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RESEARCH ARTICLE

Water loss in tree weta (*Hemideina*): adaptation to the montane environment and a test of the melanisation–desiccation resistance hypothesis

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ABSTRACT

Montane insects are at a higher risk of desiccation than their lowland counterparts and are expected to have evolved reduced water loss. *Hemideina* spp. (tree weta; Orthoptera: Anostostomatidae) have both lowland (*Hemideina femorata*, *Hemideina crassidens* and *Hemideina thoracica*) and montane (*Hemideina maori* and *Hemideina ricta*) species. *H. maori* has both melanic and yellow morphs. We use these weta to test two hypotheses: that montane insects lose water more slowly than lowland species, and that cuticular water loss rates are lower in darker insects than lighter morphs, because of incorporation of melanin in the cuticle. We used flow-through respirometry to compare water loss rates among *Hemideina* species and found that montane weta have reduced cuticular water loss by 45%, reduced respiratory water loss by 55% and reduced the molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ by 64% compared with lowland species. Within *H. maori*, cuticular water loss was reduced by 46% when compared with yellow morphs. Removal of cuticular hydrocarbons significantly increased total water loss in both melanic and yellow morphs, highlighting the role that cuticular hydrocarbons play in limiting water loss; however, the dark morph still lost water more slowly after removal of cuticular hydrocarbons (57% less), supporting the melanisation–desiccation resistance hypothesis.

KEY WORDS: Cuticular water loss, Montane, Melanism, Desiccation resistance, Alpine

INTRODUCTION

Montane insects are physiologically challenged by environmental factors including lower air density and atmospheric partial pressure of oxygen as well as increased wind speed and solar radiation (Ashby, 1997; Dillon et al., 2006; Hodkinson, 2005; Huey, 1991; Jones et al., 1987) compared with lowland habitats. Together, these environmental challenges, compounded at subzero temperatures by the high vapour pressure deficits caused by ice (Sømme, 1994), mean montane insects are at a significantly higher risk of desiccation than lowland insects and would therefore be expected to be more tolerant of desiccating environments. This increased tolerance may be achieved by surviving extreme water loss, sometimes to the point of anhydrobiosis (Watanabe, 2006); by increasing initial water content (Gibbs et al., 1997) or by absorbing water from the environment (Hadley, 1994). Most insects that remain active in desiccating environments do so by retaining water more effectively through a reduction in the rate of water loss (Gibbs and Matzkin, 2001). This is achieved through a combination of limiting excretory water losses through the mouthparts or anus (Bursell, 1960), by regulating diuresis by decreasing Malpighian tubule activity and/or increasing resorption in the hind-gut (Park, 2012), through reducing respiratory water loss (RWL) via changes in respiratory patterns (Chown et al., 2006; Marais et al., 2005; Terblanche et al., 2010) and through decreasing cuticular water loss (CWL) by reducing cuticular permeability (Chown and Nicolson, 2004).

Of these potential routes of water loss, excretory water loss accounts for only a small fraction (<6%) of total water loss in *Drosophila* (Gibbs et al., 1997), and did not differ among xeric and mesic *Drosophila* species (Gibbs et al., 2003). While respiratory water loss as a function of metabolic rate can vary widely within and among insect species (Groenewald et al., 2013), water loss can be reduced through changes in respiratory patterns (Chown, 2002). Continuous gas exchange (CGE), where spiracles do not close in a coordinated manner, is the most prevalent pattern (Marais et al., 2005). This contrasts with discontinuous gas exchange cycles (DGEs) (Chown, 2002) – a respiratory pattern that includes periods where spiracles are open and coordinated periods where spiracles are closed for prolonged periods, with no external gas exchange (Chown et al., 2006). Discontinuous gas exchange cycles are more common in xeric species (Marais et al., 2005), allowing resting or quiescent insects to minimise water loss associated with respiration (White et al., 2007). As respiratory water loss usually accounts for less than 15% of total water loss (Chown, 2002), cuticular water loss accounts for the majority of total water loss and is thus is the most significant route for preventing water loss in insects at rest (Chown and Nicolson, 2004).

Insects from xeric environments generally have reduced CWL rates compared with counterparts from more mesic environments (Addo-Bediako et al., 2001). Reduction in CWL is usually due to reduced cuticular permeability, achieved either through changes in the composition or quantity of cuticular hydrocarbons (CHCs) (Edney, 1977) or changes in the amount or extent of cuticular melanisation (Kalmus, 1941). Increased CHC quantity, longer lipid chain length, insertion of a methyl branch or ester link and unsaturation of cuticular lipids are all expected to reduce cuticular permeability (Gibbs and Pomonis, 1995; Gibbs, 1998; Rourke and Gibbs, 1999). Intraspecific (Johnson and Gibbs, 2004), interspecific (Hadley and Schultz, 1987) and sexual differences (Gibbs et al., 1997) in water loss rates have been associated with changes in CHCs. For example, a decrease in the proportion of straight-chain *n*-alkanes and an increase in the proportion of methylalkanes increased cuticular water loss in mated queen desert harvester ants, *Pogonomyrmex barbatus* (Johnson and Gibbs, 2004). In three tiger beetle species (*Cicindela*), quantity and lipid-chain length of CHCs were significantly increased in the species with the least-permeable

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List of symbols and abbreviations

a.s.l.	above sea level
CGE	continuous gas exchange
CHC	cuticular hydrocarbon
CWL	cuticular water loss
DGE	discontinuous gas exchange cycle
M_b	body mass
MCA	metabolic cold adaptation
RWL	respiratory water loss
$\dot{V}_{CO_2}$	volume of carbon dioxide released per unit time
$\dot{V}_{H_2O}$	volume of water released per unit time

cuticle (*C. oregona*) (Hadley and Schultz, 1987). Increased desiccation resistance in female *D. melanogaster* was associated with CHCs with increased lipid chain length and higher melting temperatures, both as a result of selection (Gibbs et al., 1997) and rapid desiccation hardening (Stinziano et al., 2015). Not surprisingly, given the importance of CHCs in reducing cuticular permeability, removal of cuticular lipids by organic solvents significantly increases CWL, as shown in the gall fly *Eurosta solidaginis* (Ramløv and Lee, 2000) and in *Drosophila bipectina* (Parkash et al., 2014).

Increased melanisation of the cuticle has been associated with decreased cuticular permeability and a reduction in CWL in insects (Kalmus, 1941), leading to the melanisation–desiccation resistance hypothesis, which states that darker individuals from a given population will have higher desiccation resistance and lower rates of CWL than less-melanised individuals. Increased melanisation is associated with increased desiccation resistance in *D. melanogaster* strains selected for melanism (Ramniwas et al., 2013) and increased melanism is frequently associated with both increased desiccation resistance and more arid environments both within and among *Drosophila* species (Brisson et al., 2005; Parkash et al., 2008a; Pool and Aquadro, 2007).

Melanism in insects has roles beyond desiccation resistance, and has also been linked to other physiological and/or ecological factors such as thermoregulation, immune response and diet (Dubovskiy et al., 2013; Majerus, 1998; Sword, 2001; True, 2003). For example, *Drosophila americana* from cooler environments are darker (Wittkopp et al., 2011), fitting a thermal melanism hypothesis (Clusella-Trullas and Terblanche, 2011) where darker individuals in cooler environments heat up faster than lighter-coloured insects. Melanism also forms an important innate immune defence, and darker individuals of some species are better able to resist infection (Dubovskiy et al., 2013). The diet of melanic locusts includes a higher proportion of toxic plants than non-melanic locusts, with melanism acting as a warning to deter predation (aposematism) (Sword, 2001). Although the high incidence of melanism in montane insects (Sømme, 1989) is often ascribed to thermal melanism, the melanisation–desiccation resistance hypothesis may also at least partially account for this high incidence: montane, melanic insects generally have decreased cuticular permeability and reduction in cuticular water loss (Parkash et al., 2008a,c), and montane melanism is prevalent even in predominantly nocturnal insects such as weta.

Weta belonging to the genus *Hemideina* are large, flightless, nocturnal insects found on both main islands of New Zealand. Most *Hemideina* species, including *H. crassidens* (Blanchard 1851), *H. thoracica* (White 1842) and *H. femorata* Hutton 1898 are arboreal, living in forest below the tree-line (Field and Sandlant, 2001) (Fig. 1). One species, *H. ricta* Hutton 1898, is usually arboreal but sometimes seeks refuge under rocks, while its sister species, *Hemideina maori* Pictet & Saussure 1891, the alpine tree weta, that shares morphological, behavioral and genetic similarities to *H. ricta* (Field, 1993; King et al., 2003), has adapted physiologically to a wholly terrestrial, montane lifestyle (Sinclair et al., 1999). *Hemideina maori* has two colour morphs; melanic and yellow (King et al., 2003, 1996) (Fig. 1). The melanic morph has dorsal tergites alternating black and dark purple-brown with a dark ventral surface while the yellow morph has alternating black and yellow dorsal tergites with a light ventral surface (King et al., 1996). Melanism appears to have evolved at least twice in *H. maori* (King et al., 2003). There is no evidence that repeated evolution of melanism is associated with increased immune response (Robb et al., 2003), incidence of parasitism (Robb et al., 2004) or different diets between melanic and yellow morphs (Wilson and Jamieson, 2005). Differences in cuticular water loss have not yet been investigated between melanic and yellow morphs of *H. maori*, so the melanisation–desiccation resistance hypothesis remains untested in this system.

We used flow-through respirometry to measure metabolic rate and determine several water loss parameters, including total water loss, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, CWL, RWL and proportion of total $\dot{V}_{H_2O}$ lost as RWL in montane (*H. maori* colour morphs and *H. ricta)* and lowland (*H. crassidens*, *H. thoracica* and *H. femorata*) *Hemideina* species. We did this to investigate the generality that insects in montane environments have reduced rates of water loss compared with their lowland relatives (Sømme, 1989). We hypothesise that adaptation to the montane environment reduced

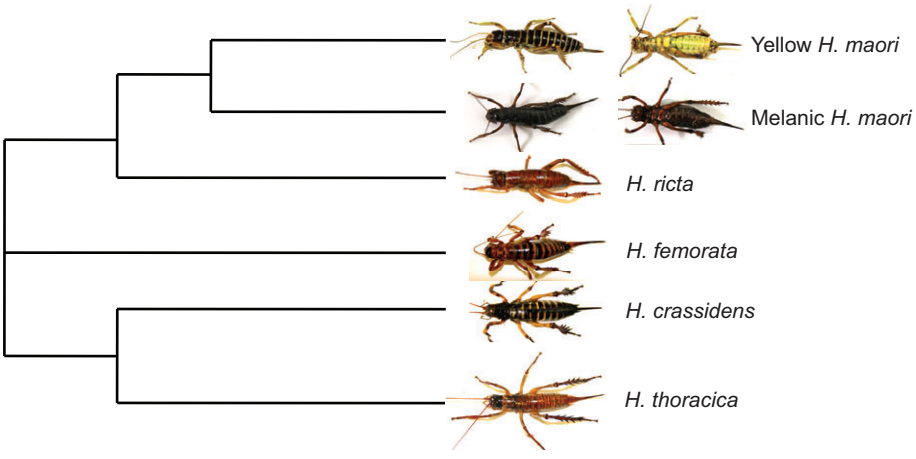

Fig. 1. Phylogeny of *Hemideina* species used in this study. Unrooted parsimonious tree from morphological and genetic data for both colour morphs of *H. maori* (showing both ventral and dorsal surfaces), *H. ricta*, *H. femorata*, *H. crassidens* and *H. thoracica*. Branch lengths are not proportional (modified from Morgan-Richards and Gibbs, 2001).

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Table 1. Gas exchange and water loss parameters in *Hemideina* species

	H. maori							
	Melanic	Yellow	*H. ricta*	Montane group	*H. crassidens*	*H. thoracica*	*H. femorata*	Lowland group
N	10	7	4	21	4	4	3	11
M_b (g)	3.4±0.3	3.9±0.3	3.6±0.2	3.6±0.2	3.8±0.2	2.6±0.2	5.7±0.4	3.9±0.3
$\dot{V}_{CO_2}$ (μmol g^{-1} h^{-1})	2.94±0.25	3.31±0.32	3.08±0.30	3.08±0.16	3.00±0.39	1.94±0.32	1.73±0.22	2.27±0.25
$\dot{V}_{H_2O}$ (μmol g^{-1} h^{-1})	0.183±0.03	0.342±0.04	0.250±0.07	0.249±0.03	0.589±0.21	0.313±0.07	0.462±0.15	0.453±0.06
Molar ratio ($\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$)	0.07±0.01	0.12±0.02	0.08±0.02	0.08±0.01	0.21±0.03	0.18±0.06	0.30±0.12	0.22±0.04
RWL (μmol g^{-1} h^{-1})	0.003±0.001	0.008±0.002	0.005±0.002	0.004±0.001	0.019±0.008	0.005±0.001	0.011±0.006	0.012±0.004
CWL (μmol g^{-1} h^{-1})	0.180±0.03	0.334±0.04	0.245±0.07	0.244±0.03	0.567±0.10	0.308±0.07	0.450±0.14	0.444±0.06
Proportion of total $\dot{V}_{H_2O}$ lost as RWL (as %)	2.43±0.7	2.40±0.8	2.82±1.1	2.50±0.5	3.68±2.0	1.21±0.3	2.20±0.4	2.40±0.8

Results presented for both colour morphs of *H. maori* and its sister species *H. ricta* (which form a montane group) and *H. crassidens*, *H. thoracica* and *H. femorata* (which form a lowland group). Only individuals that exhibited discontinuous gas cycling pattern were included. Means (±s.e.m.) are presented.

total water loss in montane weta, via decreased rates of both cuticular and respiratory water loss. We also investigated differences in water loss, and specifically differences in the rate of cuticular water loss, between melanic and yellow morphs of *H. maori*. Under the melanisation–desiccation resistance hypothesis, melanic weta should have reduced cuticular water loss rates when compared with less-melanic or non-melanic counterparts (Kalmus, 1941). We hypothesise that melanism in *H. maori* has evolved to reduce cuticular water loss. Thus, we hypothesise that melanic morphs lose significantly less total water than yellow morphs, and that this reduced rate of water loss is caused by differences in the structure of the cuticle, rather than the properties of epicuticular lipids.

RESULTS

We measured metabolic rate ($\dot{V}_{CO_2}$) and water loss parameters ($\dot{V}_{H_2O}$, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, CWL, RWL and proportion of total $\dot{V}_{H_2O}$ lost as RWL) in *Hemideina* species, using flow-through respirometry in dry air at 15°C (Table 1, Fig. 2). Both *Hemideina maori* colour morphs, *H. ricta* and *H. thoracica* employed discontinuous gas exchange (Table 2; Fig. 2B), while three out of seven *H. crassidens* and one of four *H. femorata* employed CGE (Fig. 2D). Only individuals that employed DGE were used in subsequent analyses. Activity was detected in only one *H. crassidens* individual. This activity did not coincide with the respirometry period selected for analysis in this individual and had no apparent effect on results. Activity in this weta shows that the activity detector was working, as well as confirming that weta remained largely inactive during respirometry (Fig. 2A,C).

Comparison of metabolic rate and water loss parameters among *Hemideina* species

$\dot{V}_{CO_2}$ and water loss parameters ($\dot{V}_{H_2O}$; molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$; CWL; RWL and proportion of total $\dot{V}_{H_2O}$ lost as RWL were compared among species (Table 1) with melanic and yellow morphs of *H. maori* treated as separate taxa in these analyses. Total water loss $\dot{V}_{H_2O}$ was partitioned into cuticular water loss and respiratory water loss components, with CWL estimated using the regression method (Gibbs and Johnson, 2004) and RWL calculated as the difference between $\dot{V}_{H_2O}$ and CWL.

Metabolic rate, measured as $\dot{V}_{CO_2}$, and $\dot{V}_{H_2O}$ (Table 3, Fig. 3B) differed significantly among species (Table 3, Fig. 3A,B). Molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ and CWL also differed significantly among

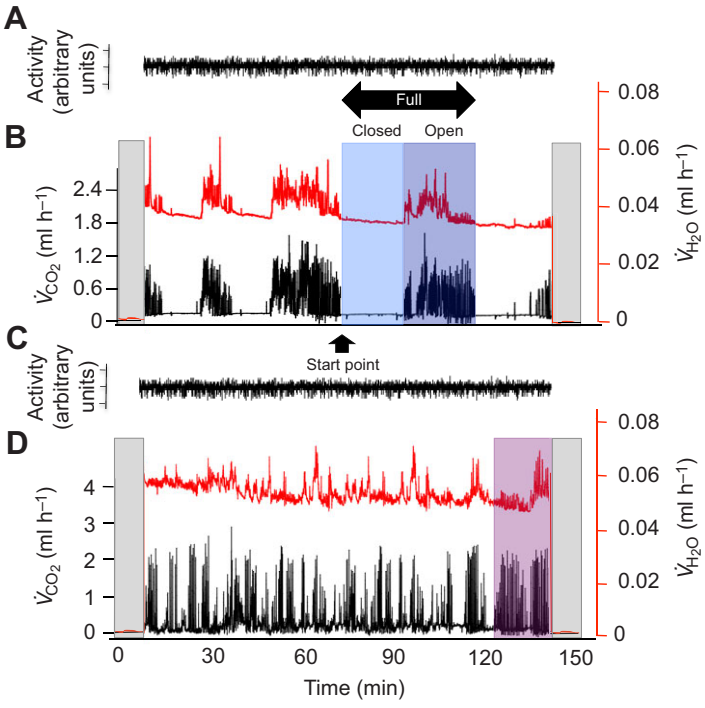

Fig. 2. Activity, CO_2 and H_2O emission traces for discontinuous gas cycle (DGE) and continuous gas exchange (CGE) patterns in *H. crassidens*. (A,C) Activity of weta exhibiting discontinuous gas exchange and continuous gas exchange, respectively. (B,D) Emission traces for weta exhibiting discontinuous gas cycling and continuous gas exchange, respectively. CO_2 is the bottom trace on the left *y*-axis (shown in black), while H_2O is the top trace in each panel on the right *y*-axis (shown in red). Baseline measurements, taken 10 min before and 10 min after respirometry, are indicated by shaded grey boxes. In B, a full phase, comprised of closed (shaded pale blue box) and open (shaded dark blue box) phases, was used for analysis. In D, the shaded purple box shows the data period (last 1227 s) used for analysis. 1227 s corresponds to the average full phase cycle length in *H. crassidens*.

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Table 2. Gas exchange characteristics in *Hemideina* species

	H. maori							
	Melanic	Yellow	*H. ricta*	Combined montane group	*H. crassidens*	*H. thoracica*	*H. femorata*	Combined lowland group
Gas exchange pattern	DGE	DGE	DGE	DGE	DGE/CGE	DGE	DGE/CGE	DGE/CGE
DGE employed	10 (10)	7 (7)	4 (4)	21 (21)	4 (7)	4 (4)	3 (4)	11 (15)
Full phase length (s)	975±134	1082±232	1316±205	1182±122	1227±313	1005±159	789±302	1045±149
Closed phase length (s)	578±128	598±159	904±181	625±91	604±226	887±115	551±199	626±90
Open phase length (s)	397±73	552±101	412±229	452±63	672±113	158±30	236±103	429±116

All *H. maori* colour morphs, *H. ricta and H. thoracica* individuals employed a discontinuous gas cycling pattern, while one of four *H. femorata* and three of seven *H. crassidens* individuals used a continuous gas cycling respiratory pattern. DGE employed described the number of individuals that employed DGE, with numbers in parentheses indicating the total number of individuals used in respirometry. No significant differences were observed among species for length of full, closed or open phases. Mean (±s.e.m.) are presented.

species (Table 3), with CWL accounting for over 95% of total water loss seen in *Hemideina* species. Neither total RWL nor the proportion of total water lost as RWL differed significantly among species (Table 3). $\dot{V}_{H_2O}$, $\dot{V}_{CO_2}$, CWL and RWL were all positively related to the fresh mass of the weta (Table 3, Fig. 3A), while a positive relationship between mass with both of the molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ and the proportion of total $\dot{V}_{H_2O}$ lost as RWL was approaching significance, and thus retained in models to account for body size (Table 3). Time spent in the respirometry chamber, taken as the starting time-point (min) for the period of respirometry selected for analysis (Fig. 2B), was not a significant covariate in any analysis (Table 3). There was no difference in $\dot{V}_{CO_2}$ or any water loss parameters ($\dot{V}_{H_2O}$; molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$; CWL; RWL and proportion of total $\dot{V}_{H_2O}$ lost as RWL) between male and female weta (Table 3).

Table 3. Results of ANCOVA for respiration and water loss parameters for comparisons among species

	Trait	d.f.	MS	*F*	*P*
$\dot{V}_{CO_2}$ (μmol h^{-1})	Mass	1	47.97	9.17	0.006**
	Species	5	23.672	4.52	0.005**
	Sex	1	0.437	0.0837	0.775
	Time	1	0.038	0.0073	0.932
	Error	23	5.23		
$\dot{V}_{H_2O}$ (μmol h^{-1})	Mass	1	0.24	18.23	0.0003**
	Species	5	0.04	3.24	0.02*
	Sex	1	0.003	0.203	0.656
	Time	1	0.032	2.42	0.13
	Error	23	0.013		
$\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$	Mass	1	0.0040	3.99	0.057
	Species	5	0.0033	3.31	0.021*
	Sex	1	0.0001	0.106	0.748
	Time	1	0.0016	1.61	0.216
	Error	23	0.0010		
CWL (μmol h^{-1})	Mass	1	0.0046	5.85	0.024*
	Species	5	0.0034	4.37	0.0062**
	Sex	1	0.0002	0.27	0.6054
	Time	1	0.0011	1.46	0.2384
	Error	23	0.0008		
RWL (μmol h^{-1})	Mass	1	0.00157	9.57	0.005**
	Species	5	0.00043	2.62	0.06
	Sex	1	0.00005	0.35	0.55
	Time	1	0.00001	0.01	0.95
	Error	23	0.00016		
Proportion of total $\dot{V}_{H_2O}$ lost as RWL	Mass	1	0.00290	4.16	0.06
	Species	5	0.00081	1.17	0.36
	Sex	1	0.00058	0.85	0.37
	Time	1	0.00007	0.10	0.75
	Error	23	0.00069		

Results for mass, species, sex and time are shown. MS, mean squares. *P<0.05; **P<0.01.

Comparison of metabolic rate and water loss parameters between montane and lowland weta

$\dot{V}_{CO_2}$ was significantly increased in montane weta when compared with lowland weta (Table 4), whereas $\dot{V}_{H_2O}$, the molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, CWL and RWL were all significantly reduced in montane weta (Table 4). Proportion of total $\dot{V}_{H_2O}$ lost as RWL did not differ significantly between groups (Table 4). All parameters were positively related to the fresh mass of the weta (Table 4). Time spent in the respirometry chamber was not a significant covariate in any analysis (Table 4). There was no difference in $\dot{V}_{CO_2}$ or any water loss parameters between male and female weta (Table 4).

Comparison of metabolic rate and water loss parameters between colour morphs of *Hemideina maori*

$\dot{V}_{H_2O}$, CWL and molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ were lower in the melanic morph of *H. maori* compared with the yellow morph (Table 5, Fig. 4), and there was also a trend toward lower RWL in melanic weta (Table 5). $\dot{V}_{H_2O}$, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, CWL and RWL were all positively related to the fresh mass of the weta. Time spent in the respirometry chamber was not a significant covariate in any analysis (Table 5). There was no difference in $\dot{V}_{CO_2}$ or any water loss parameters between male and female weta (Table 5).

Comparison of discontinuous gas exchange parameters

We compared the lengths of open, closed and full phases of discontinuous gas-exchange cycles among species, between lowland and montane groups and between *H. maori* colour morphs (Table 2). In all analyses, the lengths of open, closed or full phases did not significantly differ (P>0.1).

Comparison of water loss parameters in *Hemideina maori* colour morphs before and after removal of CHCs

Removal of CHCs significantly increased the rate of water loss for both colour morphs ($F_{1,16}$=12.33, P=0.003) (Fig. 5), with removal of CHCs increasing water loss in yellow morphs by 42% and in melanic morphs by 24%. Yellow morphs lost significantly more water than melanic morphs, both before and after removal of CHCs ($F_{1,16}$=7.15, P=0.03), and the amount of water lost was positively related to the fresh mass of the weta ($F_{1,16}$=5.6, P=0.03).

DISCUSSION

We used flow-through respirometry to determine $\dot{V}_{CO_2}$ and water loss parameters for *Hemideina* species (Table 1). Metabolic rate was significantly higher in montane weta than in lowland weta. However, several water loss parameters, including $\dot{V}_{H_2O}$, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, CWL and RWL were significantly reduced in montane weta, a group consisting of *H. maori* and *H. ricta*, two closely related species (Tables 1 and 4). Thus, it appears that

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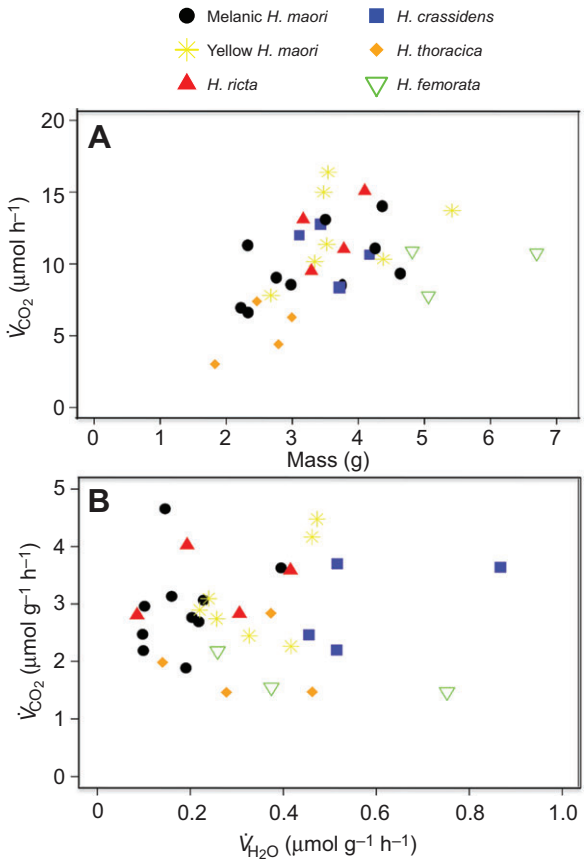

Fig. 3. Metabolic rate in *Hemideina* species. $\dot{V}_{CO_2}$ as a function of (A) mass and (B) $\dot{V}_{H_2O}$.

Table 4. Results of ANCOVA for respiration and water loss parameters for comparisons between montane and lowland groups

	Trait	d.f.	MS	*F*	*P*
$\dot{V}_{CO_2}$ (µmol h^{-1})	Mass	1	43.966	8.295	0.008**
	Group	1	61.975	11.685	0.002**
	Sex	1	0.701	0.132	0.719
	Time	1	0.038	0.007	0.933
	Error	22	5.304		
$\dot{V}_{H_2O}$ (µmol h^{-1})	Mass	1	0.265	28.32	0.00002**
	Group	1	0.090	9.61	0.005**
	Sex	1	0.0009	0.09	0.757
	Time	1	0.024	2.63	0.117
	Error	22	0.009		
$\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$	Mass	1	0.0046	5.85	0.02*
	Group	1	0.0162	20.34	0.0002**
	Sex	1	0.0002	0.27	0.60
	Time	1	0.0012	1.46	0.24
	Error	22	0.0008		
CWL (µmol h^{-1})	Mass	1	0.0046	5.85	0.02*
	Group	1	0.0162	20.34	0.0002**
	Sex	1	0.0008	0.27	0.61
	Time	1	0.0011	1.46	0.25
	Error	22	0.0008		
RWL (µmol h^{-1})	Mass	1	0.001575	9.57	0.005**
	Group	1	0.000950	5.77	0.024*
	Sex	1	0.000058	0.35	0.55
	Time	1	0.000001	0.01	0.95
	Error	22	0.000164		
Proportion of total $\dot{V}_{H_2O}$ lost as RWL	Mass	1	0.00306	3.49	0.08
	Group	1	0.00084	0.95	0.33
	Sex	1	0.0006	0.71	0.40
	Time	1	0.00003	0.03	0.85
	Error	22	0.0008		

Results for mass, group (montane or lowland), sex and time are shown. MS, mean squares. *P<0.05; **P<0.01.

montane *Hemideina* have increased desiccation resistance via decreased rates of water loss.

We also tested the melanisation-desiccation hypothesis using two colour morphs, yellow and melanic, of the polymorphic *H. maori*. We found that melanic *H. maori* morphs lost significantly less total water than yellow morphs (Tables 1 and 5, Fig. 4). This reduction was driven by a significant decrease in cuticular water loss in melanic morphs, consistent with the melanisation–desiccation resistance hypothesis, and with findings from *Drosophila* (Ramniwas et al., 2013). Removal of cuticular hydrocarbons significantly increased total water loss in both melanic and yellow morphs (Fig. 5), highlighting the role that cuticular hydrocarbons play in limiting water loss. However, the increase in water loss after removal of CHCs was less for melanic weta, showing that both melanisation and CHCs restrict CWL in this species.

Metabolic rate (estimated from $\dot{V}_{CO_2}$) was higher in montane species of weta compared with their lowland counterparts (Table 4). Increases in metabolic rate have been noted in other cold-adapted Orthoptera (Booth and Kiddell, 2007; Hadley and Massion, 1985; Rourke, 2000) and is consistent with metabolic cold adaptation (MCA) (Clarke, 1993), where species from colder climates have elevated metabolism compared with species from warmer climates. Raised metabolic rate might be a consequence of shorter growing seasons and colder temperatures (Chappell, 1982) or an adaptation to lower temperatures found at higher altitude (Hadley and Massion, 1985). Future studies could compare the metabolic rate of montane *H. maori* with either *H. maori* from lower altitudes (*H. maori* is found at around 300 m a.s.l. on two islands in Lake Wanaka; 44°61′S, 160°06′E) or with *H. maori* reared at low altitude. Although metabolic rate did not differ between low (150 m a.s.l.) and higher altitude (500–1000 m a.s.l.) populations of *H. crassidens* and *H. thoracica* (Bulgarella et al., 2015; Minards et al., 2014), *H. maori* is found at higher elevations than either *H. crassidens* or *H. thoracica*. Therefore, increased metabolic rate in montane weta could be a consequence of physiological variation and adaptation to altitude, or an ancestral feature in montane weta that facilitated alpine colonisation.

Several water loss parameters, including both cuticular and respiratory water loss, were significantly reduced in montane weta compared with their lowland counterparts (Table 4). Furthermore, the relationship between respiration and water loss, measured by the molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, appeared more costly in lowland species. Collectively, these results suggest that montane *Hemideina* have several adaptations to increase desiccation resistance and decrease water loss. Insects adapted to xeric environments have shown reduced CWL (Chown, 2002), possibly as a response to increased risk of desiccation stress (Ashby, 1997; Dillon et al., 2006; Hodkinson, 2005; Huey, 1991; Jones et al., 1987). As CWL can be reduced by changes in the chain length, methyl branching and unsaturation of cuticular hydrocarbons (Gibbs, 1998; Hadley, 1994), differences in CHCs would be expected in a comparison of cuticle from montane and lowland species. Cuticle structure, lipid content and hydrocarbon content have been examined in *H. maori* and were similar to those seen in other terrestrial insects (Hadley, 1989; Hadley et al., 1988). Removal of CHCs significantly increased water loss for both melanic and yellow morphs of

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Table 5. Results of ANCOVA for respiration and water loss parameters in *Hemideina maori* colour morphs

	Trait	d.f.	MS	*F*	*P*
$\dot{V}_{CO_2}$ (μmol h^{-1})	Mass	1	23.67	3.24	0.09
	Morph	1	11.04	1.51	0.24
	Sex	1	0.007	0.0009	0.98
	Time	1	1.14	0.157	0.70
	Error	12	7.31	14.71	0.002**
$\dot{V}_{H_2O}$ (μmol h^{-1})	Mass	1	0.0881	9.00	0.01*
	Morph	1	0.0539	2.79	0.19
	Sex	1	0.0167	1.85	0.12
	Time	1	0.0111		
	Error	12	0.0059		
$\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$	Mass	1	0.0012	10.40	0.007**
	Morph	1	0.0012	7.04	0.02*
	Sex	1	0.0004	3.56	0.08
	Time	1	0.0002	1.49	0.24
	Error	12	0.0001		
CWL (μmol h^{-1})	Mass	1	0.083	13.35	0.003**
	Morph	1	0.051	8.32	0.013*
	Sex	1	0.017	2.70	0.126
	Time	1	0.011	1.84	0.199
	Error	12	0.006		
RWL (μmol h^{-1})	Mass	1	0.00051	8.99	0.011*
	Morph	1	0.00025	4.46	0.056
	Sex	1	0.000008	0.139	0.716
	Time	1	0.00019	0.343	0.569
	Error	12	0.000057		
Proportion of total $\dot{V}_{H_2O}$ lost as RWL	Mass	1	0.0019	2.72	0.12
	Morph	1	0.0004	0.55	0.47
	Sex	1	0.0001	0.20	0.66
	Time	1	0.0004	0.64	0.44
	Error	12	0.0007		

Results for mass, morph, sex and time are shown. MS, mean squares.
*P<0.05; **P<0.01.

H. maori (Fig. 5), showing a role for CHCs in waterproofing the cuticle. The CHCs of montane and lowland *Hemideina* have not, to our knowledge, been compared and such comparisons may offer insight into how CHCs function to reduce CWL in montane weta.

Our gravimetric comparison of water loss before and after CHC removal assumes that total mass lost is a proxy for CWL. In our respirometry studies (Table 1), we found that CWL was responsible for around 95% of total water loss in *H. maori*.

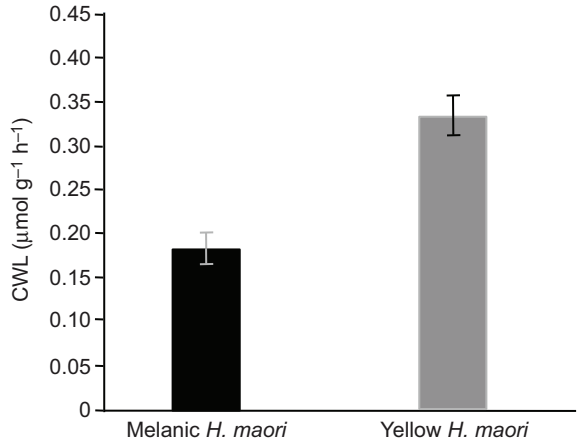

Fig. 4. Cuticular water loss in *H. maori* colour morphs. Melanic *H. maori* morphs lost significantly less cuticular water than yellow *H. maori* morphs during respirometry trials, a result consistent with the melanisation–desiccation resistance hypothesis. Means±s.e.m. are shown.

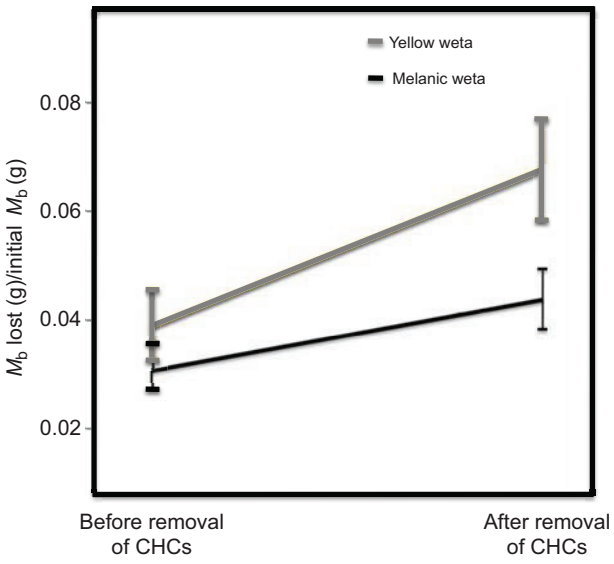

Fig. 5. Effect of cuticular hydrocarbon removal in *H. maori*. Removal of cuticular hydrocarbons (CHCs) significantly increased total water loss in both *H. maori* colour morphs during gravimetric trials, with water loss in yellow morphs significantly increased when compared with melanic morphs. Means±s.e.m. are shown.

Although we cannot rule out the possibility that handling (or hexane application) caused stress-induced upregulation of metabolic rate and consequently of respiratory water loss, this seems unlikely because: (1) the increased rate of water loss we observed was equivalent to an 11-fold increase in RWL; and (2) we controlled for handling in these experiments by applying the treatment to both yellow and black morphs, and by sham-treating weta prior to the initial measurement. In a study where the cuticle of ants was abraded, and water loss was measured using respirometry, the increased total water loss was due to increased cuticular transpiration and not via increased RWL or metabolic rate (Johnson et al., 2011). This suggests that handling does not necessarily elevate either metabolic rate or respiratory water loss in insects, and supports our assumption.

While removal of CHCs increased CWL in both yellow and melanic *H. maori*, we found that the melanic morph still had lower rates of water loss, implying that the increased incorporation of melanin in the cuticle is responsible for the lower rate of water loss in darker weta (Table 5, Figs 4 and 5). This is consistent with the melanisation–desiccation resistance hypothesis that has mainly found support in *Drosophila* (Parkash et al., 2008a,b; Ramniwas et al., 2013). Under this resistance hypothesis, melanic individuals have a selective advantage in more desiccating environments, like montane environments, than less or non-melanic individuals. Melanic *Drosophila* morphs were found in more-desiccating environments (higher altitudes) than non-melanic morphs (Parkash et al., 2008b, 2009), whereas darker abdominal pigmentation positively correlated strongly with altitude in sub-Saharan *D. melanogaster* populations (Pool and Aquadro, 2007). However, the altitudinal distribution of melanic *H. maori* differs from this pattern. In the Rock and Pillar Range, yellow weta are found mostly at altitudes above 1200 m, whereas melanic weta are generally found at lower (and presumably less desiccating) altitudes (1000–1200 m) (King et al., 1996). This distribution appears to conflict with a melanisation–desiccation resistance scenario where populations with the highest degree of melanisation should be found in more-desiccating environments, and

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suggests that desiccation resistance may not be the primary factor for melanism in this species.

While most water lost by *Hemideina* was via the cuticle, respiratory water loss was also significantly lower in the montane group (Table 4). Reduced RWL in montane species is consistent with a previous study where RWL was reduced in xeric beetle species compared with mesic species (Chown and Davis, 2003). Decreases in RWL can be associated with decreased length of the open phase in DGEs (Lehmann, 2001). However, the length of the open phase did not differ between groups (Table 2), showing that differences in RWL between montane and lowland species were due to increased volumes of H_2O released by lowland weta during the open phase, rather than the spiracles remaining open for longer. Similarly, a positive association between decreased duration of the open phase or increased duration of the closed phase of DGEs was not seen in the orthopteran species *Paracinema tricolor* when individuals were either dehydrated or exposed to conditions of low relative humidity (Groenewald et al., 2014), conditions where DGE should act to reduce RWL. While RWL made up less than 5% of total water loss, even small changes in RWL in desiccating environments may impact survival over the course of a season (Williams et al., 2010). All montane weta employed DGE, whereas two lowland species, *H. crassidens* and *H. femorata* employed both CGE and DGE (Table 2, Fig. 2B,D). While several hypotheses to explain the evolutionary pressures that lead to DGE have been proposed, one hypothesis, the hygric hypothesis, is that DGEs evolved to limit respiratory water loss by maximising the time the spiracles are closed (Chown et al., 2006). The fact that all montane weta, from a more-desiccating environment than lowland weta, exhibited DGE, while weta from two of three lowland species employed CGE, offers some support to the hygric hypothesis.

Hemideina species did not display classical DGE as $\dot{V}_{CO_2}$ never reached zero (an average of 0.004 ml min^{-1} of CO_2 was released during the 'closed' phase) (Fig. 2B). This suggests that small amounts of CO_2 may still diffuse through incompletely closed spiracles, a finding previously described in orthopterans (Groenewald et al., 2012; Hadley and Quinlan, 1993; Nespolo et al., 2007). Measuring subatmospheric intratracheal pressure in individuals that employ DGE (Groenewald et al., 2012), with a particular focus on the closed phase where limited CO_2 is still released, may offer further insight into gas exchange patterns seen in *Hemideina*.

Water loss parameters did not significantly differ by sex in any analysis (Tables 3–5). This contrasts with sex differences in desiccation resistance reported in *Drosophila* species (Gibbs et al., 1997; Sassi and Hasson, 2013). Differences in body size may account for some of the observed differences in *Drosophila* (females are typically larger than male flies). While *Hemideina* species used in this study are sexually dimorphic for certain traits (males have larger head length and width) (Koning and Jamieson, 2001), overall body size did not differ significantly between the male and female weta used in this study. Furthermore, adult *Hemideina* species are much larger (3–10 g) and long-lived (up to 3 years) (Joyce et al., 2004) in comparison to *Drosophila*, so the same trade-offs seen in *Drosophila* may not apply to *Hemideina*.

Ideally, phylogeny should also be considered in comparative studies (Felsenstein, 1985). However, the small number of *Hemideina* species precluded the use of formal phylogenetically independent contrasts in this study. Several New Zealand invertebrate groups, including giant weta (*Deinacrida*) (Trewick and Morgan-Richards, 2004), grasshoppers (Trewick, 2008), cicadas (Buckley and Simon, 2007), stick insects (Buckley et al., 2010) and cockroaches (Chinn and Gemmell, 2004), include lowland and montane species, representing multiple, independent colonisations of New Zealand's montane zone. Further analyses of physiological differences, including resistance to desiccation, using lowland and montane species pairs as the unit of replication, would offer further insight into the evolution of montane physiology and would allow physiological differences to be somewhat decoupled from phylogeny.

Conclusions

Our results support our two main hypotheses; first, montane weta have increased desiccation tolerance via reduced rates of water loss when compared with lowland species and second, CWL is reduced in melanic *H. maori* morphs when compared with yellow morphs. Montane weta have several water loss parameters including total water loss, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$, cuticular water loss and respiratory water loss that are significantly reduced when compared with lowland species. Such differences probably represent physiological changes that allowed montane weta to adapt to a more-desiccating environment than their lowland counterparts, whose environment is less harsh and where the challenges associated with water retention are reduced. Our results support the melanisation–desiccation resistance hypothesis previously tested predominantly in *Drosophila*. Total water loss was significantly less in melanic morphs than yellow morphs, driven by a decrease in CWL. This difference was also observed between colour morphs after cuticular hydrocarbon removal, showing that both CHCs and cuticular melanisation play roles in reducing water loss in weta.

MATERIALS AND METHODS

Hemideina maori of both colour morphs and sexes were hand-collected from beneath rocks in montane habitats on the Rock and Pillar Range, Otago, New Zealand (1100–1300 m a.s.l., 45°28′S, 170°02′E) in August and October, 2012 and returned to the laboratory in plastic containers on the same day. *Hemideina crassidens* of both sexes were collected from an urban garden in Wellington, New Zealand in October 2012 (<250 m a.s.l., 41°29′S, 174°78′E). *Hemideina thoracica* of both sexes were collected from an urban park in Auckland, New Zealand in December 2012 (<250 m a.s.l., 36°84′S, 174°74′E). *Hemideina femorata* and *H. ricta* of both sexes were collected from roost boxes in native forest fragments on Banks Peninsula in December 2012 and March 2013, respectively (*H. femorata*; <250 m a.s.l., 43°78′S, 173°00′E; *H. ricta*; 320–450 m a.s.l., 43°74′S, 172°98′E). Weta were kept individually in one litre plastic containers in a temperature-controlled room (10°C), with a 12 h:12 h L:D photoperiod before use and provided with food (small pieces of apple, carrot and dry cat food) and water *ad libitum*. Weta were held under laboratory conditions for at least 1 week before use in experiments. Food was withheld from weta for 24 h prior to respirometry experiments to ensure a consistent absorptive state.

Weta were weighed before and after respirometry (Sartorius BP310S, Göttingen, Germany). Weta were placed in an experimental 50 ml chamber in a PELT-5 temperature-controlled cabinet [±0.1°C; Sable Systems International (SSI), Las Vegas, NV, USA] at 15°C, and allowed 40 min equilibration time. After this period, data were recorded every second for either 90 or 150 min. Dry, CO_2-free air was pumped at 600 ml min^{-1} through the chamber [monitored by an AD-1 activity detector (SSI) via a mass flow valve (Sierra Instruments, Monterey, CA, USA)], and measured with a Mux-8 Multiplexer (SSI). CO_2 and H_2O were measured in excurrent air using a LiCor Li7000 CO_2/H_2O infrared gas analyser (LiCor, Lincoln, NE, USA). Activity was monitored using infrared activity detectors (AD-2, SSI). All instruments were interfaced to a computer using a UI2 Analog-Digital interface (SSI), and data were acquired at 1 s intervals using Expedata software (SSI). Baseline values, recorded on an empty chamber, were taken for 10 min at the beginning and end of each trial to correct for any instrumental drift. H_2O and CO_2 values were zeroed using baseline values, lag-corrected into synchrony, averaged

over 5 s, then converted into ml min^{-1} as described elsewhere (Lighton and Turner, 2004). All volumes were corrected to STPD.

To determine the relative contribution of melanisation and cuticular hydrocarbons in reducing rates of cuticular water loss, water loss rate was measured gravimetrically in melanic and yellow *H. maori* before and after removal of CHCs with hexane. Water loss was measured in a temperature-controlled room at 15°C. Weta were placed in a 50 ml chamber through which dry, CO_2-free, air was pumped (Aqua One SR 7500 air pump, Sydney, Australia) at approximately 800 ml min^{-1} for 5 h. Weta were weighed before and after this exposure (Sartorius BP310S, Göttingen, Germany). Mass lost (g) was recorded and assumed to represent CWL as CWL formed the major component (<95%) of evaporative water loss seen in earlier respirometry experiments. Each weta was measured twice. In the first treatment (essentially a handling control), the ventral abdominal tergites of each weta was rubbed with a dry cotton swab for 10 min, and water loss rate measured. Weta were then allowed 1 week of recovery, before cuticular hydrocarbons were removed by rubbing the ventral abdominal tergites for 10 minutes using a hexane-soaked cotton swab (with regular re-application of hexane to prevent the cotton swab from drying out). Excess hexane was blotted from the weta with a tissue.

Statistical analyses were performed using R v2.15.1 (R Core Team, 2012). Means±s.e.m. are reported throughout. In individuals that exhibited discontinuous gas-exchange (DGE), total $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ were calculated from the last full cycle phase in a respirometry run, corresponding to the start of a closed phase period of one cycle to the end of the open phase period of the same cycle (Fig. 2B). $\dot{V}_{H_2O}$ and $\dot{V}_{CO_2}$were converted from µl h^{-1} to mol h^{-1}, from which the molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ was calculated. Cuticular water loss was estimated using the $\dot{V}_{H_2O}$/$\dot{V}_{CO_2}$ regression method (Gibbs and Johnson, 2004), with cuticular water loss rate estimated as the intercept of this regression. Respiratory water loss rate was calculated by subtracting cuticular water loss from total water loss, while the proportion of total water lost as RWL was determined by dividing RWL by $\dot{V}_{H_2O}$.

Data were checked to ensure assumptions of normality and homogeneity were not violated, with water loss parameters ($\dot{V}_{H_2O}$, CWL, RWL, molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ and proportion of total water lost as RWL) $\log_{10}$-transformed to ensure assumptions of normality and homogeneity were met. Analyses of covariance (ANCOVAs) were then used to compare $\dot{V}_{H_2O}$ and $\dot{V}_{CO_2}$ among all *Hemideina* species (both *H. maori* colour morphs, *H. ricta*, *H. crassidens*, *H. femorata* and *H. thoracica*). We included mass as a covariate and sex as a factor in our statistical analyses as both body size (Kleynhans et al., 2014) and sex (Gibbs et al., 1997; Sassi and Hasson, 2013) can modify water loss rates. Time, taken as the time elapsed from the starting time after the initial base line to the start of a selected discontinuous gas exchange cycle was also included as a covariate to account for potential changes in $\dot{V}_{H_2O}$ or $\dot{V}_{CO_2}$ released over the course of a respirometry run. Molar ratio of $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ was compared among species using an ANCOVA with the covariates mass and time, and sex as the factor. Cuticular water loss and respiratory water loss were compared among species using an ANCOVA with mass and time as covariates and sex as a factor. Proportion of total water lost as RWL was compared among species using an ANCOVA with total $\dot{V}_{H_2O}$, mass and time as covariates and sex as a factor. Lengths of full, open and closed phases were compared using one-way ANOVA. Only individuals that exhibited DGE were included in this analysis.

Analyses of covariance (ANCOVA) were used to compare $\dot{V}_{H_2O}$ and $\dot{V}_{CO_2}$ between montane (both colour morphs of *H. maori* and *H. ricta*) and lowland groups *(H. crassidens*, *H. femorata* and *H. thoracica*), with mass and time included as covariates and group, species and sex used as factors. Molar ratio of $\dot{V}_{H_2O}$ to $\dot{V}_{CO_2}$ was compared between groups using an ANCOVA with the covariates mass and time, and the factors species and sex. Cuticular water loss and respiratory water loss were compared between groups using an ANCOVA with mass and time as covariates and species and sex as factors. Proportion of total water lost as RWL was compared between groups using an ANCOVA with total $\dot{V}_{H_2O}$, mass and time as covariates and sex as a factor. Lengths of full, open and closed phases were compared using a *t*-test. Only individuals that exhibited DGE were included in this analysis.

Analyses of covariance (ANCOVA) were used to compare $\dot{V}_{H_2O}$ and $\dot{V}_{CO_2}$ between *H. maori* colour morphs with mass and time included as covariates and sex as a factor. The ratio of $\dot{V}_{H_2O}$ to $\dot{V}_{CO_2}$ was compared between morphs using an ANCOVA with the covariates mass and time, and the factor sex to determine whether molar ratios were significantly different between morphs. Cuticular water loss and respiratory water loss were compared between morphs using an ANCOVA with mass and time as covariates and sex as a factor. Proportion of total water lost as RWL was compared between morphs using an ANCOVA with total $\dot{V}_{H_2O}$, mass and time as covariates and sex as a factor.

The effect that CHC removal had on gravimetric water loss was compared in melanic and yellow *H. maori* morphs. ANCOVA was used to compare water loss with treatment and colour morph as factors and initial body mass as a covariate (Fig. 5).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.J.K. and B.J.S. designed the experiment, interpreted the data and wrote the manuscript; K.J.K. performed the experiments.

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