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RESEARCH ARTICLE

Behavioral correction to prevent overhydration and increase survival by larvae of the net-spinning caddisflies in relation to water flow

Jay A. Yoder[1], Joshua B. Benoit[2,*], Blake W. Nelson[1], Leighanne R. Main[1] and Jon P. Bossley[3]

ABSTRACT

We report behavioral regulation of body water content in caddisfly larvae, *Hydropsyche morosa* and *Cheumatopsyche pettiti*, by selecting microhabitats with different water flow rates. The purpose of our study was to examine features necessary for survival in the same apparent habitat, because the two species co-exist in riffle areas of freshwater streams. Both species are highly sensitive to water loss as a result of high water loss rates and depend on immersion in fresh water (hypo-osmotic) to maintain water stores. In contrast to *C. pettiti*, *H. morosa* is larger, retains water more effectively, and features reduced water loss rates with suppressed activation energies. When *H. morosa* was confined to areas of low or no water flow, overhydration led to rapid mortality, whereas the same conditions favored water balance maintenance and survival in *C. pettiti*. In attraction bioassays, *H. morosa* moved and remained within areas of high water flow and *C. pettiti* preferred areas with low water flow. Because water flow rates are unlikely to directly impact water gain, the mechanism responsible for increased survival and water balance maintenance is likely related to the impact of water flow on oxygen availability, differences in feeding ecology, or other underlying factors.

KEY WORDS: Water exchange, Hyperosmotic, Attraction, Freshwater, Biological indicator

INTRODUCTION

Whole-organism water balance is an ecologically defining attribute and limits the ability of insects to function properly in the absence of water resources (Hadley, 1994). Habitat adaptation involves a complementary relationship between water balance characteristics and modifications of behavior relevant to preventing drying out or overhydrating (Hadley, 1994). To maintain water balance, water gain (m_S) must be equal to water loss (m_T) as defined by Wharton (Wharton, 1985). Net water gain occurs when $m_S > m_T$, and net water loss occurs when $m_T > m_S$. The goal is to maintain body water ($\Delta m = 0$), a condition that permits proper functioning and development through a lack of dehydration-induced stress. In a freshwater habitat, the m_S component is exceedingly large for an aquatic insect in that they are hyperosmotic to freshwater. The water activity (a_w) of the freshwater environment, $1.00a_w$, is greater than the $0.99a_w$ activity of the insect's body water [$0.990–0.997a_w$ (Wharton, 1985; Sigal et al., 1991)]. Thus, the activity gradient of freshwater results in a continuous water increase to the body by simple diffusion (Kohn, 1965; Wharton, 1985). To prevent overhydration, insects must either suppress cuticular permeability to prevent water intake or increase the rate of water removal.

Net-spinning caddisfly larvae (Trichoptera: Hydropsychidae) reside at the bottom of freshwater ponds and streams on cobblestones or limbs. They have a preference for shallow riffle areas with low to high current flow, in cooler, shaded areas (Wiggins, 1996; Bouchard, 2004). Caddisfly larvae thrive within high quality freshwater and are utilized as bioindicators [see Bonada and Williams (Bonada and Williams, 2002) and references therein]. Appreciable amounts of water are obtained from their moisture-rich food, predominately algae and detritus from the stream bottom (Snyder and Hendricks, 1995) and from drinking (Sutcliffe, 1961). The emphasis for maintaining water balance by caddisfly larvae focuses on increasing the water loss component, m_T, to counteract the continual water influx that occurs naturally from ingestion and inward diffusion by being submerged. Despite the application of caddisfly larvae in environmental studies, little work has been done on the water balance physiology of caddisfly larvae. The exception is Sutcliffe's (Sutcliffe, 1961) work on salt balance, where it was shown that caddisfly larvae are hyperosmotic and water is attracted to them.

In this paper, we determined water balance characteristics and conducted attraction bioassays in relation to water flow for larvae of two caddisfly species that regularly co-occur in freshwater streams, *Hydropsyche morosa* Hagen 1861 and *Cheumatopsyche pettiti* (Banks 1908). Few experiments have examined species comparisons based on water balance characteristics of insect larvae with the exception of mosquitoes (Bradley, 1987; Bradley, 2008) and midge larvae (Benoit et al., 2007; Kikawada et al., 2008; Elnitsky et al., 2009). Comparative studies of water balance in species that reside within the same habitats have also been minimal [fruit flies (Aggarwal et al., 2013; Parkash et al., 2011; Parkash et al., 2013), tsetse flies (Kleynhans and Terblanche, 2011; Terblanche et al., 2006; Terblanche et al., 2008), beetles (Benoit et al., 2005), mites (Benoit et al., 2008)]. Our hypothesis is that larvae of different caddisfly species may have different water balance profiles, which likely necessitate different habitat requirements to maintain water balance.

RESULTS

Analysis of stream water and flow rates

Physicochemical data of stream water were specific conductance ($524.5±12.8\ \mu S\ cm^{-1}$), dissolved oxygen ($13.6±0.59\ mg\ l^{-1}$ and 105.9±2.2%), temperature (22.06±0.11°C), and pH (8.10±0.34) (N=10 measurements for each of three sites, means ± s.e.m.). Average stream flow velocity was $4.1±1.7\ km\ h^{-1}$ taken near the bottom, middle and top of the water column (N=10 measurements

[1]Department of Biology, Wittenberg University, Springfield, OH 45501, USA. [2]Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221, USA. [3]Environmental Science Graduate Program, The Ohio State University, Columbus, OH 43210, USA.

*Author for correspondence (joshua.benoit@uc.edu)

Received 6 July 2014; Accepted 1 December 2014

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110684

Table 1. Water balance characteristics of larvae of the caddisflies *Cheumatopsyche pettiti* and *Hydropsyche morosa*

Characteristic	*C. pettiti*	*H. morosa*	Figure reference
Water content			
Initial mass, *f* (mg)	11.03±0.46	41.74±0.32	
Dry mass, *d* (mg)	3.84±0.09	13.62±0.11	
Water mass, *m* (mg)	7.19±0.36	28.12±0.44	
Water content, *m*/*d* (mg)	1.87	2.06	
Water content (%)	65.19±0.52	67.37±0.61	
Water loss			
20°C (% min^{-1})	0.45±0.02	0.27±0.05	1
Dehydration tolerance			
Water mass when moribund, m_c (mg)	5.78±0.19	21.65±0.15	
Dehydration tolerance limit (%)	19.61±0.49	23.02±0.62	
Critical transition temperature (°C)	37.1±1.2	34.7±1.8	2
Activation energy, E_a (kJ mol^{-1}) <CTT	14.5±0.5	9.1±0.7	2
Activation energy, E_a (kJ mol^{-1}) >CTT	33.2±0.9	26.4±0.6	2

Values are means ± s.e.m. (*N*=100, 10 replicates of 10 larvae each or at each of the six test temperatures in the activation energy calculation).

for each of three sites, mean ± s.e.m.). Water flow velocities for experimental airflow rates were 3 l min^{-1}=2.4±0.9 km h^{-1}, 6 l min^{-1}=5.7±0.7 km h^{-1} and 12 l min^{-1}=9.1±1.6 km h^{-1} (*N*=15 measurements, means ± s.e.m.). Oxygen content of the stream water after it was filtered and autoclaved was 14.1±0.38 mg l^{-1} and 108.3±2.7%, indicating that water in bioassays was supersaturated with oxygen.

Water balance characteristics

Hydropsyche morosa larvae were larger than *C. pettiti* larvae in initial mass, dry mass and water mass ($P<0.05$ in each pairwise comparison; Table 1). In all cases, the water mass was a positive correlate of the dry mass ($r^2 \geq 0.93$ for *H. morosa*; $r^2 \geq 0.91$ for *C. pettiti*; $P<0.001$). The two species had a similar percentage body water content of ~66% ($P>0.05$; Table 1). Thus, *H. morosa* is larger and the two species have similar relative body water pools that are available for exchange with their surroundings.

Water loss rates were more rapid for *C. pettiti* than for *H. morosa* (Fig. 1, Table 1; $P<0.05$). Once *C. pettiti* larvae lost 19.6% of their body water, although they were alive, they were unable to coordinate their movements and self-right (Table 1). The dehydration tolerance of *C. pettiti* was 19.6% and the dehydration tolerance of *H. morosa* was 23.02% ($P>0.05$; Table 1). Moribund *H. morosa* and *C. pettiti* died if placed at 100% relative humidity (RH) or in stream water, evidence that they had sustained an irreversible level of water loss at their dehydration tolerance limit.

At 37°C there is a critical transition temperature (CTT) for *C. pettiti* as denoted by the biphasic change on the Arrhenius plot (Fig. 2). The activation energy changes from 14.5 kJ mol^{-1} in the low temperature range to 33.2 kJ mol^{-1} in the high temperature range ($P<0.05$; Table 1). Water loss shows direct Boltzmann dependence in both low and high temperature ranges (r^2=0.98 and 0.94, respectively; $P<0.001$; Fig. 2). Ramp-up and ramp-down determinations yielded nearly identical activation energies as given in Table 1, producing highly reproducible CTTs at or near 36°C (data not shown). The 35°C CTT for *H. morosa* was not significantly different from the CTT for *C. pettiti* ($P>0.05$; Table 1). For *H. morosa*, there was a change in activation energy that separated the low temperature range (9.1 kJ mol^{-1}) from the high temperature range (26.4 kJ mol^{-1}) (Table 1, Fig. 2; $P<0.05$) as a regular Boltzmann temperature function ($r^2 \geq 0.92$; $P<0.001$). Activation energies were lower for *H. morosa* than for *C. pettiti* in both low and high temperature ranges ($P<0.05$; Table 1).

Survival estimates and mass changes

An experimental arena was designed using a stream water-filled specimen dish, with tubing affixed to one side of the dish (sector one) that created a high water flow current by bubbling air (Fig. 3).

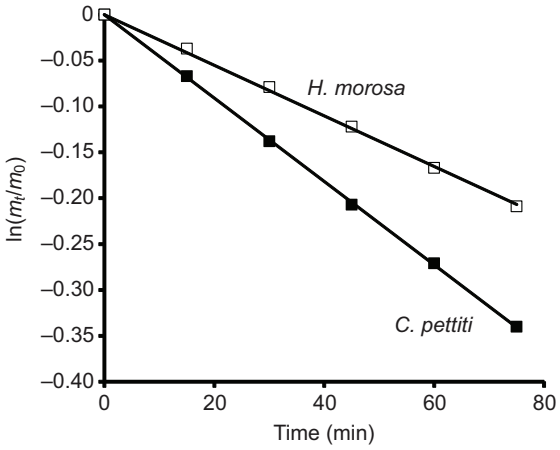

Fig. 1. Proportion of water mass lost by *Cheumatopsyche pettiti* and *Hydropsyche morosa* larvae at 20°C and 0% relative humidity (RH). The slope of the regression line is the water loss rate, m_T. m_t, water mass at any time *t*; m_0, initial water mass. Error bars lie within the symbols (±s.e.m ≤0.011). Each point is the mean of 100 larvae.

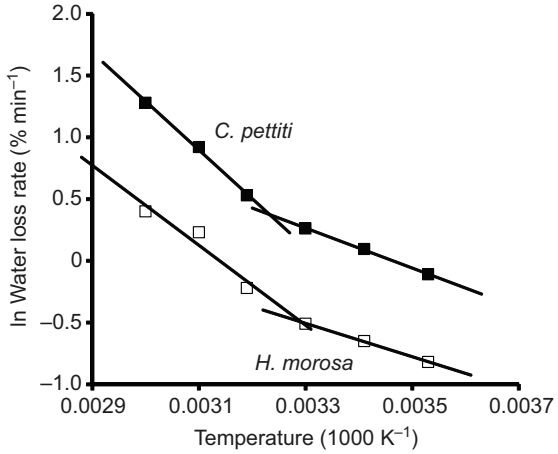

Fig. 2. Water loss–temperature relationship for freshly killed *C. pettiti* and *H. morosa* larvae. The slope of the regression is $-E_a/R$ for calculating the activation energy. K, absolute temperature; E_a, activation energy; *R*, gas constant. Error bars are within the symbols (±s.e.m. ≤0.004). Each point is the mean of 100 larvae.

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110684

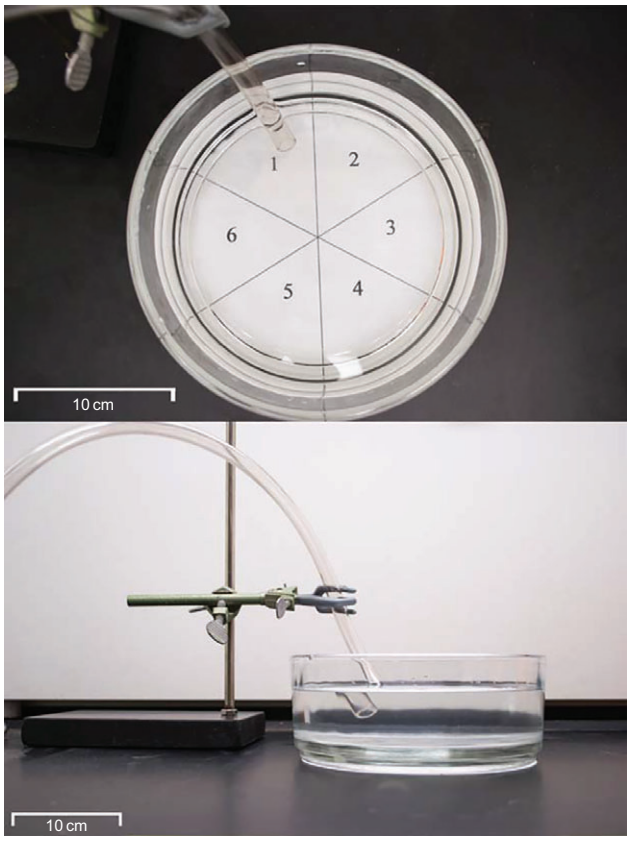

Fig. 3. Experimental set up for exposing different water flow rates to *C. pettiti* and *H. morosa* larvae. The airflow tube was fixed to an airflow detector/regulator attached to a laboratory source of filtered compressed air. Photo credit: B. Nelson and L. Main.

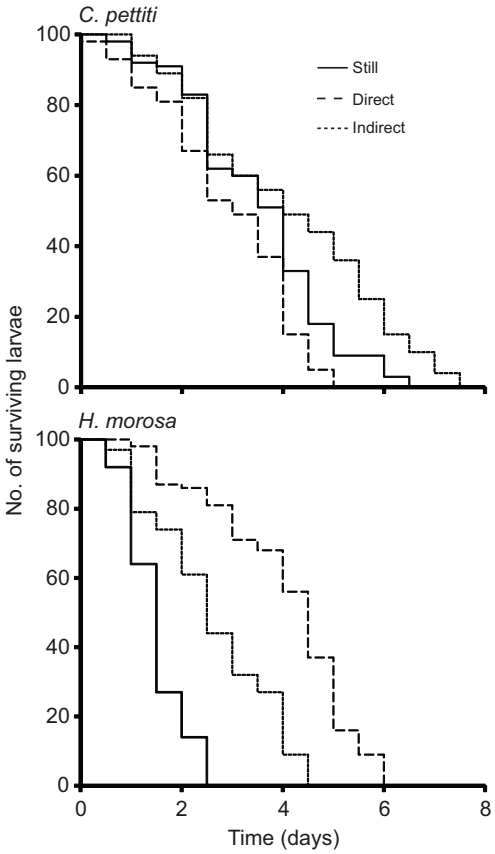

Fig. 4. Survivorship curves for starved, caged *C. pettiti* and *H. morosa* larvae exposed to different water flows. The set up described in Fig. 3 was used: water temperature 20°C, 15 h:9 h light:dark, with a flow rate of 3 l min^{-1} going into sector one. Still, no water flow (0 l min^{-1}; caged larva placed in sector one, but airflow turned off); direct, caged larva placed in sector one, exposure to high water flow from airflow tube; indirect, caged larva placed in sector four with airflow occurring in sector one. Each point is the mean of 100 larvae (±s.e.m. ≤5.8).

'Direct water flow' refers to the side of the dish (sector one) beneath the air tube where there is a high rate of water flow. 'Indirect water flow' refers to the opposite side of the dish (sector four) where the water flow is less rapid because the tube is directed into sector one.

Greatest survival for *C. pettiti* occurred in indirect water flow (sector four, Fig. 3), where larvae survived for 8.0 days (4.5 days for 50% of larvae) compared with 5.5 days (3.5 days for 50% of larvae) in direct water flow (P<0.05; Fig. 4). Still water conditions also had a positive effect on *C. pettiti* survival (survival for 7.0 days, 4.0 days for 50% of larvae), with survival of ~1 day less than in indirect water flow (P<0.05; Fig. 4). In contrast, direct water flow resulted in a greater survival for *H. morosa* compared with indirect water flow: 6.5 days (4.7 days for 50% of larvae) versus 5.0 days (2.7 days for 50% of larvae), respectively (Fig. 4; P<0.05). Survival was negatively impacted in *H. morosa* by still water, which shortened survival by approximately 2 days (survival for 3.0 days, 1.8 days for 50% of larvae) compared with survival in indirect water flow (P<0.05; Fig. 4).

Cheumatopsyche pettiti gained more water over time in the direct water flow than the indirect water flow (Fig. 5; P<0.05 in each pairwise comparison). Daily water gains in still water were between the extremes of direct flow (P<0.05 in each pairwise comparison) and indirect flow (Fig. 5; P<0.05 in each pairwise comparison). For *H. morosa*, the largest daily water gains occurred for larvae in still water (Fig. 5; P<0.05 in each pairwise comparison). There were also large amounts of daily water gain for *H. morosa* larvae held in indirect water flow compared with direct water flow (Fig. 5; P<0.05 in each pairwise comparison). Under still and indirect water flow conditions for *H. morosa*, mass measurements were discontinued once larvae died after 2–3 days (Fig. 5). Survival for *C. pettiti* did not begin to decline dramatically until after the 72 h time point (Fig. 5).

Behavioral responses emphasizing attraction

A water flow of 3 l min^{-1} resulted in larger numbers of *C. pettiti* in sector one (Fig. 3) within a day compared with untreated (no tube) or no water flow (airflow tube alone) controls (Fig. 6; P<0.05). In all cases, a slight attraction by *C. pettiti* to sector one occurred within 1 h when water was flowing at any speed compared with still water controls (P<0.05; Fig. 6). The 1 and 2 h responses of *H. morosa* to the low 3 l min^{-1} flow rate were similar to those of untreated and airflow tube-only controls (P>0.05), but increased substantially at 24 h (P<0.05; Fig. 6). Water flow at 6 and 12 l min^{-1} recruited large numbers of *H. morosa* larvae within 1 h (P<0.05), attracting and retaining nearly all of the larvae (Fig. 6). The heightened intensity of attraction to sector 1 by *H. morosa* in high speed water of 6 and 12 l min^{-1} remained elevated at 2 and 24 h (Fig. 6).

DISCUSSION

The important conclusion from this study is that larvae of *H. morosa* and *C. pettiti* display clear microhabitat preferences with regard to

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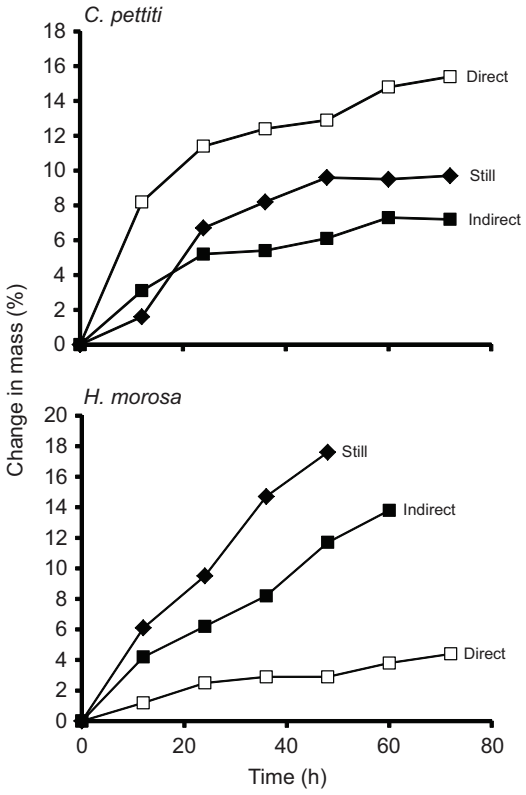

Fig. 5. Changes in body water mass under exposure to water flow for *C. pettiti* and *H. morosa* larvae. The set up described in Fig. 3 was used: water temperature 20°C, 15 h:9 h light:dark, with a flow rate set at 3 l min^{-1} going into sector one. Larvae were caged during exposure in sector one (direct water flow), sector four (indirect water flow), or sector one (still; no water flow). Larvae were taken out of cages for mass determination. Each point is the mean of 100 larvae (±s.e.m. ≤2.1).

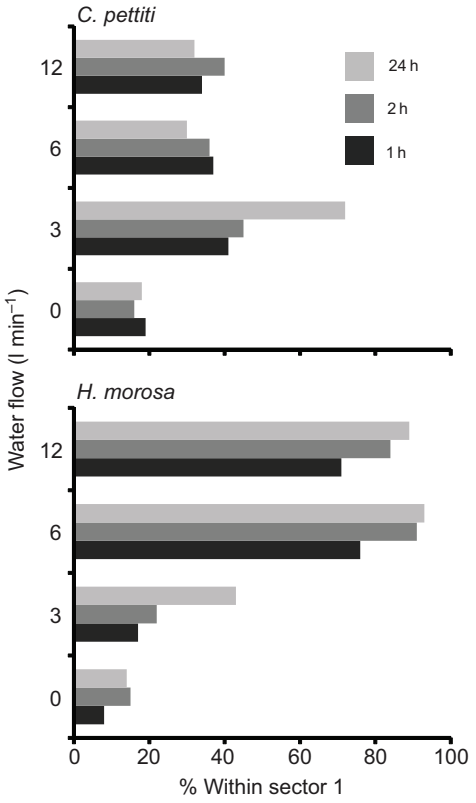

Fig. 6. Attraction of *C. pettiti* and *H. morosa* larvae to sector one in response to water flow. The set up described in Fig. 3 was used: water temperature 20°C, 15 h:9 h light:dark. Untreated, no airflow tube was inserted into the water; 0 l min^{-1}, airflow tube inserted into the water, but airflow was turned off. Larvae were introduced at the center of the arena. Each test exposure is the mean of 100 larvae (±s.e.m. ≤4.3).

water flow. When placed in non-preferred microhabitats, larvae become overhydrated, thus indicating that inability to maintain water balance could drive species-specific microhabitat preferences. Specifically, *H. morosa* has a low water loss rate and prefers to reside in areas with high water flow. For *C. pettiti*, water loss rates are higher and residence in areas with low water flow permits them to maintain water balance. There is also thermal suitability information as implied by the critical transition temperature (CTT). The CTT is within the 32–39°C of upper thermal tolerance limits for caddisfly larvae (deKozlowski and Bunting, 1981; Moulton et al., 1993); thus, mortality at high temperature can be attributed to a sharp increase in water loss, or water gain, at or above the CTT. This water balance/behavior information is relevant to the use of caddisfly larvae as bioindicators of high quality water for nutrient cycling, toxicological monitoring and thermal disturbances [see Bonada and Williams (Bonada and Williams, 2002) and references therein] as water turnover differentially affects each species.

From a water balance perspective, different flow rates are preferred for the two species. Here, we have attempted to provide a link between the water balance characteristics and preferred flow rates, if there is one. *Cheumatopsyche pettiti* and *H. morosa* rely on continuous water influx from their freshwater habitat. Their dehydration tolerance limit is unlikely to be exceeded, because water is continuously entering into the body by inward diffusion, drinking and feeding on moist food. The continuous water influx is balanced by large body water losses (water efflux) so that the larva functions properly at the body water content. *Cheumatopsyche pettiti* larvae are thus highly permeable to water and they are prevented from overhydrating by having high water loss rates. *Hydropsyche morosa* larvae, with their much lower water loss rate, require that the amount gained must be restricted, which suggests the presence of a water-proofed cuticular barrier unique to *H. morosa* that restricts the amount of water entry (water influx). The difference in water loss rate implies that the emphasis is on water elimination for *C. pettiti* and water retention for *H. morosa*. When matched with behavioral preference, the implication is that the water flow rate conditions of the microhabitat are opposite to the water turnover of the larva. This is something that we have also found for another aquatic invertebrate, the branchiobdellids (Annelida: Clitellata) that live on freshwater crayfish (Yoder et al., 2007). The species that loses water the fastest (*Cambarincola fallax*) clusters in locales with least amount of water movement on the crayfish, at subrostral sites at the base of the eyestalks. In contrast, a species that loses water the slowest (*Cambarincola ingens*) is found preferentially in the high water flow area of the gills (Yoder et al., 2007). Even though both caddisfly species have high cuticular water loss rates and require direct contact with pure water, the stream flow rates significantly impact their ability to maintain water balance.

A high water flow rate is unlikely to have an appreciable impact on the amount of water that is gained by an insect, because the supply of water available for uptake is not altered. Hydrodynamic pressure in a high flow rate is greatest on one side of the insect (the side hit directly by the current) but low on the other side of the insect (the side not exposed to current), and this would be predicted

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to yield a similar net effect on water gain (m_S) to that in still or low flow rate where the hydrodynamic pressure is more equalized over the insect surface. Indeed, we found that both *H. morosa* and *C. pettiti* can survive in still water; hence, they can evidently maintain proper body water content within tolerable limits. This is supported by field data, where *H. morosa* and *C. pettiti* have been collected in pool areas and from ponds despite the propensity by both species to be more abundant in riffle areas (Bouchard, 2004). These considerations indicate that the amount of water influx is constant whether *C. pettiti* is in a low or high water flow. The amount of water influx for *H. morosa* is less as a tradeoff for their lower water loss rate, because they cannot eliminate excess water gain as effectively as *C. pettiti*. There is enhancement of survival when water gain is lowest, and this occurs in regions of high flow for *H. morosa* and low flow for *C. pettiti*. *Hydropsyche morosa* and *C. pettiti* function better (prolonged survival) when there is at least some water flow in the area, and especially a high water flow for *H. morosa*.

The problem of desiccation impacting survival is a challenge encountered by many aquatic insects when the stream dries. Larvae of *H. morosa* and *C. pettiti* are quite active, and hence lose water rapidly, and their small body size through surface area to volume properties further exacerbates this dilemma. The high water loss rates and modest dehydration tolerance limit of *H. morosa* and *C. pettiti* necessarily imply that they are hygric-suited and adapted for a moisture-rich environment (Hadley, 1994). Many aquatic insects and other aquatic invertebrates prevent dehydration and remain viable by staying in constant, direct contact with a moisture-rich surface (Hadley, 1994). There is little interpretative value ecologically concerning body water content (i.e. not all aquatic insects have a high body water content), and for these caddisfly species it approximates the mean water content (69%) of most insects (Hadley, 1994). Under drought conditions, the most likely scenario is that the caddisfly larvae crawl underground (Wiggins, 1996; Bouchard, 2004). The differences we note in water loss rate and behavior between *H. morosa* and *C. pettiti* should also be thought of as important resistance mechanisms that promote survival of these caddisfly larvae in intermittent streams. These caddisfly larvae are driven underneath the soil surface in order to satisfy an absolute moisture requirement because of their high water loss rate.

Although our results may be a direct consequence of water balance, there are physiological functions impacted by stream flow rates that likely have an impact on survival and habitat preferences. The most likely possibility is that the choice experiments (Fig. 6) could be explained, fully or in part, by behavioral regulation of oxygen availability. A high water flow rate and increase in hydrodynamic pressure would replenish the supply of oxygen at a faster rate. Higher flowing water would bring more oxygen past the caddisfly's filamentous abdominal gills ([there are no open spiracles in Trichoptera (Wiggins, 1996)]. Perhaps the larger body size of *H. morosa* requires faster water flow rates to maintain oxygen delivery. If the oxygen requirement is not met (i.e. happens in still or low water flow), then mechanisms responsible for maintaining water balance begin to malfunction for energetic reasons. Overhydration and eventual death would be the physiological consequence of water balance mechanisms breaking down. It also seems reasonable to suggest that proper excretory function in these species requires optimal flow conditions. The chloride epithelium of caddisfly larvae likely requires specific flow rates to regulate chloride uptake, which is essential for maintaining water balance. There is insufficient information about the excretory system of caddisflies to confirm this possibility. Additionally, the negative effect on water balance in still or low flowing water could also be an artifact of some primary function that recruits *H. morosa* to higher flowing waters that could relate to behavioral differences between *C. pettiti* (preference and suitability for still or low water flow) in feeding ecology or in predator avoidance.

MATERIALS AND METHODS

Collection of caddisfly larvae and stream water

Stream locales were selected by random block design within a 3 m transect in riffle areas of a third-order segment of Buck Creek, Clark Co., OH, USA. Larvae were collected using kick nets (BioQuip Products, Rancho Dominguez, CA, USA). Larvae were identified as *C. pettiti* and *H. morosa* and were in the final, fifth instar (Merritt and Cummins, 1996; Wiggins, 1996). Slide-mounted vouchers are under specimen lot WUIC nos 1209-1229 (Wittenberg University, Springfield, OH, USA). Handling of larvae was done with an aspirator. Dead larvae were those that did not move (legs or mouthparts) and failed to respond to stimuli and crawl five body lengths when prodded when examined by 40× microscopy. Stream water was collected into autoclave-sterilized (121°C, 15 psi), 1.0 l glass volumetric flasks, and filtered (3M Aqua-Pure, 3M Co., St Paul, MN, USA) before use. Physicochemical data of the stream water were collected on site with a water quality sonde (YSI Environmental, Yellow Springs, OH, USA) using a Flowprobe (Global Water, White Plains, NY, USA) to determine flow rate.

Equipment, instrumentation and experimental conditions

RH was maintained in glass desiccators (6.0 l; Fisher) containing anhydrous $CaSO_4$ for 0% RH (1.5×10^{-2}% RH; W. A. Hammond Drierite Co., Xenia, OH, USA) (Toolson, 1978) and deionized double-distilled (DI) water for 100% RH. RH was measured with a hygrometer (s.d. ±0.5% RH; Thomas Scientific, Philadelphia, PA, USA). An electrobalance (CAHN, Ventron Co., Cerritos, CA, USA; precision and accuracy were s.d. ±0.2 µg and ±6 µg at 1 mg, respectively) was used for measuring mass changes of larvae. Larvae were dried to complete dryness (constant mass for 3 days) in a 90°C drying oven (Blue M Electric Co., Chicago, IL, USA) (Hadley, 1994). Basic observations were conducted at 20±1°C, 15 h:9 h light:dark photoperiod. The temperature for other studies varied less than ±0.5°C.

Fig. 3 shows the six-sector bioassay that we modified from a statistically valid, short range Petri plate attraction bioassay developed previously (Arlian and Vyszenski-Moher, 1995; Allan and Sonenshine, 2002). A straight-sided flat bottom specimen dish (19 cm i.d.×7 cm deep=1986 l) was placed over the top of a paper template that had been scored into six equal 47.2 cm^2 sectors. The specimen dish was filled with 1.0 l of filtered stream water. A 1.0 cm Tygon tubing (Fisher) attached to a source of compressed air and flowmeter for regulation (Rochester Gauges, Dallas, TX, USA) created a bubbling water flow into sector one. No water flow, i.e. flow of 0 l min^{-1} (still water conditions), served as a control. An additional control was a specimen dish without the airflow tube. Stream water, freshly autoclave-sterilized culture dishes and unconditioned larvae that had not been previously used in bioassays were utilized for each experiment.

All experiments were replicated 10 times, 10 larvae per replicate (total N=100 larvae), with each replicate coming from a different collection site within the stream. Data are means ± s.e.m.

Determination of water balance characteristics

Following Wharton's (Wharton, 1985) standard gravimetric methods and equations, the rate of water loss at 0% RH is an accurate measure of the water loss rate as it would occur while submerged in water [experimental determination using ^{3}HOH (Arlian and Staiger, 1979)]. Each larva was monitored individually, without anesthesia or enclosure, and weighed in less than 1 min. All experiments use 4–6% pre-desiccated larvae so that mass changes reflect changes in body water levels (Arlian and Eckstrand, 1975; Wharton, 1985).

To determine water content and water loss rate, a larva was weighed (fresh, initial mass, f), placed at 20°C and 0% RH, and re-weighed for five readings of mass. The larva was then dried to constant mass (d=dry mass)

in the 90°C drying oven. Dry mass was subtracted from each mass measurement to convert the mass measurement into the water mass (m):

$$m = f - d\,. \quad (1)$$

Percentage body water content was calculated as:

$$\%\, m = 100\,(f - d)\,/\,f\,. \quad (2)$$

Water loss rate (integumental plus respiratory water loss) was calculated by fitting mass measurements to Eqn 3:

$$m_t = m_0 e^{-kt}\,, \quad (3)$$

where m_t is the water mass at any time t, m_0 is the initial water mass and $-k$ is the water loss. Fresh mass, dry mass, water mass and percentage body water content were based on the same cohort of 100 larvae. The water loss rate was based on a different cohort of 100 larvae.

To determine dehydration tolerance, a larva was weighed, held at 0% RH and 20°C, and re-weighed, each time checking the larva for its ability to self-right and crawl five body lengths. At the critical mass (m_c), the larva could move but could not coordinate its movements and failed to self-right and crawl when placed at 100% RH or in 15 ml of water. Percentage change in m was calculated as:

$$\%\Delta m = 100\,(m_c - m_0)\,/\,m_0\,, \quad (4)$$

where m_0 is the initial water mass, and was used to calculate the dehydration tolerance limit. A separate cohort of 200 larvae was used for determining the dehydration tolerance limit: 100 larvae for a rescue attempt by placing larvae at 100% RH and a separate group of 100 larvae for a rescue attempt by placing larvae in stream water.

To obtain activation energy (E_a) for water loss, water loss rates were determined (Eqn 3) with freshly killed larvae and temperature was varied. Larvae were killed by the freeze–thaw method. Water loss rate was determined for the same larva experiencing a temperature increase (ramp-up) or a temperature decrease (ramp-down). The Arrhenius equation (Eqn 5) was used to determine the activation energy:

$$k = A e^{-E_a/(RT)}\,, \quad (5)$$

where k is the passive water loss rate, E_a is activation energy, R is the universal gas constant, T is absolute temperature and A is the steric frequency factor (Gibbs, 2002). The significance of activation energy is controversial (Yoder et al., 2005), but there is agreement on the occurrence of a CTT when the activation energy changes (Gibbs, 2002). Water loss rate accelerates abruptly above the CTT (Gibbs, 2002). Each water loss rate determination was based on a cohort of 100 larvae in the activation energy calculation to total N=600 larvae. The ramp-up and ramp-down experiment each utilized 100 larvae, tracking the water loss rate for the same cohort of 100 larvae going up, or down, the temperature scale.

Determination of survival and attraction potential

The cage for housing a single larva was made with a 1.5 ml polypropylene microcentrifuge tube (Fisher) perforated with 30 holes to permit water entry. The cage was anchored with a spot of glue (Loctite low-odor; Henkel Co., Rocky Hill, CT, USA) on to a plain glass microscope slide (76.2×25.4×1 mm; Fisher) to keep the cage submerged while in the bioassay arena (Fig. 3). The larva was out of test conditions for less than 1 min for weighing and examination of motor activity, and the ability to self-right and crawl five body lengths at 40×. Percentage change in mass was calculated as:

$$\%\Delta m = 100\,(m_t - m_0)\,/\,m_0\,, \quad (6)$$

where m_t is the water mass at any time t. Treatments included placing the caged larva in non-moving water (0 l min^{-1} flow rate) in sector one in the bioassay arena (Fig. 3), as well as in moving water with a flow rate of 3 l min^{-1}. We selected 3 l min^{-1} flow rate because this produces a more localized flow in sector one with little to no flow in sector four compared with a flow rate of 6 or 12 l min^{-1}, which is too high. In the bioassay arena (Fig. 3), a comparison was done where the caged larva was placed in sector four (indirect water flow), thereby preventing the larva from being close to the source of moving water from the tubing located in sector one (direct water flow). Survivorship curves and mass measurements were done 20 times, five larvae at a time, each in a separate cage, to total N=100.

Additionally, uncaged larvae were introduced, 10 at time, at the center of the bioassay arena (Fig. 3). Counts of larvae in sector one (Fig. 3) were made after 1, 2 and 24 h during the photophase. Treatments included exposure of larvae to 3, 6 and 12 l min^{-1} water flows. There were two still water controls, one with the airflow tube in the water with 0 l min^{-1} airflow and one with no airflow tube in the water to rule out potential right–left bias. Data are the responses of 100 larvae, based on 10 replicates of 10 larvae.

Statistics

The alpha value was adjusted to a level of significance of 0.05. Arcsin transformation was done for percentage data. Water balance data were compared with an analysis of covariance (ANCOVA). Water loss rates and activation energies were compared with a test for the equality of slopes of several regressions (Sokal and Rohlf, 1995). The number of larvae in sector one in the attraction bioassays was analyzed by a means comparison, paired t-test following an arcsin transformation (Sokal and Rohlf, 1995). We did not want to have confounding effects if the survival data were non-parametric, so we utilized the Kaplan–Meier survival curve with a log rank test after an Abbott's correction (Sokal and Rohlf, 1995). Statistical software were SPSS 14.0 for Windows (IBM, Armonk, NY, USA), Microsoft Excel (Redmond, WA, USA) and Minitab (Chicago, IL, USA).

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.A.Y. conceived, designed and supervised experiments, collected and analyzed data, interpreted the results, and wrote the manuscript. J.B.B., B.W.N. and L.R.M. carried out experiments, collected specimens, analyzed data, and revised and checked the manuscript. J.P.B. did the initial taxonomic identification of the caddisfly larvae and was involved in checking the manuscript. All authors have approved this manuscript with confirmation in writing.

Funding

Funding for this project was provided, in part, by research grants to B.W.N. and L.R.M. from Wittenberg University.

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