

# Changes in water chemistry can disable plankton prey defenses

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The effectiveness of antipredator defenses is greatly influenced by the environment in which an organism lives. In aquatic ecosystems, the chemical composition of the water itself may play an important role in the outcome of predator–prey interactions by altering the ability of prey to detect predators or to implement defensive responses once the predator’s presence is perceived. Here, we demonstrate that low calcium concentrations (<1.5 mg/L) that are found in many softwater lakes and ponds disable the ability of the water flea, *Daphnia pulex* to respond effectively to its predator, larvae of the phantom midge, *Chaoborus americanus*. This low-calcium environment prevents development of the prey’s normal array of induced defenses, which include an increase in body size, formation of neck spines, and strengthening of the carapace. We estimate that this inability to access these otherwise effective defenses results in a 50–186% increase in the vulnerability of the smaller juvenile instars of *Daphnia*, the stages most susceptible to *Chaoborus* predation. Such a change likely contributes to the observed lack of success of daphniids in most low-calcium freshwater environments, and will speed the loss of these important zooplankton in lakes where calcium levels are in decline.

prey vulnerability | morphological defenses | kairomones | phenotypic plasticity | multiple stressors

Natural selection has produced a finely tuned array of defenses in organisms to keep them relatively safe when confronting their predators. These defenses evolve in the context of specific environmental conditions, and thus changes in an organism’s environment may disrupt the effectiveness of its defenses and its ability to deal with predators. In aquatic environments, animals often use chemicals released by predators (kairomones) to judge the type and degree of predation risk present and to then respond with an appropriate adjustment in their behavior, morphology, or life history to lessen the impact of predation (1–3). The chemical properties of the pond, stream, lake, or ocean may play an important role in the implementation of this antipredator strategy, either by influencing the detection of kairomones by prey or the ability of the animal to respond in an effective manner. Thus, changes in water chemistry may reduce the effectiveness of antipredator defenses, compromising the ultimate success of an animal and restricting its distribution in aquatic environments.

Here, we examine how the concentration of calcium in a lake or pond influences the vulnerability of the water flea *Daphnia pulex* to one of its major predators, larvae of the phantom midge *Chaoborus americanus*. *Daphnia* species form an important component of freshwater zooplankton communities and their ability to defend themselves from an array of potential predators is critical to their success in these systems. *D. pulex* and *C. americanus* commonly co-occur in fishless lakes and ponds throughout temperate North America (4–6), with predation by

*Chaoborus* often constituting the major source of mortality for *Daphnia* (7).

*Chaoborus* larvae are tactile, ambush predators that remain motionless in the water column, and attack approaching *Daphnia* and other zooplankton prey, ingesting them whole (8, 9). Vulnerability of *D. pulex* to these gape-limited predators is largely determined by three morphological features of this animal: body size, neck spines, and carapace rigidity, each of which is influenced by the presence of predator kairomones and may be affected by the calcium concentration in the lake or pond. *Daphnia* body size plays a critical role in its vulnerability to *Chaoborus*, because prey whose size exceeds the mouth gape diameter of this predator are difficult to ingest (10). This results in a linear decrease in *Chaoborus* strike efficiency (probability of ingestion of prey following an attack) with increasing *Daphnia* body length (11–13). The formation of neck spines (also referred to as neckteeth) in juvenile instars of *D. pulex* also substantially reduces the ability of *Chaoborus* to successfully manipulate and ingest these prey, greatly decreasing their vulnerability (13–15). This defensive structure, which is an extension from the dorsal margin of the head that projects into one or more pointed “teeth” (Fig. S1), is induced specifically by kairomones released by *Chaoborus* (16–19). Finally, *Daphnia* that have a carapace with greater rigidity and strength will be less vulnerable to *Chaoborus* predation, because this makes it very difficult for the predator to crush and ingest relatively large prey (10). *Daphnia* have taken advantage of this by developing the ability to increase the strength and hardness of their carapace in response to the presence of *Chaoborus* kairomones, thus producing a more effective defensive armor (20).

Calcium is a critical element for *Daphnia* and many other crustaceans. In addition to its role in a variety of physiological processes, a sufficient amount of calcium is essential for calcification of the exoskeleton, which provides structural integrity for the animal. *Daphnia* have relatively high calcium demands among freshwater zooplankton as a consequence of their heavily calcified exoskeleton and the large loss of calcium that occurs during ecdysis (21–24). Because *Daphnia* acquire the great majority of their calcium directly from the water, low ambient calcium concentrations, as are found in many softwater lakes and

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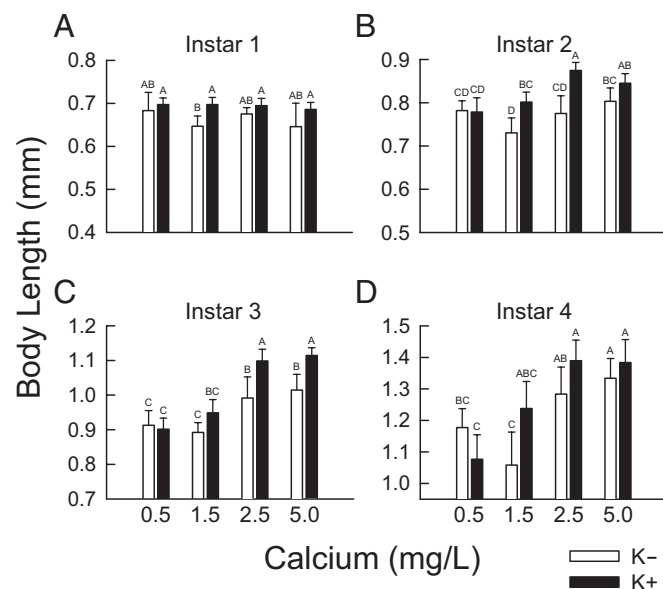
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ponds, may have significant impacts on their physiology and influence a variety of ecological interactions (24–26). While the calcium concentration in many lakes is well above the saturation point for *Daphnia* (~5–10 mg/L), in others it is below this level, producing an environment that is physiologically and ecologically suboptimal (24). This is especially the case in softwater lakes and ponds in areas such as Scandinavia and the Canadian Shield of North America, where a high percentage of lakes have calcium concentrations <1.5 mg/L (22, 27, 28). Low levels of calcium in these softwater systems may alter one or more of the morphological features described above that influence the vulnerability of *Daphnia* to *Chaoborus* predation. Such conditions may reduce the growth and body size of *Daphnia* (24, 25), produce a carapace that is less rigid, or interfere with the production of neck spines. Low ambient calcium concentrations may produce one or more of these effects either directly (if calcium uptake is reduced or metabolic costs associated with calcium uptake are increased) or indirectly (if this interferes with the normal induction of antipredator defenses in response to *Chaoborus* kairomones).

We analyze the joint effect of calcium concentration (0.5, 1.5, 2.5, and 5.0 mg/L) and *Chaoborus* kairomones (present or absent) on the morphological features used by *D. pulex* to reduce *Chaoborus* predation by examining growth, neck spine development, and carapace strength in the juvenile instars of this species in a full factorial experiment. We demonstrate individual calcium and kairomone effects on the development of these defenses as well as an interaction between the two in which calcium concentration affects the response of *D. pulex* to its predator.

## Results

The concentration of calcium in the water had significant effects on *Daphnia* growth and body size (Fig. 1 and Table 1). While lower calcium concentrations did not affect size at birth in *D. pulex* (Fig. 1A and Table 1), they did reduce subsequent growth, resulting in smaller body sizes in instars 2–4. In instar 2, this



**Fig. 1.** Effect of calcium concentration and *Chaoborus* kairomones on the body length of the first four instars of *Daphnia pulex*. (A) Instar 1, (B) instar 2, (C) instar 3, and (D) instar 4. White bars, *Chaoborus* kairomones absent (K-); black bars, *Chaoborus* kairomones present (K+). Error bars represent 95% CI. Within each instar, treatments that do not have a letter in common are significantly different from one another ( $P < 0.05$ ). Note that the scale for body length is different in each instar panel.

**Table 1.** Results of two-way ANOVA on body length for the first four instars of *Daphnia pulex*

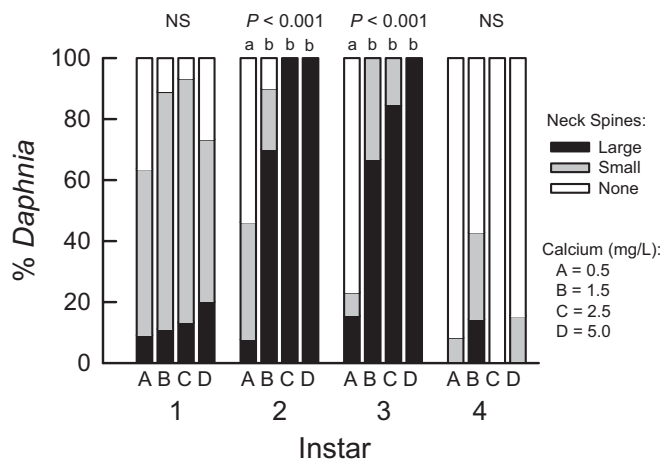
Instar	Source	df	MS	F	P
1	Calcium	3	0.00218	2.29	0.0849
	Kairomone	1	0.01773	18.64	<0.0001
	Calcium × Kairomone	3	0.00145	1.52	0.2149
	Error	78	0.00095		
2	Calcium	3	0.02040	10.54	<0.0001
	Kairomone	1	0.06117	31.60	<0.0001
	Calcium × Kairomone	3	0.01060	5.48	0.0017
	Error	84	0.00194		
3	Calcium	3	0.16909	39.87	<0.0001
	Kairomone	1	0.09792	23.09	<0.0001
	Calcium × Kairomone	3	0.01969	4.64	0.0046
	Error	92	0.00424		
4	Calcium	3	0.33872	24.58	<0.0001
	Kairomone	1	0.07569	5.49	0.0215
	Calcium × Kairomone	3	0.07251	5.26	0.0022
	Error	84	0.01378		

reduction in body size was limited to individuals exposed to *Chaoborus* kairomones, whose body length was reduced by ~5–10% (Fig. 1B). In the older juvenile instars, however, the effect of low calcium was apparent in all *Daphnia*, regardless of kairomone exposure, with body length reduced by 8–19% in instar 3 (Fig. 1C) and by 8–22% in instar 4 (Fig. 1D). A calcium threshold for juvenile growth at or slightly above 1.5 mg/L appears to exist, below which body size is significantly reduced. This threshold is manifested in the differences in body lengths of instar 3 and 4 individuals at low (0.5–1.5 mg/L) versus high (2.5–5.0 mg/L) calcium concentrations (Fig. 1C and D). Because neonate size was not significantly affected by calcium, differences in the body size of later instars were due entirely to a slower rate of growth at lower calcium concentrations.

The presence of *Chaoborus* kairomones significantly increased *D. pulex* body size in each of the four juvenile instars (Fig. 1 and Table 1). In instars 2–4, however, this effect only occurred at calcium concentrations  $\geq 1.5$  mg/L. The kairomone-induced increase in body size in the later juvenile instars appears to be a function of both a larger size at birth (Fig. 1A) and a higher subsequent growth rate. However, this size increase did not occur at the lowest (0.5 mg/L) calcium levels (Fig. 1B–D).

Neonate body length was unaffected by calcium concentration, but increased on average from 0.663 mm to 0.694 mm (4.7%) when the animals were exposed to *Chaoborus* kairomones (Fig. 1A). This increase was consistent in all calcium treatments (ranging from 2.0 to 7.8%), resulting in the lack of a significant interaction between calcium and predator kairomones (Table 1). Significant interaction effects did occur, however, in instars 2–4 (Table 1). These resulted from the lowest calcium level (0.5 mg/L) preventing a kairomone-induced increase in body length that was apparent at higher calcium concentrations ( $\geq 1.5$  mg/L), where increases varied from 5.2–12.8% in instar 2 (Fig. 1B), 6.3–10.8% in instar 3 (Fig. 1C), and 3.7–17.0% in instar 4 (Fig. 1D).

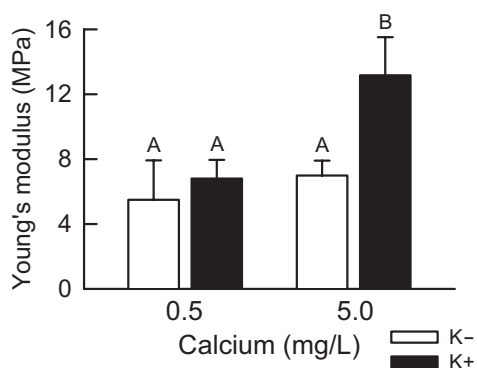
Neck spine development in the presence of *Chaoborus* kairomones was strongest in instars 2 and 3 (Fig. 2), a pattern that is common for *D. pulex* (13, 15, 17). Most instar 1 individuals produced only small neck spines and there were no significant differences in the development of these structures among the experimental calcium treatments (Fig. 2). The presence of neck spines was largely absent in instar 4 *Daphnia* (a few individuals formed small neck spines), with no significant calcium effect on their development (Fig. 2). The degree of neck spine development in both instars 2 and 3, however, was significantly influenced by calcium concentration (Fig. 2). Most or all of these



**Fig. 2.** Effect of calcium concentration (0.5, 1.5, 2.5, and 5.0 mg/L) on neck spine development (percentage of individuals in each of the three neck spine categories) in each of the four juvenile instars of *Daphnia pulex* raised in the presence of *Chaoborus* kairomones. Results of exact contingency table tests: differences within instar 1 ( $P = 0.541$ ) and instar 4 ( $P = 0.071$ ) are not significant; significant differences exist within instar 2 ( $P < 0.001$ ) and instar 3 ( $P < 0.001$ ). Within instars 2 and 3, calcium treatments that do not have a letter in common are significantly different from one another (pairwise comparisons with Sidak's correction,  $P \leq 0.009$ ).

individuals raised at calcium levels  $\geq 1.5$  mg/L formed large neck spines, but these defensive structures were either absent or greatly reduced at the lowest (0.5 mg/L) calcium concentration. Neck spines produced at 1.5 mg/L in the second and third instars appear to be somewhat less developed than at higher calcium levels, but these differences were not significant. Nevertheless, it is clear that the development of prominent neck spines as a defense against *Chaoborus* predation is greatly inhibited at very low calcium concentrations.

These same very low calcium concentrations (0.5 mg/L) also prevent *D. pulex* from strengthening its carapace in response to *Chaoborus* kairomones (Fig. 3). At the relatively high calcium level of 5.0 mg/L, the presence of *Chaoborus* kairomones significantly increased the strength (= rigidity or stiffness) of the carapace of third-instar *D. pulex* by 88%, an induced response



**Fig. 3.** Effect of calcium concentration (0.5 and 5.0 mg/L) and *Chaoborus* kairomones on carapace strength (rigidity or stiffness, measured as Young's modulus in megapascals) of third-instar *Daphnia pulex*. White bars, *Chaoborus* kairomones absent (K-); black bars, *Chaoborus* kairomones present (K+). Error bars represent 1 SE. Treatments that do not have a letter in common are significantly different from one another ( $P < 0.05$ ) based on a one-way ANOVA (3 df,  $F = 4.09$ ,  $P = 0.0162$ ) followed by post hoc pairwise comparisons among treatments using Tukey's HSD test.

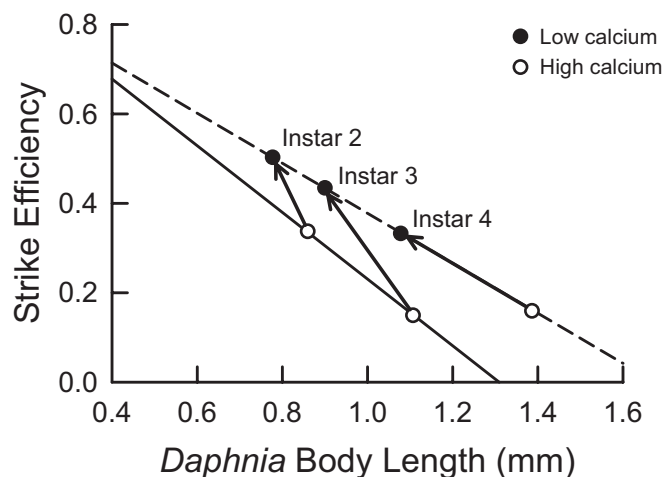
that was absent at 0.5 mg Ca/L. In the absence of these kairomones, however, this higher calcium level did not by itself result in a stronger carapace. Thus, the presence of *Chaoborus* kairomones results in a stronger and more rigid carapace in *Daphnia* that would be more difficult for a predator to crush (see also ref. 20), but only if there is sufficient calcium available to effect this change.

### Discussion

Lakes and ponds with very low ambient calcium concentrations appear to be especially dangerous environments for *Daphnia*. The normal array of defenses that protect these zooplankton from *Chaoborus* and other invertebrate predators (e.g., carnivorous copepods) are greatly weakened under these conditions. Low calcium levels directly reduce *Daphnia* body size with an apparent calcium threshold at or slightly above 1.5 mg/L (Fig. 1). They also block the kairomone-induced development of adaptive defensive responses to *Chaoborus*, which would normally involve an increase in body size (Fig. 1), the formation of large neck spines (Fig. 2), and an increase in carapace strength (Fig. 3). These changes will greatly increase the vulnerability of *Daphnia* to *Chaoborus* predation. We estimated this effect by modeling the impact of these changes on predator strike efficiency (an indicator of prey vulnerability), using the results of Riessen and Trevett-Smith (13) to calculate differences between *C. americanus* strike efficiencies on *D. pulex* at high (induced defenses present) and low (induced defenses absent) calcium concentrations (Fig. 4). This analysis indicates that the effect of low calcium levels on *Daphnia* morphology (smaller body size, absence of neck spines, inability to strengthen the carapace) is ecologically important, increasing the vulnerability of juvenile instars of *D. pulex* to *C. americanus* by 50–186% (Fig. 4).

Calcium is essential for the production of the calcified exoskeleton of crustaceans and is especially important in *Daphnia*, whose exoskeletons have a high calcium content relative to other zooplankton taxa (22–24, 29). This high calcium demand when coupled with the high loss rate that occurs as the exoskeleton is shed during ecdysis requires a large uptake of calcium by *Daphnia* from the water during molting (21, 24). Two different processes involving calcium uptake from the water may account for reduced *Daphnia* growth and body size when the animals are exposed to low ambient calcium concentrations. First, *Daphnia* may expend more energy on calcium uptake under these conditions, which will increase metabolic costs and reduce the amount of energy that can be allocated to growth (25). Second, low concentrations of available calcium may restrict growth following a molt by limiting the amount of new exoskeleton that can be sufficiently calcified.

The means by which low calcium concentrations interfere with the normal kairomone-induced responses of *Daphnia* are less clear. One possibility involves a physiological effect. Very low ambient levels of calcium may disable the animal's ability to detect predator kairomones (*Daphnia* becomes unaware of predator's presence) or not allow for normal transmission of information from receptors, thus preventing *Daphnia* from increasing body size, forming neck spines, and increasing carapace strength. On the other hand, a more likely scenario is that this failure of *Daphnia* to respond to the predator may simply be due to a structural limitation imposed by low calcium. The inability to induce an increase in body size at a calcium concentration of 0.5 mg/L (Fig. 1) may be a result of these very low calcium levels imposing an upper size limit for a given instar that cannot be exceeded. In addition, the failure of second- and third-instar *D. pulex* to develop large neck spines and to increase the strength of their carapace at this same low calcium concentration (Figs. 2 and 3) may be the result of the need for extra calcium to produce these defensive adaptations. Strengthening of the cuticle may require the use of more calcium than is available under these



**Fig. 4.** Model estimating the effect of low calcium on vulnerability of juvenile *Daphnia pulex* (instars 2–4) to *Chaoborus americanus* predation. Prey vulnerability measured as predator strike efficiency (probability of ingestion following an attack). Lines represent the relationships between *C. americanus* strike efficiency and *D. pulex* body size established by Riessen and Trevett-Smith (13) for *Daphnia* that exhibit induced defensive responses in the presence of *Chaoborus* kairomones (solid line:  $y = 0.977 - 0.746x$ ) and those not exposed to these kairomones (dashed line:  $y = 0.938 - 0.560x$ ). We superimpose on these strike efficiency relationships the characteristics of *D. pulex* in our study (body size, presence or absence of neck spines, relative carapace strength) for instars 2–4 raised at high (2.5–5.0 mg/L) and low (0.5 mg/L) calcium concentrations in the presence of *Chaoborus* kairomones. This model assumes that the low calcium *Daphnia* in our experiment, which are unable to develop induced defenses, are phenotypically equivalent (in terms of vulnerability to predation) to the uninduced *Daphnia* in Riessen and Trevett-Smith's ref. 13, and therefore are placed on the dashed line. Open circles, *Daphnia* raised at high calcium concentration: body sizes are mean values from 2.5 mg/L and 5.0 mg/L treatments (Fig. 1); neck spines present in instars 2 and 3, but not instar 4 (Fig. 2). Closed circles, *Daphnia* raised at low calcium concentration: neck spines absent in instars 2–4 (Fig. 2). Increase in strike efficiency at low calcium (arrows) = 50% for instar 2, 186% for instar 3, and 108% for instar 4. For instars 2 and 3, the changes in strike efficiency take into account the smaller body size of *Daphnia* and its inability to produce neck spines and strengthen its carapace at low calcium concentrations when exposed to *Chaoborus* kairomones. For instar 4 (which does not form large neck spines in this *D. pulex* clone, but may be able to strengthen its carapace under high calcium concentrations), the increase in vulnerability at low calcium levels is due to small body size alone, but may be an underestimate because the dashed line represents individuals that do not have a strengthened carapace and the strike efficiency value at high calcium (open circle) will be below this line if the carapace is in fact strengthened.

low calcium conditions to increase structural support. This process is likely energetically costly, and thus would only be used in situations (presence of predators) where its benefits (reducing mortality by inhibiting predator handling and ingestion) are substantial.

While other studies have demonstrated an effect of changes in water chemistry (e.g., increased levels of Cu and CO<sub>2</sub>) on the development and functioning of antipredator defenses in aquatic animals, these have involved changes outside of the normal range found in nature (30–32). Here we demonstrate that changes in calcium concentration that fall within the spatial and temporal variation commonly found in lakes and ponds are great enough to affect the ability of aquatic animals to properly defend themselves against their predators. A large number of softwater lakes in eastern North America and Scandinavia currently have calcium concentrations  $\leq 1.5$  mg/L, the apparent threshold level for growth and neck spine development of juvenile *D. pulex* (Figs. 1 and 2). This includes nearly 50% of lakes in Norway (27) and 12–51% of lakes in various regions of the

Canadian Shield in Ontario (28). These lakes have a greatly reduced *Daphnia* fauna compared with their historical populations prior to a decline in calcium levels, or to similar lakes that have higher ambient calcium concentrations (27, 28, 33). Although lakes and ponds with low calcium concentrations are inhospitable to *Daphnia* for a variety of reasons (24–27), the loss of effective antipredator defenses (resulting in increased mortality from predators) is likely a major contributor to their poor performance in these commonly *Chaoborus*-rich environments (34). Further, the threat to *Daphnia* will likely increase as the number of low calcium lakes increases in response to logging cycles and ongoing changes in atmospheric acid deposition (35). These changes will be long lasting as there is no reason to assume a negative feedback of the loss of daphniids on *Chaoborus* (potentially leading to a cycling of predator and prey) because this predator can simply switch to other taxa once the daphniids are gone (8, 10, 36).

Marine plankton also face the prospect of reduced calcification, in this case as an indirect consequence of ever increasing concentrations of CO<sub>2</sub> in the oceans (37, 38). Much like their freshwater counterparts, many marine plankton also may find themselves increasingly vulnerable to a variety of predators. Thus, the indirect effect of changes in water chemistry on predator–prey interactions in both freshwater and marine communities may play an important role in determining the ultimate success of species in these environments.

## Methods

We conducted a full factorial experiment to examine the effect of calcium concentration (0.5, 1.5, 2.5, and 5.0 mg/L) and *Chaoborus* kairomones (present or absent) on growth and body size, neck spine development, and carapace strength in the juvenile instars (instars 1–4) of *Daphnia pulex*. The experiment was conducted over a 6-d period in January 2010 and was initiated in each of the eight treatments with the isolation of a cohort of between 45 and 55 newborn (<19 h old) *D. pulex*. In each treatment, 40 of the neonates were placed individually in 40-mL vials containing the treatment medium with added algae (see below), and the remaining individuals ( $n = 5$ –15) were preserved in 75% ethanol for later analysis of instar 1. Daily examination of each individual in each treatment determined (via the presence or absence of an exuvium in the vial) whether or not it had molted to the next instar. A subset of the individuals entering a subsequent juvenile instar was preserved in 75% ethanol for later analysis. Those individuals not preserved were transferred to new vials with fresh media and food. This process was continued each day until instar 4, when all remaining *Daphnia* were preserved, and resulted in 7–14 (usually 12–13) preserved *Daphnia* in each of the second, third, and fourth juvenile instars in each treatment. *Daphnia* mortality during the 6-d experiment was very low (2%); however, a few individuals did not develop normally and 9 were lost due to an experimental error, all of which resulted in slightly lower than planned instar sample sizes in a few of the treatments.

This experiment examined a single clone of *D. pulex*, which was isolated from Sherborne Road Pond, a shallow (maximum depth = 0.5–1.0 m) forest pond near Dorset, ON, Canada that contains *Chaoborus* larvae (*C. americanus* and *C. trivittatus*) and has a relatively high calcium concentration of 24 mg/L (13). Mothers of the *Daphnia* used in the experiment were separated shortly after birth into the eight different experimental media treatments (four calcium concentrations, with or without *Chaoborus* kairomones) and raised under these conditions into adulthood. The cohorts of newborn *D. pulex* used in the experiment were from the third clutch of these mothers.

The media used in the experimental treatments was a modification of the softwater FLAMES medium (39) that was adjusted to calcium concentrations of 0.5, 1.5, 2.5, and 5.0 mg/L. These calcium levels were chosen to represent an extremely low concentration that is just sufficient for *D. pulex* survival and reproduction (0.5 mg/L), a low concentration below which *Daphnia* reproduction is reduced (1.5 mg/L), and two progressively higher concentrations (2.5 and 5.0 mg/L) (26). The four treatments in which *Chaoborus* kairomones were present used the media described above, which was then modified by the addition of fourth-instar *C. americanus* at a density of 20/L for 24 h before use, along with approximately two small *Daphnia* per *Chaoborus*, which were added as food for the predators. A comparable number of small *Daphnia* were also added to the nonkairomone treatment media (without *Chaoborus*) to serve as a control. All treatment media (with

or without kairomones) was filtered through a 1.2- $\mu\text{m}$  Whatman filter to remove all particulate matter before use, and was prepared daily to ensure that the kairomone media maintained a high concentration of *Chaoborus* kairomones. All treatments received the same algal food, an equal mixture of *Scenedesmus obliquus* (0.5 g C/L) and *Pseudokirchneriella subcapitata* (0.5 g C/L) that formed a final concentration of 1.0 g C/L. The algae was grown in batch culture in Bold's basal medium, harvested during log phase, allowed to settle in centrifuge tubes, and resuspended in the appropriate experimental media before use. All treatments were kept in a Conviron E7/2 temperature-controlled chamber at 20 °C under a 16:8 light:dark cycle.

All preserved *D. pulex* were viewed under a Leica MZ12 stereomicroscope and digitally photographed at either 80 $\times$  or 100 $\times$  with an attached Nikon DS-Fi1 camera. The body length of each *Daphnia* was measured from these photographs as the distance from the top margin of the head to the base of the tail spine. These measurements were made using Nikon NIS-Elements Documentation software (version 3.0, SP5; Laboratory Imaging) after calibration with a 5-cm stage micrometer with 0.1-mm gradations. Each *Daphnia* was also examined for neck spine size, which was scored as either "none" (no neck spine: dorsal margin of head completely smooth; Fig. S1A), "small" (poorly developed neck spine: usually one, but occasionally two, small spike(s) on the dorsal margin of the head, but with no pedestal), or "large" (well-developed neck spine: two or more spikes at apex of a distinct pedestal that bulges from the dorsal margin of the head; Fig. S1B). The small and large neck spine categories used here are equivalent to the "weak" and "strong" neck spines, respectively, described by Riessen and Trevett-Smith (13).

Carapace strength (which can also be interpreted as carapace rigidity or stiffness) was measured using atomic force microscopy (AFM) along with a force mapping procedure. We limited our analysis to third-instar *D. pulex* from the lowest (0.5 mg/L) and highest (5.0 mg/L) calcium treatments. Individuals from four treatments were therefore measured: (i) 0.5 mg/L, kairomones absent ( $n = 5$ ), (ii) 0.5 mg/L, kairomones present ( $n = 9$ ), (iii) 5.0 mg/L, kairomones absent ( $n = 9$ ), and (iv) 5.0 mg/L, kairomones present ( $n = 8$ ). Instar 3 individuals were selected for measurement because they were relatively large and demonstrated an induced response to *Chaoborus* kairomones in the development of defensive neck spines. Individual *Daphnia* were mounted on glass slides using double sticky tabs and selected points on the carapace were indented with a defined force using a pyramidal tip that was mounted at the end of a cantilever spring. The maximum indentation force at each point was set to 10 nN, which resulted in an indentation depth of  $\sim 100$  nm, ensuring that only the stiffness of the first cuticular layer was

probed. From each of these selected points, a force-indentation curve was produced, which was used to extract the mechanical properties of the sample surface (40). Measurements were obtained from 40 to 65 points within a  $75 \times 75 \mu\text{m}$  grid in the central region of the carapace of each individual, which were used to calculate the mean elastic modulus of the cuticle for each *Daphnia*. The elastic modulus (or Young's modulus, measured in megapascals), is a measure of the material properties of the cuticle that relates to its stiffness, rigidity, or tensile strength. Details of the procedure and calculations are provided in *SI Methods*.

Differences in *Daphnia* body length among treatments for each of the four juvenile instars were analyzed with a two-way analysis of variance to test for the main and interactive effects of calcium and *Chaoborus* kairomones (Statistix 9; Analytical Software). We then made post hoc pairwise comparisons among treatments using Tukey's honestly significant difference (HSD) test. The effect of calcium concentration on kairomone-induced neck spine development in each *D. pulex* instar was analyzed by comparing the distribution of individuals in the three neck spine categories (none, small, and large) among the four calcium treatments. This comparison was made using an exact contingency table test (an extension of Fisher's exact test for contingency tables with greater than  $2 \times 2$  dimensions) for each instar (4 calcium treatments  $\times$  3 neck spine categories) (41, 42). We made post hoc pairwise comparisons among treatments using the same procedure and adjusted the significance level with Sidak's correction factor ( $m = 6$  pairwise comparisons, critical value of  $P = 0.009$ ). Finally, differences in carapace strength of third-instar *D. pulex* at low (0.5 mg/L) and high (5.0 mg/L) calcium concentrations, with and without the presence of *Chaoborus* kairomones, were analyzed with a one-way analysis of variance followed by post hoc pairwise comparisons among treatments using Tukey's HSD test (Statistix 9; Analytical Software).

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