***
repro	Cu:Antibiotic	5.29E-04	1	8.41E-04	0.977	
repro	Cu:Predation:Antibiotic	1.53	1	2.42	0.123	
morph	Cu	69.9	1	0.376	0.541	
morph	Predation	23300	1	125	3.62E-19	***
morph	Antibiotic	927	1	4.99	0.0277	*
morph	Cu:Predation	29.1	1	0.156	0.693	
morph	Predation:Antibiotic	1930	1	10.4	0.00172	**
morph	Cu:Antibiotic	30.5	1	0.164	0.686	
morph	Cu:Predation:Antibiotic	1370	1	7.39	0.00777	**

lipid	Cu	12.8	1	1.01	0.318	
lipid	Predation	118	1	9.31	0.00295	**
lipid	Antibiotic	0.0582	1	0.0046	0.946	
lipid	Cu:Predation	25.2	1	1.99	0.162	
lipid	Predation:Antibiotic	2.47	1	0.195	0.66	
lipid	Cu:Antibiotic	116	1	9.15	0.00318	**
lipid	Cu:Predation:Antibiotic	6.91	1	0.546	0.462	

565
566

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572

573 **Conflict of interest**

574 The authors have no conflicts of interest associated with this publication.

575

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