

1 **Osmoregulatory capacity at low temperature is critical for insect cold**
2 **tolerance**

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14

15 **Abstract**

16 At low temperature many insects lose extracellular ion homeostasis and the capacity to mitigate
17 homeostasis imbalance determines their cold tolerance. Extracellular homeostasis is ensured by the
18 osmoregulatory organs and recent research has emphasized key roles for the Malpighian tubules and
19 hindgut in modulating insect cold tolerance. Here we review the effects of low temperature on transport
20 capacity of osmoregulatory organs and outline physiological processes leading from cold exposure to
21 disruption of ion homeostasis and cold-injury in insects. We show how cold adaptation and cold acclimation
22 are associated with physiological modifications to transport capacity in Malpighian tubules and hindgut.
23 These responses mitigate loss of homeostasis and we highlight how further study of molecular and cellular
24 mechanisms are critical to fully appreciate the adaptations that facilitate insect cold tolerance.

25

26 **Introduction**

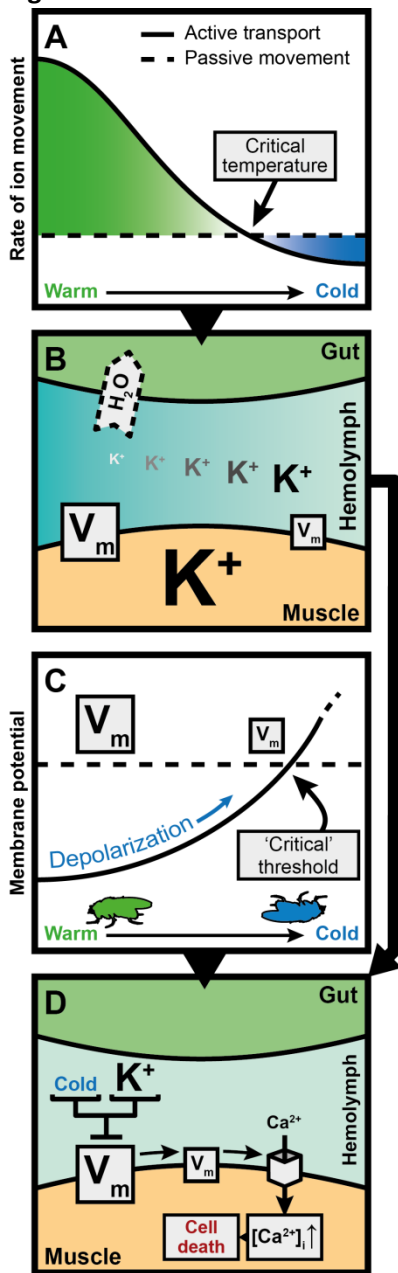
27 Most insects have limited ability for physiological regulation of body temperature (but see [1]) and rely
28 partially on behaviour to avoid extreme environmental temperatures [2,3]. Nevertheless, insects invariably
29 experience variations in body temperature and there are considerable differences in the ability of species
30 to resist or tolerate adverse temperature conditions including cold extremes [4-7]. Insect cold tolerance has
31 historically been associated with the ability to avoid or tolerate ice formation [8,9], however, most insects
32 from tropical and temperate regions, are “chill-susceptible” and succumb to cold at temperatures above
33 the freezing temperature of their body fluids [10-12]. The cold tolerance of chill-susceptible species is
34 therefore determined by their ability to maintain physiological homeostasis at low temperature rather than
35 their ability to resist or tolerate freezing [9,11,13].

36 The central physiological processes leading to chill injury and defining the current understanding of
37 insect chill tolerance is outlined in **Fig. 1**. Briefly, as temperature decreases, active transport of solutes in
38 cells and tissues is slowed to a level that is insufficient to counterbalance passive movement of solutes and
39 water leading to loss of homeostasis (**Fig. 1A**) [14,15]. Reduced active transport leads to loss of hemolymph
40 volume and disturbance of ion balance, notably an increase in extracellular $[K^+]$ (i.e. hyperkalemia)
41 triggering cold-induced cellular depolarization (**Fig. 1B**) [16-19]. This cold depolarization, which is
42 exacerbated by the loss of electrogenic potential, ultimately leads to the loss of neuronal and muscular
43 excitability inducing chill coma (**Fig. 1C**) [20,21]. Cold-induced depolarization may also activate voltage-
44 dependent Ca^{2+} channels and promote Ca^{2+} -activated apoptosis and necrosis (**Fig. 1D**), suggesting there is a
45 mechanistic link between loss of ion balance and cold-induced cell death [22-24].

46 There are still many outstanding questions in relation to this physiological model of insect chill
47 tolerance but as evident from **Table 1**, loss of ion and water balance has consistently been associated with
48 chill injury in several insect orders which places studies on temperature effects on osmoregulatory organs
49 (e.g. Malpighian tubule and hindgut) at the centre of our understanding of insect chill tolerance. In this
50 review, we outline general effects of temperature on the transport capacity of insect osmoregulatory
51 organs and subsequently discuss how cold adaptation and cold acclimation has been shown to modify this
52 capacity to secure homeostasis at low temperature. Finally, we discuss putative cellular processes of
53 importance for these physiological responses and suggest that future research should consider these
54 cellular and molecular processes in relation to insect cold tolerance.

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Figure 1



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57 **Figure 1 – Conceptual model of the physiological processes leading to chill-related injury.** The process
 58 leading from critical cold exposure to the development of chill injury starts with **(A)** low temperature
 59 slowing active transport below what is necessary to counterbalance passive movement of ions and water
 60 (dashed line) (i.e. maintain homeostasis) (adapted from [14]). **(B)** During prolonged exposure to cold, water
 61 (and Na^+ ; not depicted) will tend to move away from the hemolymph resulting in an up-concentration of K^+
 62 remaining in the hemolymph [16-18]. **(C)** This hyperkalemia and the slowing of electrogenic pumps cause
 63 depolarization of excitable tissues which is often directly linked to chill coma phenotypes [14,20,25]). **(D)**
 64 Depolarization of membrane potential (V_m) may also cause opening of voltage-gated Ca^{2+} channels leading
 65 to cell death *via* a catastrophic overload of intracellular Ca^{2+} [22-24].

66

67 **Table 1**

68 Evidence for loss of osmoregulatory capacity linked to chill injury during cold stress in insects.

Physiological consequence of cold	Blattodea	Coleoptera	Diptera	Hemiptera	Lepidoptera	Orthoptera
Loss of ion balance	+	+	+	+	+	+
Loss of water balance	+		+	+	-	+
Chill-related injury	+	+	+	+		+
Reference(s)	[26]	[27]	[17,18,28-31]	[15,27]	[32]	[16,33-38]

69 The loss of osmoregulatory capacity is here indicated by either a loss of hemolymph ion balance, a loss of
 70 water balance (mostly reported as a loss of hemolymph volume), or both. In this table, chill-related injury
 71 refers to the loss of the ability to move or onset of mortality associated with loss of ion and water balance.
 72 '+' indicates evidence that balance is disturbed during cold exposure, '-' indicates no evidence, and if
 73 nothing is written in the table it has not been directly investigated. Note that the non-significant changes in
 74 Lepidoptera hemolymph volume were qualitatively similar to what has been observed in other insect
 75 families.

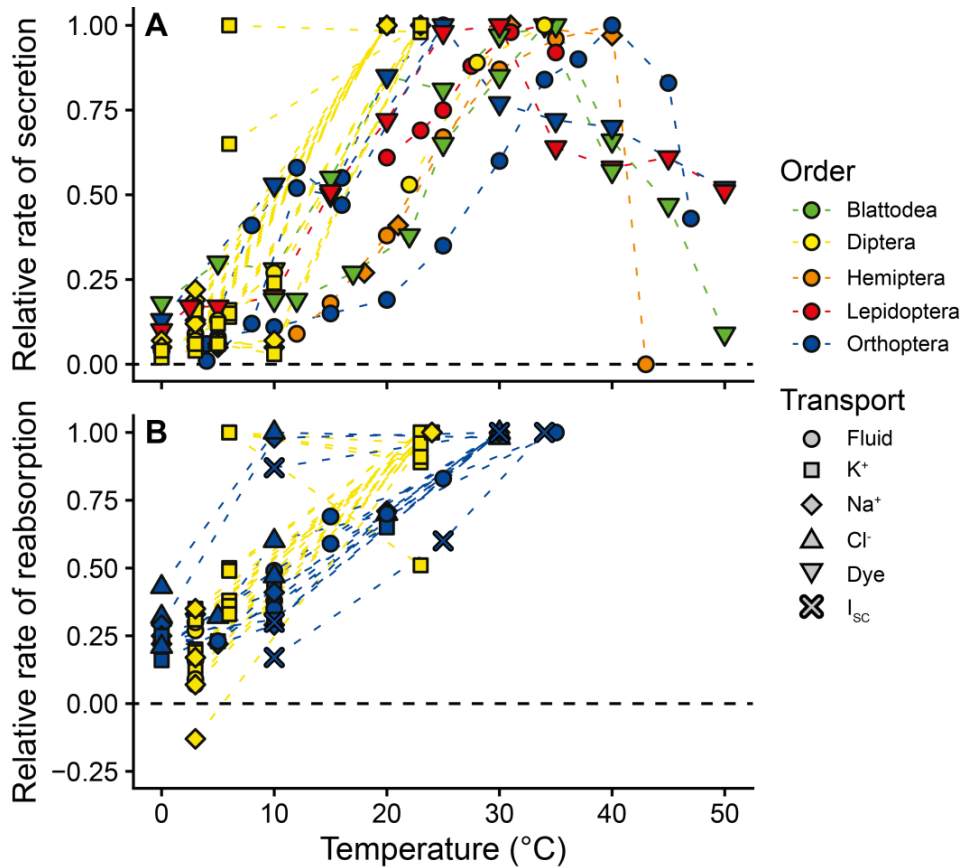
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77 **The influence of temperature on osmoregulatory capacity**

78 Temperature directly affects the activity of ion pumps and therefore also transepithelial transport by
79 insects' osmoregulatory organs. **Figure 2** reviews temperature-dependent transport rates measured in
80 isolated preparations of Malpighian tubules [17,29,39-46] and hindgut [29,37,39,47-50] (data can be found
81 in the **Supplementary Data**). These studies include measurements of secretion (Malpighian tubules) or
82 reabsorption (hindgut) of fluid, K⁺, Na⁺, Cl⁻, and dye in several insect orders (Blattodea, Diptera, Hemiptera,
83 Lepidoptera and Orthoptera) and measurements of short-circuit current (I_{sc}) are also included as an
84 estimation of active transport. The rates reviewed in **Fig. 2** often represent bulk (*net*) transport, making it
85 difficult to distinguish between active transport and passive movement (that are often opposed [51]) when
86 they are assessed using *in vitro* methods such as the Ramsay assay, gut preparations, or SIET
87 [17,29,37,39,47,49,52-54]. These methods are used to investigate specific aspects of transepithelial
88 transport and *in vitro* measurements from isolated preparations can therefore involve "unnatural"
89 conditions with respect to epithelial ion gradients and hormonal factors [55-57]. This complicates direct
90 interpretation of transport capacity in relation to *in vivo* conditions where physiological feed-back
91 regulation is involved in homeostatic regulation of secretion and reabsorption. To fully understand the
92 influence of cold on osmoregulatory capacity it is therefore important to consider a multi-faceted
93 methodological approach, investigating both secretory and reabsorptive tissues and their regulation to
94 evaluate the *in vitro* results in relation to the osmoregulatory disturbance observed *in vivo* (*sensu* **Table 1**).

95 As seen in **Fig. 2**, the general temperature-response for transport rates in Malpighian tubules fit with
96 a classical 'thermal performance curve' [58,59] with a slow decline towards lower temperature, an
97 optimum between 20°C and 40°C, and an abrupt decline of transport rates above 40°C (**Fig. 2A**). Similarly,
98 reabsorption at the hindgut (and ileum) is optimized between 20°C and 30°C with a slow decline towards
99 lower temperature (**Fig. 2B**; rates of reabsorption above 35°C remain to be investigated). Thermal
100 performance curves of "osmoregulatory capacity" are therefore consistent with those measured for many
101 other physiological traits that directly or indirectly rely on homeostatic regulation (i.e. metabolic rate,
102 reproductive capacity, activity, growth, and feeding) [60-63].

103 With respect to cold, it is clear that a marked reduction in osmoregulatory function may result in
104 insufficient capacity to maintain homeostasis of extracellular ion balance (see **Table 1**). Nevertheless, and
105 as evident from **Fig. 2**, great variation in the response to cold exists: Some species/transport systems
106 maintain relatively high transport capacity for solutes at 10°C while others have almost lost all transport
107 capacity at this temperature. Specifically, studies of dipterans and orthopterans have shown interspecific
108 (acclimation) and intraspecific (adaptation) variation in transport that relate well to the observed
109 differences in the capacity to maintain extracellular homeostasis at low temperature.



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112 **Figure 2 – The influence of temperature on osmoregulatory capacity in insects.** The direct effect of
 113 temperature on **(A)** secretion of Malpighian tubules and **(B)** reabsorption of the hindgut (and ileum) in five
 114 insect orders; Blattodea (green), Diptera (yellow), Hemiptera (orange), Lepidoptera (red), and Orthoptera
 115 (blue). Rates of fluid (circles), Na⁺ (diamonds), K⁺ (squares), Cl⁻ (triangles), and dye (inverted triangles) are
 116 normalized to the highest measured value for each species and acclimation regime for each study.
 117 Measurements of reabsorption at the hindgut also include measurements of short-circuit current (*I_{sc}*,
 118 crosses) which is an indirect measure of active transport. Different observations within an order/transport
 119 combination may represent interspecific or intraspecific variations. The black, dashed line indicates no
 120 transport, and the points below this line represent reversals of the transport direction. Data stems from
 121 references [17,29,37,39-50], was extracted using WebPlotDigitizer 3.8 software [64], and can be found in
 122 the **Supplementary Data**.

123 **The influence of thermal acclimation and adaptation on osmoregulatory capacity**

124 It is expected that inter- and intraspecific differences in cold tolerance will be manifested by adaptive
125 differences in osmoregulatory capacity. Responses that could help to preserve extracellular homeostasis in
126 the cold include reduced leak and/or changes to active transport systems to counteract the build-up of
127 hemolymph hyperkalemia. Within the last 10 years a number of studies have investigated this question in
128 different comparative model systems of cold tolerant and intolerant insects (referenced below). Most of
129 these studies use the aforementioned *in vitro* assays and often discuss relative changes in transport (i.e. if
130 transport generally decreases with low temperature it still remains important if K⁺ transport changes
131 relative to Na⁺ transport). Despite these methodological considerations, it is clear that the experimental
132 evidence has overwhelmingly confirmed that cold adaptation (evolutionary processes) and cold acclimation
133 (phenotypic plasticity) elicit critical modifications to osmoregulatory capacity at low temperature to
134 increase cold tolerance of insects (**Fig. 3**).

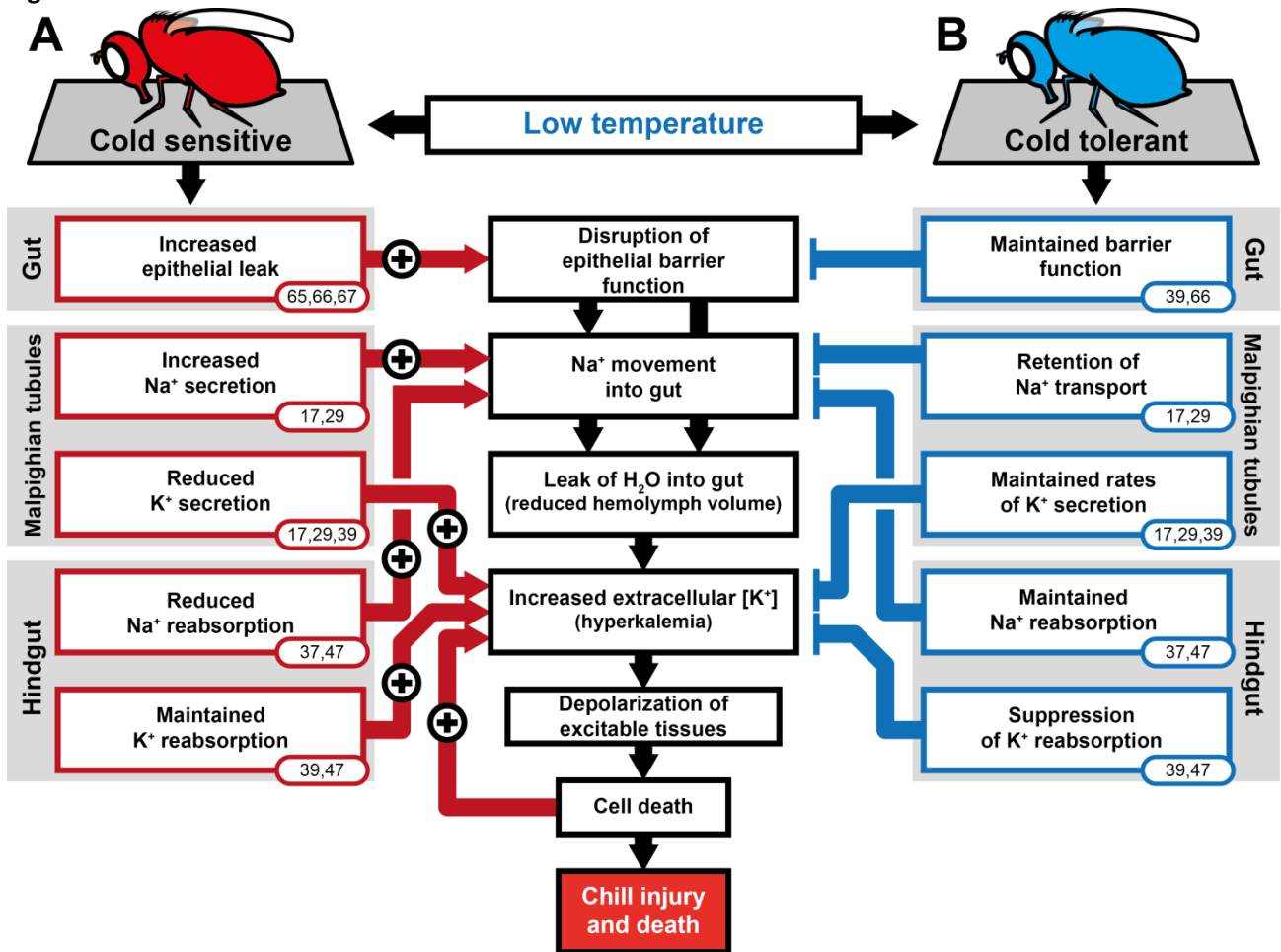
135 Ion and water leak between the hemolymph and gut is at the centre of the cascade leading to
136 hyperkalemia and hence cold adaptive responses (**Fig. 1**). For both *Drosophila* [39,65,66] and *Locusta* [67] it
137 has been shown that epithelial barrier function is reduced at low temperature causing increased leak in
138 cold sensitive species (**Fig. 3A**). The adaptive response to cold would include a tighter gut epithelium
139 [51,68] and support of reduced leak has been reported for both cold tolerant *Drosophila* species [39] and
140 cold-acclimated *Drosophila melanogaster* [66] during cold exposure (**Fig. 3B**). Similarly, cold acclimation
141 increases the electrical resistance across the gut (i.e. rectal) epithelium of *Locusta migratoria* [48].
142 Furthermore, epithelial leak can be prevented by reducing gradients across the gut, which was
143 demonstrated for cold tolerant *Drosophila* species, where the Na⁺ gradient was reduced by “substituting”
144 Na⁺ for compatible osmolites in the hemolymph [18,69], and similar evidence has been found in Orthoptera
145 [70]. Thus, mitigating transepithelial leak is a key adaptation underlying improved cold tolerance in several
146 insect species.

147 The osmoregulatory organs balance passive leak and active transport associated with secretion and
148 reabsorption [71,72]. To prevent cold-induced hyperkalemia, the osmoregulatory organs must maintain K⁺
149 clearance and/or retain Na⁺ and fluid to preserve haemolymph volume and [K⁺] at low temperature (*sensu*
150 **Fig. 1 and 3**). Again, cold tolerant insects appear to exhibit the expected adaptive responses: Relative
151 maintenance of K⁺ secretion by the Malpighian tubules during cold exposure has been shown in cold-
152 acclimated *D. melanogaster* and cold-adapted *Drosophila* species [17,29,39] (**Fig. 3B**). Interestingly, cold
153 tolerant drosophilids appear to favour maintenance of K⁺ clearance over fluid and Na⁺ retention at the
154 Malpighian tubules [17], which could be viewed as maladaptive unless balanced with improved fluid and
155 Na⁺ reabsorption in the hindgut. However, using a novel hindgut assay, Andersen and Overgaard [47]

156 recently found that cold tolerance in these drosophilid species was associated with such upregulation of
157 Na⁺ and water reabsorption. Furthermore, cold tolerant drosophilids suppress hindgut K⁺ reabsorption in
158 the cold [39,47] which will also act to prevent hyperkalemia (**Fig. 3B**). Similar to *Drosophila*, cold-acclimated
159 locusts (*Locusta migratoria*) maintain active ion and water transport in the hindgut (i.e. reabsorption)
160 during cold exposure [37,48] and again this reabsorption is associated with a relative increase in Na⁺
161 reabsorption compared to K⁺, which facilitates fluid balance and prevents hyperkalemia [37]. Another
162 orthopteran, *Gryllus pennsylvanicus*, appears to arrest active secretion when cold-acclimated [42], which is
163 similar to freeze tolerant species going into winter dormancy [73], suggesting that adaptations relating to
164 ion and water homeostasis maintenance during cold exposure might be species- and cold-tolerance-
165 strategy-specific (i.e. chill vs. freeze tolerant). Thus, it is important to consider that adaptations may differ
166 for species facing seasonal cold or diurnal cold exposure.

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Figure 3



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Figure 3 – Conceptual model of the differential effects of cold on the osmoregulatory organs of cold sensitive and cold tolerant insects. (A) In sensitive insects, cold compromises epithelial barrier function promoting transepithelial leak. Ion secretion at the Malpighian tubules is generally decreased but the ratio of Na⁺ and K⁺ secretion is affected in a manner that favours movement of Na⁺ into the gut and retention of K⁺ in the hemolymph (i.e. Na⁺ secretion increases relative to K⁺ secretion). In sensitive insects, cold also reduces rates of hindgut reabsorption in an unfavourable manner: K⁺ reabsorption remains relatively unaffected compared to a major reduction in Na⁺ reabsorption, contributing to the loss of hemolymph Na⁺ and increase in hemolymph [K⁺]. Thus, cold sensitive species are unable to mitigate/prevent the cascade of events leading to hyperkalemia and the secondarily depolarization-induced cell death. **(B)** The increased cold tolerance of cold-adapted or cold-acclimated insects is generally associated with modified osmoregulatory function at low temperature. Cold tolerant insects are often able to maintain epithelial barrier function, limiting transepithelial leak of ions and water. The Malpighian tubules of cold tolerant insects maintain higher transport rates in general but also balance secretion better such that K⁺ secretion remains higher relative to Na⁺ secretion. Similarly, the hindgut of cold tolerant insects defends active transport in general and is therefore better able to maintain Na⁺ reabsorption and, *via* unknown means, suppress K⁺ reabsorption. These hindgut responses mitigate loss of Na⁺ from the hemolymph and counteract hemolymph hyperkalemia. Numbers in the figure represent example studies that demonstrate links between osmoregulatory capacity and the conceptual model for insect chill tolerance; see the full text for a comprehensive list of references.

189 **Molecular changes underlying increased homeostatic capacity in the cold**

190 Above we reviewed the strong links between insect cold tolerance and the ability to maintain
191 osmoregulatory (homeostatic) capacity at both the organismal (**Table 1, Fig. 1**) and tissue level (**Fig. 3**).
192 However, to advance our understanding further we also need to identify and understand the cellular and
193 molecular basis for the ability to maintain osmoregulatory function, and by extension extracellular
194 homeostasis, in the cold.

195 It is likely that cold acclimation and adaptation facilitate maintenance of osmoregulatory capacity by
196 modifications to membrane transport proteins and pathways. A number of studies have investigated the
197 transcriptomic and proteomic changes associated with insect cold tolerance (see for example [74] and
198 [75]). However, most studies have used a whole-organism approach and few accounted for tissue-
199 specificity, making interpretation difficult. We argue that organ-specific approaches could help to identify
200 key proteins and regulatory mechanisms associated with thermal acclimation and adaptation. For example,
201 cross-referencing whole-organism transcriptomic data from cold-acclimated *D. melanogaster* [76] with
202 organ-specific enrichment data from FlyAtlas [77-79] indicates differential expression of a number of
203 transporters and ion channels in the Malpighian tubule and/or hindgut that could be involved (V-type H⁺
204 ATPase, Na⁺/K⁺ ATPase, K⁺ channels, Na⁺/H⁺ exchangers, aquaporins, and Cl⁻ channels). From tissue-specific
205 studies there is also evidence for differential roles of septate junction proteins in promoting epithelial
206 tightness [66], and for a Na⁺/Cl⁻ co-transporter in facilitating hindgut fluid reabsorption [80]. In another
207 tissue-specific transcriptomic study of *G. pennsylvanicus*, cold acclimation was found to downregulate
208 genes related to Malpighian tubule secretion (carbonic anhydrase, V-type H⁺ ATPase) and upregulate genes
209 associated with hindgut reabsorption (Na⁺/K⁺ ATPase) [81]. However, the specific mechanisms by which
210 protein up- or downregulation modulates tolerance remains largely unknown, and we encourage future
211 studies to investigate this using an integrative approach combining physiological measurements of
212 transport with transcriptomic analysis as well as pharmacological and/or genetic interventions (e.g.
213 knockout flies).

214 Neuroendocrine regulators (diuretic and antidiuretic factors) and other signaling molecules can also
215 exert control over the transport rates of the osmoregulatory organs [82,83], and therefore also cold
216 tolerance. Currently, there is evidence that members of the *capability* neuropeptide family (CAPA)
217 modulate cold tolerance *via* effects on the Malpighian tubules [56] and that this effect is dose-dependent;
218 high amounts of CAPA improve cold tolerance by stimulating K⁺ secretion and *vice versa* [55]. However,
219 cellular and molecular effects mediated by peptides at low temperature remain unexplored, with one
220 exception: By measuring active transport across the *L. migratoria* rectum, Gerber et al. [48] demonstrated
221 that cold-acclimated locusts were able to maintain active transport in the cold *via* continued stimulation of

222 cGMP-signaling pathways (a secondary messenger in ion transport peptide signal transduction [84], for
223 which genes were upregulated in the hindgut of cold-acclimated *G. pennsylvanicus* [81]). These results
224 suggest that neuroendocrine factors could play a critical role in insect cold tolerance, but more research is
225 needed.

226 Lastly, it is well-known that thermal adaptation results in modifications of the cell membrane
227 phospholipid composition (i.e. homeoviscous adaptation), that act to defend the physical properties of the
228 membrane and function of the membrane-imbedded proteins [85]. These responses have been repeatedly
229 demonstrated in whole-insect studies in response to cold (reviewed in [86]), but to our knowledge the only
230 tissue-specific study on osmoregulatory organs found no support for homeoviscous adaptation in the
231 rectum of cold-acclimated *L. migratoria* [48]. Even so, we argue that more lipidomic studies could assess
232 membrane modifications of osmoregulatory tissues by cold acclimation and adaptation.

233 In summary, we find strong evidence that chill injury in insects is linked to osmoregulatory failure and
234 loss of extracellular homeostasis at low temperature. In support of this “paradigm” we show how beneficial
235 modifications in epithelial leak, secretion and reabsorption of osmoregulatory organs are consistently
236 found in association with cold adaptation and cold acclimation of insects. These findings are backed by
237 emerging molecular and cellular studies and we therefore argue that further integrative studies of cold
238 tolerance and osmoregulatory capacity (combining molecular, cellular, organ and organismal responses)
239 will be critical to understand the physiology dictating insects cold tolerance, distribution and performance.

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448

449 **Highlighted references**

450 * of special interest

451 ** of outstanding interest

- 452 1. [11]* Literature review detailing the conceptual model of insect chill tolerance. Of particular
453 interest is the proposition that cold is linked to loss of ion balance and injury through interactions
454 on the membrane potential.
- 455 2. [15]** The first study to link the onset of chill-related injury to the loss of ion and water balance at
456 low temperature. This study further demonstrated that prolonged cold resulted in hemolymph
457 hyperkalemia and that this could be mitigated by prior cold acclimation.
- 458 3. [16]** This study demonstrated that hemolymph hyperkalemia was caused by loss of hemolymph
459 volume during cold exposure as a consequence of water and Na⁺ leak towards the gut.
- 460 4. [17]** Using a comparative *Drosophila* model system this study demonstrates the link between
461 cold tolerance, ion and water homeostasis, and secretion capacity of Malpighian tubules at low
462 temperature. Cold tolerant *Drosophila* species are capable of maintaining ion transport rates and
463 ratios during cold exposure which is strongly correlated with their ability to maintain ion balance
464 and survive cold exposure.
- 465 5. [29]* This study reveals links between transport capacity of the osmoregulatory organs and cold
466 tolerance in cold- and warm-acclimated *Drosophila melanogaster*. Cold-acclimated flies maintain
467 ion secretion rates and ratios at the Malpighian tubules and hindgut, and the study link these
468 modifications to changes in ion transporter activity and morphology of the Malpighian tubules.
- 469 6. [37]* Using 'everted gut sac' preparation of the locust hindgut this study links cold-induced loss of
470 ion and water homeostasis to a loss of reabsorptive capacity of the hindgut. It further
471 demonstrates that cold-acclimated locusts maintain superior hindgut reabsorption at low
472 temperature which promotes cold tolerance.
- 473 7. [39]* Using the SIET technique in a comparative *Drosophila* species model system this study
474 evaluates the effects of cold adaptation on K⁺ transport of both Malpighian tubules and hindgut.
475 Cold tolerant *Drosophila* species are characterised by maintained K⁺ secretion and reduced K⁺
476 reabsorption as well as reduced epithelial leak during cold exposure.
- 477 8. [47]** This study introduces a novel assay for quantitative measurement of ion reabsorption rates
478 in hindgut of *Drosophila*. Using this assay the study shows that cold tolerant drosophilids better
479 maintain hindgut reabsorption rates and ratios in the cold.

- 480 9. [55]* This study shows that different concentrations of the CAPA neuropeptide have opposing
481 effects on secretion at the Malpighian tubules. By injecting CAPA into flies, this study further
482 demonstrates that CAPA's effects on the Malpighian tubules directly impacts cold tolerance.
- 483 10. [56]* This study uses targeted silencing of the *capa* gene in transgenic *Drosophila* lines in
484 combination with *in vitro* measurements of Malpighian tubule transport rates to demonstrate links
485 between insect cold tolerance, osmoregulatory function, and neuroendocrine regulation.
- 486 11. [66]* This study demonstrates that cold exposure causes transepithelial leak of solutes across the
487 gut in *Drosophila melanogaster*. Cold acclimation improved cold survival *via* differential expression
488 of septate junction proteins which mitigate transepithelial leak and increases epithelial tightness.
- 489 12. [81]* This study investigates tissue-specific changes in gene expression and identify candidate
490 genes associated with insects cold tolerance in the osmoregulatory organs. Of particular interest
491 are changes associated with increased epithelial tightness, suppression of secretion by the
492 Malpighian tubules, and improved hindgut reabsorption.

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