Elevated pCO₂ affects behavioural patterns and mechano-sensation in predatory phantom midge larvae Chaoborus obscursipes

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Aquatic acidification is a major consequence of fossil fuel combustion. In marine ecosystems it was shown, that increasing pCO₂ levels significantly affect behavioural and sensory capacities in a diversity of species. This can result in altered predator and prey interactions and thereby change community structures. Just recently also CO₂ dependent acidification of freshwater habitats has been shown. Also here, increased levels of pCO₂ change organisms' behaviour and sensory capacities. For example, the freshwater crustacean Daphnia’s ability to detect predators and accurately develop morphological defences was significantly reduced, rendering Daphnia more susceptible to predation. It was speculated that this may have cascading effects on freshwater food webs. However, for a comprehensive understanding of how increased levels of CO₂ affect trophic interactions, it is also important to study how CO₂ affects predators. We tested this using the dipeteran phantom midge larva Chaoborus obscursipes, which is a world-wide abundant inhabitant of freshwater impoundments. We monitored activity parameters, predation parameters, and predation rate. Chaoborus larvae are affected by increased levels of pCO₂, as we observed an increase in undirected movements and at the same time, reduced sensory abilities to detect prey items. This is likely to affect the larvae’s energy budgets. Chaoborus is a central component of many freshwater food-webs. Therefore, CO₂ effects on predator and prey levels will likely have consequences for community structures.

The earth’s climate is currently changing at a fast rate due to the ongoing release of greenhouse gases like CO₂ into the atmosphere. A large portion of this CO₂ is taken up by the oceans, changing seawater chemistry and reducing pH with consequences for marine ecosystems1-4. Lately it was shown, that CO₂ also accumulates in freshwater habitats, also changing pH1-3. This probably has been long overlooked as in freshwater environments CO₂ conditions are highly diver5. Here they depend on the geographic location and respective climatic regimes, heterotrophic activity in combination with a complexity of abiotic and biotic interactions, which is further complicated by soil respiration rates, and terrestrial productivity7. All these factors contribute to the overall freshwater pCO₂ which can therefore also be higher than atmospheric pCO₂. In fact, pCO₂ in freshwater lakes world-wide ranges from 3.1-fold below to 16-fold above atmospheric pCO₂, with a mean of ~1000 µatm in 20078,9. Moreover, in freshwater systems pCO₂ is often not stable throughout the day and throughout the season10. Regardless, authors have discussed1, prognosticated5 and shown3 that also freshwaters acidify with ongoing fossil fuel combustion. It is further discussed that pCO₂ peak periods intensify under climate change scenarios3,7.

Elevation of environmental pCO₂ levels accompanied by changes in aquatic pH has detrimental effects on organism fitness. Ocean acidification not only affects calcifying organisms where it reduces calcification abilities and growth rates7 but also affects development11, reproduction12, metabolic rate13, sensory abilities and behaviour in a range of non-calcifying species14,15. Especially, when sensory abilities are impeded, this can change species interactions as organisms are hampered in their ability to detect conspecifics and heterospecifics. For example, sensory cues passing between predator and prey cannot be correctly interpreted and anti-predatory responses are often suppressed which may result in altered community dynamics. This has been displayed in a range of marine fish where pCO₂ dependent reductions in pH affect sensory abilities. As a result, this can impair the detection of...
of pCO2 primary produces to higher trophic levels), it was shown that sensory abilities are impaired by elevated levels of pCO2. These effects become less straightforward as predation rates have not been shown. To uncover this, we here investigated the effect of increased pCO2 levels on the activity patterns of Daphnia, a key freshwater predator at different trophic levels including many fish species, the larvae themselves prey on ciliates, copepods, and cladocerans like Daphnia. For example, pink salmon larvae Oncorhynchus nerka show alterations in olfactory responses and anti-predator behaviour towards elevated pCO2. Similarly, shelter seeking behaviour in crayfish is affected. More explicitly, in the freshwater crustacean Daphnia (which is a keystone species as it has a disproportional large effect on its natural environment as it links primary producers to higher trophic levels), it was shown that sensory abilities are impaired by elevated levels of pCO2. In two species (i.e. D. pulex and D. longicephala) the ability to sense predators and develop accurate morphological defences was hampered, which renders them more susceptible to predation. This was discussed to have far reaching effects for the ecosystem as an inadequate defence expression may have cascading effects on all trophic levels. However, the increasing prey vulnerability is just one side of pCO2 impacts on predator-prey systems. To our knowledge, possible effects of constantly elevated pCO2 levels on freshwater predators and their predation rates have not been shown. To uncover this, we here investigated the effect of increased pCO2 levels on one central predator preying on first level consumers. The phantom midge larvae Chaoborus (diptera) is a typical inhabitant of standing freshwater bodies world-wide. While they serve as an important food source for higher trophic levels including many fish species, the larvae themselves prey on ciliates, copepods, and cladocerans like Daphnia. If the predator is affected by elevated levels of pCO2, and predation effectivity is reduced the overall food web effects become less straightforward.

### Results

**Activity patterns.** At elevated pCO2, larvae showed significantly increased total activity levels ~1.5 fold from 49.55 ± 21.84 (mean ± StD.) movements in the control condition to 76.40 ± 47.50 (mean ± StD.) movements in the elevated pCO2 condition (Table 1, Fig. 1A). The larvae performed significantly more turns increasing ~1.5 fold from 6.92 ± 5.05 (mean ± StD.) turns to 10.55 ± 8.83 (mean ± StD.) turns in the elevated pCO2 condition (Table 2 Fig. 1B). Also the number of twitches significantly increased ~1.5 fold from 19.43 ± 11.04 (mean ± StD.) to 28.86 ± 20.30 (mean ± StD.) in the elevated pCO2 condition (Table 1, Fig. 1C). The number of moves significantly increased ~1.2 fold from 26.01 ± 19.49 (mean ± StD.) to 30.98 ± 25.87 (mean ± StD.) in the elevated pCO2 condition (Table 1, Fig. 1D). Predation parameters and predation rate. We found that pCO2 exposed Chaoborus made significantly fewer strikes at their prey (Table 2, Fig. 2A). Larvae exposed to elevated levels of pCO2 stroke on average ~0.7 fold less in comparison to the larvae of the control conditions. While pCO2 exposed larvae performed

| Model | Variable | Estimate | S.E. | z-value | d.f. | P value |
|-------|----------|----------|------|---------|------|---------|
| Generalized linear mixed model (poisson) | Total activity | 4.125 | 0.044 | 93.11 | 162 | <0.001*** |
|       | Intercept | 0.207 | 0.019 | 11.00 | 162 | <0.001*** |
| Generalized linear mixed model (beta) | Turns | 2.056 | 0.094 | 21.79 | 162 | <0.001*** |
|       | Intercept | 0.281 | 0.052 | 5.452 | 162 | <0.001*** |
| Generalized linear mixed model (poisson) | Twitches | 3.054 | 0.072 | 42.45 | 162 | <0.001*** |
|       | Intercept | 0.296 | 0.031 | 9.464 | 162 | <0.001*** |
| Generalized linear mixed model (poisson) | Moves | 3.284 | 0.036 | 92.49 | 162 | <0.001*** |
|       | Intercept | 0.148 | 0.029 | 5.09 | 162 | <0.001*** |
| Generalized linear mixed model (poisson) | Spins | 0.693 | 0.077 | 9.038 | 162 | <0.001*** |
|       | Intercept | −0.155 | 0.115 | −1.352 | 162 | 0.176 |
| Generalized linear mixed model (poisson) | Dodges | −0.441 | 0.145 | −3.053 | 162 | <0.01 ** |
|       | Intercept | 0.061 | 0.191 | 0.318 | 162 | 0.750 |
| Generalized linear mixed model (poisson) | Brushes | 1.160 | 0.154 | 7.544 | 162 | <0.001*** |
|       | Intercept | −0.067 | 0.087 | −0.87 | 162 | 0.440 |

Table 1. Statistical results of activity patterns. Generalized linear mixed model, with time as a random effect component. S.E.: Standard Error, d.f.: degree of freedom of the residuals, P values, with significance levels p ≤ 0.05*, p ≤ 0.01**, p ≤ 0.001***.
only 19.63 ± 7.06 (mean ± StD.) strikes at their prey, larvae of the control conditions stroke 27.38 ± 13.47 (mean ± StD.) times. From the performed strikes, we did not observe difference in capture performance, i.e. strikes were similarly effective between both treatments and led to no changes in the amount of captures (Table 2, Fig. 2B). Similarly, ingestions following captures were not significantly different (Table 2, Fig. 2C).

The predation rate was significantly reduced in CO2 exposed larvae. Larvae exposed to elevated levels of pCO2 consumed ~0.6 less prey; on average only 5.29 ± 2.69 (mean ± StD.) Daphnia, while control larvae consumed 9.00 ± 3.06 (mean ± StD.) Daphnia (Fig. 3).

Figure 1. Differences in activity patterns in Chaoborus larvae under control and elevated pCO2 conditions. (A) Total activity is significantly increased in elevated pCO2 exposed Chaoborus larvae. (B) The number of turns, and (C) the number twitches, are significantly increased in elevated pCO2 exposed larvae. (D) the number of moves, is significantly increased under elevated pCO2 conditions in comparison to the control. Similarly, (E) the number of spins, (F) the number of dodges, and (G) the number of brushes remain unaffected by increased levels of pCO2. Statistics displayed in Table 1.
However, are also affected by pCO₂ as their ability to adequately develop defences is decreased and thereby of publications investigating pCO₂ dependent effects in freshwater taxa24–26 and community structures27. In line increased spontaneous firing rate which induced epileptic- like neuronal activity35. Such neuronal activities stem-Chaoborus other invertebrates like dipteran larvae.

... are the dominant inhibitory receptors in many organisms, coupled to an ion channel permeable for chloride... Α receptor antagonist gabazine on... This negatively affects their energy budget, and in combination with the possibly higher energy demand, will have... 

### Table 2. Statistical results of predation parameters. Generalized liner model for each parameter (intercept, control and elevated pCO₂) with specified distributions. D.f.: Degree of freedom of the residuals, S.E.: Standard Error, Pr (>|z|): P values of z statistics, with significance levels p ≤ 0.05*; p ≤ 0.01**, p ≤ 0.001.

| Model                      | Variable | d.f. | Estimate | Std. Error | Z value | Pr (>|z|) |
|----------------------------|----------|------|----------|------------|---------|----------|
| Generalized linear model   | Strikes  | 31   | 3.310    | 0.0478     | 69.266  | <0.001***|
|                           | Intercept| 31   | −0.333   | 0.074      | −4.501  | <0.001***|
| Beta regression model      | Catches %| 31   | −0.013   | 0.081      | −0.161  | 0.872    |
|                           | Control vs. pCO₂ | 31 | −0.039 | 0.114 | −0.342 | 0.732 |
| Beta regression model      | Ingestions %| 31 | 0.995   | 0.274      | 3.637   | 0.00276*** |
|                           | Intercept| 31   | 0.367    | 0.0516     | 6.05   |          |
|                           | Control vs. pCO₂ | 31 | −0.199 | 0.376 | −0.516 | 0.605 |

**Discussion**

While there is a wealth of research focussing on the effect of ocean acidification on species interactions, only little is known about the effects of elevated pCO₂ levels in freshwater ecosystems. Up to now there are only a handful of publications investigating pCO₂ dependent effects in freshwater taxa24–26 and community structures27. In line with these previous observations, we here observe that Chaoborus larvae exposed for 24 h to high levels of pCO₂ are significantly affected in their behavioural patterns. In these 4th instar larvae we observe behavioural changes in form of increased activity levels accompanied with reduced predatory strikes that result in a reduction of predation rate.

**Increased activity levels.** It is already well known, that elevated levels of pCO₂ alter behavioural patterns in a diversity of marine species (reviewed in28). Similarly, some freshwater species showed changes in behaviour19,29 while others did not29. Lepomis macrochirus showed increased swimming velocities30, and Oncorhynchus nerka was shown to reduce anxiety31, while Gasterosteus aculeatus showed decreased boldness and curiosity during pCO₂ elevated conditions29. Not only vertebrates are affected by pCO₂ also other invertebrates, e.g. the fresh-water mussel Lampsilis siliquidea shows a reduction of valve movement. Crayfish Procambarus clarkii similarly reduced overall activity32. Our data contribute to these observations showing that behaviour is also affected in other invertebrates like dipteran larvae. Chaoborus larvae exposed to increased pCO₂ levels increase their overall activity patterns resulting from an increased number of turns and twitches. Directed movements such as forward movements and cleaning patterns (i.e. brushes) or dodges away from conspecifics were not affected. A reason for these increased activity levels may be that larvae try to escape these unfavourable environmental conditions, but this has to be tested in future experiments.

Importantly, our results show that pCO₂ effects cannot be inferred from other species as increasing and decreasing activity levels are observed. It is quite plausible that such pCO₂ induced higher activity levels incur energetic costs and higher energy demands.

**Reduced sensory abilities affect predation rate.** We find that Chaoborus strike less when exposed to elevated pCO₂ conditions. However, if they strike the probability of prey capture and prey ingestion is not changed. This indicates that not prey handling but prey detection is impaired. Chaoborus detect their prey using mechano–sensation32,33, which when impaired could explain for the reduced number of strikes. In consequence, we observe that predation rate is significantly reduced, i.e. larvae catch less prey during the same time period. This negatively affects their energy budget, and in combination with the possibly higher energy demand, will have implications for the larvae's life history parameters, and could affect population growth rates causing changes in community structures. In addition, it is plausible that larvae become more visible for their own predators.

In deed, this may suggest that predation pressure on the prey organism Daphnia is reduced. Daphnia itself however, are also affected by pCO₂ as their ability to adequately develop defences is decreased and thereby become more prone to predation1. How this will change population dynamics will probably depend on who of the two partners is affected more.

**Mode of action.** At present, the precise way how CO₂ mechanistically affects organisms is still controversial and there are several plausible hypotheses. For example, CO₂ especially at high concentrations can have narcotic effects on nervous system functionality and could either affect the whole nervous system or only parts that are especially sensitive, thereby disbalancing motor actions and sensory modalities33,34.

Another hypothesis focusses on a change in GABAₐ receptor functioning, where the inhibitory action of GABA is reversed and becomes excitatory15. This can result in an increased excitability of the overall nervous system and has the potential to lead to the larvae's hyperactivity34. In an experiment mimicking GABAₐ receptor malfunctioning with the help of the GABAₐ receptor antagonist gabazine on Danio rerio brains showed an increased spontaneous firing rate which induced epileptic- like neuronal activity29. Such neuronal activities stemming from neuronal hyperexcitability could on the behavioural level cause the larva's increase in undirected movements. An alternative hypothesis, discusses changes to glycine receptor functioning34. Glycine receptors are the dominant inhibitory receptors in many organisms, coupled to an ion channel permeable for chloride ions and carbonate HCO₃⁻ acting in a similar manner like the GABAₐ system. It thus, represents an additional.
explanation of our observations. Which of these hypotheses holds true needs to be subject in future investigations using dedicated strategies e.g. as suggested by34.

Conclusion

Predator - prey interactions are powerful drivers of community dynamics very often regulated via sensory cues passing between predators and prey28. As predator and prey, both gather information about the presence of the other, the effects of pCO₂ increase on predator-prey dynamics will strongly depend on which participant is more compromised. However, the effect of CO₂ on organismal behaviour is not straightforward but defined by the CO₂ mode of action which is probably determined by the evolutionary history of the explicit species.

There is strong evidence, that when predator - prey interactions are impeded by anthropogenic stressors such as CO₂, this may destabilize food-webs and lead to changes in biodiversity.

Figure 2. Predation parameters in 4th instar Chaoborus larvae under control and elevated pCO₂ conditions. (A) Number of strikes is significantly reduced by elevated pCO₂. (B) Based on the number of performed strikes, the percentage of successful strikes is not significantly different in larvae exposed to elevated pCO₂ conditions. (C) Similarly, the percentage of successful prey ingestion is not affected by elevated pCO₂. Statistics displayed in Table 2.
Material and Methods

Animal cultures. Chaoborus larvae hatch from eggs deposited in freshwater and pupate into adult midges after processing through four larval stages that are increasing in body size. Due to the gape limitation of their catching basket, they are size selective in their prey choice, and the smaller instars feed on smaller prey items like ciliates, while the larger instars feed on copepods and cladocerans like D. pulex. To rule out size selection effects, we choose 4th instar larvae as a representative instar, as these have been well investigated for preying on D. pulex in the 2nd juvenile instar. This predator-prey system has been well established in the past. We anticipate that the results of this instar are well transferable to the other instars. These instars have the same predator capabilities, with the only exception that they prey on smaller items.

All experiments were conducted between September and December of 2018. Chaoborus obscuripes larvae of the 4th juvenile instar were caught in the ponds of the Ruhr University’s botanical gardens maximally 5 days prior to the experiments. During this season the ponds have a depth-dependent temperature range of 4 °C to 17 °C. During the summer, when larvae are most active, temperatures can reach up to 25 °C. To acclimate larvae to laboratory conditions, we gradually increased temperature by transferring the larvae from 4 °C via 15 °C to 22 °C in temperature-controlled rooms.

In detail, larvae were isolated from the ponds and twenty individuals were transferred into 1.5 L glass beakers (WECK, Germany) filled with artificial M4 media (pH 8.0, with a pCO2 of ~1,200 µatm, at 4 °C) see Table 3, and fed with 50 D. pulex juveniles daily. Larvae were first transferred to a cold room at 4 °C ± 1.0 °C for 24 h (16:8 day:night cycle). Subsequently, they were transferred to a room of 15 °C ± 1.0 °C for 48 h, where the medium warmed gradually to carefully acclimate the larvae. They were then transferred to a climatized laboratory set to 22 °C ± 1.0 °C again for gradual acclimation for 48 h. Larvae were not fed 24 h prior to the experiment.

As prey, we used age-synchronized D. pulex (also collected from the botanical gardens, but had been in the department’s animal culture already since 2017). Daphnia were also kept in 1 L beakers in M4 at 20 °C ± 0.1 °C (16:8 day:night cycle) in densities of 30 animals per litre. D. pulex were fed every 48 h with the green algae Acutodesmus obliquus. Beakers were cleaned and water was exchanged on a weekly basis. To match 4th instar Chaoborus larva’s prey spectrum, all experiments were conducted with D. pulex that had reached the second juvenile instar.

PCO2 conditions and experimental set-up. We set control conditions to a pCO2 of ~1,300 µatm (Table 4) with a pH of ~8.0 and elevated pCO2 conditions ~12,000 µatm (Table 4) with a pH of ~6.6 as published earlier. These, in comparison to the ocean, high values in the control condition were selected based on the global mean pCO2 in freshwater habitats. Similarly, we selected the treatment condition of ~12,000 µatm based
We analysed activity patterns based on recorded videos. Sequences were viewed and analysed using iMovie (Mac OS Mojave Version 10.14.6, Apple Inc.). The larvae display distinctive activity patterns, which we categorized into movement categories. A ‘move’ was defined as a forward movement of a larva. A ‘turn’ was defined as a 180° change in orientation, while a ‘spin’ was defined as a full 360° turn around the body axis. A ‘twitch’ was defined as a sudden, undirected convulsive movements. A ‘dodge’ describes the movement, when larvae tried to avoid contact to other larvae. A brush describes a movement in which the larvae clean their tail fan. The category total activity level comprises the sum of all movement categories of the experimental population.

### Predation parameters.

During the one hour observation period, we counted all strikes, catches, and ingestions of the larval attacks (according to 22) and thereby determined the population’s predation parameters. We then calculated the proportion of strikes that led to catches (in %) and the proportion of catches (%) during this one hour.

### Predation rate.

To analyse the effects of pCO2 on the predation rate of Chaoborus, we reared one Chaoborus larvae for 24 h in 250 mL M4 either in the control condition or aerated with CO2 (Table 4). The experiment started on currently observed pCO2 maxima of ~10,000 μatm, resulting from the diel and seasonal fluctuations40. The elevated pCO2 condition was achieved via bubbling and setting the pH to 6.6 prior to the experiments using pH and temperature probes (by Aqua Medic, Germany), documenting temperature levels alongside being stable at ~22 °C. 200 mL of all media were titrated using a Titrino (Methrohm, Switzerland) after the experiments to validate pCO2 and temperature conditions. We determined temperature, pH as well as acid and base capacity for pCO2 calculation via Phreeqc3,41 (see Table 4). The control and the elevated pCO2 condition were both tested on the same day but consecutively. To rule out day-time and circadian rhythm dependent effects, we randomized the sequence in which the two treatments were measured. Each experimental trial started between 9 and 10 a.m. for the first condition and between 12 and 1 p.m. for the alternative condition with the exposure of three Chaoborus larvae to control and three Chaoborus larvae to elevated pCO2 conditions for 24 h in custom made water tanks (12.5 cm × 2 cm × 10.5 cm). Tanks were covered airtight by sealing the lid with parafilm to prevent outgassing. All experiments were performed at a constant temperature (see Table 4) in a temperature-controlled room in above mentioned water tanks. On the following day, i.e. 24 h post exposure (i.e. between 9 and 10 a.m. and between 12 and 1 p.m.), the experiments started with the addition of 100 second juvenile instar D. pulex. Predator and prey were allowed to acclimater for 10 min. Subsequently, larvae predation parameters were monitored for 1 h. During this monitoring period we additionally recorded 5 film sequences of 10 min using an iPhone 7 (Mac iOS 12.4.2 Apple Inc.) interspaced by 2 min. breaks. For that the iphone was fixed in 13 cm distance from the tank using a tripod (KobraTech, Germany). Iphone camera orientation was positioned in parallel to the frontal plane of the tank. To ensure homogeneous illumination, a diffusor plate (customized translucent PVC plate) was positioned behind the tank illuminated by a 15 W LED lamp (IP 65, LE, Germany). As the larvae are about 1.7 to 2.0 cm in size, this allowed us to record activity patterns and predation parameters in the glass tanks over the experimental period. All experimental trials were replicated 17 times.

### Analysis of activity patterns.

To analyse the effects of pCO2 on the predation rate of Chaoborus, we reared one Chaoborus larvae for 24 h in 250 mL M4 either in the control condition or aerated with CO2 (Table 4). The experiment started
with the addition of 20 second juvenile instar *D. pulex*. After 4 h we counted remaining *Daphnia* and determined the number of consumed animals. We replicated this experiment 7 times.

**Statistics.** In total, we performed 17 experimental replicates in the control condition and 17 experimental replicates in the pCO₂ condition. In the pCO₂ condition one replicate had to be excluded due to instabilities in pCO₂ (therefore Ncontrol = 17; NpCO₂ = 16). Activity patterns and predation parameters were calculated as the summed activity of all three larvae and therefore represent the population's total activity. We recorded activity parameters (i.e. total activity, turns, twitches, dodges, spins, moves and brushes) 5 times (for 10 min) within one hour observation time (Ncontrol = 85 and NpCO₂ = 80). To determine if elevated pCO₂ has a significant effect on activity patterns we performed generalized linear mixed models (GLMMs) in combination with a poisson distribution for count data, where the different activity parameters were used as response variables, and treatment (control, elevated pCO₂) was used as fixed effect. As we measured 5 times per 1 h, we included time as a random factor (to reflect a repeated measures design). We fitted the GLMMs using the glm function implemented in the lme4 package in R (www.raproject.org).

To analyse count data obtained in the predation parameter 'strike' and predation rate, we performed linear mixed models using the glm function and a poisson regression in R. Percent data (i.e. relative catches, and relative ingestions) were analysed using a beta regression using the betareg function in the Betareg package in R according to [1]. As relative ingestion data contained 0 and 1, data was transformed as suggested by [2] using formula $x' = (x(N - 1) + s)/N$ (with $N = sample\ size$ and $s = 0.5$). All models were validated by visual inspection of the normalised residuals based on the REML fit against fitted values to identify possible violation of homogeneity, according to [3,4]. We tested for overdispersion; a dispersion value of $<2$ was considered not overdispersed [5]. None of our data was overdispersed.

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L.C.W. and R.T. conceived the study, A.A.K. and N.K., performed the experiments, A.A.K. and L.C.W. analysed the data, L.C.W. and R.T. wrote the manuscript. All authors contributed to and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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