

Dear evaluation committee,

I write to ask your consideration for the Graduate Student Research Prize. The basis of my application is my recently accepted article entitled *Crush-resistant air-sacs allow insect larvae to exploit aquatic habitat at extreme depth*, which will soon appear in the journal *Science*.

My research reveals how the aquatic larvae of a lake fly from Lake Malawi (*Chaoborus edulis*) has adapted its respiratory system into actively regulated, air-filled hydrostatic organs (air-sacs) that resist implosion to depths of half a kilometer, making it the world's deepest freely diving insect. The larvae of *Chaoborus* are unique among aquatic insects as they can regulate their position in the water column by controlling the volume, and buoyancy, of their air-sacs. To do this, the semirigid air-sac wall must resist collapsing under the hydrostatic pressure of deep dives, but must also be able to expand against it to regulate buoyancy. Before I began my graduate research the physiological mechanisms underpinning air-sac function were unknown, and had baffled August Krogh, one of the founders of the field of comparative physiology, when he investigated this problem in 1911. My work shows the mechanical behaviour of *Chaoborus* air-sacs at depth, how the air-sac wall generates force to expand, and how these adaptations have been developed to an extreme level in *C. edulis* as a consequence of this insect's need to escape predatory fish by diving into Lake Malawi's anoxic depths during the day.

Chaoborus air-sacs transduce chemical potential energy, specifically changes in pH, into mechanical work to expand their volume against hydrostatic pressure. I measured the mechanical capacity of air-sacs from the deep diving species *C. edulis* by first increasing their pH, causing them to expand, then applying hydrostatic pressure and measuring the mechanical work required to reverse the pH induced swelling. This provides a basis for future comparative work which explores the diversity of such mechanochemical systems. The article named above also provides a first step into this exploration by examining the maximum depth and air-sac morphology of *Chaoborus* species from lakes of different depth.

This article was recently accepted for publication in *Science* but, with their embargo timeline, a full reprint could not be provided. However, the editors have kindly allowed me to send an author accepted manuscript version. This and the editor's confirmation of acceptance are included in my application package. Sacha Vignieri (svignier@aaas.org), editor at *Science*, is available to verify this if needed.

Sincerely,

Evan McKenzie

From: Aneera Dobbins <science_editors@aaas.org>
Sent: May 5, 2026 3:01 PM
To: Matthews, Philip <pmatthews@zoology.ubc.ca>
Cc: adobbins@aaas.org; svignieri@aaas.org
Subject: Acceptance of your Science Manuscript aed0667

[CAUTION: Non-UBC Email]

05-May-2026

Manuscript number: aed0667

Dear Dr. Matthews,

I am pleased to accept your paper "Crush-resistant air-sacs allow insect larvae to exploit aquatic habitat at extreme depth" for publication in *Science*. Thank you for submitting this exciting work to our journal. Please carefully review the following information:

PUBLICATION PROCESS

- Please alert us immediately if you are presenting this work at an upcoming conference or have specific information pertinent to the timing of publication.
- You will be contacted if the accepted version of your paper is selected for online publication via First Release. First Release papers are typically posted 2 to 3 weeks after acceptance and prior to copyediting or galley proof distribution. The First Release version is superseded by the final version when it is published in an issue.
- Once your paper is scheduled in an issue, a Content Production Editor will copyedit your manuscript and send you a file marked with changes and questions. You will have an opportunity to approve editing changes and respond to queries during review of galley proofs. Our composition vendor will contact you by email when the galley proofs are available for viewing. Instructions for approving your proofs will be provided.
- We consider the author list in the currently accepted version of the manuscript to be final. Please check that every author is appropriately listed and included there. This is especially important for papers with large author lists or group authors. Any necessary changes to listed authors require the approval of all authors and should be conveyed to us immediately; the galley proof stage is too late to efficiently ensure requisite approvals. Addition or removal of authors or modification of their order after First Release or final publication is generally not feasible.

FEES AND REPRINTS: With your galley proofs, you will be given instructions for ordering paper

reprints and paying your figure fees. For Research Articles in print, the cost of color images in the main text is **\$650 for the first color figure and \$450 for each additional color figure**. Please note that if you uploaded color figures at revision, they will be printed in that format. There is an additional cost for paper reprints. We also provide a free electronic reprint (e-print) service through which you will receive a link for seamless access to the final published article up to 2000 times. This link, which may be posted to your personal or institutional website, will be emailed to you from aaas@alerts.sciencepubs.org within 24 hours after your article is published.

PRESS COMMUNICATIONS: Your paper is under embargo until 2:00 pm ET on the day of publication. The *Science* Press Package team will be contacting you about a week before publication with detailed information regarding the embargo that will ensure that your paper is covered maximally and accurately in the popular press. They will also be requesting any multimedia or striking visuals that they could distribute to the press. If, prior to the week of publication, you receive any press requests for copies of your paper or for interviews, please refer them to Meagan Phelan, Communications Director, *Science* Journals (mphelan@aaas.org or 202-326-6436). Alternately, you can send an email to scipak@aaas.org.

ACCESS POLICIES: Authors can share their work in the following ways and under the following conditions:

1. Through the AAAS e-print link service. Authors may take advantage of the AAAS e-print link service in order to provide free access to the Final Published Version via their personal or institutional website. AAAS will provide authors with one free e-print link per article, which may be used up to 2000 times. This link will be emailed to you from aaas@alerts.sciencepubs.org within 24 hours after your article is published.
2. Authors of peer-reviewed research, reviews, and commentary can post the Accepted Version of their manuscript to their personal website or institutional repository, immediately following publication by AAAS. In such cases, authors must include a hyperlink to the Final Published Version on the *Science* website, along with the following notice: "This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published in [Science Journal Title] on [date], DOI: [insert DOI number]."
3. Authors of peer-reviewed research articles can also post the Accepted Version of their manuscript to the public repository of their choice for public release immediately upon publication by AAAS. Authors must include a hyperlink to the Final Published Version on the *Science* website, along with the following notice: "This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published in [Science Journal Title] on [date], DOI: [insert DOI number]."
4. Authors of peer-reviewed research submitted to *Science* on or after 1 January 2023 who are required by their employer or funder to distribute their accepted manuscript under a CC BY license may do so, immediately upon publication, with inclusion of a hyperlink to the Final Published Version on the *Science* website. Similarly, a CC BY license may be applied, upon publication, to the Accepted Version of articles that have immediate relevance to public health or that report the reference sequence of a genome, provided that a hyperlink to the Final Published Version on the *Science* website is included. Authors of cOAlition S funded peer-reviewed research submitted to a *Science* subscription journal on or after 1 January 2021 may, upon publication, distribute the Accepted Version, with inclusion of a hyperlink to the Final Published Version on the *Science* website, under a CC BY license or a CC BY-ND license (if permitted by the funder).

Please contact me if you have any questions. Thanks again for this excellent contribution.

Sincerely,
Sacha Vignieri, Ph.D.

Editor, Life Sciences

Science

svignier@aaas.org

Title: Crush-resistant air-sacs allow insect larvae to exploit aquatic habitat at extreme depth

Authors: Evan K. G. McKenzie¹, Maxon J. R. Ngochera², Joseph Chombo², Hayley McLennan³, Roland Proud^{3,4}, Tahnee Ames¹, Philip G. D. Matthews^{1*}

Affiliations:

1. Department of Zoology, University of British Columbia, Vancouver, B.C., V6T 1Z4, Canada.
2. Department of Fisheries, Ministry of Natural Resources and Climate Change, P.O. Box 593, Lilongwe, Malawi.
3. Pelagic Ecology Research Group, School of Biology, Scottish Oceans Institute, Gatty Marine Laboratory, University of St Andrews, Fife, KY16 8LB, UK.
4. Cupar Analytics Ltd, Cupar, United Kingdom.

*Corresponding author. Email: pmatthews@zoology.ubc.ca

Abstract: The absence of aquatic insects from pelagic marine habitats has been attributed to their air-filled tracheal respiratory system, which could implode at depth while undertaking diel vertical migrations to escape predatory fish. However, we found that the aquatic larvae of the lake fly *Chaoborus edulis* in Lake Malawi have modified their tracheal system into reinforced buoyancy-regulating air-filled sacs, allowing them to escape predatory fish by undertaking these migrations well over 200 m deep into the lake's anoxic hypolimnion during the day. The crush-depth of their air-sacs increases with each instar, with final instars resisting implosion at depths greater than half a kilometer. Contrary to expectations, these insects adapt to the extreme hydrostatic pressures of deep-water habitats, allowing them to co-exist with pelagic fish.

Main Text: Insects represent the most successful expansion of a terrestrial animal lineage into the aquatic environment, constituting more than 60% of all freshwater aquatic animal species (1). But despite their success in both freshwater and land-locked hypersaline habitats, no known aquatic insects live underwater in the open ocean. In this environment insects must contend with the presence of fish – voracious predators of pelagic invertebrates. Zooplankton survive in pelagic environments by performing diel vertical migrations (DVMs): they hide in deep, dark water during the day and only swim up from the depths at night to feed under cover of darkness (2). However, because aquatic insects retain the same air-filled tracheal respiratory system as their terrestrial ancestors, it has been hypothesized that they cannot dive deeply enough to escape predatory fish during the day, as they would die from tracheal collapse before reaching the safety

of twilight-zone depths (3). The aquatic larvae of the lake fly *Chaoborus edulis*, which thrive in one of the deepest lakes in the world (Lake Malawi/Nyasa/Niassa), challenge this assumption.

The larvae of *C. edulis*, like those of all *Chaoborus* species, exploit the open water of lakes by modifying their tracheal system into two pairs of air-filled sacs that function as hydrostatic organs (4). Although derived from the insect's respiratory system, the closed air-sacs play no respiratory role and *Chaoborus* larvae rely entirely on cutaneous respiration to breathe underwater (4). The air-sacs, however, employ a unique mechanochemical system for buoyancy control whereby bands of the protein resilin, laminated between bands of hard cuticle in the air-sac wall, are driven to expand and contract in response to changes in pH generated by the air-sac's epithelium (5). Each air-sac transduces pH change into mechanical work to regulate its volume against hydrostatic pressure. As the wall of the air-sac is gas permeable (4, 6), gases within it exist in equilibrium with gases dissolved in the surrounding water. In air-equilibrated water, this pressure equals the atmospheric pressure at the water's surface, increasing slightly with depth in the same manner as pressure varies within a vertical column of gas (7). The air-sacs are neither supported nor inflated by internal gas pressure. Rather, they must be sufficiently rigid to resist the total hydrostatic pressure at depth without collapsing, and be able to expand their resilin bands against this pressure.

C. edulis larvae are extraordinarily abundant in Lake Malawi (8), despite it being deep, clear and inhabited by a diverse pelagic fish fauna (9, 10). They survive in this challenging aquatic environment by undertaking deep migrations daily (11). Plankton trawls revealed that the larvae dive progressively deeper as they develop through four larval instars. They descend over 200 m into Lake Malawi's permanently anoxic hypolimnion each morning to escape predatory fish before returning to shallower water at night to hunt zooplankton (12). However, the depth of their daily descent and the maximum depth they can withstand were previously unknown. Our objectives were to i) determine the depth of *C. edulis*' DVMs in Lake Malawi and relate their daily migration to the distribution of fish and the depth of the hypolimnion; ii) determine the capacity of *C. edulis* air-sacs to withstand failure and regulate buoyancy at depth; and iii) compare these mechanical parameters with those measured from the air-sacs of four additional *Chaoborus* species: *C. anomalus* and *C. pallidipes* from Lake Victoria, Kenya, an 80 m deep, non-stratified lake; and *C. americanus* and *C. trivittatus* from Shirley Lake, BC, Canada, an 8 m deep fish-free lake.

Hydroacoustic monitoring of *C. edulis* DVM and pelagic fish

Chaoborus larvae can be monitored using hydroacoustics because their air-sacs scatter sound waves (13, 14)(fig. S2). To quantify the pattern of daily migrations of *C. edulis* in Lake Malawi, we deployed a multi-frequency sonar system with an upward-facing transducer suspended 20 m above the lake floor in water 295 m deep, approximately 1.6 km offshore from Nkhata Bay, Malawi (Fig. 1A, B). A 250-meter vertical transect of water quality parameters was recorded at the same location, revealing a sharp decline in dissolved oxygen (DO) levels from 6.17 to 0.18 mg/L between 150 and 200 m, coincident with a decrease in temperature of 0.8 °C and pH, from 8.15 to 7.37 (fig. 1D).

Layers of *C. edulis* descending into Lake Malawi's hypolimnion became detectable (i.e. above background noise levels) between 02:22 and 11:13, sinking at a mean rate $\pm$ SD of 7.56 ± 6.48 m h^{-1} . The maximum depth recorded for any *C. edulis* layer was 258.6 m, and the overall weighted

mean depth (depth weighted by echo intensity, a proxy for *C. edulis* density) of vertically static layers, which all occurred during daytime, was 213.7 ± 13.6 m. The mean shallowest depth of any static layer was 196.8 m. The ascent started between 13:35 and 16:20, and layers became undetectable between 17:09 and 18:29 at the end of their dusk ascent. The mean ascent rate was 19.8 ± 15.2 m h⁻¹. The upward migration started slowly with a mean ascent rate of 15.48 ± 6.48 m h⁻¹, increasing to 23.76 ± 19.8 m h⁻¹ (fig. 1D). These measured rates exceeded the maximum rates of vertical movement recorded from passively sinking and floating larvae in a pressure chamber exposed to hydrostatic pressures between 0 to 282 m depth (-5.54 and 7.23 m h⁻¹, respectively fig. 1C), suggesting that the migration involved some active swimming. Larvae were observed swimming in short bursts lasting 0.75 ± 0.8 s at an average speed of 105.8 ± 51 m h⁻¹ ($\pm$ SD). Thus, intermittent swimming with passive floating/sinking can adequately account for vertical movement rates measured hydroacoustically. However, as fish easily swim an order of magnitude faster than this (15), swimming is clearly no defense against predation.

A total of 394,644 individual fish tracks were detected over the survey area, with only 1,605 (0.4%) having a mean depth >196.8 m, the shallowest mean depth of static *C. edulis* layers observed during the day. Fish detections had a mean depth of 91.5 ± 51.1 m, with 99% of all fish detections occurring <189.9 m, where the water had a DO of 0.41 mg L⁻¹ (fig. 1D).

Air-sac morphology

Chaoborus larval air-sacs are curved fusiform tubes that vary substantially in geometry between species and across development (16) (fig. 2). The dimensions of 4th instar air-sacs were measured from profile images of excised air-sacs placed in pH 6 buffer (the in vivo pH of neutrally-buoyant *C. americanus* air-sacs at zero depth (5)). Maximum *C. edulis* air-sac diameter was 0.223 mm, and the maximum air-sac length was 1.099 mm (both measurements from anterior air-sacs). Volumes of *C. edulis* air-sacs under the same conditions as above were estimated by modelling them as truncated cones capped on either end by prolate ellipsoid hemispheres (fig. S5). The mean volume of an anterior air-sac $\pm$ SD was 19.5 ± 1.7 nL ($n = 7$) and the mean volume of a posterior air-sac was 11.6 ± 2.3 nL ($n = 21$). The estimated total air-sac volume of 62.2 nL displaced sufficient water to bring a 4th instar *C. edulis* larva to, or very near, neutral buoyancy.

Diameters of 4th instar posterior air-sacs varied substantially between species. The mean diameter $\pm$ SD of *C. edulis* air-sacs ($n = 52$) was 0.163 ± 0.014 mm, which was not significantly different from the 0.165 ± 0.005 mm diameter of *C. anomalus* air-sacs ($n = 13$), while all other differences were significant (fig. 2). *C. pallidipes* air-sacs, at 0.195 ± 0.004 mm ($n = 4$), were slightly wider. The air-sacs of *C. americanus* ($n = 26$) and *C. trivittatus* ($n = 17$) were significantly wider than all others, with means of 0.271 ± 0.022 mm and 0.307 ± 0.017 mm (fig. 2).

Crush-depth of *Chaoborus* larvae and pupae

Intact larvae (2nd to 4th instar) and pupae (*C. edulis* and *C. pallidipes* only) were placed in a water-filled pressure chamber (fig. S6) and exposed to stepwise increases in pressure. The pressure increment at which the first air-sac or pupal respiratory horn (a pair of static air-filled structures used for buoyancy and respiration) imploded indicated depth of failure. For *C. edulis*,

mean crush-depth $\pm$ SD increased across development, from 242 ± 24 m in the 2nd instar, to 324 ± 39 m in the 3rd instar, and 463 ± 75 m in the 4th instar (fig. 2). There was no significant difference between the crush depths of 4th instar larvae and pupae (fig. 2). In comparison, the crush depths of 4th instar *C. anomalus* and *C. pallidipes* from Lake Victoria (~80 meters deep) were far shallower (97 ± 27 and 114 ± 39 m, respectively), and those of *C. americanus* and *C. trivittatus* from Shirley Lake (~8 meters deep) were shallower still (16 ± 2 and 22 ± 2 m, respectively).

From 4th instar data we calculated air-sac safety factors as the ratio of mean crush depth to maximum depth occupied, a metric which indicates the air-sac's capacity to withstand hydrostatic pressures beyond those routinely encountered. 4th instar *C. americanus* and *C. trivittatus* have been recorded at maximum depths of 10 and 20 m, respectively, in a 42 m-deep, fish-free lake adjacent to Shirley Lake (17), giving a safety factor of 1.6 for *C. americanus* and 1.1 for *C. trivittatus*. The maximum depth of Lake Victoria was taken to be the maximum depth occupied for both *C. anomalus* and *C. pallidipes*, giving safety factors of 1.2 and 1.4. Assuming the maximum detected *C. edulis* layer depth of 258.6 m represents the voluntary dive limit of this species, their air-sac safety factor was 1.8.

Air-sac compression at depth

While air-sacs vary in shape, the structure of the air-sac wall is consistent, comprising alternating bands of resilin and stiff cuticle arranged transverse to the air-sac's long axis (Fig. 3, S8) (5). This arrangement means that air-sacs change volume uniaxially, varying in length but not diameter (5). To quantify their mechanical properties at depth, freshly excised air-sacs from 4th instar larvae of *C. edulis*, *C. pallidipes*, *C. anomalus* and *C. americanus* were exposed to stepwise increases in hydrostatic pressure in the same chamber used above, but filled with insect physiological saline, again buffered to pH 6. Air-sacs were contained within shallow wells attached to the inside surface of the pressure chamber's clear acrylic window, allowing imaging from above. The profile area (mm²) of each air-sac was measured from images taken following each pressure increase until failure (movie S1-S3). Because the air-sac's wall constrains profile area to change in direct proportion with long-axis length (5), % area change was plotted against pressure as % length change to describe the air-sacs' lengthwise stiffness in units of MPa. However, as air-sacs are hollow and not uniform in cross-section, this relationship is not a true compressive modulus, but rather a measure of the whole air-sac structure's stiffness (fig. 3).

The compression curves of the air-sacs from these four species are J-shaped, with approximately linear initial decreases in air-sac length and increasing stiffness at higher pressures (fig. 3C). The initial stiffness of the four species' air-sacs corresponded with the maximum depth of their environment. Thus, air-sacs of *C. edulis* from Lake Malawi had a modelled stiffness and 95% CI of 5.17 ± 0.53 MPa, followed by the two species from Lake Victoria, *C. anomalus* and *C. pallidipes*, being 2.63 ± 0.59 and 2.80 ± 0.84 MPa, respectively. Finally, *C. americanus*, from Shirley Lake, had the most compliant air-sacs (1.17 ± 0.53 MPa). Between species, only the co-occurring *C. anomalus* and *C. pallidipes* had air-sacs that did not differ significantly in their stiffness (fig. 3).

With increased pressure, air-sacs either imploded (*C. americanus*) or increased in stiffness, likely due to compression of the cuticular bands against one another before failure (movie S3). This increase was most dramatic in *C. edulis* air-sacs, which had a stiffness $\pm$ SD before failure of

76.36 ± 22.82 MPa, nearly a 15-fold increase over their initial stiffness. In comparison with the other species, the maximum stiffness of *C. edulis* air-sacs was 5-fold greater than those of *C. pallidipes* (14.69 ± 9.27 MPa), 18-fold greater than those of *C. anomalus* (3.86 ± 1.03 MPa), and 24-fold greater than those of *C. americanus* (3.21 ± 0.78 MPa). Despite these large differences in stiffness, the degree of linear compression at failure was similar for all species: between 0.21 ± 0.03 (*C. americanus*) to 0.33 ± 0.03 (*C. edulis*) (fig. 3), following the trend of dive depth between species.

Air-sacs and mechanical work

The osmotic work done by resilin expanding the air-sac wall following a pH increase is approximately equal and opposite to the mechanical work performed by an increase in hydrostatic pressure compressing the air-sac wall as, neglecting losses, the free energy change required to chemically induce an expansion of the air-sac wall should be equivalent to the mechanical energy associated with its recompression (18). Manipulating pH and pressure while measuring change in air-sac length indicated the air-sac's capacity to convert pH changes into mechanical work. Air-sacs from 4th instar *C. edulis* larvae were placed in pH 6 buffer and secured inside the pressure chamber. They were then exposed to two 1-unit increases in pH, up to pH 8. Stepwise pressure increases were then applied while the air-sac profile area was recorded. We found that the hydrostatic pressure at a depth of 52.1 ± 4.74 m (± 95% CI) was sufficient to compress the air-sacs back to their unpressurized length at pH 6 (fig. 4A). Thus, increasing the hydrostatic pressure by 509.42 kPa did the same amount of work compressing the air-sacs as they could perform expanding against this pressure following a 6 to 8 pH increase. Factoring in the average cross-sectional area of the air-sac in the direction of expansion, we calculated this work (± 95% CI) to be 1.051 ± 0.083 mJ m⁻¹, i.e., work per meter of initial air-sac length. The results of this experiment suggest that *C. edulis* can only regulate their air-sac volume and buoyancy at depths less than ~50 m. However, this species may have the capacity to vary the pH of their air-sac wall over a greater range than 6 to 8 to do work at greater depths. But as air-sac volume change was greatest within this pH range (fig. 4B), only a minor gain in volume control would be achieved by going outside of these limits.

Conclusions

Our research suggests that the tracheal system of *C. edulis* has evolved into a robust hydrostatic organ that uniquely enables exploitation of a deep-water pelagic environment. This adaptation, coupled with the unusual ability of *Chaoborus* larvae to respire anaerobically by converting stores of malate to succinate (19), are key to their survival in Lake Malawi, allowing them to feed on zooplankton in surface waters at night before diving into the persistently anoxic hypolimnion to escape predatory fish during the day. This predation pressure explains why *C. edulis* larvae undertake a DVM that is likely the deepest of any freshwater invertebrate, more than three times deeper than that performed by the lake's crustacean zooplankton (12), and tolerate ~10 h each day in near total anoxia. Although unable to pursue the larvae into the anoxic hypolimnion, fish congregate between 140-180 m, within the hypoxic thermocline just above this region (fig. 1D). Several deep-water cichlid genera in Lake Malawi possess hemoglobin mutations associated with enhanced O₂ binding (10, 20), suggesting strong selection pressure to enable these fish to pursue and feed upon the high concentrations of *Chaoborus* larvae as they

enter and leave their anoxic refuge. Stomach contents of pelagic cichlids confirm that they feed on *C. edulis* at dawn and dusk, while also revealing that 4th instar larvae and pupae are their principal prey (21). This explains why daytime plankton trawls within the lake's upper 150 m collected 2nd and 3rd instars, while 4th instars and pupae were rarely encountered at these depths (11). Thus, while pre-4th instar larvae remain above the hypolimnion, they still increase their air-sac crush depth as they develop (fig. 2), ensuring that when they become most vulnerable to predation as 4th instars they are equipped to escape into the anoxic depths.

The ability of *Chaoborus* larvae to undertake a DVM requires that their air-sacs retain some capacity to regulate their volume, and thus the larvae's buoyancy, while also resisting implosion at depth. As we show here, the ability of *C. edulis* resilin to convert a change in pH into mechanical work is limited: the hydrostatic pressure at 50 m depth would prevent it from expanding when exposed to a biologically reasonable change in pH from 6 to 8. Any deeper, and the air-sac volume changes little as the resilin bands become fully compressed and the cuticular bands abut one another, increasing the stiffness of the air-sac wall and allowing maintenance of approximately constant buoyancy down to ~200 m (figs. 3, 4). Even when compressed to minimum volume, air-sacs still substantially reduce the larvae's density, slowing their sinking rate and reducing the energetic cost of holding position at depth. We propose that *C. edulis* larvae use a hybrid buoyancy control strategy across their DVM: regulating buoyancy in shallow water where pH-driven control of air-sac volume is possible, then relying on air-sac rigidity to reduce body density at depths beyond 50 m.

The mean air-sac and respiratory horn crush depths of the four *Chaoborus* species from lakes without permanent anoxic layers closely correspond to the maximum depth of the lake where they live, indicating that these species can find refuge from fish in the lake's sediment. However, both the 4th instar larvae and pupae of *C. edulis* have crush depths substantially below the maximum recorded depth of the anoxic hypolimnion (fig. 2; ~279 m (22)), indicating that this species could dive far deeper than it does. The safety factor of 1.8 calculated for this species is also the highest measured here, while the other four species have a mean safety factor of 1.3 ± 0.3 . The only animals with rigid air-filled hydrostatic organs comparable to *Chaoborus* air-sacs are marine cephalopods. Curiously, *Sepia*, *Nautilus* and *Spirula* also have safety factors of ~1.3 despite diving to very different maximum depths (150, 700 and 1,750 m, respectively) (23-25).

The extreme hydrostatic pressures *C. edulis* experience have driven adaptive changes to their hydrostatic organ morphology. Compared with all other species, their air-sacs are narrow and show the least axial curvature (fig. 2), while the respiratory horns of the pupa are almost spherical, rather than spindle-shaped as in other *Chaoborus* species (26). These geometric changes reduce air-sac wall stress (27) and appear to have evolved comparatively recently, as Lake Malawi filled to its current depth less than 75,000 years ago (28).

Our research offers new insights into the depth limits of free-living aquatic insects. While seal lice retain the record as the deepest-diving insects, they are essentially air-breathing insects with typical tracheal systems that endure intermittent deep submergence while immobile and clinging to their mammal hosts (29, 30). In contrast, *C. edulis* larvae swim, feed, grow and pupate in open water before surfacing to eclose as adult midges. By completing their entire lifecycle in and over deep water, they prove that depth per se does not exclude insects from pelagic habitats. Their success further indicates that a key requirement for insect survival in open water is a fish-free refuge. Freshwater lakes are not the only bodies of water with suitable conditions. For example, if *Chaoborus* could tolerate the osmotic challenges of seawater, they could conceivably survive

in the Black Sea, which is persistently anoxic below 125 to 200 m over most of the basin (31). The ability of *Chaoborus* larvae and pupae to live in deep freshwater lakes indicates that physiological and ecological factors other than depth must be considered to explain the absence of insects from open-water marine environments.

5

References and Notes:

1. K.-D. B. Dijkstra, M. T. Monaghan, S. U. Pauls, Freshwater biodiversity and aquatic insect diversification. *Annu. Rev. Entomol.* **59**, 143-163 (2014).
- 10 2. G. C. Hays, A review of the adaptive significance and ecosystem consequences of zooplankton diel vertical migrations. *Hydrobiologia* **503**, 163-170 (2003).
3. S. H. Maddrell, Why are there no insects in the open sea? *J. Exp. Biol.* **201**, 2461-2464 (1998).
4. A. Krogh, On the hydrostatic mechanism of the corethra larva with an account of methods of microscopical gas analysis. *Skand. Arch. Physiol* **25**, 183-203 (1911).
- 15 5. E. K. G. McKenzie, G. T. Kwan, M. Tresguerres, P. G. D. Matthews, A pH-powered mechanochemical engine regulates the buoyancy of *Chaoborus* midge larvae. *Curr. Biol.* **32**, 927-933.e925 (2022).
6. S. Teraguchi, Correction of negative buoyancy in the phantom larva, *Chaoborus americanus*. *J. Insect Physiol.* **21**, 1659-1670 (1975).
7. W. O. Fenn, Partial pressure of gases dissolved at great depth. *Science* **176**, 1011-1012 (1972).
- 20 8. K. Irvine, in *The Fishery Potential and Productivity of the Pelagic Zone of Lake Malawi/Niassa*, A. Menz, Ed. (Natural Resources Institute, Chatham, UK, 1995), chap. 4, pp. 109-140.
9. G. F. Turner, R. L. Robinson, B. P. Ngatunga, P. W. Shaw, G. R. Carvalho, in *Management and Ecology of Lake and Reservoir Fisheries*, I. G. Cowx, Ed. (2002), chap. 29, pp. 353-366.
10. C. Hahn, M. J. Genner, G. F. Turner, D. A. Joyce, The genomic basis of cichlid fish adaptation within the
- 25 11. K. Irvine, Food selectivity and diel vertical distribution of *Chaoborus edulis* (Diptera, Chaoboridae) in Lake Malawi. *Freshw. Biol.* **37**, 605-620 (1997).
12. K. Irvine, in *The Fishery Potential and Productivity of the Pelagic Zone of Lake Malawi/Niassa*, A. Menz, Ed. (Natural Resources Institute, Chatham, Kent, UK, 1995), chap. 3, pp. 85-108.
- 30 13. T. G. Northcote, Use of a high-frequency echo sounder to record distribution and migration of *Chaoborus* larvae. *Limnol. Oceanogr.* **9**, 87-91 (1964).
14. F. R. Knudsen, P. Larsson, P. J. Jakobsen, Acoustic scattering from a larval insect (*Chaoborus flavicans*) at six echosounder frequencies: Implication for acoustic estimates of fish abundance. *Fisheries Research* **79**, 84-89 (2006).
- 35 15. R. Bainbridge, The speed of swimming of fish as related to size and to the frequency and amplitude of the tail beat. *J. Exp. Biol.* **35**, 109-133 (1958).
16. K. S. Bardenfleth, R. Ege, On the anatomy and physiology of the air-sacs of the larva of *Corethra plumicornis*. *Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening* **67**, 25-42 (1916).
17. A. Y. Fedorenko, M. C. Swift, Comparative biology of *Chaoborus americanus* and *Chaoborus trivittatus* in
- 40 18. Eunice Lake, British Columbia. *Limnol. Oceanogr.* **17**, 721-730 (1972).
19. W. Kuhn, A. Ramel, D. H. Walters, G. Ebner, H. J. Kuhn, in *Fortschritte Der Hochpolymeren-Forschung*. (Springer, 1960), pp. 540-592.
19. F. Scholz, I. Zerbst-Boroffka, Environmental hypoxia affects osmotic and ionic regulation in freshwater midge-larvae. *J. Insect Physiol.* **44**, 427-436 (1998).
- 45 20. J. I. Camacho García *et al.*, Widespread genetic signals of visual system adaptation in deepwater Cichlid fishes. *Mol. Biol. Evol.* **42**, (2025).
21. E. H. Allison, K. Irvine, A. B. Thompson, B. P. Ngatunga, Diets and food consumption rates of pelagic fish in Lake Malawi, Africa. *Freshw. Biol.* **35**, 489-515 (1996).
22. M. K. Vollmer, R. F. Weiss, H. A. Bootsma, in *The East African Great Lakes: Limnology, Palaeolimnology and Biodiversity*, E. O. Odada, D. O. Olago, Eds. (Springer Netherlands, Dordrecht,
- 50 23. 2002), vol. 12, pp. 209-233.
- P. Ward, L. Greenwald, F. Rougeriye, Shell implosion depth for living *Nautilus macromphalus* and shell strength of extinct cephalopods. *Lethaia* **13**, 182-182 (1980).

24. E. J. Denton, Croonian Lecture, 1973: On Buoyancy and the Lives of Modern and Fossil Cephalopods. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **185**, 273-299 (1974).
25. A. J. Dunstan, P. D. Ward, N. J. Marshall, Vertical distribution and migration patterns of *Nautilus pompilius*. *PLOS One* **6**, e16311 (2011).
- 5 26. O. A. Saether, *Nearctic and Palaearctic Chaoborus (Diptera: Chaoboridae)*. Bulletins of the Fisheries Research Board of Canada (Fisheries Research Board of Canada, Ottawa, 1970), vol. 174, pp. 57.
27. C. T. F. Ross, *Pressure vessels under external pressure*. (Elsevier Applied Science, England, 1990).
28. C. A. Scholz *et al.*, East African megadroughts between 135 and 75 thousand years ago and bearing on early-modern human origins. *Proceedings of the National Academy of Sciences* **104**, 16416-16421 (2007).
- 10 29. M. S. Leonardi *et al.*, Under pressure: the extraordinary survival of seal lice in the deep sea. *J. Exp. Biol.* **223**, (2020).
30. A. Preuss *et al.*, The ectoparasitic seal louse, *Echinophthirius horridus*, relies on a sealed tracheal system and spiracle closing apparatus for underwater respiration. *Communications biology* **8**, 852 (2025).
31. J. W. Murray *et al.*, Unexpected changes in the oxic/anoxic interface in the Black Sea. *Nature* **338**, 411-413 (1989).
- 15 32. J. Verbeke, in *Exploration du Parc National Albert. Mission G.F. de Witte (1933-1935)*. (Institut des Parcs Nationaux du Congo Belge, Brussels, 1958), vol. Fascicule 94, pp. 1-57.
33. J. Green, J. O. Young, The larva of the lake fly *Chaoborus pallidipes* Theobald (Diptera: Chaoboridae) from Kenya. *J. Nat. Hist.* **10**, 463-467 (1976).
- 20 34. J. Verbeke, in *Exploration Hydrobiologique des Lacs Kivu, Édouard et Albert (1952-1954)*. (Mem. Inst. Roy. Sc. Nat. Belg., Brussels, 1957), vol. III, Fasc. 2, pp. 187-203.
35. O. A. Saether, *Nearctic and palaearctic Chaoborus (Diptera: Chaoboridae)*. (Fisheries Research Board of Canada, 1970).
36. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. *Nat. Meth.* **9**, 671-675 (2012).
- 25 37. E. J. La Row, G. R. Marzolf, Behavioral differences between 3rd and 4th instars of *Chaoborus punctipennis* Say. *The American Midland Naturalist* **84**, 428-436 (1970).
38. D. Lemon *et al.*, in *2012 Oceans*. (IEEE, 2012), pp. 1-6.
39. W.-J. Lee *et al.*, Echopype: A Python library for interoperable and scalable processing of water column sonar data for biological information. *arXiv preprint arXiv:2111.00187*, (2021).
- 30 40. A. De Robertis, I. Higginbottom, A post-processing technique to estimate the signal-to-noise ratio and remove echosounder background noise. *ICES J. Mar. Sci.* **64**, 1282-1291 (2007).
41. G. Patterson, O. Kachinjika, in *The Fishery Potential and Productivity of the Pelagic Zone of Lake Malawi/Niassa*, A. Menz, Ed. (Natural Resources Institute, Chatham, UK, 1995), chap. 1, pp. 1-68.
- 35 42. A. Menz, *The Fishery Potential and Productivity of the Pelagic Zone of Lake Malawi/Niassa*. (Natural Resources Institute, Chatham, UK, 1995).
43. R. Proud, M. J. Cox, S. Wotherspoon, A. S. Brierley, A method for identifying sound scattering layers and extracting key characteristics. *Methods in Ecology and Evolution* **6**, 1190-1198 (2015).
44. A. Ariza, R. Blackwell, S. Fielding, E. Pauthenet, X. Wang. (2025).
- 40 45. C. Truong, L. Oudre, N. Vayatis, Selective review of offline change point detection methods. *Signal Processing* **167**, 107299 (2020).
46. D. N. MacLennan, A. Menz, Interpretation of *in situ* target-strength data. *ICES J. Mar. Sci.* **53**, 233-236 (1996).
47. D. P. Bertsekas, The auction algorithm for assignment and other network flow problems: A tutorial. *Interfaces* **20**, 133-149 (1990).
- 45 48. S. S. Blackman, *Multiple-target tracking with radar applications*. (Artech House, Inc., Dedham, MA, 1986), pp. 463.
49. R. V. Lenth, J. Piaskowski. (2026).
50. M. Burrows, M. Dorosenko, Rapid swimming and escape movements in the aquatic larvae and pupae of the phantom midge *Chaoborus crystallinus*. *J. Exp. Biol.* **217**, 2468-2479 (2014).
- 50 51. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. *Journal of statistical software* **67**, 1-48 (2015).
52. F. Baty *et al.*, A Toolbox for Nonlinear Regression in R: The Package nlstools. *Journal of Statistical Software* **66**, 1 - 21 (2015).

Acknowledgments

Our thanks to Malawi's National Commission for Science and Technology (NCST) for approving the research, and Evance Mataka, Joseph Chipalamoto and Yusufu Muhammad of the Nkhata Bay District Fisheries Office for invaluable fieldwork assistance. We also thank Bruce Gillespie, Dept of Zoology workshop, UBC, for construction of the pressure chamber, WSP Wild Water Productions Limited for assisting with travel and logistics in Kenya and Robert Shadwick for feedback on an earlier draft of this paper.

Funding:

NSERC Discovery project and Accelerator grant RGPIN-2020-07089 (PGDM)

Company of Biologists (COB) Travelling Fellowship JEBTF23051050 (EKGM)

The Society for Integrative and Comparative Biology Fellowship of Graduate Student Travel (FGST) (EKGM)

ASL Environmental Sciences, B.C., Canada, 2023 Acoustic Zooplankton Fish Profiler (AZFP) award (MJRN, PGDM)

Author contributions

Conceptualization: PGDM, EKGM

Methodology: PGDM, EKGM, MJRN, RP, HM

Investigation: PGDM, EKGM, TA, MJRN, JC, HM, RP

Visualization: PGDM, EKGM, HM

Funding acquisition: PGDM, MJRN, EKGM

Project administration: PGDM

Supervision: PGDM, RP

Writing – original draft: PGDM, EKGM, RP, HM

Writing – review & editing: PGDM, EKGM, TA, MJRN, JC, HM, RP

Competing interests

Authors declare that they have no competing interests.

Data and materials availability

All data are available on Dryad: <https://doi.org/10.5061/dryad.4tmpg4fqk>

Supplementary Materials

5 **Materials and Methods**

Figs. S1 to S6

Tables S1 to S2

References (32-52)

Movies S1 to S3

10 **Data S1 to S4**

Figures:

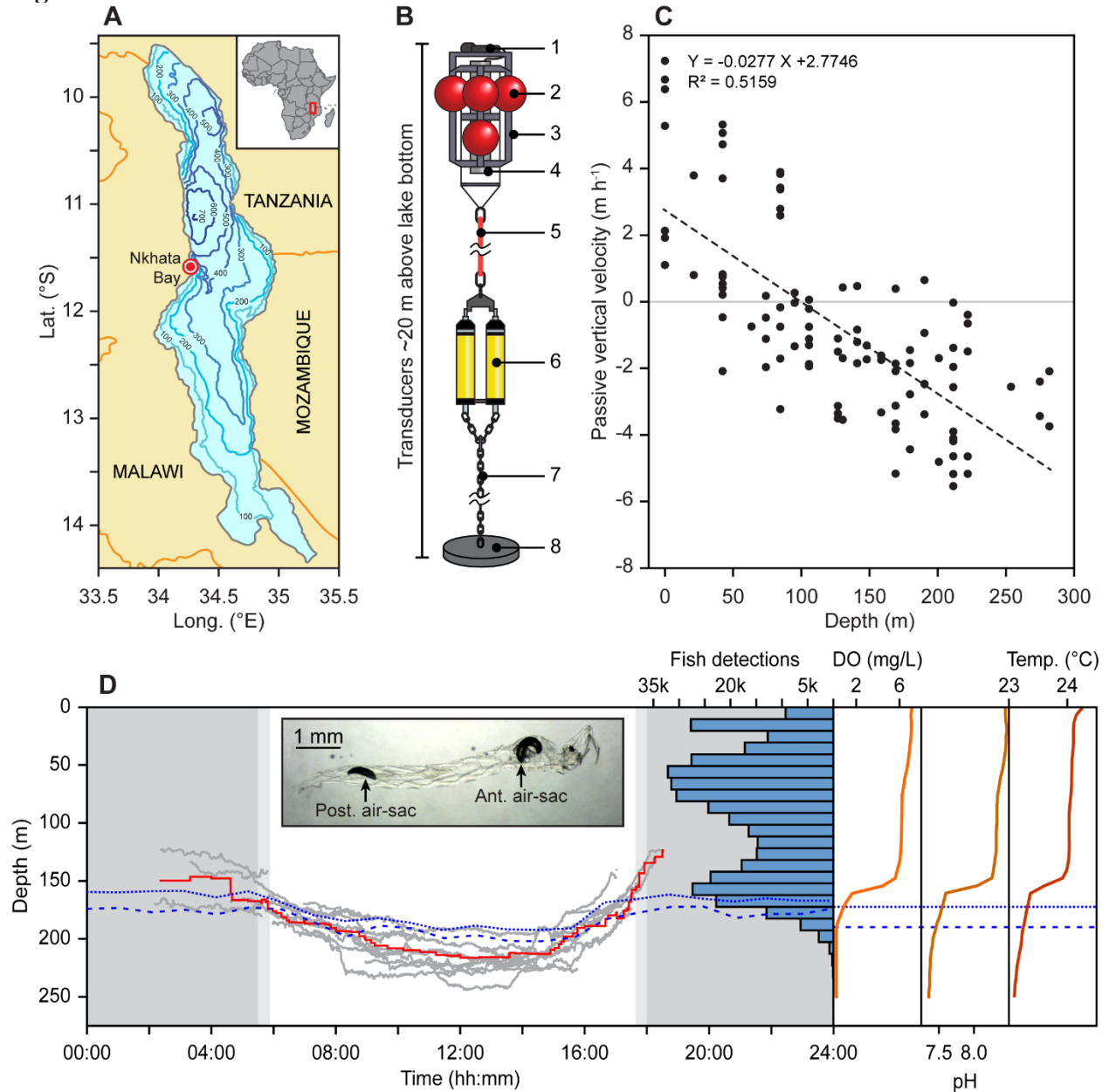


Fig. 1. *Chaoborus edulis* DVM in Lake Malawi. A) Bathymetric map of Lake Malawi showing study location, inset showing location in East Africa. B) Quad-frequency Acoustic Zooplankton Fish Profiler (AZFP): 1. 67, 120, 200, 455 kHz transducers, 2. 6× ABS floats, 3. Cage, 4. AZFP, 5. 12.3 m rope, 6. Acoustic releases, 7. 3 m drop chain, 8. 120 kg iron ballast. C) Passive vertical velocities of larvae as a function of depth. D) The deepest portion of the DVM of *C. edulis* and its relationship to the vertical distribution of fish and water parameters. Night, twilight and day indicated by dark grey, light grey and white panels, respectively. Dark grey lines show mean depth of all detected *C. edulis* layers, red line shows the overall mean. Blue lines show depth of 95th (dotted) and 99th (dashed) percentiles of fish detections. Histogram shows the distribution of all fish detections in 10 m depth bins. Panels to the right show dissolved oxygen (DO), pH and water temperature measured at the sonar deployment site. Blue lines show depth of 95th and 99th

percentiles of all fish detections over deployment period. Inset shows a 4th instar *C. edulis* larva, with posterior and anterior air-sacs indicated.

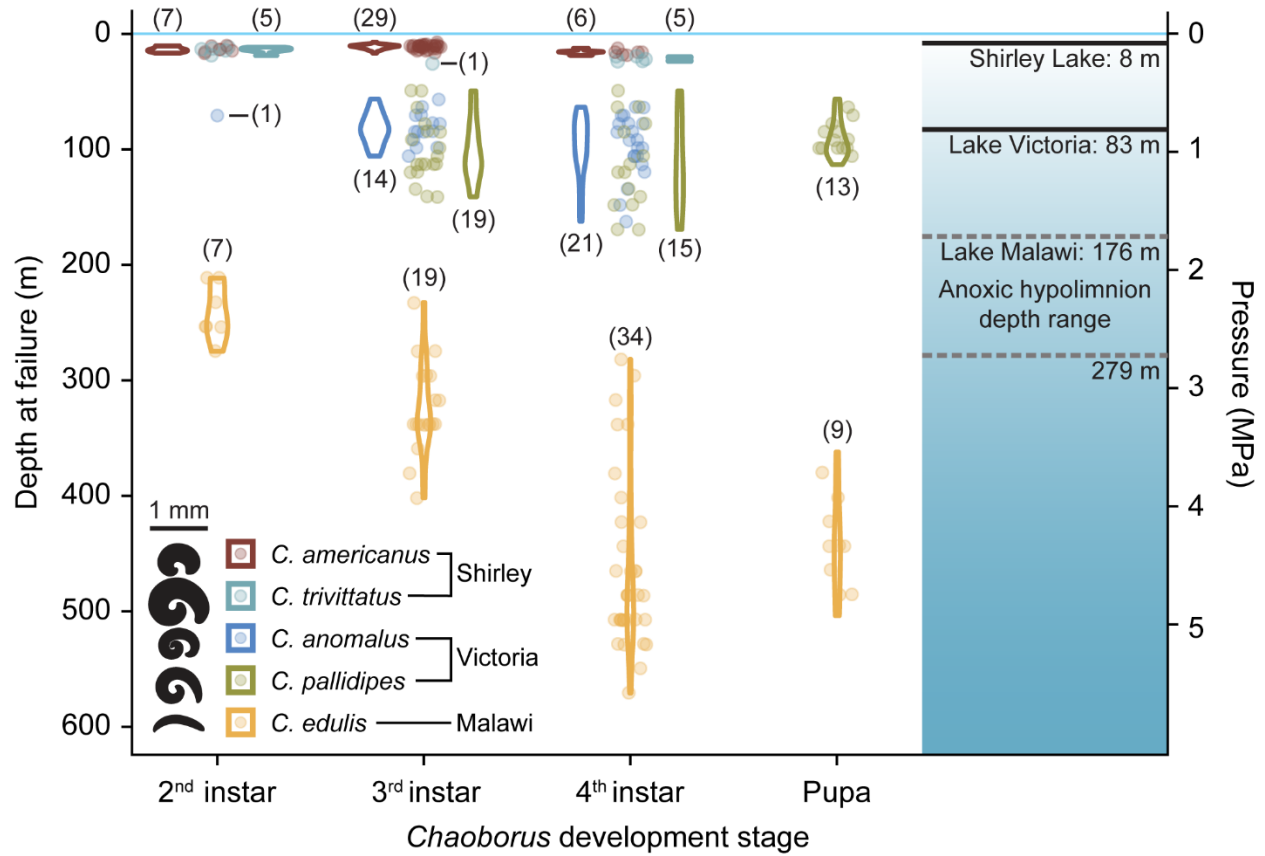


Fig. 2. Implosion depths of larval air-sacs and pupal respiratory horns *in vivo*. Five species of *Chaoborus* were exposed to stepwise increases in hydrostatic pressure until the first air-sac or respiratory horn imploded. Pupae were only available for *C. edulis* and *C. pallidipes*. Their habitat depth (from shallowest to deepest) was: *C. americanus* (red) and *C. trivittatus* (teal), both from Shirley Lake; *C. anomalus* (blue) and *C. pallidipes* (green), both from Lake Victoria; and *C. edulis* from Lake Malawi (yellow). Sample size indicated in brackets. The depth scale on the right indicates maximum lake depth or the depth range of the anoxic hypolimnion in Lake Malawi (22). *C. edulis*, *C. anomalus*, and *C. trivittatus* all showed significant positive trends of depth tolerance with instar (ANOVA: $F = 106.0$, $p < 0.0001$; $F = 4.8$, $p = 0.036$; and $F = 12.4$, $p = 0.006$, respectively). Through larval development, *C. edulis* gained 119.02 ± 11.56 m depth tolerance instar⁻¹ ($F = 106$, $p < 0.0001$). Depth of failure for *C. edulis* 4th instar larvae and pupae were not significantly different (Welch Two Sample t-test: $t = 1.2$, $df = 28.0$, $p = 0.2$) but in *C. pallidipes* pupae failed at shallower depths than 4th instar larvae (Welch Two Sample t-test: $t = 2.3$, $df = 16.9$, $p = 0.035$). Air-sac diameters were compared using Tukey adjusted pairwise comparison of estimated marginal means, with the only nonsignificant difference being between *C. edulis* and *C. anomalus* ($t = 0.7$, $p > 0.9$). Air-sac silhouettes show a 4th instar posterior air-sac from each species to scale and *in vivo* orientation.

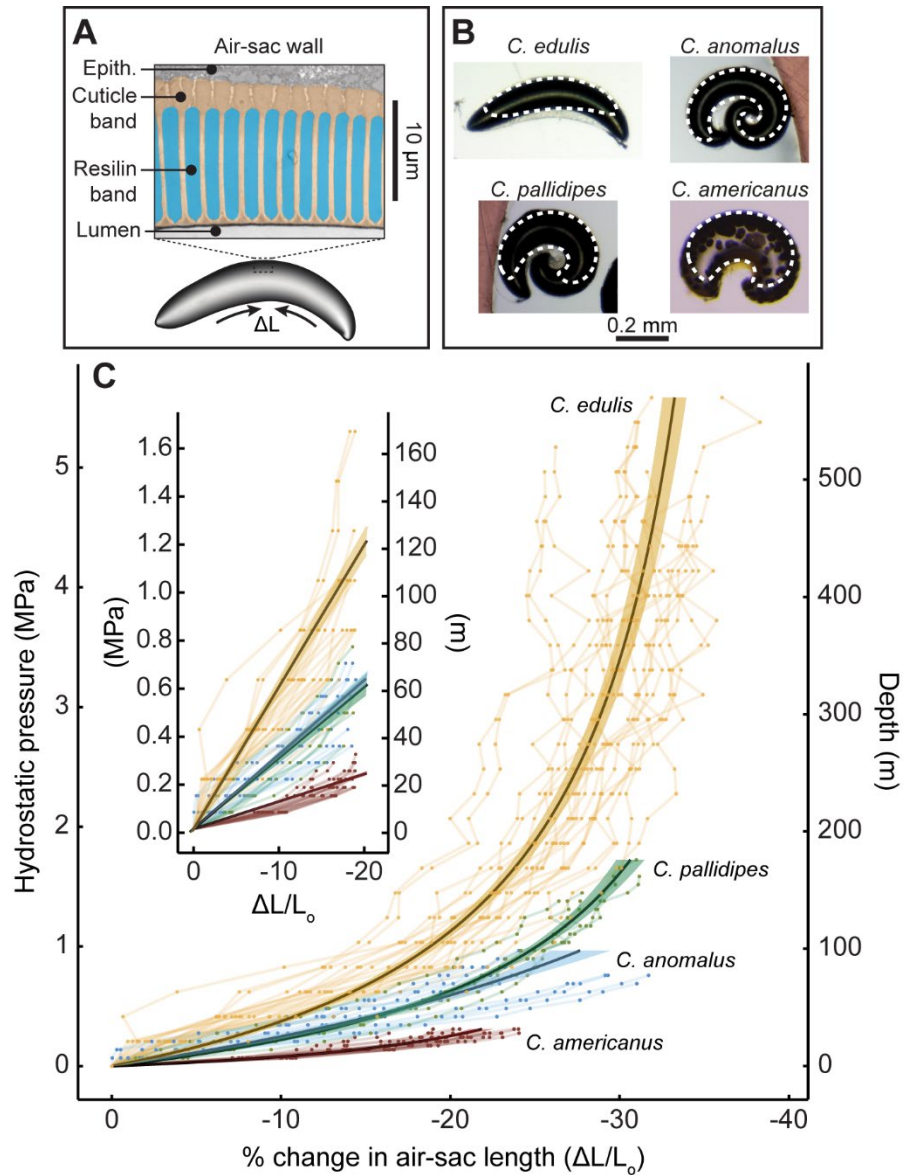


Fig. 3. Air-sac compression and stiffness under hydrostatic pressure A) TEM of longitudinal section through 4th instar *C. edulis* air-sac wall showing arrangement of alternating resilin and cuticle bands, air-sac epithelium (epith.) and direction of air-sac compression. B) Photos of *Chaoborus* air-sacs at atmospheric pressure with dashed outlines indicating degree of compression before failure. C) The change in excised air-sac length in pH 6 buffer with increasing hydrostatic pressure indicates compressive stiffness. Bands show 95% CI. Air-sac length was normalized to the initial unpressurized length. Inset shows the initial, linear phase of each air-sac stiffness curve. A Tukey post-hoc comparison of estimated marginal means was used to compare stiffness between species. Only *C. anomalus* (n = 13) and *C. pallidipes* (n = 4) were not significantly different (t = 0.32, p = 0.99); *C. edulis* (n = 21) and *C. americanus* (n = 18) had stiffnesses significantly different from all other species (t ≥ 4.7, p ≤ 0.016).

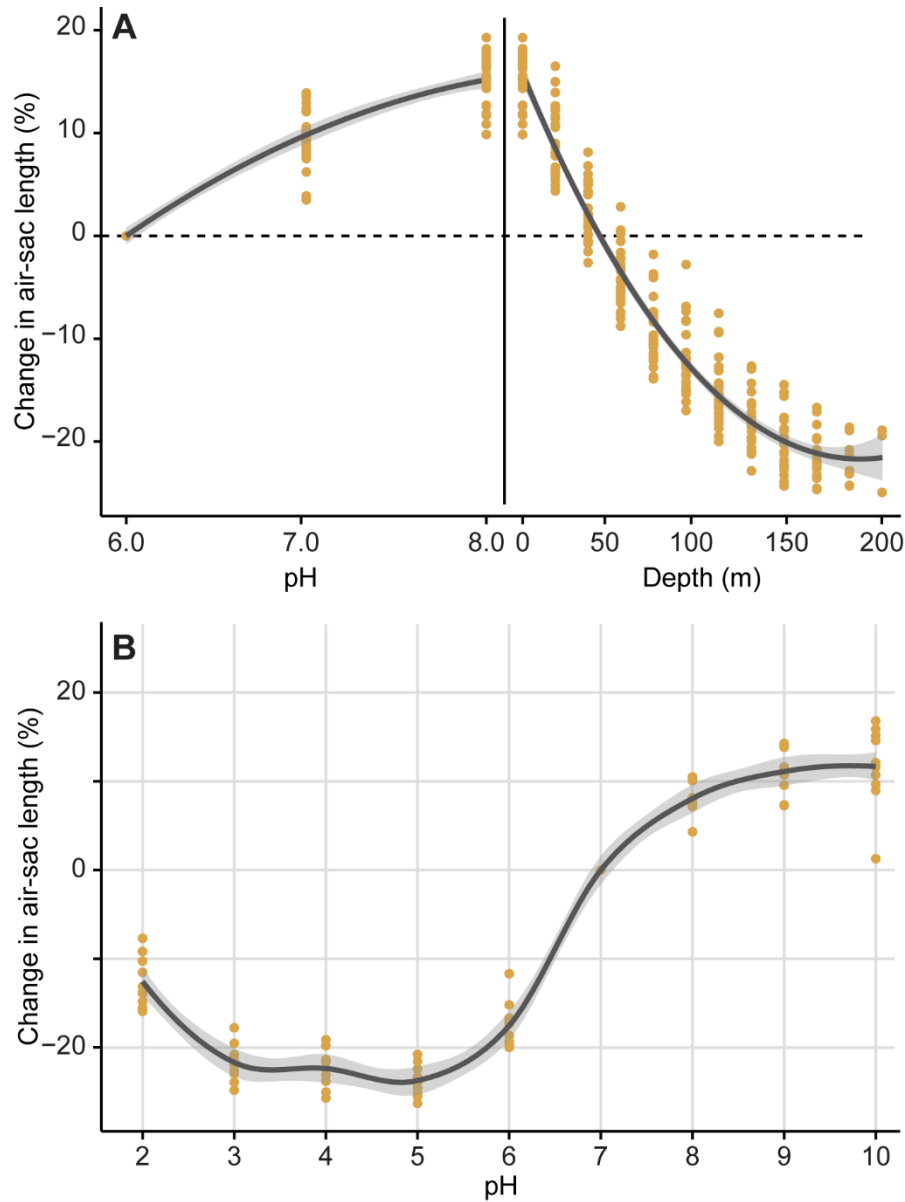


Fig. 4. Relationship between *C. edulis* air-sac length, pH and hydrostatic pressure. A) Expansion of unpressurized air-sacs following an increase in pH from 6 to 8 (LHS), and the mechanical work done in compressing the air-sacs at pH 8 by increasing hydrostatic pressure (RHS) (n = 11). B) Percent change in length of excised *C. edulis* air-sacs in relation to pH of the bathing medium (n = 11) (measured from change in profile area and normalized to pH 7). The greatest change occurs between pH 6 and 8. Bands show 95% CI.

Supplementary Materials for

Crush-resistant air-sacs allow insect larvae to exploit aquatic habitat at extreme depth

Evan K. G. McKenzie¹, Maxon J. R. Ngochera², Joseph Chombo², Hayley McLennan³, Roland Proud^{3,4}, Tahnee Ames¹, Philip G. D. Matthews^{1*}

Corresponding author: pmatthews@zoology.ubc.ca

The PDF file includes:

Materials and Methods
Figs. S1 to S8
Tables S1 to S2
References (32-52)

Other Supplementary Materials for this manuscript include the following:

Movies S1 to S3
Data S1 to S4

Materials and Methods

***Chaoborus* larva collection and maintenance:**

C. edulis collection

All work on *C. edulis* was carried out in Nkhata Bay, Malawi, between August 5th and 27th, 2023. *Chaoborus edulis* larvae and pupae were collected from Lake Malawi within a 3.4 km radius of Nkhata Bay, Malawi (11° 36' 32.1948" S 34° 17' 59.5716" E). The identity of this species was verified using adult flies that emerged from collected pupae using information in (32). Larvae and pupae were collected after sunset (which began at 17:40, dusk at 18:00) using a conical plankton net 12" diameter, 42" long, 243 µm mesh and with a 500 ml PP collecting bottle on the cod end (Wildco, FL, USA). Vertical hauls were made to a maximum depth of 100 m, with deeper trawls being used before 19:00 as 4th instars were not caught in the top 50 m just after sunset. Plankton samples were immediately decanted from the net into 1 L Nalgene bottles and brought back to the field lab. Larvae and pupae were then sorted into a bucket containing 10 L of lake water collected from the surface at the same location as the collection. Larvae survived more than 3 days in this water, while pupae would eclose, allowing adult flies to be collected.

C. anomalus and *C. pallidipes* collection

All work on *C. anomalus* and *C. pallidipes* was carried out on Rusinga Island, Kenya, between November 6th and 14th, 2023. Larvae and pupae were collected from Lake Victoria within a 13 km radius of Rusinga Island, Kenya (0° 24' 38.4984" S 34° 8' 32.1828" E). Depth of the collection locations never exceeded 45 m as determined using a commercial fish finder/depth sounder (STRIKER Vivid 4cv and a GT20-TM transducer, Garmin International, KA, USA). Larvae and pupae were collected using the same conical plankton net as described previously. Larvae were identified to species using the information in (33, 34). Plankton samples were decanted into 1 L Nalgene bottles and brought immediately back to the field lab. Larvae and pupae were then sorted from the crustacean zooplankton and placed in plastic trays 3 cm deep, filled with lake water.

C. americanus and *C. trivittatus* collection

The larvae of *C. americanus* and *C. trivittatus* were collected from Shirley Lake in UBC's Malcolm Knapp Research Forest, B.C., Canada (49° 20' 42.9576" N 122° 33' 40.9428" W) between July 1st 2022 and November 1st 2022. *C. americanus* was collected again from the same lake on December 13th 2023 for determining crush pressure of excised air-sacs in pH 6 buffer - the same assay conducted using the three African *Chaoborus* species. Shirley Lake is fish-free and has a maximum depth of 8 m. Larvae were collected using the same conical plankton net as described previously, transferred into 1 L Nalgene bottles and immediately brought back to the lab at UBC, Vancouver. They were then transferred to a 20 L aquarium containing dechlorinated tap water and held at 4 °C within a constant temperature (CT) cabinet. Larvae were identified to species using the keys in Saether (35), and drawings within Fedorenko and Swift (17). Experiments using intact larvae were performed at lab temperature in dechlorinated Vancouver tap water, while experiments on excised air sacs were performed in a saline solution buffered to the appropriate pH.

Identification of larval instars

All *Chaoborus* develop through 4 larval instars before undergoing pupation. Every larva used for data collection was identified to instar by measuring its head capsule length from an image taken with a digital microscope (AM73915MZTL Dino-Lite Edge 3.0, Dunwell Tech, CA, USA). Briefly, either the head or the whole larva was placed in a droplet of water in a Petri dish containing a layer of clear silicone (Sylgard 184, Dow Corning, MI, USA) and illuminated from below. A digital image including a scale bar was then taken and the length of the head capsule was measured using ImageJ (36) by drawing a straight line from the anterior-most tip of the rostrum to the posterior-most edge of the head capsule (fig. S1). Head capsule measurement is the standard method for determining *Chaoborus* larval instar because, unlike body length, this structure only increases in size during molting, and thus head capsule lengths of different instars do not overlap (17, 37).

Lake Malawi water parameter vertical transects:

A calibrated water quality sonde (EXO1, YSI Inc., OH, USA) was used to measure depth, temperature, conductivity, pH, oxidation-reduction potential, and chlorophyll to a depth of 250 m. The sonde was lowered in 10 m increments using a pre-marked rope and left for 1 min at each depth. The water parameters were logged continuously at 10 s intervals. The coordinates of each measurement location were recorded using a handheld GPS device (GPSMAP 67i, Garmin International, KA, USA). Transects were recorded at 6 locations within a 5.5 km radius of Nkhata Bay between the 7th and 18th of August, 2023, including a measurement directly over the sonar deployment site on the 14th of August.

Lake Malawi acoustic data collection and analysis:

Lake Malawi sonar equipment and deployment

A multi-frequency scientific sonar system transmitting at 455, 200, 120 and 67 kHz (Acoustic Zooplankton and Fish Profiler (AZFP), ASL Environmental, B.C., Canada) was deployed on 12th August, 2023 at 1:30 pm at 11° 36' 3.474" S 34° 18' 43.2828" E and recovered on August 25th at 1:30 pm for a total duration of 13 days. Calibration of the sonar system was performed before deployment using a 12.7 mm diameter tungsten-carbide (WC) sphere as a target in a freshwater tank according to standard methods (38). A depth sounder (STRIKER Vivid 4cv and a GT20-TM transducer) indicated an approximate depth of 286 m at this location. The AZFP sonar system was configured as follows: Pulse length 1000 us, burst interval 5 s, 1 ping per burst, digitization 20 kS/s. The AZFP sonar system was enclosed in a cage equipped with 6 UBE center-hole floats (CT-368b ABS, Nichimo Co., Japan), which suspended the sonar transducers facing upward 20 m above the lake floor. A 12.3 m long length of rope connected the bottom of the cage to a pair of acoustic releases (Push Off Release Transponder, Edgetech, MA, USA) which held 3 m of drop chain attached to 120 kg of iron ballast. Subsequent analysis of the sonar traces indicated a deployment depth of 275 m.

Preprocessing of acoustic data

Acoustic data were processed in Python. Raw files were read using the 'echotype' python package (39) and processed by calendar day of the survey. Background noise was

removed following De Robertis and Higginbottom (40). No further noise removal was carried out based on visual examination of the data.

Detection of *C. edulis* layers

Lake Malawi is the 2nd deepest lake in Africa after Lake Tanganyika, with an average depth of 292 m and a maximum depth of 706 m (41). It is generally oligotrophic as well as being meromictic, with a persistent hypolimnion that becomes anoxic at a depth of ~280 m at the southern end of the lake and ~176 m at the northern end (22). *C. edulis* are an important and abundant component of the lake's ecology, occurring at densities between 400 to >12,000 larvae m⁻² (8). Their high abundance means that they play a central role shaping the zooplankton community, as well as acting as a key food source for fish, particularly the deepwater demersal fish community (21). They are also the only taxon known to inhabit the lake's anoxic zone (11). Previous sampling indicates that fourth-instar *C. edulis* larvae form dense, vertically discrete layers in this region during daylight hours (11). We therefore assumed that sound-scattering layers (SSLs) observed within the anoxic zone represented aggregations of *C. edulis* larvae. Because these larvae are small relative to the acoustic wavelengths used (1.25 cm at 120 kHz; 0.75 cm at 200 kHz), they behave as Rayleigh scatterers, with backscattered intensity dependent on target volume rather than shape. Accordingly, we expect stronger volume backscattering strength (S_v , dB re 1 m⁻¹) values to be observed at 200 kHz than 120 kHz for *C. edulis*.

Visual inspection of echograms confirmed that SSLs in the anoxic zone had relatively high frequency-differential values ($S_{v200} - S_{v120} > 13$ dB, based on the maximum observed dB difference) and adequate signal-to-noise ratios (SNR > 10 dB at 200 kHz) for reliable detection. Mean SSL S_v values recorded at 200 kHz (S_{v200}) were typically around -80 dB re 1 m⁻¹. However, at lower backscattering levels ($S_v < -90$ dB), SNR often fell below 10 dB, causing estimates of S_v (and consequently of dB difference) to become unreliable. To ensure robust estimation of SSL properties, we set a minimum threshold of -90 dB re 1 m⁻¹ for SSL detection at 200 kHz. Although small zooplankton could theoretically produce similar Rayleigh-type scattering, previous sampling indicates that crustacean zooplankton rarely occur below 80 m in Lake Malawi (42). Layers meeting these criteria and migrating into the anoxic zone were therefore classified as *C. edulis* larval layers.

SSLs were extracted from the data using a method adapted from the Sound Scattering Layer Extraction Method (SSLEM (43)). Echosounder observations were integrated over a grid (0.2 m depth by 12 pings/1 minute) using functions from *echopy* (44) and SSLs were extracted from both the 200 kHz and dB difference ($S_{v200} - S_{v120}$) data. A maximum filter was applied to the $S_{v200} - S_{v120}$ data using a 5 column by 5 row kernel, prior to layer detection to amplify features of interest. After thresholding, cells classified as SSLs in both the 200 kHz and the $S_{v200} - S_{v120}$ data were designated as *C. edulis* layers. The parameters of SSL detection are as shown in Table S1 and an example of the workflow is given in fig. S2.

Layers were split into different phases of the diel vertical cycle by applying window-based change point detection to the layers vertical speed (45). The vertical speed was calculated as the difference between the mean layer depth between samples and smoothed using a median filter over 60 columns (1 hour). The window used for change point detection was 30 columns/30 minutes. The number of predicted breakpoints was set to the length of the layer in hours divided by 3, rounded up to the nearest integer, allowing at least one break point for every three hours that the layer persisted (fig. S3).

Metrics were calculated to describe each layer, including start time, end time, mean depth, overall weighted mean depth (WMD, weighted by the backscattering intensity at each depth within a column), WMD at the start of the layer, and WMD at the end of the layer. The ascent and descent rate of each layer was calculated as the WMD at the end of the layer minus the WMD at the start of the layer, divided by the total duration of the layer in seconds.

Single target detection and fish tracking

Single target detection was run in Echoview version 15.1 using the single beam method 2 operator to detect echoes from individual large fish at 120 kHz with a threshold of -65 dB re 1 m² (see Table S2 for full details of parameters). MacLennan and Menz (1996) (46) found a peak of -45 dB re 1 m² in single target fish detections at 120 kHz in Lake Malawi, but all single targets in their survey had target strength (TS) > -65 dB re 1 m², so this threshold was applied to detect all resolvable single targets likely to represent individual fish within the acoustic data. A fish track detection algorithm (47, 48) was applied to these single targets, and all detected fish tracks across the survey period were exported. The mid-points of fish tracks were used to identify which fish overlapped with which *C. edulis* layer in both space and time.

***Chaoborus* 4th instar air-sac geometry:**

Calculation of 4th instar *C. edulis* air-sac volume

Air-sac volumes were estimated from images of excised 4th instar air-sacs used in the mechanical work experiment (see below). Zero applied pressure and pH 6 were chosen for this measurement, as previous work showed that the pH of neutrally buoyant and unpressurized *C. americanus* air-sacs was approximately 6 (5). Volume was estimated separately for the air-sac midsection which was split at its approximate widest point to be measured as two truncated cones, and the air-sac tips were measured as prolate ellipsoids (fig. S5). Truncated cone volumes were calculated by the formula

$$V = (1/3) \times \pi \times h \times (r^2 + r \times R + R^2) \quad \text{Eq. S1}$$

where r and R are the two radii that define a truncated cone, and h the height of the cone. The hemisphere volume of a prolate ellipsoid was calculated as

$$V = 1/2 (r^2 \times h \times \pi \times 4/3) \quad \text{Eq. S2}$$

where r is the radius and h is the height of the ellipsoid. The sum of the two prolate spheroid hemispheres and two truncated cone sections provided the estimated total volume of the air-sac (fig. S5). The total volume of all the air-sacs within a typical 4th instar *C. edulis* larva was then calculated as the sum of $2 \times$ the mean anterior air-sac volume and $2 \times$ the mean posterior air-sac volume.

Comparison of air-sac diameter across species

To assess air-sac diameter between species and incorporate *C. edulis* air-sac data from multiple experiments, a linear model was used to assess all available excised air-sac diameters from each species used in this report. A comparison between species was made by applying the

Tukey method to this linear model using the emmeans package (49). Experimental variation is shown as standard deviation around mean values, and testing level (alpha) for significance is 0.05.

5 **Pressure chamber experiments:**

Pressure chamber details and mode of operation

A portable pressure chamber was constructed to determine the crush depth of intact larvae and pupae, the compressibility and crush depth of excised air sacs, and rates of vertical ascent and descent of intact larvae. The chamber body was milled from a circular aluminium block, 110 mm in diameter and 25 mm thick (fig. S6). A cylindrical cavity 45 mm in diameter was bored through its center to provide a volume of 39.8 ml. Grooves to house 50 mm-diameter O-rings were milled around each opening. Two 17.6 mm thick clear acrylic windows, 95 mm in diameter, sealed each side of the cylindrical cavity by being bolted in place using 6 × M8, 40 mm long stainless-steel bolts. The chamber was pressurized using a hand-operated hydraulic pressure pump (Additel 925W Hydraulic Pressure Test Pump, Additel Corporation, CA, USA) filled with distilled water. A calibrated 6895 kPa (1000 psi) digital pressure gauge (Additel 680 GP1K, Additel Corporation) on one of the pump's two pressure ports was used to monitor the pressure to within 0.7 kPa ± 0.25% FS. The second pressure port was connected to the pressure chamber by a 5 ft length of polyamide hose with a ¼" NPT fitting (ADT100-HTK, Additel Corporation, CA, USA).

Vertical rate of ascent and descent, and swimming velocity, of *C. edulis*

The pressure chamber was filled with lake water and 3 to 8 *C. edulis* larvae of various instars (2nd to 4th). The chamber was filled completely, sealed and then oriented on its side so the acrylic windows were vertical. To determine rates of ascent and descent, videos up to 1 min long were recorded using the camera in an iPhone 11 (Apple Inc., CA, USA) as the pressure in the chamber was gradually increased in increments of 413 kPa (60 psi), each pressure increase occurring over a 2 min period. The phone was propped up vertically on the table such that the entire circular cross-section of the chamber was visible in the camera frame. Short videos between 20 s and 1 min 15 s long were recorded at each pressure. A total of 3 pressure-increasing trials with different larvae yielded 63 videos for analysis. Once recorded, each video was analyzed in ImageJ (36). First the image size was calibrated using the known diameter of the chamber, then an individual larva was identified and followed throughout the video. Periods where it was clearly either sinking or floating passively at a constant rate were identified, and markers were manually placed on its head in the video frame at the beginning and end of each passive vertical movement period. The coordinates of these points and the elapsed durations between them were exported into a spreadsheet, and the differences in the Y coordinates (in mm) were divided by the difference in time (s) between the frames to yield vertical velocity. This was repeated for all larvae in each video. During sinking, particularly at higher pressures, it was noticed that the larvae sank head-first, their bodies being more than 65° from their usual horizontal attitude.

The same process as above was used to determine swimming velocity, except that a marker was placed on the head of the swimming larva every 5 frames to produce a multisegmented track of the distance travelled. Dividing this swimming bout track distance by time elapsed yielded

average swimming velocity. Swimming velocities measured this way were entirely consistent with those measured from another, similarly sized species, *Chaoborus crystallinus* (50).

As individual larvae were often recorded multiple times within a video, and because larvae could not be reliably distinguished across a video recording, these values illustrate the range
5 sinking, floating and swimming velocities encountered across a range of larval instars, and how they vary with hydrostatic pressure.

Whole larvae and pupae crush depth measurements

The pressure chamber was placed lying flat with the acrylic window on the top of the
10 chamber removed. It was then filled with fresh lake water (*C. edulis*, *C. anomalus* and *C. pallidipes*) or dechlorinated Vancouver tap water (*C. americanus*, *C. trivittatus*). A plastic insert with multiple hexagonal chambers (7 chambers used for *C. edulis*, 13 for *C. anomalus* and *C. pallidipes* Fig. S7) was submerged in the chamber up to its top edge, dividing the chamber into multiple shallow hexagonal cells. The bottom of each cell had a hole in the center, through which
15 a length of 1 mm outer diameter PTFE tube projected vertically 2 mm up from the floor of the cell. This ensured pressure equalization within each cell while preventing the larva from escaping down through the hole. A 3.5 ml disposable transfer pipette was used to individually place a larva or pupa into each cell. The acrylic lid was then placed on top and the main screw on the hydraulic pressure pump was turned to completely flood the gap between the top of the cells
20 and the acrylic window. The window was then bolted down, closing the open tops of the hexagonal chambers while the vent on the hydraulic pump was opened to prevent compression from the window from pressurizing the chamber. Once the chamber window was secured the vent was closed and the chamber was moved to the stage of an EXS-210 stereo microscope with 1× and 3× objectives, 10× eyepieces, and illuminated from below (Accuscope, NY, USA).
25 Pressurization proceeded in a stepwise fashion, with the pressure ramped by 207 kPa (30 psi) over a minute, then the larvae or pupae were observed for signs of air-sac or respiratory horn implosion for a further 3 minutes. Air-sac collapse was generally accompanied by the larva thrashing vigorously for a few seconds and the damaged air-sac was clearly visible through the insect's transparent cuticle. Respiratory horn collapse resulted in the horn detaching while pupae
30 sank onto the cell floor. The pressure at which the first implosion was observed (and for larvae, the anterior or posterior location of the imploded sac) was noted for each individual. The loss of a single air-filled structure (a larva's air-sac or a pupa's respiratory horn) was considered the biologically relevant failure point. The pressure continued to be increased until all individuals had experienced structural failure. Individuals were then removed and imaged using a 10 - 220×
35 digital microscope (AM73915MZTL Dino-Lite Edge 3.0, Dunwell Tech, CA, USA) and larval head capsule length was measured to determine instar.

The effect of instar progression on air-sac crush pressure in intact larvae was tested using an ANOVA to conduct a type I F-test for each species (Fig 2). A Tukey post hoc comparison performed on a linear model consisting of air-sac collapse pressure responding to instar and
40 species was used for a comparison of mean air-sac or respiratory horn crush pressure between larvae and pupae (also using the emmeans package in R (49)).

Excising air-sacs

Air-sacs were excised from live *Chaoborus* larvae by placing them individually into a 35
45 mm diameter silicone-lined Petri dish filled with pH 6 buffer (HEPES: 8 mM, Bis-Tris: 8 mM, NaCl: 300 mM, and Mannitol: 306 mM, titrated using 1 M HCl). This buffer was also used to

immerse excised air-sacs during experimentation. Fine forceps (#5 Dumostar Biologie forceps, No. 11295-10, Dumont, Switzerland) were used to hold the larva, while Vannas spring scissors with a 2.5 mm cutting edge (No. 15000-08, Fine Science Tools, CA, USA) were used to cut it in half. The air-sacs were then gently squeezed out of the open end of the abdominal segment.

Larval heads were imaged side on for later confirmation of stage (instar/pre-pupa) using the Dino-Lite Edge 3.0 digital microscope. Only pairs of air-sacs from 4th instar larvae were used in subsequent experiments.

Calculation of change in air-sac length

We have shown previously that, because *Chaoborus* air-sacs are constrained to expand and contract in only one dimension along their long axis, a change in air-sac length is proportional to a change in both its area and volume dimensions (5). The air-sacs of all species examined here display this same property as demonstrated by testing for changes in air-sac diameter during the crush depth experiments. The effect of maximum hydrostatic pressure applied on air-sac diameter was modeled with a linear mixed effect model, accounting for species, individual, and air-sac repeated measures using lmer() (51). This revealed that the maximum hydrostatic pressure preceding failure had no significant effect on air-sac diameter in any of the species, according to a Tukey post-hoc comparison of initial and final air-sac diameters using emmeans (49). Therefore, percent changes in air-sac profile area obtained from digital images using ImageJ (36) were proportional to percent changes in air-sac length and volume:

$$\Delta A/A_0 = \% \Delta A = \% \Delta L = \% \Delta V \quad \text{Eq. S3}$$

where ΔA is the absolute change in air-sac profile area (μm^2), A_0 is the initial profile area (μm^2), and $\% \Delta A$, $\% \Delta L$ and $\% \Delta V$ are the percent change in air-sac area, length and volume, respectively. For experiments where air-sac work was calculated, however, the absolute change in air-sac length in μm was needed, rather than percent change. To obtain this, the initial length of the air-sac's long axis (L_0) was found for the same air-sac image used to measure A_0 by drawing a line between the air-sac's anterior and posterior tips along the midline of its long axis using ImageJ's segmented line tool. Multiplying L_0 by the $\% \Delta A$ calculated for any subsequent image then allowed absolute change in length (ΔL in μm) to be determined:

$$\Delta L = L_0 - (L_0 \times \% \Delta A) \quad \text{Eq. S4}$$

Compression of excised air-sacs at pH 6

Excised air-sac pairs were transferred into droplets of pH 6 buffer contained within shallow wells mounted on the inside of the pressure chamber window. The wells were either rings of 3.175 mm I.D. tubing (Bev-a-line IV plastic tubing) cut ~ 1 mm thick with a razor blade and stuck on with poster putty (UHU tak) as used for *C. edulis* (n = 12), or 3.5 mm I.D. circular holes milled into an acrylic disc, 1.7 mm thick acrylic and 43 mm in diameter, also stuck onto the inside of the pressure chamber window using a thin film of poster putty, used with *C. americanus* (n = 10), *C. anomalus* (n = 8), and *C. pallidipes* (n = 3). The putty created a seal around the edge of each well, rendering it watertight. The predominantly posterior air-sacs, with some anterior air-sacs included for *C. edulis*, from an individual larva were gently transferred into a well using fine forceps, where they floated up against the surface of the droplet. Air-sacs are extremely delicate and were held in the surface tension between the forceps arms without being directly grasped.

Once air-sacs had been transferred into all the wells, the pressure chamber window was lifted off the bench and quickly inverted, allowing all the air-sacs to float up against the upturned window within their wells. The window was slowly lowered against the opening of the pressure chamber, which had been filled with the same pH 6 solution. Similar to the procedure for intact larvae, the window was first placed onto the chamber with the wells and adhering droplets suspended just above the solution surface. The chamber was then slowly filled completely by advancing the hand crank of the pressure pump until contact was made between the droplets and the solution in the chamber, with this done slowly enough to allow the elimination of any air spaces between the wells.

Once sealed in the chamber, the air-sacs were left to equilibrate with the pH of the chamber solution, with no pressure applied, for approximately one hour. This period was followed by two images of the air-sacs taken ten minutes apart to confirm no or minimal remaining change in air-sac size. Once this was determined, the last set of images taken was used to show air-sac size at 0 kPa, after which pressure was applied in 207 kPa increments. The pressure steps were advanced 10 minutes apart to ensure that the transient increase in gas pressure within the air-sacs caused by our addition of hydrostatic pressure was allowed to dissipate. After each time interval following a pressure step had elapsed, profile images of the air-sacs from each well were taken, followed by another increase in pressure until all the air-sacs had either imploded or more slowly flooded. Air-sac area was normalized to initial area so that fractional area change, which is equivalent to fractional length change (% ΔL) as described in Eq. S3, could be plotted against pressure as % ΔL , with whole air-sac stiffness calculated as:

$$\text{Stiffness} = \Delta P / (\% \Delta L) \quad \text{Eq. S5}$$

Where ΔP is the increase in hydrostatic pressure (MPa) and % ΔL is the % change in length. Differences in air-sac stiffness between species as determined from the slope of the initial, linear phase of compression were analyzed by ANOVA using a linear mixed effect model (51) fitting the effects of compression, species, and their interaction, and including larval identity (as each contributed multiple air-sacs) as a random factor (Fig 3). A Tukey post hoc comparison of these slopes allowed direct species comparisons using the emmeans package in R (49).

Using a Nonlinear Least Squares, nls() function in R (52), the overall curved shape of air-sac applied pressure and fractional change in length was modeled. The equation for a rectangular hyperbola captured the nonlinearity of the air-sac pressure – strain response. The stiffness of an air-sac, or slope at a given point along this nonlinear relationship, is provided by the equation's first order derivative, including maximum stiffness before failure. To evaluate maximum stiffness, air-sacs were each modelled separately, allowing the resultant regressions to truncate at the real point of failure. Thus, maximum stiffness and its variation are shown as means $\pm$ SD of stiffness at mechanical failure for each air-sac of a given species.

Mechanical work required to compress alkalized *C. edulis* air-sacs

To quantify the mechanical work performed by the air-sacs as they transduced changes in pH into changes in air-sac volume, we used a two-part experiment, first driving excised air-sacs at zero hydrostatic pressure to expand in response to a known increase in pH, then applying a hydrostatic pressure to compress the air-sac back to its starting volume. This was achieved as follows: Air-sacs from 11 live 4th instar *C. edulis* larvae were excised and placed into the same experimental pressure chamber described above, and individual heads were imaged and

documented in the same manner as described above. Here, however, the air-sacs were exposed to three stepwise changes in pH (through pH 6, 7, and then 8) with no hydrostatic pressure applied, followed by the same method of incremental pressure increase as above: 207 kPa every ten minutes until air-sac failure. All pH buffers were of the same composition described above, with differing amounts of HCl titrating pH to the desired value. The air-sacs were left to equilibrate at each pH for 2 h to reach their final sizes before imaging. For pHs 6 and 7, the air-sacs were exposed to the subsequent pH by removing the pressure chamber lid with air-sacs still attached, filling the chamber with the next solution, then carefully reattaching the lid and air-sacs for equilibration, with pressure applied after equilibration and imaging at pH 8.

The effect of pH on the length of unpressurized air-sacs was fitted to a quadratic linear model chosen via iterative model selection using AIC. Subsequent compression values had a cubic spline applied to show the varying degrees of compression over the range of pressures applied. Plotted side by side with the same y-axis scale of % air-sac length change, the results of this two-part experiment allow a comparison of the work resulting from a decrease in length caused by pressurizing the air-sac at a constant pH of 8 with the increase in length caused by its initial alkalization from pH 6 to 8 under constant pressure at zero depth/hydrostatic pressure. The total mechanical work (W) done on the pH 8 air-sac by the stepwise increases in hydrostatic pressure was calculated by summing the work done on the air-sac by each step increase in pressure:

$$W = \sum F \Delta L \quad \text{Eq. S6}$$

where F is the force (N) applied during each pressure increase and ΔL the resulting change in air-sac length. As pressure, and not linear force, was used to compress the air-sacs, F in Eq. S6 was first obtained by multiplying the pressure increment by the air-sac's transverse cross-sectional area (i.e., the area perpendicular to its long axis; its axis of compression). But because an air-sac's cross-sectional area varies along its long axis, so too does the magnitude of the force exerted by the pressure perpendicular to this axis. To account for this, the mean air-sac radius (r), was calculated from the profile area and length of the air-sac as

$$r = (A_0/L_0)/2 \quad \text{Eq. S7}$$

and this r was then used to calculate the mean circular transverse cross-sectional area (A_{cs}): the area over which pressure (P) acts to compress air-sac length. From this, F could be calculated as

$$F = A_{cs} \times P \quad \text{Eq. S8}$$

Near the beginning of most elastic deformations, there is a period where length changes linearly with pressure applied. Here, beginning with the unpressurized air-sac length at pH 8, this period of linear compression encompasses the air-sacs' initial length at pH 6 during the unpressurized experimental phase, corresponding to a depth range of from 0 m to 84.4 m. This range of data was used to establish the relationship between applied pressure and air-sac length necessary for the calculation of mechanical work. Linear compression was supported by AIC model comparison, where a linear mixed effects model assuming a linear relationship between length and depth change performed better than one which includes a quadratic term to accommodate curvature. A quadratic linear mixed effects model using the nlme() package in R

(51) was used to model work done on *C. edulis* air-sacs in mJ m^{-1} . Larval identity was modeled as a random factor and increase in variance over the experiment's progression was incorporated using the varExp() function to apply an exponential variance structure.

Percent change in length of excised *C. edulis* air-sacs over a range of pH from 2 to 10

The posterior air-sac pair was dissected from the body cavity of a larva submerged in pH 2 buffer (Hydrion, Micro Essential Laboratory Inc. Brooklyn, USA). Undamaged air-sacs not obscured by attached chromatophores or debris following dissection were selected for use. 11 air-sacs from 11 individuals were loaded singly into square walled borosilicate capillary tubes, 1 mm $\times$ 1 mm in cross section, 55 mm long. Tubes were initially filled with pH 2 solution and held vertically over the air-sac, allowing it to float upward into the tube. The tube was then turned horizontal, arresting its ascent. The tube was then placed in a bracket which allowed for flowthrough of subsequent pH buffers and imaging using a with a digital microscope (AM73915MZTL Dino-Lite Edge 3.0. The data is shown using a LOESS function performing local polynomial regression fitting.

Image processing and data handling

As pressure was increased in a stepwise fashion, followed by a 10-minute equilibration period, an air-sac may still be intact during imaging, but then collapse during imaging of the air-sacs in the other wells, before the pressure for all was increased once more. Alternatively, an air-sac may collapse during the process of increasing the pressure, or immediately before it was imaged at that pressure. Therefore, the maximum pressure at which each air-sac is shown in the data may either be the true maximum pressure it reached shortly before collapsing, or it may be 207 kPa less than the pressure at which it ultimately collapsed.

Our field arrangement of microscope and chamber led to there being some lighting and focus variation, sometimes affecting the air-sac area recorded via thresholding. Brightness differences were minimised by individually adjusting the brightness and contrast for each image in the sequence using a reference from that set of images. ImageJ's image brightness histogram was used to aid human judgement. This prevented increases in brightness from reducing the threshold area, which would artificially reduce the measured air-sac size. Variation in threshold area caused by obscuring chromatophores, debris, or lack of contrast was reduced with manual spot drawing using the ImageJ brush tool (kept to a minimum). Focus variation is more difficult to compensate for without more extensive, and bias prone, touching up of the air-sac boundaries. Therefore, some variation due to this error remains in the data set. Focus varied little over the experiments, however.

Throughout the main text, where we refer to observed biological variation, standard deviation ($\pm$ SD) is shown. Where we refer to the confidence of a modelled estimate, a 95% confidence interval (95% CI) is shown. Variation around maximum air sacs stiffness is shown as $\pm$ SD because the spread of the termination points of each regression represents observed biological variation, not the fit of a model.

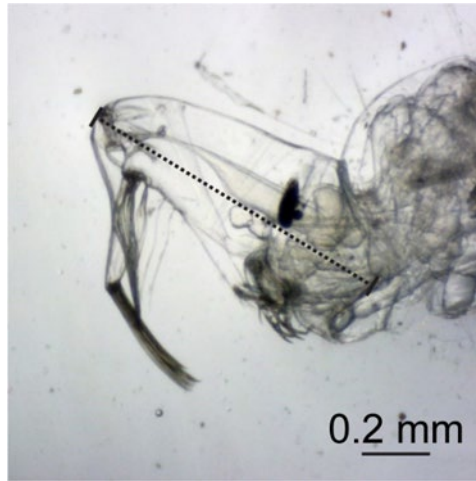


Fig. S1.

4th instar *Chaoborus edulis* showing location of head capsule length measurement (dashed line).

5

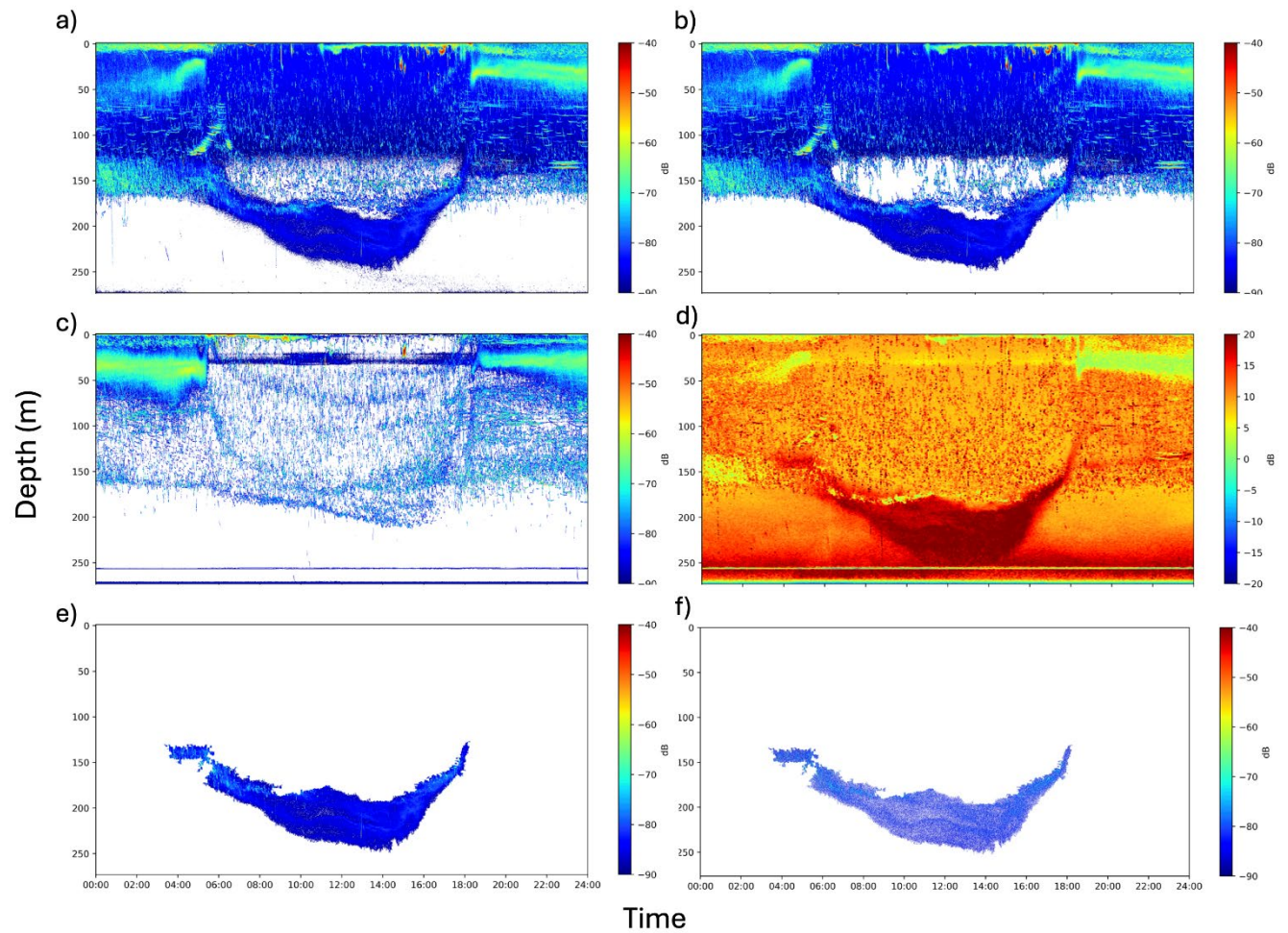


Fig. S2.

- 5 Processing workflow for detecting *C. edulis* layers showing a) 200 kHz echogram smoothed over a 12 ping by 0.2 m grid, b) Results of layer detection run on the echogram in a), c) 120 kHz echogram smoothed over the same grid size, d) $S_{v200}-S_{v120}$ echogram with maximum filter applied over a 5 column by 5 ping grid, e) 200 kHz echogram with a mask applied where layers > -90 dB re 1 m^{-1} detected in 1 and layers > 13 dB detected in d), and f) the same mask applied
- 10 to the 200 kHz data at full resolution. The y axis shows range from the transducer in metres while the x axis shows time in columns of 12 pings (60 seconds) for a) to e) and 1 ping (5 seconds) in f).

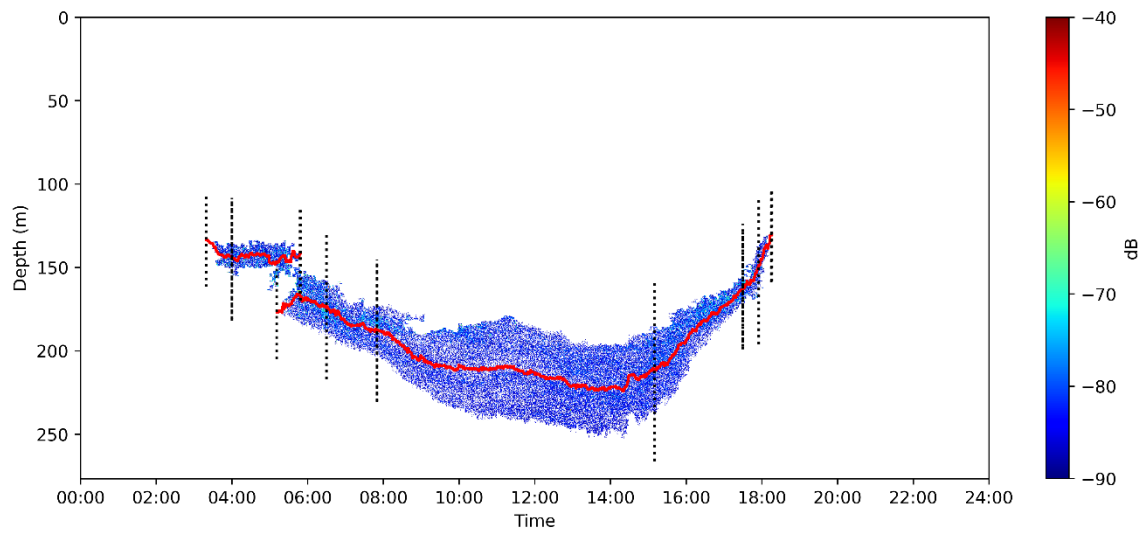


Fig. S3.

Example of the results of change point detection on *C. edulis* layers. The red line shows the mean depth of the layer and the dotted lines show the predicted break points.

5

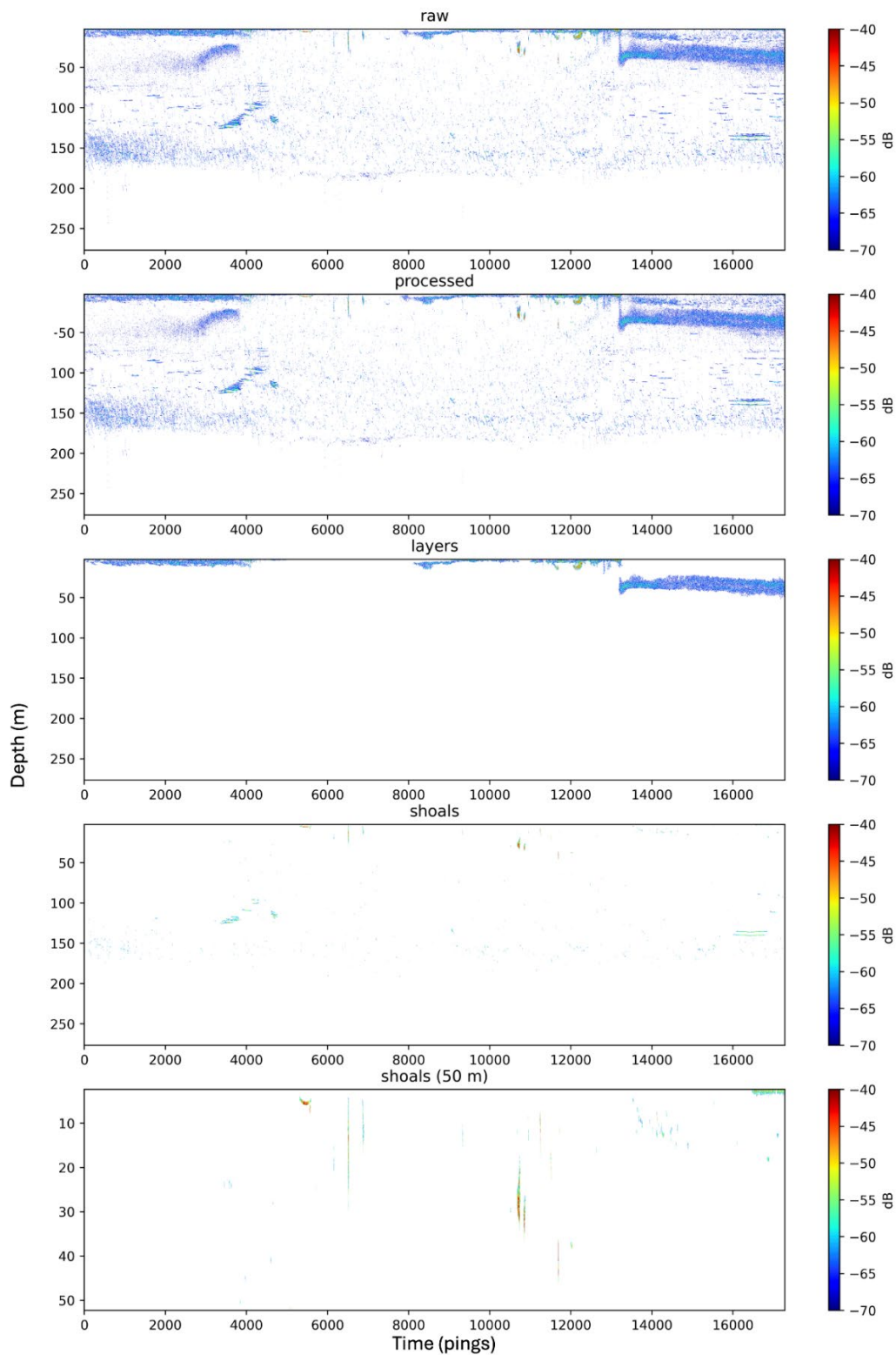


Fig. S4.

Processing workflow showing the raw 120 kHz echogram, the cleaned echogram with noise removed, the detected layers and the detected fish shoals.

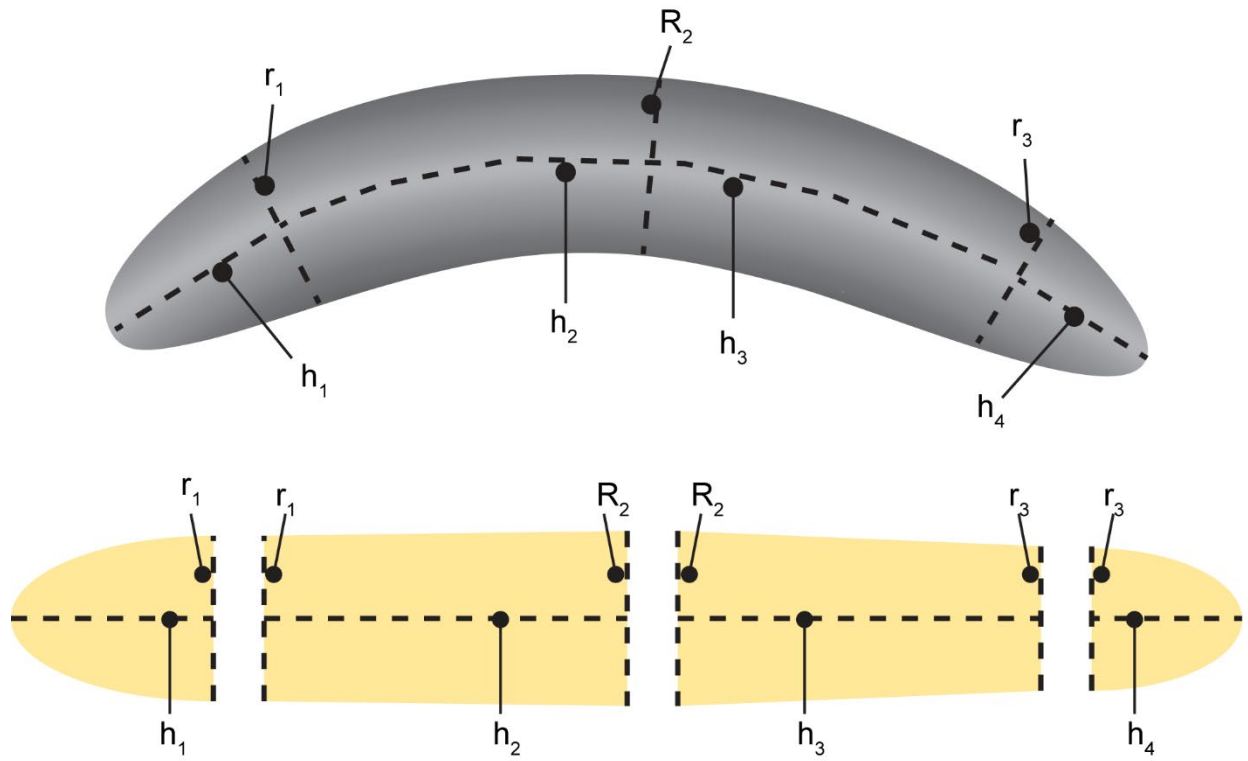


Fig. S5.

- 5 To estimate the volume for a *C. edulis* air-sac it was divided into four segments: two prolate ellipsoids hemispheres (the air-sac tips) and two truncated cones (the mid-section). Air-sacs were divided as indicated by the locations of the radii r_1 , R_2 and r_3 . The length of each segment provided the height h_{1-4} for each section.

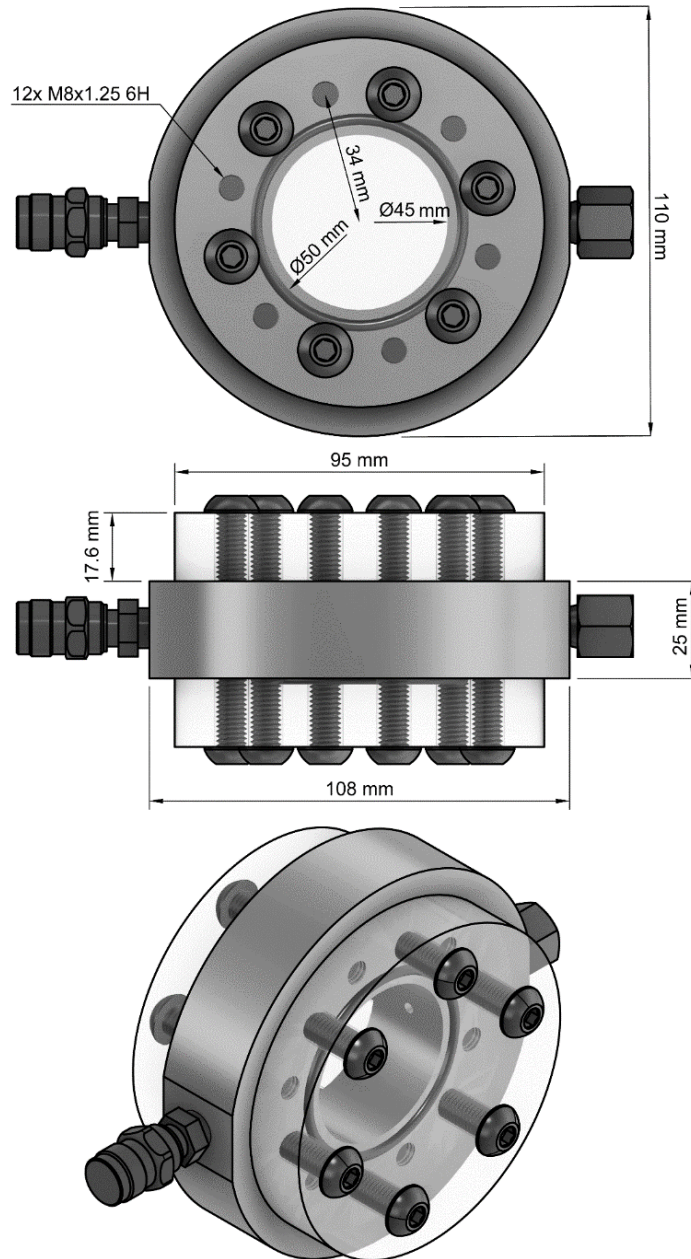


Fig. S6.

Pressure chamber with viewing windows for simulating dives to depth. The chamber is a block of aluminum machined with a central cavity that is closed on either side by clear acrylic windows sealed by O-rings. A female $\frac{1}{4}$ " NPT fitting (RHS) allowed the chamber to be connected to a screw pressure pump. A bleed valve (LHS) allowed air bubbles to be purged and the chamber to be filled.

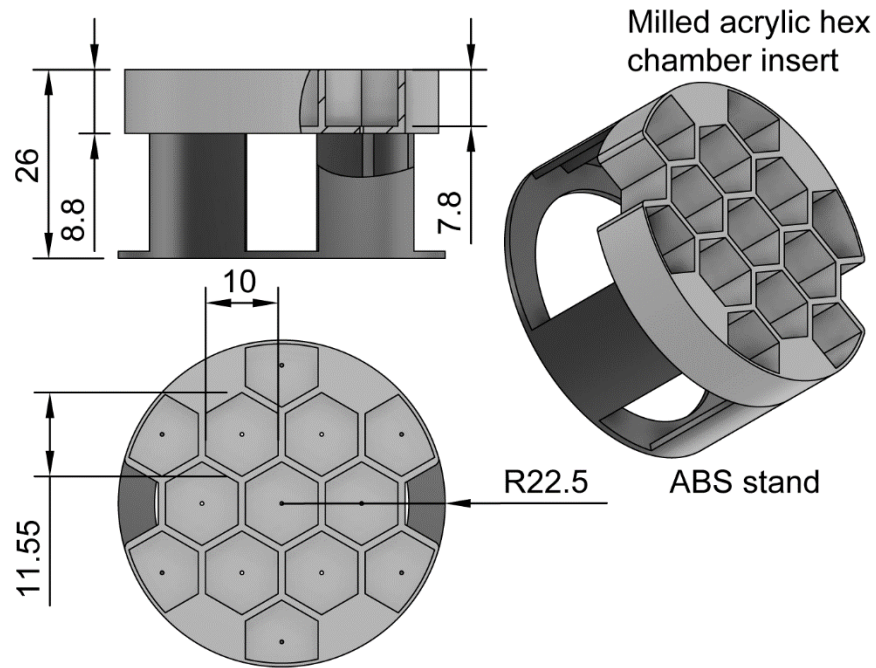


Fig. S7.

Insert with hexagonal chambers and stand used to contain individual larvae and pupae within the pressure chamber during crush-depth experiments. The 13-cell chamber was milled from clear acrylic. A 3D printed stand made of ABS plastic was used to position the insert level with the top of the pressure chamber such that the pressure chamber window would close the open tops of the chambers.

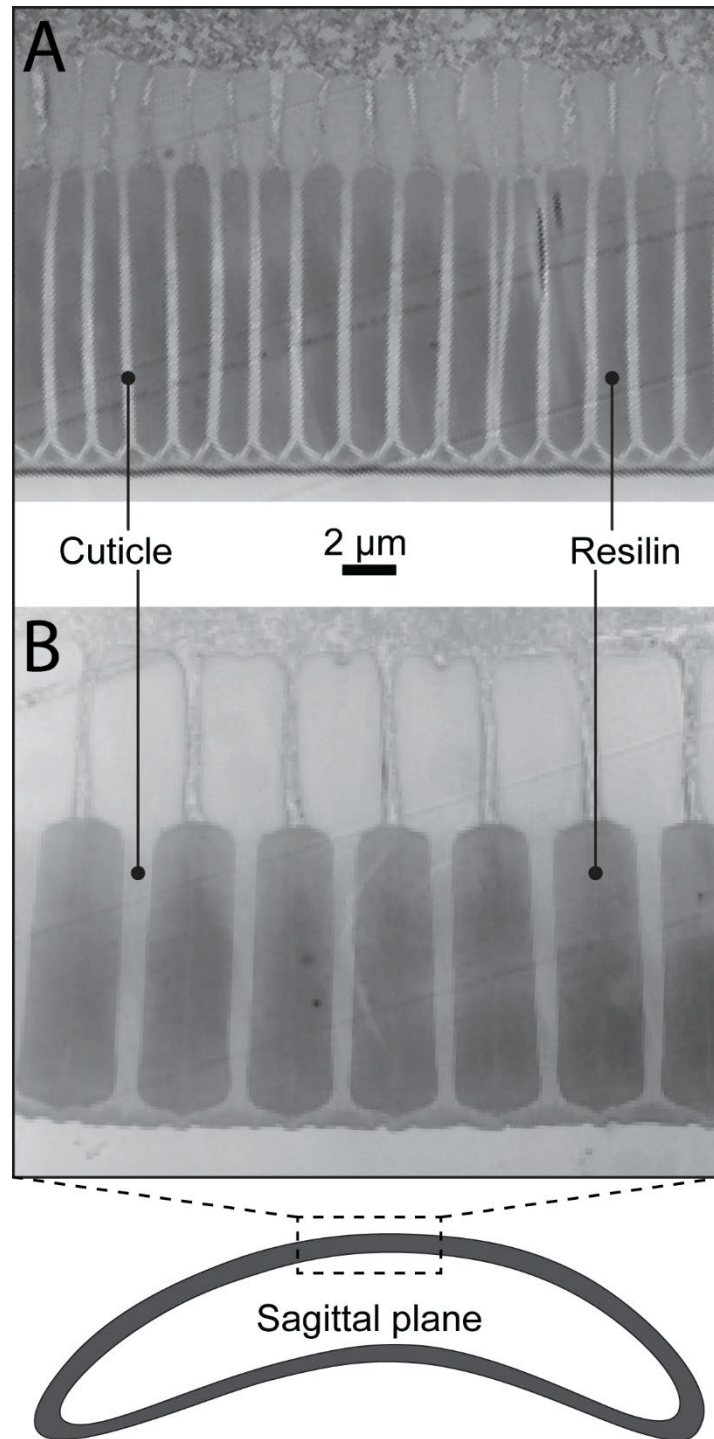


Fig. S8.

Transmission electron micrographs (TEM) of sagittal sections through *C. edulis* (A) and *C. americanus* (B) air-sac walls revealing the bands of cuticle and resilin in cross-section. Air-sac exterior surface and epithelium are seen at the top of each image, and inner air-sac lumen is seen at the bottom.

Table S1. Sound scattering layer detection parameters.

Parameter	Value
Minimum duration	2 hours
Minimum thickness	3 m
Maximum thickness	90 m
Minimum separation	0.5 m
S _v threshold	-90 dB re 1m ⁻¹ at 200 kHz / S _{V200} -S _{V120} > 13 dB

Table S2. Parameters used for single target detection and fish track detection.

Process	Parameter	Value
Single target detection	Target strength threshold	−65 dB re 1 m ²
	Pulse length determination level	6 dB
	Minimum normalised pulse length	0.7
	Maximum normalised pulse length	1.5
	Maximum beam compensation	4 dB
Fish track detection	Alpha	Major: 0.7 Minor: 0.7 Range: 0.7
	Beta	Major: 0.5 Minor: 0.5 Range: 0.5
	Target gates (Exclusion)	Major: 4 m Minor: 4 m Range: 0.7 m
	Target gates (Missed ping expansion)	Major: 0% Minor: 0% Range: 0%
	Weights (Group A)	Major: 30 Minor: 30 Range: 40 TS: 0 Ping gap: 0
	Weights (Group B)	Major: 0.6 Minor: 0.6 Range: 0.8 TS: 0 Ping gap: 0
	Minimum targets per track	3
	Minimum pings per track	3
	Maximum gap between single targets	5 m

Movie S1.

An excised *C. edulis* air-sac in pH 6 buffer solution being exposed to increasing hydrostatic pressure before imploding at a pressure of 5.647 MPa, equivalent to a depth of 577 m in Lake Malawi.

5

Movie S2.

An excised *C. edulis* air-sac in pH 6 buffer solution being exposed to increasing hydrostatic pressure before imploding at a pressure of 4.964 MPa, equivalent to a depth of 507 m in Lake Malawi.

10

Movie S3.

Timelapse of a pair of excised *C. edulis* air-sacs showing their characteristic decrease in length, but not diameter, during exposure to stepwise increases in pressure from 0.21 to 4.76 MPa (21 to 486 m depth). Each frame corresponds to a 207 kPa increase in pressure. The lower air-sac is the same as in Movie S2.

15

Data S1. (separate file)

File: Data_S1_Lakefly_layers: *Chaoborus edulis* larval layers detected in Lake Malawi using a moored active acoustic instrument in August 2023.

20

Data S2. (separate file)

File: Data_S2_fish_tracks: Individual fish detected in Lake Malawi using a moored active acoustic instrument in August 2023

Data S3. (separate file)

File: Data_S3_Water_quality_sonde: 250 m deep vertical water quality transect recorded using YSI EXO1 sonde in Lake Malawi in August 2023

25

Data S4. (separate file)

File: Data_S4_Chaoborus_airsac: Air-sac experiments and *in vivo*/excised depth of failure data for all *Chaoborus* species

30

THE UNIVERSITY OF BRITISH COLUMBIA



Dr Philip G. D. Matthews
6270 University Boulevard
Vancouver, B.C., Canada V6T 1Z4
Tel: 604-827-2773
Fax: 604-822-6973
pmatthews@zoology.ubc.ca

Reference Letter for Evan McKenzie

To the selection committee,

I am delighted to recommend my PhD student Evan McKenzie (who successfully defended his dissertation in November 2025) for the 2026 Canadian Council of University Biology Chairs Graduate Student Research Prize to recognize his outstanding research paper *Crush-resistant air-sacs allow insect larvae to exploit aquatic habitat at extreme depth*, recently accepted for publication in the prestigious journal *Science*. This paper is the culmination of his PhD research elucidating the physiology and biomechanics of a remarkable buoyancy regulation system possessed by the aquatic larvae of the *Chaoborus* midge: hydrostatic air-sacs derived from the insect's tracheal system. These larvae are the only aquatic animals, other than teleost fish, that regulate their buoyancy by controlling the volume of internal gas-filled sacs. However, while the ability of *Chaoborus* larvae to control their buoyancy has been investigated since the turn of last century, the actual mechanism they use to control their air-sac volume remained unresolved until Evan's 2022 paper in *Current Biology*. This work described an entirely new pH-powered hydrostatic control mechanism, where the air-sac's gas-filled volume is regulated by bands of the protein resilin within the air-sac wall expanding or contracting in response to pH change. His *Science* paper builds on this work by exploring the limits of *Chaoborus* air-sac function at depth. The bulk of this research focuses on the larvae of *Chaoborus edulis*, a species native to Lake Malawi in East Africa, one of the deepest lakes in the world. Evan's curiosity, exceptional experimental skills and incredible hard work allowed him to characterize the crush resistance and work capacity of their air-sacs, while the deployment of a sonar system demonstrated that *C. edulis* larvae routinely dive over 200 m each day, making them the world's deepest free-diving insect. Furthermore, his work suggests that the absence of aquatic insects from the open ocean cannot simply be attributed to hydrostatic pressure causing collapse of their air-filled tracheal system, as has been suggested previously. As *C. edulis* demonstrate, insects can evolve air-filled tracheae resistant to implosion at great depth.

All of Evan's work on *C. edulis* was completed during a 1-month research trip to Nkhata Bay, Malawi, in August, 2023. With funding from The Society for Integrative and Comparative Biology (a prestigious Fellowship of Graduate Student Travel) and the Company of Biologists (Travelling Fellowship), and with the assistance of two researchers from Malawi's Department of Fisheries, Evan successfully conducted experiments on *C. edulis* larvae collected from Lake Malawi to determine the biomechanical properties of their air-sacs. Using apparatus and experimental approaches he developed, Evan discovered that their air-sacs have the capacity to transduce changes in pH into mechanical work up to a maximum depth of ~50 m but can compress and resist implosion up to an astonishing 500 meters deep. He also assisted with the deployment and retrieval of a multi-frequency sonar system at a depth

THE UNIVERSITY OF BRITISH COLUMBIA

of 300 m in Lake Malawi, confirming that the larvae routinely descend over 200 m into the lake's anoxic hypolimnion during the day before returning to the surface waters to hunt zooplankton at night. The sonar data was analyzed by collaborators at St Andrews in the UK. To compare the biomechanical properties of *C. edulis* air-sacs with those from shallower-diving species, Evan also investigated air-sacs from two Canadian *Chaoborus* species which live in shallow lakes and ponds, while I collected data from an additional two species from Lake Victoria in Kenya, a lake with an intermediate maximum depth of ~80 m. Evan performed all the data extraction and analysis.

In my view, Evan's development of new experimental techniques and apparatus to interrogate the function of *Chaoborus* air-sacs, as well as the truly novel findings that his work has produced, spanning physiology, ecology and biomechanics, indicates a combination of imagination, insight and experimental skills which set him apart from many of his peers and marks him out as a researcher with a bright future. I wholeheartedly recommend that his paper be given the strongest possible consideration for the CCUBC Graduate Student Research Prize.

Sincerely,

A handwritten signature in black ink, appearing to read 'P. G. D. Matthews', with a stylized, cursive script.

Assoc. Prof. Philip G. D. Matthews, 20th May 2026