

SHORT COMMUNICATION

Rapid stress hardening in the Antarctic midge improves male fertility by increasing courtship success and preventing 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 potentially damaging stress. In this study of the Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae), we examined how rapid hardening in response to dehydration (RDH) or cold (RCH) improves male pre- and post-copulatory function 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 and mating success following sublethal cold exposure were enhanced. Egg viability decreased following direct exposure of the mating males to sublethal cold but improved following RCH and RDH. Sublethal cold exposure reduced the 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.

KEY WORDS: Pre-copulation, Competition, Post-copulation, Performance, Stress exposure

INTRODUCTION

The Antarctic midge, *Belgica antarctica*, is the only insect endemic to maritime Antarctica (Convey and Block, 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 and Block, 1996; Potts et al., 2020; Sugg et al., 1983). Larval development extends over 2 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 larval and adult stages and are synthesized by multiple organs. Consequently, stress exposure during larval life can reduce female reproductive output by reducing the accumulation of larval-derived storage proteins as well as proteins associated with the gel matrix (Finch et al., 2020).

During the austral summer, mating occurs in swarms where numerous males court and attempt to mate (Convey and Block, 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 likely 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 quite a few insect systems (Chakir et al., 2002; Lacoume et al., 2007; Rinehart et al., 2000; Singh et al., 2015; Vollmer et al., 2004).

Rapid cold hardening (RCH) is a highly responsive protective process documented in many insect systems against cold injury (Teets et al., 2019; Teets et al., 2020). RCH can also be induced by short periods of dehydration, which we refer to as rapid dehydration hardening (RDH) (Levis et al., 2012). Mechanisms underlying processes of RCH and prolonged cold acclimation include numerous biochemical and molecular features (reviewed in (Teets and Denlinger, 2013; Teets et al., 2020). Importantly, RCH and cold acclimation offer beneficial effects allowing biological processes such as movement, feeding and mating to occur at lower temperatures or to enhance performance when conditions become more favorable (Kelty et al., 1996; Shreve et al., 2004; Srithiphaphirom et al., 2019; Teets et al., 2019). During summer, males of the Antarctic midge are freeze intolerant and unlikely to be exposed to lethal temperatures (−8 to −10°C) because summer temperatures in their habitat commonly range from −5 to 5°C. In summer-acclimated freeze-tolerant larvae, RCH improves recovery performance following sub-lethal freezing injury and facilitates energy saving (Teets et al., 2019). The impact of RCH on adult reproductive performance has yet to be examined. Adults are active and moving on the surfaces of the buffered habitats where the larvae reside. In this study of *B. antarctica*, we examined the impact of RCH and RDH on male performance following exposure to sublethal cold stress. Specifically, we assessed pre- and post-copulatory function related to male performance and fertility.

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MATERIALS AND METHODS

Midge collection

Belgica antarctica Jacobs 1900 adults and larvae were collected from the same location, on off-shore islands near Palmer Station, Antarctica. Larvae within organic debris were returned to Palmer Station and extracted with a modified Berlese funnel at 20°C (Benoit et al., 2007b). Larvae were stored with substrate from their natural habitat (rocks, soils, moss and the alga *Prasiola crista*, which serves as a food source for *B. antarctica*) at 2–4°C for at least 1 week. Larvae were shipped to the University of Cincinnati in their natural substrate at –5°C and stored under similar conditions until they emerged as adults. Adults were collected every 12 h for use in the studies described. Males and females were separated based on their morphological characters.

Survival assessment

Survival following cold exposure was based on previous methods (Benoit et al., 2007a; Lee et al., 2006) but adapted for these specific experiments. Adult males held at 4°C were placed into individual 2 ml microcentrifuge tubes and transferred from 4°C to either –5°C for 1 h (sublethal cold exposure) or –10°C for 1 h (lethal cold exposure), based on temperatures determined in previous studies (Lee et al., 2006). RCH was induced by exposure to –2°C for 1 h before direct transfer to the treatment temperature. Temperature changes occurred at 0.5°C min^{–1} and were accomplished using temperature-controlled water baths. RDH was accomplished by exposing adults to 75% relative humidity (RH), using a saturated solution of sodium chloride, until a loss of 5–8% of their water content (Benoit et al., 2007a, 2009). Survival was assessed following a 24 h recovery at 4°C. Adults were deemed alive if they could move at least 5 cm. Three groups with 8 replicates were examined for each treatment. A one-way ANOVA was used to examine significance and a Tukey's test was used to identify differences between treatments.

Activity

A Locomotor Activity Monitor (TriKinetics Inc., Waltham, MA, USA) in conjunction with DAMSystem3 Data Collection Software (TriKinetics) was used to assess activity of adult males. Individuals were placed in standard *Drosophila* vials (25 mm diameter and 95 mm height) following 6 h of recovery at colony conditions of 4°C. Tubes were placed horizontally in the monitor and the entire system was placed within a plastic container held at 93% RH using a saturated salt solution of potassium nitrate. Temperature was held at 4°C within an environmental chamber. After a 2 h acclimation, general activity was measured for 4 h under continuous light. For each treatment, 15–20 males were monitored, and locomotion was quantified as detected movements (infrared beam breaks as the males move within the vials) per minute. A one-way ANOVA was used to examine significance and a Tukey's test was used to identify differences between treatments.

Male mating competition

Competitive mating assays were conducted following cold exposure or rapid hardening followed by cold exposure. Cold level for exposure was sublethal (–5°C for 1 h). RCH was induced by exposure to –2°C for 1 h before direct transfer to the treatment temperature. Following treatment, males were returned to colony conditions and allowed to recover for 6 h prior to mating assays to match studies on activity. The mating assays were accomplished by releasing a virgin female into an arena with two males. Males were distinguished by partial removal of either the left or right antennae

(randomized between each assay). The first male to copulate with the female was scored as a successful copulation. Each assay was replicated 50 times and significance was determined with a Chi-squared (χ^2) test. New females and males were used for each assay.

Fertility assay

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

RNA-seq and qPCR analyses of male accessory proteins

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

RNA was extracted from accessory glands by homogenization (BeadBlaster 24, Benchmark Scientific) in Trizol reagent (Invitrogen), following the manufacturer's protocols with slight modification of an additional centrifuge step to remove cuticle debris based on other studies of invertebrates. Extracted RNA was treated with DNase I (Thermo Scientific) and cleaned with a GeneJet RNA Cleanup and Concentration Micro Kit (Thermo Scientific) according to the manufacturer's protocols. RNA concentration and quality were examined with a NanoDrop 2000 (Thermo Scientific).

qPCR analyses were conducted based on previously developed methods (Finch et al., 2020; Hagan et al., 2018; Meibers et al., 2019). RNA was extracted as described previously for independent biological replicates. cDNA was generated with a DyNamo cDNA Synthesis Kit (Thermo Scientific). Each reaction used 250 ng RNA, 50 ng oligo (dT) primers, reaction buffer containing dNTPs and 5 mmol l^{–1} MgCl₂, and M-MuLV RNase H⁺ reverse transcriptase. KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich, St Louis, MO, USA) along with 300 nmol l^{–1} forward and reverse primers, cDNA diluted 1:20, and nuclease-free water were used for all reactions. Primers were designed using Primer3 based on contigs obtained from the transcriptome analysis (Table S1). qPCR reactions were conducted using an Illumina Eco quantitative PCR system. Reactions were run according to previous studies (Finch et al., 2020; Hagan et al., 2018; Meibers et al., 2019). Four biological replicates were examined for each gene. Expression

levels were normalized to *rpl19* using the $\Delta\Delta C_q$ (Finch et al., 2020; Teets et al., 2013). A one-way ANOVA was used to examine significance followed by a Tukey's test for differences between treatments.

RESULTS AND DISCUSSION

Rapid hardening does not impact survival of males

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

Increased survival during lethal cold stress is a major factor associated with rapid hardening, induced by dehydration or cold (Benoit et al., 2009; Teets et al., 2019, 2020). For the Antarctic midge, RCH significantly improves larval survival when exposed to freezing temperatures (Lee et al., 2006; Teets et al., 2008). But the increased survival appears to be stage specific in the Antarctic midge as adults do not improve survival following RCH (this study; Lee et al., 2006). Rapid hardening in other insect systems, such as *Drosophila*, shows differences between developmental stages, specifically in relation to survival (Colinet and Hoffmann, 2012; Jensen et al., 2007; Teets et al., 2020; Terblanche et al., 2007). Increased survival generated by RCH may lack ecological relevance for these short-lived adult midges because they are rarely exposed to lethal temperatures during their active period in the austral summer (Lee et al., 2006; Teets et al., 2019).

Rapid hardening improves male locomotor activity and competition during female courtship

Sublethal cold exposure resulted in a significant reduction in locomotor activity ($F_{3,72}=12.07$, $P=1.73\times 10^{-6}$), but activity was retained in RCH-treated males (Tukey's test, $P=0.246$, Fig. 1B). Locomotor activity was not significantly different between control males and those that experienced RCH. These results indicate that RCH allows males to maintain locomotor activity following cold stress.

Results from the competitive mating assays demonstrated that sublethal cold exposure drastically impaired male mating ($N=50$, χ^2 , $P=8.16\times 10^{-5}$; Fig. 1C). RCH or RDH without sublethal cold stress did not alter male mating ($N=50$, χ^2 , $P>0.26$; Fig. 1C). Males first exposed to RDH or RCH before sublethal cold stress showed increased mating compared with those that experienced only the sublethal cold stress ($N=50$, χ^2 , $P<0.01$, Fig. 1C). These results suggest that RDH and RCH can ameliorate the cold-induced reduction in pre-copulatory interactions in males, allowing for increased mating success.

A key aspect of RCH is organismal performance following exposure to sublethal temperatures (reviewed in Teets et al., 2020). Three key aspects related to sublethal cold stress are a lowering of the critical thermal minimum temperature (CT_{min}), increased recovery from the CT_{min} , and fitness/energetic advantages following cold stress (Benoit et al., 2021; Coulson and Bale, 1992; Findsen et al., 2013; Rinehart et al., 2000; Shreve et al., 2004; Teets et al., 2019, 2020). We did not determine whether RCH reduces the CT_{min} or recovery time, but rapid hardening did improve the male activity levels to near those of controls following sublethal

cold exposure. RCH likely prevents protein and membrane damage including resting potential depolarization that would normally occur following sublethal cold stress (Košťál et al., 2004, 2006; Overgaard and MacMillan, 2017). Damage as a result of sublethal stress is likely to include negative impacts on muscle and nervous tissues (Andersen et al., 2015; Košťál et al., 2006; MacMillan et al., 2014; Overgaard and MacMillan, 2017), which in turn can be expected to significantly impact general activity levels, as observed for males in our study.

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

Rapid hardening prevents reductions in male fertility

The total number of eggs produced did not vary in response to the treatment of the males (one-way ANOVA, $P=0.456$). But, egg viability significantly differed among treatments (Fig. 2A; one-way ANOVA, $F_{3,46}=14.12$, $P=1.18\times 10^{-6}$). In particular, egg viability was significantly reduced (by nearly 40%) when males were exposed to sublethal cold (Tukey's test, $P<0.001$). This reduction in egg viability was prevented when males were pretreated with RCH (Fig. 2A; $P=0.717$). RCH before the sublethal cold exposure thus allowed males to maintain fertility, as indicated by egg viability (Fig. 2A; $P=0.371$ versus control, $P=0.0009$ versus sublethal cold). Thus, both pre-copulatory and post-copulatory aspects of reproduction are impacted when males are exposed to sublethal cold stress.

To determine a putative mechanism for the reduced fertility, we examined expression patterns of four accessory gland proteins (Acps) that are expressed exclusively in the male accessory gland (Fig. 2B). When exposed to sublethal cold stress, expression of all four Acps was significantly reduced (Fig. 2C, one-way ANOVA, $P<0.03$ in all cases). When subjected to RCH prior to sublethal cold exposure, expression of three Acps increased in comparison to expression levels seen following sublethal cold exposure without RCH (Fig. 2C). These results suggest a reduction in factors associated with seminal fluid production could underlie the observed reduction in male fertility.

Similar to our results, when pharate adult flesh flies, *Sarcophaga crassipalpis*, are exposed to cold stress, male fertility is reduced, and it is recovered by RCH (Rinehart et al., 2000). Among potential mechanisms that could contribute to this impairment, we noted that expression of previously identified male Acps (Finch et al., 2020) was reduced substantially. In *B. antarctica*, RCH prompted an increase in fertility and a recovery in expression of transcripts encoding the Acps. Two of these proteins are putative metalloproteases previously implicated in the processing of seminal proteins, sperm competition and sperm storage (Avila and Wolfner, 2017; LaFlamme et al., 2012; Laflamme et al., 2014). Along with sperm viability, female sexual receptivity increases when specific proteases are inhibited (LaFlamme et al., 2012). This could have

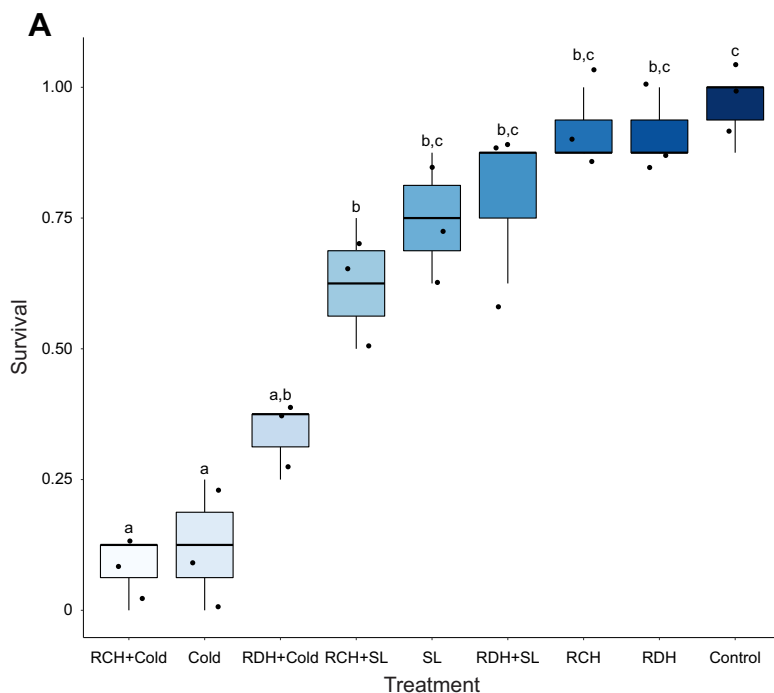
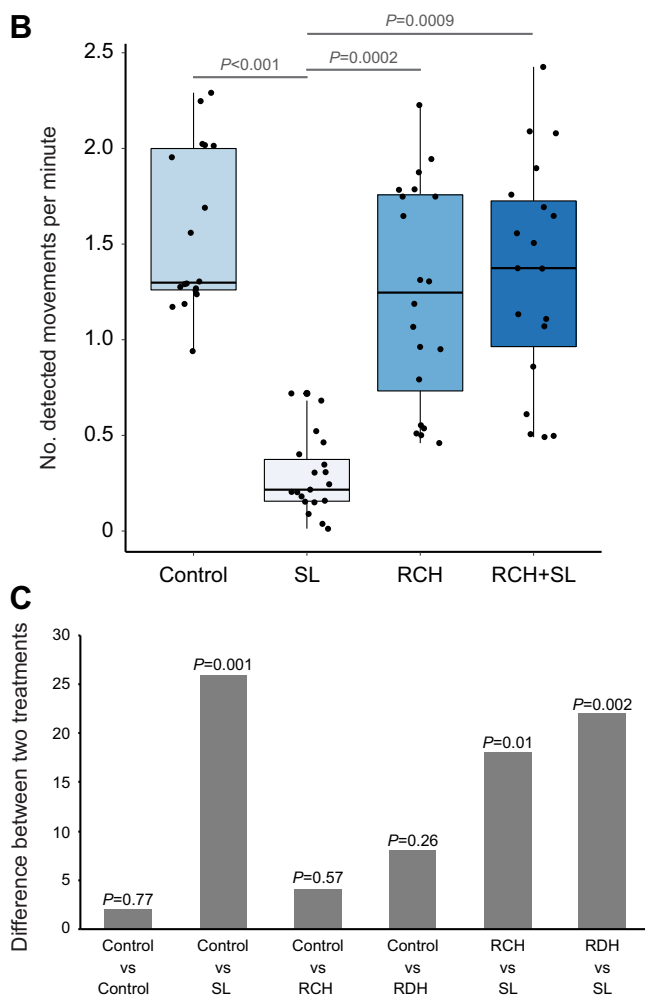


Fig. 1. Effect of rapid hardening on male survival, locomotor activity and mating competition of the Antarctic midge following cold exposure. (A) Survival of males in the indicated treatment groups: RCH+Cold, rapid cold hardening before damaging cold exposure; Cold, damaging cold exposure; RDH+Cold, rapid dehydration hardening before damaging cold exposure; RCH+SL, rapid cold hardening before sublethal cold stress; SL, sublethal cold stress; RDH+SL, rapid dehydration hardening before sublethal cold stress; RCH, rapid cold hardening; RDH, rapid dehydration hardening; Control, untreated group. Different letters indicate significant (one-way ANOVA, Tukey's honest significant difference test, $P < 0.05$) differences between treatment groups. (B) Activity differences between Control, SL, RCH and RCH+SL groups. Significance was determined by ANOVA followed by Tukey's test for differences between treatments. (C) Success in mating competitions of Control or RCH/RDH males compared with those in the specified treatment below (reported as an increase in success of the former versus the latter). Zero indicates no difference. Significance was determined with a χ^2 test.



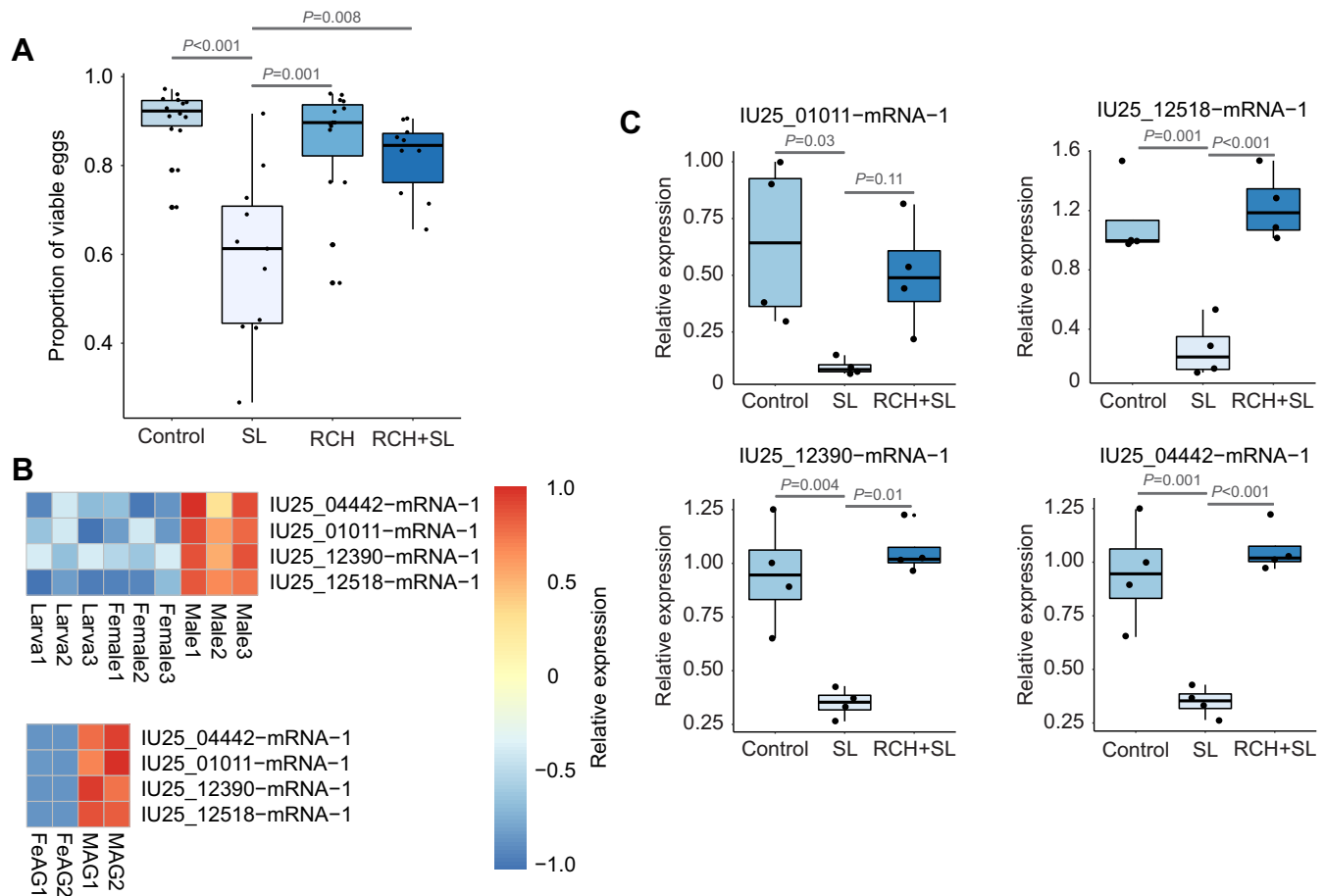


Fig. 2. Effect of rapid hardening of males on egg viability and transcript level analysis of male accessory gland proteins during cold exposure. (A) Differences in egg viability between Control, SL, RCH and RCH+SL groups. Significant differences between treatment groups were determined with a one-way ANOVA followed by Tukey's test for differences between treatments. The total number of eggs produced by females did not vary between treatments ($P = 0.456$). (B) Expressional analysis to identify male accessory gland (MAG) protein targets based on previous RNA-seq studies underlying reproductive biology of the Antarctic midge (Finch et al., 2020). FeAG, female accessory glands. (C) qPCR analysis of Control, RCH and RCH+SL groups. Significant differences between treatment groups were determined with a one-way ANOVA followed by a Tukey's test for differences between treatments.

a substantial impact in species such as *B. antarctica*, where mating occurs in swarms and females mate multiple times (Finch et al., 2020). The two other Acps examined here have less clear functions; one has an unknown function and the other is related to salivary-associated antigen 5-like allergens (King and Spangfort, 2000). From our studies, we cannot rule out direct damage to sperm as an underlying cause for females producing fewer viable eggs, as

documented in other insect systems (Colinet and Hance, 2009; Levie et al., 2005; Rinehart et al., 2000). Both the reduction in transcript levels for Acps and sperm damage may contribute to reproductive impairment by low temperature, but regardless of the immediate cause, RCH appears to be an effective mechanism for protecting not only pre-copulatory activity but also post-copulatory factors impacting fertility in the Antarctic midge.

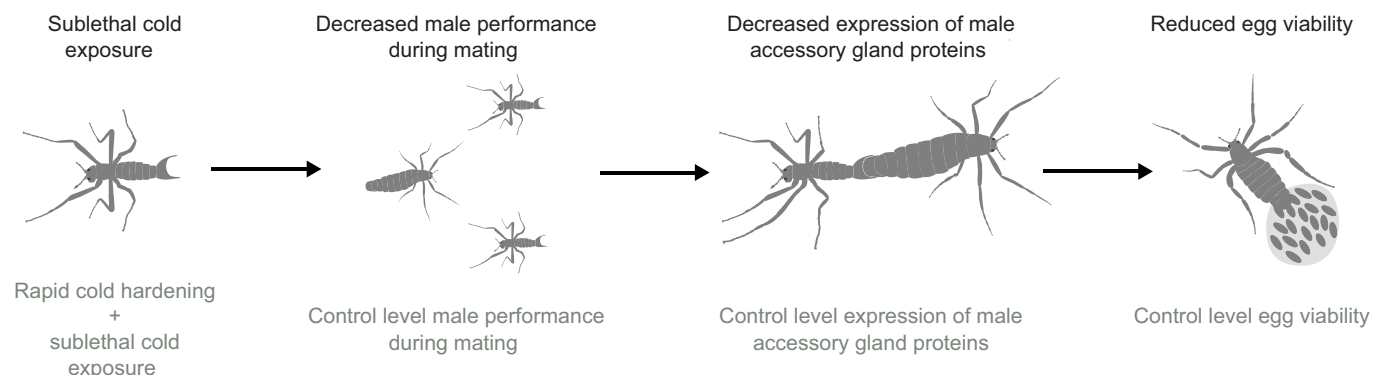


Fig. 3. Summary of the impact of sublethal cold exposure and RCH on pre-copulatory and post-copulatory features of male fertility and performance.

Conclusions

This study highlights the critical role of rapid hardening in relation to male performance in the Antarctic midge, as summarized in Fig. 3. Hardening had very little impact on survival of males but allowed them to retain high levels of activity. This increased activity is likely a major underlying factor allowing males to be more competitive in their ability to copulate with females. Male exposure to sublethal cold stress resulted in a significant reduction in fertilization rate, as reflected in the production of fewer viable eggs. This impairment was recovered when males were subjected to RCH prior to cold exposure. This reduction in fertility could be due, in part, to a major reduction in expression of specific accessory gland proteins, which are critical for males to maintain maximum fecundity. The combined impact is that rapid hardening can promote male fitness in two ways: (1) by allowing high performance during pre-copulatory interactions, and (2) by allowing males to maximize fertility. Interactions between pre- and post-copulatory factors contribute to male fertility (Birkhead and Pizzari, 2002; Polak et al., 2021), which we show are both negatively impacted by exposure to stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B.B.; Methodology: J.D.G., G.F., J.B.B.; Formal analysis: O.M.A., G.F., J.B.B.; Data curation: O.M.A.; Writing - original draft: O.M.A., J.B.B.; Writing - review & editing: O.M.A., J.D.G., G.F., R.E.L., D.L.D., J.B.B.; Visualization: O.M.A., J.B.B.; Supervision: R.E.L., D.L.D., J.B.B.; Project administration: R.E.L., D.L.D., J.B.B.; Funding acquisition: R.E.L., D.L.D.

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Data availability

Sequencing information is available in association with the NCBI Bioproject PRJNA576639. All other data are directly included within the paper.

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