Article

Flight Capacity and Response to Habitat Drying of Endemic Diving Beetles (Coleoptera: Dytiscidae) in Arkansas (USA)

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Abstract: The ability to colonize new habitat is essential for wild populations affected by disturbance or other forms of habitat change. For aquatic insects in small streams, overland flight is an important strategy for dispersal when barriers to in-stream migration exist and when populations are isolated in upland habitats. Two Ozark-endemic water beetles (*Heterosternuta sulphuria* and *Heterosternuta phoebeae*) have shown little overlap in distributions, with the former frequently occurring in small upland watersheds and the latter occurring in aquatic habitats farther downstream in larger watersheds. Because *H. sulphuria* has been associated with perennial aquatic habitats, we hypothesized that *H. sulphuria* individuals could exhibit low capacity for flight, thereby affecting population distributions over time. Laboratory flight observations showed that zero individuals of *H. sulphuria* flew ($n = 67$), whereas 17 of 76 individuals of *H. phoebeae* were observed to fly. Stream habitat drying experiments provided further evidence of the weak capacity for flight and overland migration of *H. sulphuria*, with low probabilities of survivorship in microhabitats exposed to drying. Weak flight capacity and apparent intolerance to habitat drying have important implications for the evolutionary history and conservation of *H. sulphuria* in small Ozark streams exposed to variable flow regimes and stream margins vulnerable to disturbances.

Keywords: diving beetles; ecohydrology; disturbance; headwaters; Ozark

1. Introduction

Heterosternuta sulphuria Matta and Wolfe, 1979 and *Heterosternuta phoebeae* Wolfe and Harp, 2003 are endemic predaceous diving beetles occurring in streams on the Ozark Plateau [1–3], located within the Interior Highlands in the mid-continental United States. Because of limited historical records and a high priority ranking in the Arkansas Wildlife Action Plan, *H. sulphuria* was the focus of surveys throughout northern Arkansas where the goal was to identify additional occurrences of this species of concern. Updated distributional records for *H. sulphuria* in 2009 included 48 occurrences across 14 counties in northwestern Arkansas [3]. In addition to new distributional information for *H. sulphuria*, a total of six other hydroporines have been collected from historical surveys: *Sanfilippodytes* (sp.) Franciscolo, 1979; *Hydrocolus* (sp.) Roughley and Larson 2000, *Heterosternuta wickhami* Zaitzev 1908, *H. ouachita* Matta and Wolfe 1979; *H. pulchra* LeConte 1855; and *H. phoebeae* Wolfe and Harp 2003. Among these, *H. phoebeae* [2] is also a species of greatest conservation need (SGCN) in Arkansas because of its narrow distribution across only a few counties [2,3].

Distributional records for *H. sulphuria* and *H. phoebeae* have shown ecological segregation between these congeners in shared watersheds, with *H. sulphuria* occupying aquatic habitats in the upper portions of watersheds (median watershed area = 1.75 km^2, $n = 42$) that are often associated with perennial flows, whereas *H. phoebeae* has primarily been collected from larger watersheds (median watershed area = 13.84 km^2, $n = 13$) [3,4] associated with greater water volumes and more variable flows. These species have been found

together at only two sites [3,4], and from a historical survey of large Ozark streams, only 3 individuals out of over 1100 total dytiscid specimens collected were *H. sulphuria* (G.L. Harp, in lit.).

The occurrence of *H. sulphuria* in small upland watersheds and its close association with perennial rather than ephemeral habitats indicates that the capacity for overland flight could be limited, thus exposing populations to threats associated with disrupted flows in small streams (including periods of zero-flow days) [5] and rendering them vulnerable to disturbances that affect the aquatic-terrestrial margins, a habitat that hydroporines prefer and the important terrestrial pupation habitat for these beetles. To better understand environmental flow relationships and assess threats to these endemic species of concern, this study was conducted to determine the flight capacities of these endemic beetles and to investigate responses to experimental habitat drying.

2. Materials and Methods

Heterosternuta sulphuria individuals were collected from five different Ozark streams and *H. phoebeae* individuals were collected from one stream (because of low occurrences of *H. phoebeae* across sampled sites). We collected *H. sulphuria* individuals across multiple watersheds to account for potential variation in flight capacity among potentially disconnected populations, a basis for our premise of low flight capacity for this species. Individuals of *H. phoebeae* were only able to be collected from one location because of the relatively limited distribution of this species. Individuals were collected using either a small plastic pipette or a D-frame net by gently sweeping the substrate at stream margins. Live individuals collected were placed into a cooler with water and substrate from the stream. After transporting to the laboratory, beetles were placed in groups of 10–15 individuals in small plastic aquariums with approximately 8 cm of water prior to individual flight observations. Following observations of flight and the habitat drying experiment, individuals were killed in 70% ethanol and stored in glass vials and species were confirmed; data associated with species that were neither *H. sulphuria* nor *H. phoebeae* were excluded from further analysis.

2.1. Flight Observations

Individual flight was observed using a small shallow pan (35 cm L × 23 cm W × 5 cm H) with a piece of cardboard fastened to the bottom of the pan, similar to the design used in a previous study [6] (Figure 1). The sides of the pan were sufficiently steep and slippery to prevent any escape by crawling beetles. An incandescent 60 W light and a white curtain were hung above and enclosed the observation area to slightly increase the ambient laboratory temperature while also providing soft lighting to mimic sunlight.

Two individuals of the same species were placed into the testing pan and flight activity was recorded during a 20 min observation period for each pair of beetles. While interactions among individuals in this setup could occur and potentially affect flight, we did not observe any apparently antagonistic interactions and individuals were observed to move independently and typically with minimal to zero contact between individuals. Because we wanted to make sure the 20 min timeframe was sufficient, following the 20 min observation period individuals were placed into small dry pots (7 cm diameter × 3.5 cm height) and individual pots were placed into a small shallow pan as described above, with this pan filled to 8 cm with water. The setup was covered by a net that allowed individuals to fly from the clay pots yet be retained within the water in the bottom of the pan, if and when individuals flew after the 20 min observation. Beetles in these post-observation tests were held in place for 24 h and individuals were recorded as flight capable if they occurred in the water or on the pot-enclosure net. Therefore, individual flight capacity per individual was recorded from (1) flight within our 20 min observation window and (2) from the number of individuals recovered from water substrate or clinging to the enclosure net during or at the termination of the 24 h post-observation period. For both species, the numbers of flying individuals were tallied across both 20 min and 24 h observational periods and summed to gain the total number of flight-capable individuals per species

across all observations (Table 1). Each individual was tested only once. Following flight observations, all individuals were killed and stored in 70 percent ethanol. Individuals were removed from ethanol for determination of body size (L × W), species confirmations, and sex. Body length was measured using a lens micrometer fitted to a stereomicroscope, with length (L, mm) measured as the distance from the front clypeal margin of the head to the apex of the elytra, and width (W, mm) measured as the distance across the widest point of the elytra. The sex of each individual was determined through observation or dissection of genitalia. Flight capacity among species was compared between the two species using a chi-square test in SYSTAT version 13.00.05.

Figure 1. To assess flight of *Heterosternuta sulphuria* and *H. phoebeae* (**top left**), beetles were placed in small shallow pans (35 cm L × 23 cm W × 5 cm H, top left) with a piece of cardboard fastened to the bottom of the pan. The sides of the pan were sufficiently steep and slippery to prevent any escape by crawling beetles. An incandescent 60 W light and a white curtain were hung above and enclosed the observation area to slightly increase the ambient laboratory temperature while also providing soft lighting to mimic sunlight. Small, plastic disposable funnels (26 cm D × 30.5 cm H) filled with sand substrate were used to represent wet and drying habitats (**bottom left**, and images on **right**). Photographs by Scott Longing.

Table 1. Locations where individuals of either *Heterosternuta sulphuria* or *H. phoebeae* were collected and the number of male and female individuals that flew during laboratory observations.

Stream	GPS	# Observed	# Flew (Male)	# Flew (Female)
H. sulphuria				
Wildcat Creek	36.160991–94.308550	36	0	0
Unnamed stream	35.995116–94.136201	14	0	0
Unnamed stream	36.126359–94.184033	11	0	0
Unnamed stream	36.156602–93.547469	6	0	0
Total		67	0	0
H. phoebeae				
Shop Creek *	35.952400–93.243328	71	8	7

* one individual *of undetermined sex* flew, to total 16 individuals that flew.

2.2. Habitat Drying Experiment (H. sulphuria)

For the habitat drying experiment, *H. sulphuria* individuals were collected from the middle prong of Sneeds Creek, a perennial, spring-fed stream in the Ponca Wilderness Area of the Buffalo National River. A blocked experimental design was used with one 10-gallon aquarium representing 1 block (15 blocks total). One aquarium was treated as one block containing both treatments because of the differential exposure of aquariums to minor HVAC airflow in the laboratory, which resulted in different rates of habitat drying among blocks. Each aquarium was filled with approximately 5 cm of water, with one "drying" treatment microhabitat and one control microhabitat positioned at the bottom and opposite ends of each aquarium. Small, plastic disposable funnels (26 cm D × 30.5 cm H) were used to represent habitats. Each funnel was filled approximately 2/3 full with sand substrate. To inhibit beetles crawling out of the funnel yet still allow an open top for beetles to fly/escape, one additional funnel of the same size was cut, inverted, and fastened to the funnel containing sand substrate and beetles. A small piece of Nalgene tubing with a stopcock was attached to the bottom funnel tube to regulate water flow out of the funnel (i.e., to simulate habitat drying). To facilitate potential movement/flight out of the microcosms, we extended the tops of the aquariums using 40 cm pieces of cardboard fastened to each side of the aquariums and placed a mesh top on the cardboard extenders (Figure 1).

In each of the fifteen aquariums, eight beetles were transferred from small plastic aquariums to each of the microcosm funnels (two funnels per aquarium, with four beetles per drying or control treatment). Therefore, we exposed a total of 120 beetles to either a permanently wet control (*n* = 60 beetles) or a microhabitat drying treatment (*n* = 60 beetles). Beetles in both drying and control funnels were allowed to acclimate for 24 h before the experiment. The acclimation period resulted in no beetle mortality or escape from funnels (beetles did not fly from open funnels). Following the acclimation period, water began draining from the drying treatment over a period of 144 h, at which time the sand substrate was completely dry. At 24 h intervals, we recorded the number of dead individuals within each funnel and the number of individuals that flew from either control or drying treatments (beetles that ended up in the water at the base of the 10-gallon aquarium). Data were analyzed using survival analysis in SYSTAT version 13, where probabilities of survivorship were calculated for both the control and drying treatments. The effect of block (i.e., aquarium) was included as a covariate in the analysis. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

3. Results

3.1. Body Size Comparison

The average body length and width were significantly different among *H. sulphuria* and *H. phoebeae* individuals (*H. phoebeae* mean L = 39.070 mm, mean W = 19.451 mm; *H. sulphuria* mean L = 33.448 mm, mean W = 16.552 mm) (*p* = 0.000) (Figure 2). These differences in size

are concordant with differences in aquatic habitat size (i.e., stream channel width) occupied by the two endemic congeners, with the smaller species (*H. sulphuria*) occupying upstream aquatic habitats and the larger species (*H. phoebeae*) occupying larger downstream habitats.
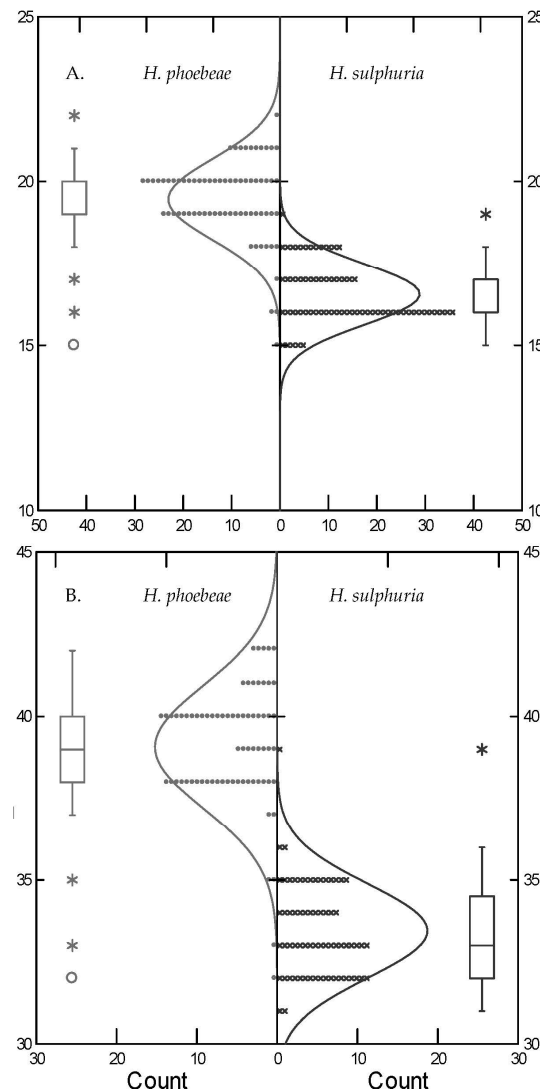

Figure 2. Differences in body size between *Heterosternuta sulphuria* and *H. phoebeae*, shown by measurements of length (mm *y*-axis, count of individuals: *x*-axis) (**A**) and width (**B**) for 67 and 71 individuals, respectively. Both variables were significantly different among the two species based on a two-sample *t*-test. Box plots depict the minimum, first quartile, median, third quartile, and maximum, with outliers depicted as single points. * These appear to be sample data points outside the quartiles.

3.2. Flight Observations

Sixty-seven *H. sulphuria* and 72 *H. phoebeae* individuals were observed for flight. During flight observation periods, 17 *H. phoebeae* individuals were observed to fly (23.6 percent) (Table 1). Nine *H. phoebeae* individuals flew during both the 20 min and 24 h observations, seven beetles flew only during the 24 h observation period, and one beetle flew only during the 20 min observation period. Across sexes, approximately equal numbers of males and females were observed to fly (Table 1). Zero *H. sulphuria* individuals flew between both the direct and 24 h observations, so frequencies of flight were highly significantly different between *H. sulphuria* and *H. phoebeae* (Pearson chi-square $\chi^2 = 16.826$, *p*-value = 0.000).

3.3. Habitat Drying Experiment (H. sulphuria)

Highly significant differences in survival between control and drying treatments were indicated by chi-square statistics: Mantel ($\chi^2 = 103.67$, $p = 0.000$), Breslow–Gehan ($\chi^2 = 91.06$, $p = 0.000$), and Tarone–Ware ($\chi^2 = 96.87$, $p = 0.000$) (Figure 3). All *H. sulphuria* individuals exposed to habitat drying ($n = 60$) died within 144 h whereas five beetles in control treatments died. The numbers of beetles in the drying treatment were observed to significantly decline after approximately 75 h of drying, whereas little mortality was observed within 24 or 48 h when the substrate still contained some moisture. Mortality reached 100% in the drying experiment after approximately three days, when the sand substrate was completely dry. Zero beetles were observed to fly during the experiment from the open microcosms, across both drying and wet treatments. Moreover, there was a significant block effect attributed to the positions of blocks: Mantel ($\chi^2 = 6.52$, $p = 0.952$), Breslow–Gehan ($\chi^2 = 11.69$, $p = 0.631$), and Tarone–Ware ($\chi^2 = 8.99$, $p = 0.832$); several adjacent aquariums were located in a position where they received more air flow, so drying and subsequent mortality occurred approximately 24 h earlier in these aquariums.
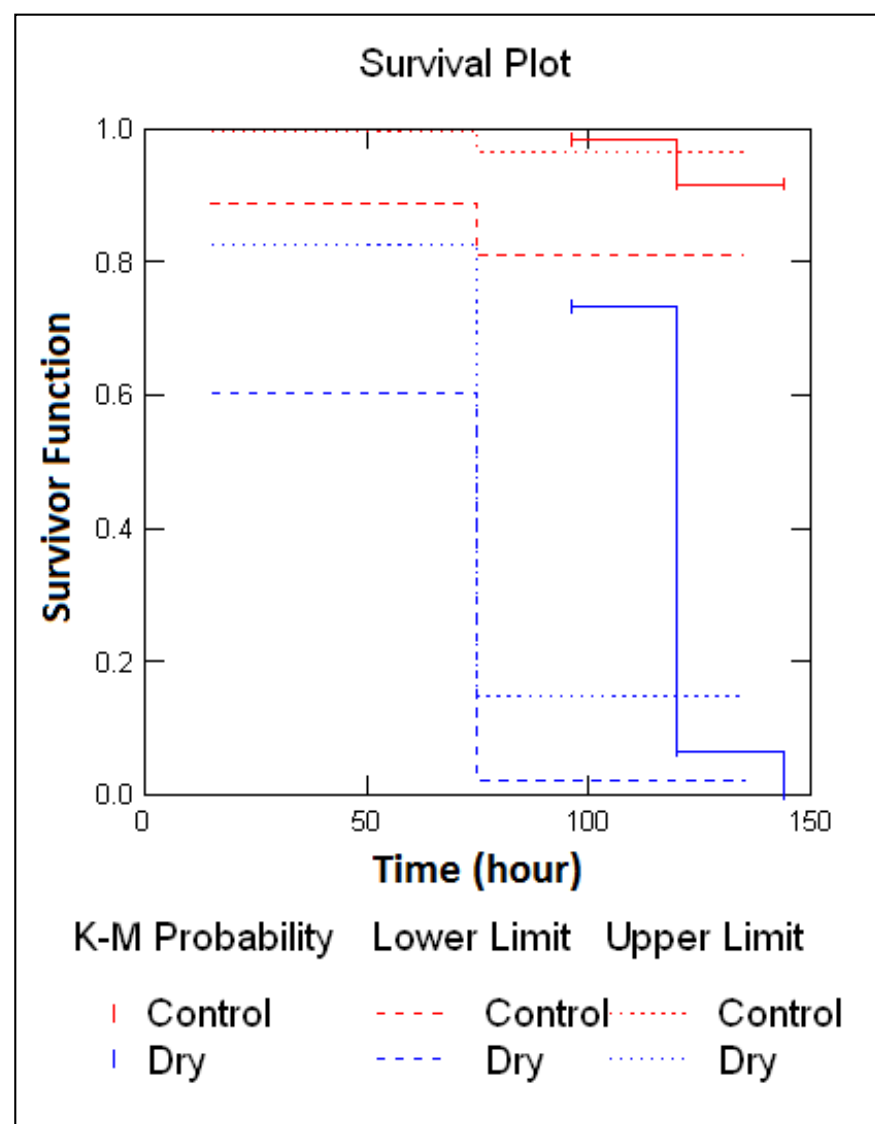

Figure 3. Survival analysis using the Kalan-Meier model probability survivor function and chi-square test of survivorship over the length of the drying experiment (144 h) (SYSTAT ver. 13). Data show a dramatic decrease in probability of survivorship of *H. sulphuria* individuals from drying mesocosm habitats compared to control habitats where mesocosms remained wet.

4. Discussion

In this study, we found a low capacity for flight by the endemic Sulphur Springs diving beetle, *H. sulphuria*. The occurrence of *H. sulphuria* in perennial aquatic habitats suggests that populations could have undergone evolutionary adaptation towards flightlessness because individuals lack the need to disperse to new habitats. This has serious implications regarding the conservation of *H. sulphuria* populations in Ozark stream networks, especially from threats affecting perennial flows that could alter the flow-ecology relationships [7] of vulnerable populations containing flightless individuals. Evidence of flightlessness in *H. sulphuria* is supported by past studies in related fauna showing variation in flight capacity among small water beetles, with differences in flight musculature explaining some of the variation [6].

Differences in body size between *H. sulphuria* and *H. phoebeae* are associated with flight capacity, since the former has a larger body and is also more capable of flight based on our observations. Some studies have demonstrated that increasing body size enhances flight ability for dispersal; among terrestrial insects, larger insects have been shown to contain more energy storage that enables longer flight durations and greater distances [8,9]. While variable sizes of hydrophilid water beetles persist among diverse aquatic habitats, the persistence and stability of habitats have been shown to drive dispersal in addition to differences in wing morphologies within species complexes [10]. Among diving beetles, dispersal is linked as well to habitat persistence and the species' ability to cope with changing environments [11], and this can be independent of body size, although to date relatively small hydroporines such as those tested in the current study have been shown to exhibit flightlessness ([6], this study). Moreover, the energetic cost of flight has been shown to decrease with increasing body size in some insects [12]. For *H. sulphuria*, the conditions of small body size, narrow distribution (towards upland aquatic habitats), and flightlessness represent a combination of factors that could present difficulties in sustaining populations over time among threatened habitats. In a study investigating upper thermal tolerances of diving beetles, Calosi et al. [13] found that small hydroporine beetles with lower upper thermal tolerances also had lower capacities to adapt to changing temperatures, and these were the diving beetles with narrower distributions and occurring in isolated mountain habitats. Thermal tolerances for *H. sulphuria* are unknown but represent a key piece of information for developing conservation actions and modelling distributions and risks associated with climate change and changing freshwater systems in the U.S. Interior Highlands.

Based on studies of diving beetles in the genus *Heterosternuta* [2,4,14], it is expected that *H. sulphuria* and *H. phoebea* are univoltine, with adults overwintering and ovipositing in early spring and larvae completing development by late spring to late summer. This means that dispersal would be necessary for both species to escape to refugia during severe seasonal drought or other disturbances [7]. Especially in isolated habitats, the flight ability of *H. phoebeae* would be a positive trait for migration and could be associated with their potentially high tolerance of habitat changes as compared to *H. sulphuria.* Moreover, the size of the watersheds occupied by these species shows ecological segregation, with *H. sulphuria* occupying smaller mean watershed areas and *H. phoebeae* occupying larger ones, with little overlap in occurrences. Therefore, habitats in perennial upland streams are likely more stable where groundwater inputs keep stream channel margins static, while downstream reaches can be prone to drying and greater water fluctuations in aquatic-terrestrial margins compared to stable perennial flows. These are conditions that could likely lead to a greater propensity for dispersal when conditions become unfavorable.

One idiosyncratic note related to these conditions is that the flightless *H. sulphuria* is more widespread geographically than is the flight-capable *H. phoebeae* [2–4]. However, *H. phoebeae* might use its capacity for flight only for short-range dispersal to maintain populations and to avoid potential risks, such as metabolic costs, predation, and dealing with more dynamic flow habitats (i.e., greater discharge) in larger watersheds [15]. For *H. sulphuria*, flightlessness could have more selective advantages in permanent aquatic habitats such as groundwater-mediated, small perennial streams [16]. Furthermore, alter-

native dispersal strategies could include transportation by passive dispersal using wind and animal vectors [17] or flooding [6].

Bert's predaceous diving beetle (*Sanfilippodytes bertae*), an endangered species in Canada, was suggested to have likely used groundwater conduits as a means of dispersal among surface-water, isolated spring habitats [18]. In Britain, flightless water beetles are understood to migrate between populations via wet intervening regions [19], including subsurface aquatic habitats. In our microcosm drying experiments, dying and dead individuals were mostly found head-down within the substrate and therefore it might be hypothesized that some individuals accidentally or purposely could enter groundwater systems and disperse to new locations. The weak flight capacity observed for *H. sulphuria* coupled with its occurrence in isolated mountaintop habitats suggests that this species could experience a substantial extinction debt and localized extirpations if stream drying occurs and where connections to groundwater habitats are severed. These evolutionary processes are highlighted with the subterranean fauna from Australia, where diving beetles have undergone major speciation in underground habitats, resulting from continental aridification during the late Miocene and early Pliocene [20], which led to dramatic extinctions but also the colonization of new groundwater habitats [21]. In North America, five subterranean diving beetles are known (with four of five occurring in Texas at southerly latitudes and in arid zones), where phylogenetic analyses revealed close associations with surface water genera including *Heterosternuta* [22]. Although the ecological and environmental flow-driven processes associated with surface and subterranean fauna in North America are unknown (especially comparted to what is known of the fauna in Australia), this ecological template represents a natural evolutionary laboratory where aridification in the southern United States could lead to changes in belowground aquatic biodiversity. Furthermore, Forest et al. [23] stated that a goal of biodiversity conservation should involve maintaining the evolutionary potential of biology. Accordingly, and in the case of *Heterosternuta* diving beetles, understanding influential habitat conditions including environmental flows and fluctuations in the aquatic-terrestrial and surface-subsurface ecozones remains a critical need for supporting *H. sulphuria* populations in affected small watersheds.

References

1. Matta, J.F.; Wolfe, G.W. New species of Nearctic *Hydroporus* (Coleoptera: Dytiscidae). *Proc. Biol. Soc. Wash.* **1979**, *92*, 287–293.
2. Wolfe, G.W.; Harp, G.L. A new species of predacious diving beetle, *Heterosternuta phoebeae* (Coleoptera: Dytiscidae), from the Ozark Mountains of Arkansas. *Coleopt. Bull.* **2003**, *57*, 117–121. [CrossRef]
3. Longing, S.D.; Bacon, P.; Harp, G.L. Distribution, conservation, and status of three endemic *Heterosternuta* (Coleoptera: Dytiscidae) in Arkansas. *J. Ark. Acad. Sci.* **2013**, *67*, 177–180.
4. Longing, S.D.; Haggard, B.E. New distribution records of an endemic diving beetle, *Heterosternuta sulphuria* (Coleoptera: Dytiscidae: Hydroporinae), in Arkansas with comments on habitat and conservation. *Southwest. Nat.* **2009**, *54*, 357–361. [CrossRef]
5. Leasure, D.; Magoulick, D.D.; Longing, S.L. Natural flow regimes of the Ozark and Ouachita Mountain regions. *River Res. Appl.* **2016**, *32*, 18–35. [CrossRef]

6. Jackson, D.J. Observations on the capacity for flight of water beetles. *Proc. R. Entomol. Soc. Lond.* **1952**, *27*, 57–70. [CrossRef]
7. Lynch, D.T.; Leasure, D.R.; Magoulick, D.D. The influence of drought on flow-ecology relationships in Ozark Highland streams. *Freshw. Biol.* **2018**, *63*, 946–968. [CrossRef]
8. Dingle, H.; Arora, G. Experimental studies of migration in bugs of the genus *Dydercus*. *Oecologia* **1973**, *12*, 119–140. [CrossRef]
9. Dingle, H.; Blakeley, N.R.; Miller, E.R. Variation in body size and flight performance in milkweed bugs (*Oncopeltus*). *Evolution* **1980**, *34*, 372–385.
10. Arribas, P.; Velasco, J.; Abellan, P.; Sanchez-Fernandez, D.; Andujar, C.; Calosi, P.; Millian, A.; Ribera, I.; Bilton, D.T. Dispersal ability rather than ecological tolerance drives differences in range size between lentic and lotic waterbodies (Coleoptera: Hydrophilidae). *J. Biogeogr.* **2012**, *39*, 984–994. [CrossRef]
11. Bilton, D.T. Dispersal in dytiscidae. In *Ecology, Systematics, and the Natural History of Predaceous Diving Beetles (Coleoptera: Dytiscidae)*; Springer International Publishing: Cham, Switzerland, 2023; pp. 505–528.
12. Roff, D.A. Dispersal in dipterans: Its costs and consequences. *J. Anim. Ecol.* **1977**, *46*, 443–456. [CrossRef]
13. Calosi, P.; Bilton, D.T.; Spicer, J.I. Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biol. Lett.* **2008**, *4*, 99–102. [CrossRef] [PubMed]
14. Hilsenhoff, W.L. Dytiscidae and Noteridae of Wisconsin (Coleoptera). VI. Distribution, habitat, life cycle, and identification of species of *Hydroporus* Clairville *sensu lato* (Hydroporinae). *Gt. Lakes Entomol.* **1998**, *28*, 1–23.
15. Rankin, M.A.; Burchsted, J.C.A. The cost of migration in insects. *Annu. Rev. Entomol.* **1992**, *37*, 533–559. [CrossRef]
16. Jarvinen, O.; Vepsalainen, K. Wing dimorphism as an adaptive strategy in water-strider (*Gerris*). *Hereditas* **1976**, *84*, 61–68. [CrossRef]
17. Bilton, D.T.; Freeland, J.R.; Okamura, B. Dispersal in freshwater invertebrates: Mechanisms and consequences. *Annu. Rev. Ecol. Syst.* **2001**, *32*, 159–181. [CrossRef]
18. Environment and Climate Change Canada. *Recovery Strategy for the Bert's Predaceous Diving Beetle (Sanfilippodytes bertae) in Canada*; Species at Risk Act Recovery Strategy Series; Environment and Climate Change Canada: Ottawa, ON, Canada, 2017; pp. 6–21.
19. Balfour-Browne, F. *British Water Beetles*; Ray Society: London, UK, 1958.
20. Leys, R.; Watts, C.H.; Cooper, S.J.; Humphreys, W.F. Evolution of subterranean diving beetles (Coleoptera: Dytiscidae Hydroporini, Bidessini) in the arid zone of Australia. *Evolution* **2003**, *57*, 2819–2834. [PubMed]
21. Toussaint, E.F.; Condamine, F.L.; Hawlitschek, O.; Watts, C.H.; Porch, N.; Hendrich, L.; Balke, M. Unveiling the diversification dynamics of Australasian predaceous diving beetles in the Cenozoic. *Syst. Biol.* **2015**, *64*, 3–24. [CrossRef] [PubMed]
22. Miller, K.B.; Jean, A.; Alarie, Y.; Hardy, N.; Gibson, R. Phylogenetic placement of North American subterranean diving beetles (Insecta: Coleoptera: Dytiscidae). *Arthropod Syst. Phylogeny* **2013**, *71*, 75–90.
23. Forest, F.; Grenyer, R.; Rouget, M.; Davies, T.J.; Cowling, R.M.; Faith, D.P.; Balmford, A.; Manning, J.C.; Proches, S.; Van der Bank, M.; et al. Preserving the evolutionary potential of floras in biodiversity hotspots. *Nature* **2007**, *445*, 757–760. [CrossRef] [PubMed]