Rapid stress hardening in the Antarctic midge improves male fertility by increasing courtship success and preventing the decline of accessory gland proteins following cold exposure

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Abstract

Rapid hardening is a process that quickly improves an animal's performance following exposure to a potentially damaging stress. Features of reproduction can be improved by rapid hardening, but little is known about how rapid hardening may contribute to physiological responses in the cold environment of Antarctica. In this study of the Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae), we examine how rapid hardening in response to dehydration (RDH) or cold (RCH) improves male pre- and post-copulatory function related to fertility when the insects are subsequently subjected to a damaging cold exposure. Neither RDH nor RCH improved survival in response to lethal cold stress, but male activity following sublethal cold exposure was enhanced. Both RCH and RDH improved mating success of males compared to those subjected directly to a sublethal bout of cold. Egg viability decreased following direct exposure to sublethal cold, but improved following RCH and RDH. Sublethal cold exposure reduced expression of four accessory gland proteins, while expression remained high in males exposed to RCH. Though rapid hardening may be cryptic in males, this study shows that it can be revealed by pre- and post-copulatory interactions with females.

Keywords: Male fertility, competition, cold exposure, performance, Antarctic midge

Introduction

The Antarctic midge, *Belgica antarctica*, is the only insect endemic to maritime Antarctica (Convey et al., 1996; Sugg et al., 1983). It has a sporadic distribution along the western coast of the Antarctic Peninsula and the South Shetland Islands, where the wingless adults may form aggregations of thousands under favorable conditions (Convey et al., 1996; Potts et al., 2020; Sugg et al., 1983). Larval development extends over two years; developmental progression occurs during the austral summer, and larvae overwinter frozen or immobile within the ice (Usher and Edwards, 1984). Females produce a single batch of eggs or sometimes two smaller clutches (Finch et al., 2020; Harada et al., 2014). The eggs are deposited within a gel matrix produced by the female accessory gland (Finch et al., 2020), and numerous proteins from the accessory gland are critical for nourishing developing larvae, preventing egg/larval dehydration, and thermally buffering the developing larvae. These proteins are produced during both the larval and adult stages and are synthesized by multiple organs. Consequently, stress exposure during larval stages can reduce reproductive output of the females (Finch et al., 2020).

During the austral summer, mating occurs in swarms where numerous males court and attempt to mate (Convey et al., 1996; Finch et al., 2020; Sugg et al., 1983). Males produce a suite of accessory proteins that are transferred to the females during mating, and based on sequence similarity to accessory gland proteins from other insects, these proteins are likely to impact female fertility and male sperm viability along with competition (Avila et al., 2011; Chapman et al., 2000; Simmons, 2019; Sitnik et al., 2016; Wolfner, 1997). As with females, stress exposure of male larvae impacts subsequent male fertility (Finch et al., 2020). It is not known how cold exposure impacts fertility when males are exposed to cold as adults. Impacts could be revealed during pre-copulation competition between males or post-copulation by a reduction in accessory gland protein or viable sperm. Cold exposure reduces male fertility in multiple insect systems (Chakir et al., 2002; Lacoume et al., 2007; Rinehart et al., 2000; Singh et al., 2015; Vollmer et al., 2004).

68 Rapid cold hardening (RCH) is a highly-responsive protective process documented in many insect
69 systems against cold injury (Teets et al., 2019; Teets et al., 2020). RCH can also be induced by short
70 periods of dehydration, which we refer to as rapid dehydration hardening (RDH) (Levis et al., 2012).
71 Mechanisms underlying processes of RCH and prolonged cold acclimation include synthesis and
72 accumulation of specific cryoprotectants, changes in the composition of cell membranes, inhibition of
73 apoptotic pathways, changes in stress protein abundance, protein phosphorylation shifts, and metabolic
74 differences (reviewed in (Teets and Denlinger, 2013; Teets et al., 2020). Importantly, RCH and cold
75 acclimation offer beneficial effects allowing biological processes such as movement, feeding, and mating
76 to occur at lower temperatures or to enhance performance when conditions become more favorable
77 (Kelty et al., 1996; Shreve et al., 2004; Srithiphaphirom et al., 2019; Teets et al., 2019). During summer,
78 males of the Antarctic midge are unlikely to be exposed to lethal temperatures (-8 to -10°C) because
79 summer temperatures in their habitat commonly range from -5 to 5°C. In summer-acclimated larvae,
80 RCH improves recovery performance following sub-lethal freezing injury and facilitates energy saving
81 (Teets et al., 2019). The impact of RCH on adult reproductive performance has yet to be examined even
82 though adults are active and moving on the surfaces of the buffered habitats where the larvae reside.

83 In this study, we examined the impact of RCH and RDH on male performance of *B. antarctica*
84 following exposure to sublethal cold stress. Specifically, we assessed pre- and post-copulatory function
85 related to male performance and fertility. Males exposed directly to sublethal cold stress suffered
86 significant declines in mating competitions with untreated males and subsequent fertility was reduced.
87 These impairments were reduced by RCH or RDH prior to mating. The RCH-induced enhancement of
88 male competitiveness is likely due to a general increase in activity, and the boost in fertility is likely due
89 to retention of the capacity to express seminal fluid proteins. These physiological responses suggest that
90 cold hardening may be essential for allowing males to retain fertility during the austral summer.

91

92 **Materials and Methods**

93 Midge collection

94 Adults and larvae were collected from the same location, on off-shore islands near Palmer Station,
95 Antarctica. Larvae within organic debris were returned to Palmer Station and extracted with a modified
96 Berlese funnel (Benoit et al., 2007b). Following recovery, larvae were stored with substrate from their
97 natural habitat (rocks, soils, moss, and the alga *Prasiola crispa*, which serves as a food source for *B*.
98 *antarctica*) at 2-4°C. Larvae were shipped to the University of Cincinnati and stored under similar
99 conditions until they emerged as adults and were used in the studies described. Males and females were
100 separated based on their morphological characters.

101

102 Survival assessment

103 Survival following cold exposure was based on previous methods (Benoit et al., 2007a; Lee et al., 2006)
104 but adapted for these specific experiments. Adult males held at 4°C were placed into individual 2 ml
105 microcentrifuge tubes and transferred from 4°C to either -5°C for 1 h (sublethal cold exposure) or -
106 10°C for 1 h (lethal cold exposure), based on temperatures determined in previous studies (Lee et al.,
107 2006). RCH was induced by exposure to -2°C for 1 h before direct transfer to the treatment
108 temperature. RDH was accomplished by exposing adults to 75% RH until a loss of 5-8% of their water
109 content (Benoit et al., 2007a; Benoit et al., 2009) before cold exposure. Survival was assessed following
110 a 24 h recovery at 4°C. Adults were deemed alive if the individual could move at least 5 cm. Three
111 groups with 8 replicates were examined for each treatment. A one-way ANOVA was used to examine
112 significance and a Tukey's test was used to identify differences between treatments.

113

114 Activity

115 A Locomotor Activity Monitor (TriKinetics Inc., Waltham, MA, USA) in conjunction with DAMSystem3
116 Data Collection Software (TriKinetics) was used to assess activity of adult males. Individuals were placed
117 in standard *Drosophila* vials (25 mm diameter and 95 mm height) following 6 h of recovery at colony
118 conditions of 4°C. Tubes were placed horizontally in the monitor and the entire system was placed
119 within a plastic container held at 93% RH using a saturated salt solution of potassium nitrate.
120 Temperature was held at 4°C within an environmental chamber. After a two-hour acclimation, general
121 activity was measured for 4 h under continuous light. For each treatment, 15-20 males were monitored.
122 A one-way ANOVA was used to examine significance and a Tukey's test was used to identify differences
123 between treatments.

124

125 Male mating competition

126 Competitive mating assays were conducted following cold exposure or rapid hardening followed by cold
127 exposure. Cold level for exposure was sublethal (-5°C for 1 h). RCH was induced by exposure to -2°C for
128 1 h before direct transfer to the treatment temperature. Following treatment, males were returned to
129 colony conditions and allowed to recover for 6 h prior to mating assays to match studies on activity. The
130 mating assays were accomplished by releasing a virgin female into an arena with two males. Males were
131 distinguished by partial removal of either the left or right antennae (randomized between each assay).
132 The first male to copulate with the female was scored as a successful copulation. Each assay was
133 replicated 50 times and significance was determined with a Chi-squared (χ^2) test. New females and
134 males were used for each assay.

135

136

137 Fertility assay

138 To determine the impact of thermal exposure on male fertility, males exposed to thermal stress were
139 allowed to mate with either two females based on previously developed methods (Finch et al., 2020).
140 Briefly, virgin females were collected immediately after adult eclosion and stored at 4°C and 93% RH,
141 with access to moist substrate. Males were exposed to thermal stresses as described in the survival
142 assessment section. Following treatment, males were evaluated for survival and allowed to mate with
143 two females consecutively. Following mating, females were returned to colony conditions of 4°C and
144 93% RH and observed for deposition of eggs. Eggs, held at 4°C and 93% RH, remained attached to the
145 wet substrate and were observed for larval emergence for 60 days (Finch et al., 2020; Harada et al.,
146 2014). Each treatment was replicated with 8-12 males. A one-way ANOVA was used to examine
147 significance followed by a Tukey's test for differences between treatments.

148

149 RNA-seq analysis to identify highly enriched male accessory glands genes for targeted studies

150 To establish putative underlying factors that could impact fertility, we re-examined RNA-seq data on
151 male and male accessory glands (Finch et al., 2020), with the goal of identifying four targets for
152 subsequent analyses following cold exposure and RCH. The desired targets were selected on the basis of
153 having high expression in males and male accessory glands compared to females/larvae and female
154 accessory glands. The four targets were characterized by BLAST comparison to other genes in other
155 insect systems. The four selected targets included: IU25_04442, a *Belgica* specific gene; IU25_01011, a
156 seminal metalloprotease; IU25_12390, an antigen 5-like allergen; IU25_12518, a seminal
157 metalloprotease.

158

159 qPCR analyses of male accessory proteins

160 RNA was extracted from the accessory glands by homogenization (BeadBlaster 24, Benchmark Scientific)
161 in Trizol reagent (Invitrogen), using manufacturer's protocols with slight modification based on other
162 studies of invertebrates. Extracted RNA was treated with DNase I (Thermo Scientific) and cleaned with a
163 GeneJet RNA Cleanup and Concentration Micro Kit (Thermo Scientific) according to the manufacturer's
164 protocols. RNA concentration and quality were examined with a NanoDrop 2000 (Thermo Scientific).

165 qPCR analyses were conducted based on previously developed methods (Finch et al., 2020;
166 Hagan et al., 2018; Meibers et al., 2019). RNA was extracted as described previously for independent
167 biological replicates. Complementary DNA (cDNA) was generated with a DyNAmo cDNA Synthesis Kit
168 (Thermo Scientific). Each reaction used 250 ng RNA, 50 ng oligo (dT) primers, reaction buffer containing
169 dNTPs and 5 mmol•l^{-1} $MgCl_2$, and M-MuLV RNase H+ reverse transcriptase. KiCqStart SYBR Green qPCR
170 ReadyMix (Sigma Aldrich, St Louis, MO, USA) along with 300 nmol l^{-1} forward and reverse primers, cDNA
171 diluted 1:20, and nuclease-free water were used for all reactions. Primers were designed using Primer3
172 based on contigs obtained from the transcriptome analysis (Table S1). qPCR reactions were conducted
173 using an Illumina Eco quantitative PCR system. Reactions were run according to previous studies (Finch
174 et al., 2020; Hagan et al., 2018; Meibers et al., 2019). Three biological replicates were examined for each
175 sex, and two biological replicates were examined for each accessory gland. Expression levels were
176 normalized to *rpl19* using the ΔΔCq (Finch et al., 2020; Teets et al., 2013). A one-way ANOVA was used
177 to examine significance followed by a Tukey's test for differences between treatments.

178

179

180

181 **Results**

182 Rapid hardening does not impact survival of males

183 Significant differences in mean survival were noted for the different treatment groups as determined by
184 one-way ANOVA ($F_{8,18}$ = 34.25, *p-value* = 2.38 x 10^{-9}). Sudden exposure of males to the extreme cold
185 treatment significantly decreased survival in comparison with controls, but survival was not improved by
186 RCH (Fig. 1). Although the value for male survival was higher when extreme cold exposure was
187 preceded by RDH, this increase was not significant (Fig. 1). Neither RCH nor RDH improved survival in
188 those exposed to a sublethal stress (Fig. 1). Thus, we observed no evidence that rapid hardening
189 enhanced survival of male adults.

190

191 Rapid hardening improves male activity and competition during female courtship

192 Sublethal cold exposure resulted in significant reduction in activity ($F_{3,72}$ = 12.07, *p-value* = 1.73 x 10^{-6}),
193 but activity was retained in RCH-treated compared to control males (Tukey's test, *p-value* = 0.246, Fig.
194 2a). Activity was not significantly different between control males and those that experienced RCH.
195 These results indicate that RCH allows males to maintain activity following cold stress.

196 Results from the competitive mating assays demonstrated that sublethal cold exposure
197 drastically impaired male mating (N = 50, χ^2, *p-value* = 8.16 x 10^{-5}, Fig. 2b). RCH or RDH without sublethal
198 cold stress did not alter male mating (N = 50, χ^2, *p-value* > 0.26, Fig. 2b). Males first exposed to RDH or
199 RCH before sublethal cold stress showed increased mating compared to those only experiencing
200 sublethal cold stress (N = 50, χ^2, *p-value* < 0.01, Fig. 2b). These results suggest that RDH and RCH will
201 likely improve pre-copulatory interactions in males, allowing for increased mating success.

202

203 Rapid hardening prevents reductions in male fertility

204 The total number of eggs produced did not vary among treatment groups (one-way ANOVA, *p-value* =
205 0.456). But, egg viability significantly differed among treatments (Fig. 3.; one-way ANOVA, $F_{3,46} = 14.12$,
206 *p-value* = 1.18×10^{-6}). In particular, egg viability was significantly reduced (by nearly 40%) when eggs
207 were exposed to sublethal cold (Tukey's test, *p-value* < 0.001) . This reduction in egg viability was
208 prevented when males were pretreated with RCH (Fig. 3; *p-value* = 0.717). RCH before the sublethal cold
209 exposure thus allowed males to maintain fertility, as indicated by egg viability (Fig. 3; *p-value* = 0.371 vs.
210 control, *p-value* = 0.0009 vs. sublethal cold). Thus, both pre-copulatory and post-copulatory aspects of
211 reproduction are impacted when males are exposed to sublethal cold stress.

212 To determine a putative mechanism for reduced fertility, we examined expression patterns of
213 four accessory gland proteins (ACPs) that are expressed exclusively in the male accessory gland (Fig. 4a).
214 When exposed to sublethal cold stress, expression of all four ACPs was significantly reduced (Fig. 4b,
215 one-way ANOVA, *p-value* < 0.03 in all cases). When subjected to RCH prior to sublethal cold exposure,
216 expression of three ACPs increased in comparison to expression levels seen following sublethal cold
217 exposure without RCH (Fig. 4b). These results suggest that a reduction in factors associated with seminal
218 fluid production could underlie the observed reduction in male fertility.

219

220 **Discussion**

221 This study highlights the critical role of rapid hardening in relation to male performance in the
222 Antarctic midge, as summarized in Figure 5. Hardening had very little impact on survival of males but
223 allowed them to retain higher levels of activity. This increased activity is likely a major underlying factor
224 allowing males to be more competitive in their ability to copulate with females. Male exposure to
225 sublethal cold stress resulted in a significant reduction in fertilization rate, as reflected in production of

226 fewer viable eggs. This impairment was recovered when males were subjected to RCH prior to cold
227 exposure. This reduction in fertility could be due, in part, to a major reduction in expression of specific
228 accessory gland proteins, which are critical for males to maintain maximum fecundity. The combined
229 impact is that rapid hardening can promote male fitness in two ways: 1) by allowing high performance
230 during pre-copulatory interactions, and 2) by allowing males to maximize fertility. Interactions between
231 pre- and post-copulatory factors work together in contributing to male fertility (Birkhead and Pizzari,
232 2002; Polak et al., 2021). In this study, we also show that both aspects can be negatively impacted by
233 exposure to stress.

234 Increased survival during lethal cold stress is a major factor associated with rapid hardening,
235 induced by dehydration or cold (Benoit et al., 2009; Teets et al., 2019; Teets et al., 2020). For the
236 Antarctic midge, RCH significantly improves larval survival when exposed to freezing temperatures (Lee
237 et al., 2006; Teets et al., 2008). But, the Increased survival appears to be stage-specific in the Antarctic
238 midge. While larvae show improved survival following RCH, adults do not (Lee et al. 2006). Our results
239 are consistent with these earlier observations showing that rapid hardening has little impact on adult
240 survival of the midge. Rapid hardening in other insect systems, such as *Drosophila*, also shows
241 differences between developmental stages, specifically in relation to survival (Colinet and Hoffmann,
242 2012; Jensen et al., 2007; Teets et al., 2020; Terblanche et al., 2007). Increased survival generated by
243 RCH may lack ecological relevance for these short-lived adult midges because they are rarely exposed to
244 potentially lethal temperatures during their active period in the austral summer (Lee et al., 2006; Teets
245 et al., 2019).

246 A key aspect of RCH is organismal performance following exposure to sublethal temperatures
247 (reviewed in (Teets et al., 2020)). Three key aspects related to sublethal cold stress are a lowering of the
248 critical thermal minimum temperature (CT_{min}), increased recovery from the CT_{min}, and fitness/energetic
249 advantages following cold stress (Benoit et al., 2021; Coulson and Bale, 1992; Findsen et al., 2013;

250 Rinehart et al., 2000; Shreve et al., 2004; Teets et al., 2019; Teets et al., 2020). We did not determine
251 whether RCH reduces the CT_{min} or recovery, but rapid hardening did improve the male activity levels to
252 near those of controls following sublethal cold exposure. RCH likely prevents protein and membrane
253 damage including resting potential depolarization that would normally occur following sublethal cold
254 stress (Koštál et al., 2004; Koštál et al., 2006; Overgaard and MacMillan, 2017). Damage due to sublethal
255 stress is likely most pronounced in muscle and nervous tissues (Andersen et al., 2015; Koštál et al., 2006;
256 MacMillan et al., 2014; Overgaard and MacMillan, 2017), which in turn can be expected to significantly
257 impact general activity levels, as observed for males in our study.

258 The overall increased level of activity is likely a major factor underlying the RCH-generated
259 improvement in male copulatory behavior. *B. antarctica* mates in swarms (Finch et al., 2020; Sugg et al.,
260 1983), thus there is likely significant pre-copulatory competition between males, and improved male
261 performance could represent a significant advantage if there are periods of unexpected cold during the
262 summer. In *Drosophila melanogaster*, courtship behavior is preserved by RCH at low temperatures
263 (Shreve et al., 2004), but whether this improves courtship abilities following more extreme stress is not
264 known. In larvae of the Antarctic midge, RCH does improve performance when larval locomotion is
265 monitored (Teets et al., 2019). Studies from multiple systems suggest that RCH improves general activity
266 during cold exposure, especially in chill susceptible systems (Overgaard and MacMillan, 2017)

267 Along with a reduction in pre-copulatory performance, male fertility declined following cold
268 exposure as evidenced by a nearly 40% reduction in egg viability. This reduction was prevented when
269 males were exposed to RCH before the sublethal cold stress. When pharate adult flesh flies, *Sarcophaga*
270 *crassipalpis*, are exposed to cold stress, there is also a reduction in male fertility that can similarly be
271 recovered by RCH (Rinehart et al., 2000). Among potential mechanisms that could contribute to this
272 impairment, we noted that expression of previously identified male ACPs (Finch et al., 2020) were
273 reduced substantially. RCH yielded a substantial recovery in expression of transcripts encoding the

274 ACPs. Two of these proteins were identified as putative metalloproteases previously implicated in
275 processing of seminal proteins, sperm competition, and sperm storage (Avila and Wolfner, 2017;
276 LaFlamme et al., 2012; Laflamme et al., 2014). Along with sperm viability, female sexual receptivity also
277 increases when specific proteases are inhibited (LaFlamme et al., 2012). This could have a substantial
278 impact in species such *as B. antarctica* where mating occurs in swarms and females mate more than
279 once (Finch et al., 2020). The two other ACPs examined here include one that does not have an assigned
280 function and one that is related to antigen 5-like allergens. Antigen-like proteins are common in salivary
281 glands in many insect systems (King and Spangfort, 2000), but the specific role for this protein in the
282 Antarctic midge remains unknown. From our studies, we cannot rule out direct damage to sperm as an
283 underlying cause for females producing fewer viable eggs, as documented in other insect systems
284 (Colinet and Hance, 2009; Levie et al., 2005; Rinehart et al., 2000). Both the reduction in transcript
285 levels for ACPs and sperm damage may contribute to reproductive impairment by low temperature, but
286 regardless of the immediate cause, RCH appears to be an effective mechanism for protecting not only
287 pre-copulatory activity but also post-copulatory factors impacting fertility in the Antarctic midge.

288

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295

296 **Figure and table legends**

297 **Figure 1** - Effect of rapid hardening on male survival of the Antarctic midge. Different letters indicate
298 significant (one-way ANOVA, Tukey's Honest Significant Difference test, *P-value* < 0.05) differences
299 between treatment groups. (RCH+Cold = rapid cold hardening before damaging cold exposure; Cold =
300 damaging cold exposure; RDH+Cold = rapid dehydration hardening before damaging cold exposure;
301 RCH+SL = rapid cold hardening before sublethal cold stress; Sublethal = sublethal cold stress; RDH+SL =
302 rapid dehydration hardening before sublethal cold stress; RCH = rapid cold hardening; RDH = rapid
303 dehydration hardening, Control = untreated group).

304

305 **Figure 2** - Effect of rapid hardening on competition between males of the Antarctic midge during
306 courtship. (A) Activity differences in control, sublethal cold exposure, and rapid cold hardening (RCH)
307 followed by sublethal cold exposure. Significance determined by ANOVA followed by a Tukey's test for
308 differences between treatments. (B) Difference between the choice of control or RCH males compared
309 to the specific treatment. Significance was determined with χ^2 test.

310

311 **Figure 3** - Effect of rapid hardening of males on viability of eggs produced by females. Treatments are
312 control, cold exposure, rapid cold hardening (RCH), rapid cold hardening before sublethal cold exposure
313 (RCH-Cold). Significant differences between treatment groups were determined with a one-way ANOVA
314 followed by a Tukey's test for differences between treatments. The total number of eggs produced by
315 females did not vary between treatments (P = 0.456).

316

317 **Figure 4** - Transcript level analysis of male accessory gland proteins during cold exposure. (A)
318 Expressional analysis to identify male accessory gland protein targets based on previous RNA-seq studies
319 underlying reproductive biology of the Antarctic midge (Finch et al., 2020). (B) quantitative PCR analysis

320 following cold exposure and rapid cold hardening (RCH). Significant differences between treatment
321 groups were determined with a one-way ANOVA followed by a Tukey's test for differences between
322 treatments.
323
324 **Figure 5** - Summary of the impact of sublethal cold exposure and rapid cold hardening on pre-copulatory
325 and post-copulatory features of male fertility and performance.
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327 **Table S1** - Primers utilized for quantitative PCR studies.
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Figure 1

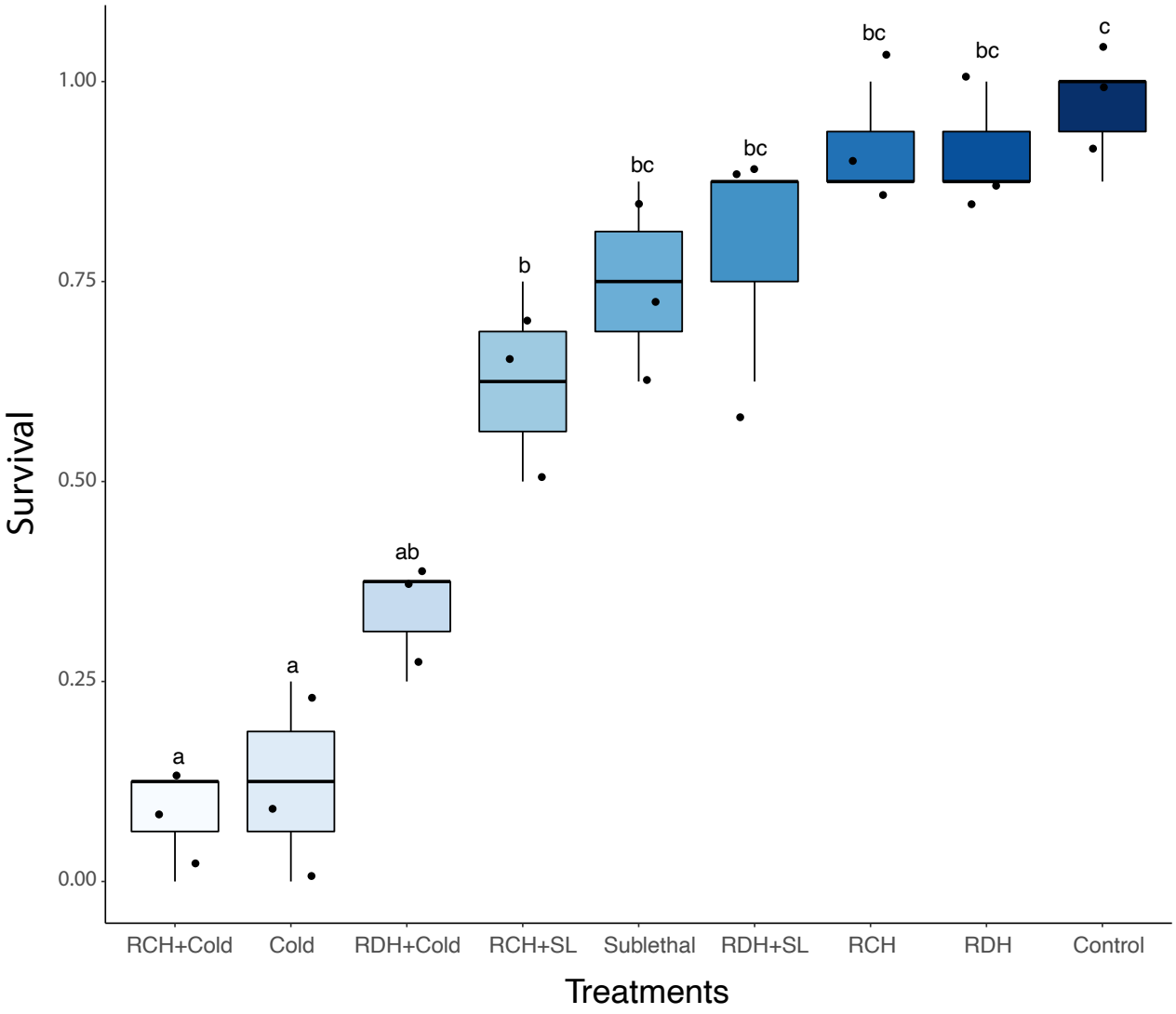
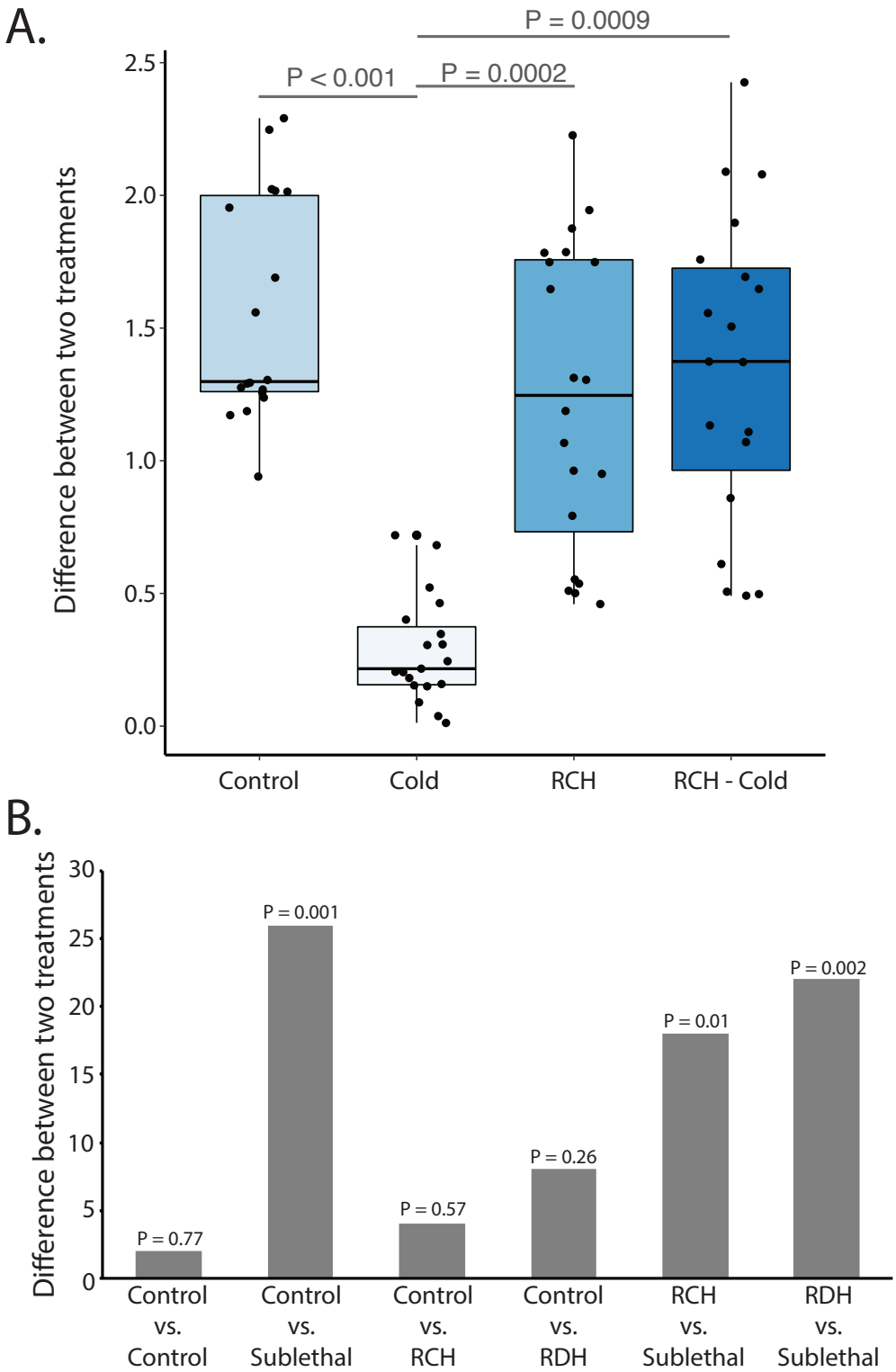

Survival
1.00
0.75
0.50
0.25
0.00
a
a
ab
b
bc
bc
bc
bc
c
RCH+Cold
Cold
RDH+Cold
RCH+SL
Sublethal
RDH+SL
RCH
RDH
Control
Treatments

Figure 2


A.
P = 0.0009
P < 0.001
P = 0.0002
Difference between two treatments
2.5
2.0
1.5
1.0
0.5
0.0
Control
Cold
RCH
RCH - Cold
B.
Difference between two treatments
30
25
20
15
10
5
0
P = 0.77
P = 0.001
P = 0.57
P = 0.26
P = 0.01
P = 0.002
Control vs. Control
Control vs. Sublethal
Control vs. RCH
Control vs. RDH
RCH vs. Sublethal
RDH vs. Sublethal

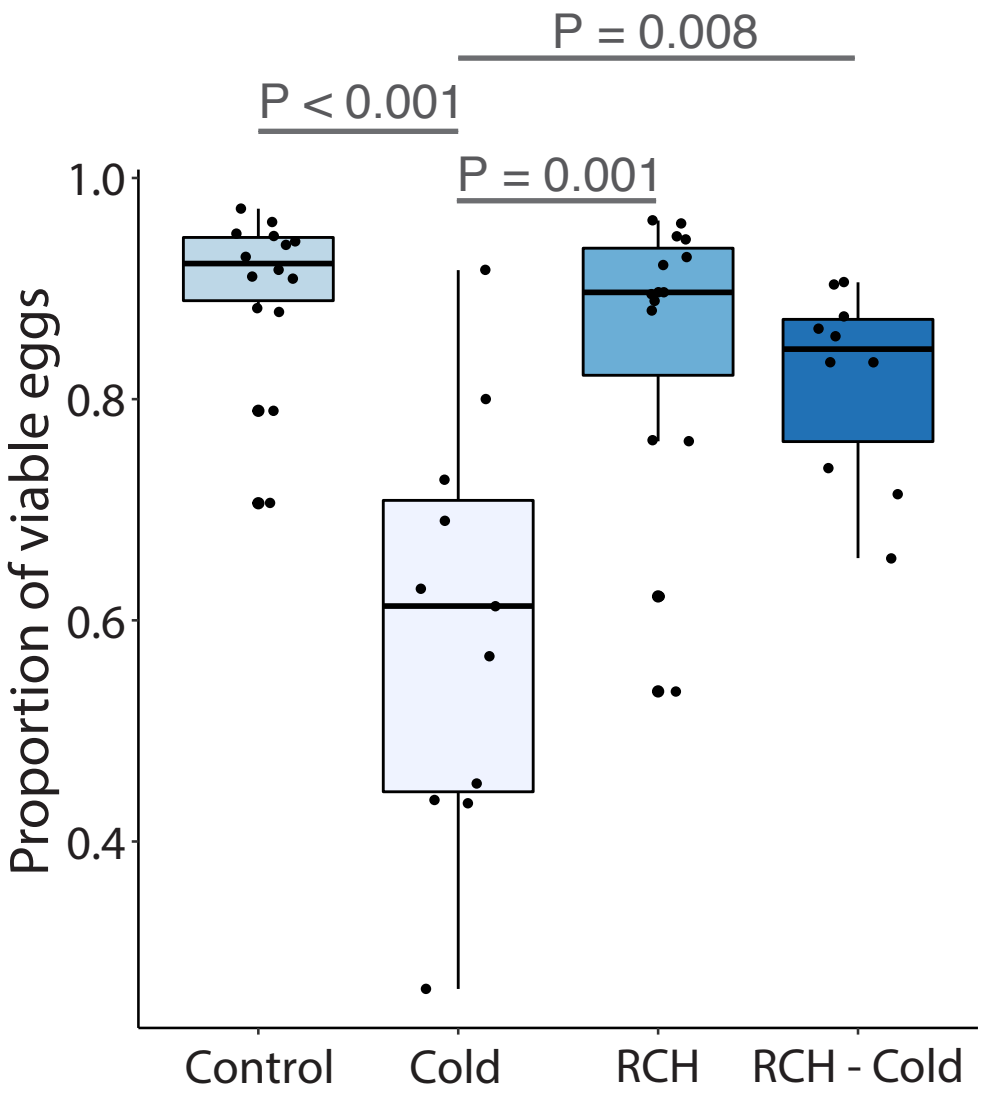

P = 0.008
P < 0.001
P = 0.001
1.0
0.8
0.6
0.4
Proportion of viable eggs
Control
Cold
RCH
RCH - Cold

Figure 4

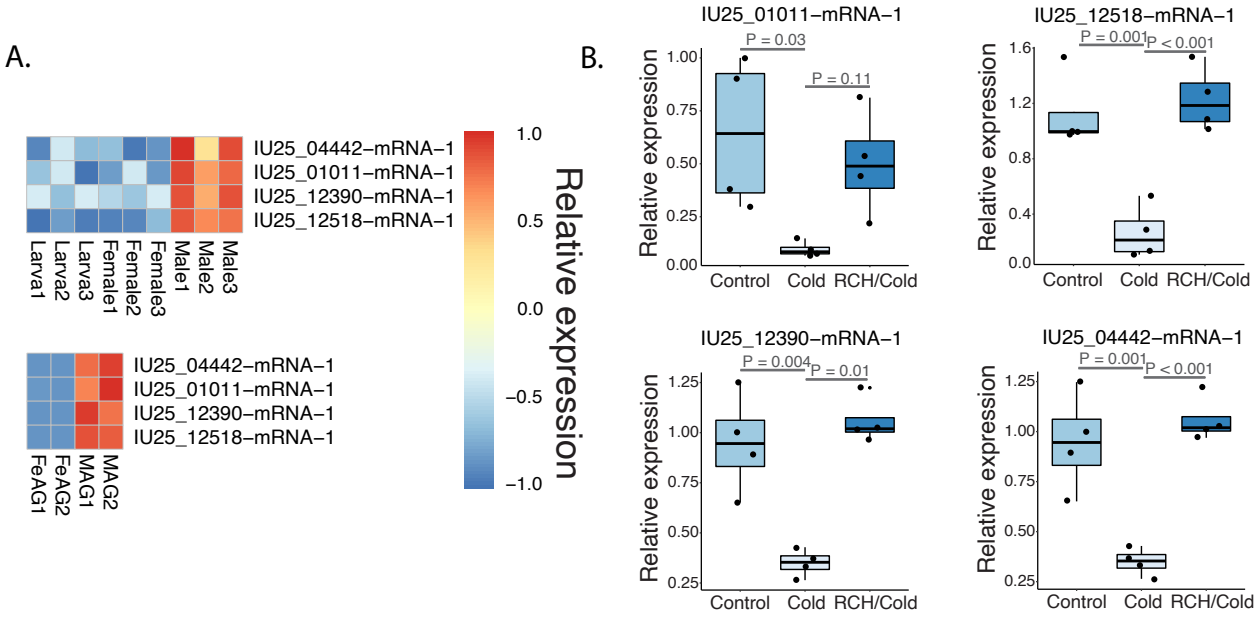

A.
IU25_04442−mRNA−1
IU25_01011−mRNA−1
IU25_12390−mRNA−1
IU25_12518−mRNA−1
Larva1
Larva2
Larva3
Female1
Female2
Female3
Male1
Male2
Male3
FeAG1
FeAG2
MAG1
MAG2
1.0
0.5
0.0
−0.5
−1.0
Relative expression
B.
IU25_01011−mRNA−1
P = 0.03
P = 0.11
IU25_12518−mRNA−1
P = 0.001
P < 0.001
IU25_12390−mRNA−1
P = 0.004
P = 0.01
IU25_04442−mRNA−1
P = 0.001
P < 0.001
Relative expression
Control
Cold
RCH/Cold

Figure 5

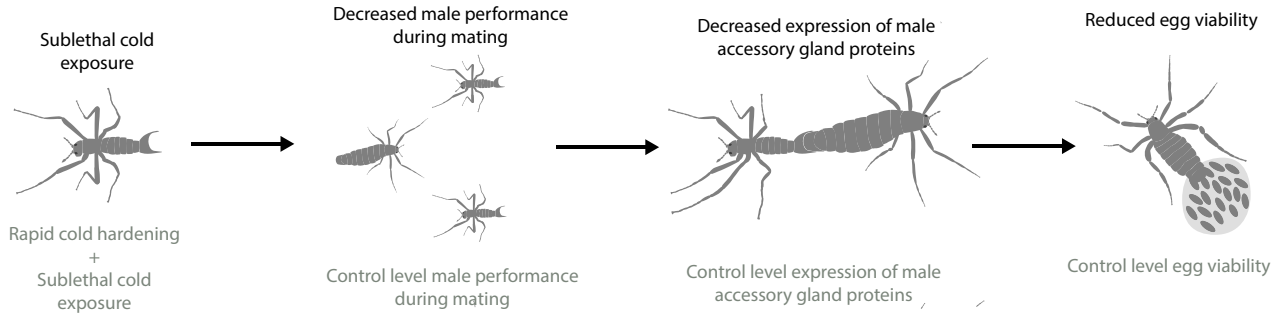

Sublethal cold exposure
Rapid cold hardening
+
Sublethal cold exposure
Decreased male performance during mating
Control level male performance during mating
Decreased expression of male accessory gland proteins
Control level expression of male accessory gland proteins
Reduced egg viability
Control level egg viability

Table S1

	Forward	Reverse
IU25_04442-mRNA-1	CGTCTTCAAGCCTTACAGGA	GTCTCGCAGAGTGATCGTTT
IU25_01011-mRNA-1	CACAATAAGTGGGGCTGAAC	TGATCACTCTGAAGCAACCA
IU25_12518-mRNA-1	CACAGCTTGGATTCCTTAAC	CTGATGCATGTGCATTAAAC
IU25_12390-mRNA-1	ATTACTGTGACCCAATCCTG	TGTTGTCTTCTGTCAGTTCG