Cryopreservation produces limited long-term effects on the nematode *Caenorhabditis elegans*

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Abstract

Cryopreservation, the freezing and later warming of biological samples with minimal loss of viability, is important in many scientific disciplines. For some applications, particularly those where there is limited available material, it is critical to ensure the maximal survival rates of cryopreserved materials. Most of the challenges encountered with such techniques take place after the warming process where cryodamage affects cell viability and future development. Here we have used the nematode *Caenorhabditis elegans* to investigate the effects of cryodamage caused by slow-freezing. We find that freezing results in the death of some worms, with an approximately 40% reduction in the number of worms that develop in the frozen populations, but that the effects on worms that survive are limited. For example, there are no differences in the lifetime fecundity or in lifespan between frozen and control worms, although early fecundity and body size was reduced in frozen worms. Similarly, analyses of body wall muscle structure and of pharyngeal function indicates that muscle development and function are not significantly affected by freezing. We do however determine that freezing increases the rates of matricidal hatching, where progeny hatch within the mother. Overall, these results indicate that, for worms that survive, cryopreservation produces limited long-term effects, but do indicate that some phenotypes could be used in further analyses of the cellular damage induced by cryopreservation.

Keywords

C. elegans, Freezing, Cryopreservation, Cryodamage

Abbreviations

LRS, lifetime reproductive success

NGM, nematode growth media

1. Introduction

Cryopreservation, the freezing of cells, biological tissues, organs and whole organisms at low temperatures, whilst maintaining their viability is crucially important in biotechnology and medicine. When cells are exposed to low enough temperatures, all chemical and enzymatic activities cease, thereby protecting cells from harm [2]. The ability to keep biological samples frozen over long periods has revolutionised many fields including regenerative medicine, the use of stem cells and reproductive medicine. One such example is the freezing of oocytes before radical chemo- and/or radiotherapy, an approach that has later resulted in healthy births [12] and that is now a standard approach in such situations.

There are two main categories of cryopreservation, slow-freezing and vitrification [8]. The slow freezing technique was the first to be developed and involves cells being exposed to a low concentration of permeating cryoprotectants, cooled in straws to between -5 and -7°C where they are kept for several minutes to equilibrate, and are then subsequently cooled slowly at 0.3 to 0.5°C per minute to -30 to -65°C. After this slow process, the straws are plunged into liquid nitrogen to reach their final storage temperature of -196°C [34]. During this process, ice crystals are formed. The process of vitrification on the other hand involves the solidification of solutions at low temperatures, without ice crystal formation, facilitated by an extreme increase in the viscosity of the solution. Slow freezing of human oocytes has been associated with lower survival rates due to physical damage by ice crystal formation and potential chemical damage by changes in the intracellular fluid due to osmotic changes [19,21]. The potential harm of slow freezing methods can be somewhat ameliorated using cryoprotectants such as glycerol, ethanediol, ethylene glycol, dimethyl sulfoxide and propanediol [25,26]. Such substances have high permeability and can withstand very low temperatures, but can damage cells directly and are not enough to avoid all damage associated with freezing and thawing. Vitrification avoids mechanical damage caused by ice crystals with rapid cooling and liquid solidification resulting in the formation a solid glass-like state, defined by high viscosity but without

crystallisation, that keeps the cell structure intact and fixed in its original shape [10]. Despite many improvements in cryopreservation approaches, the biggest issues continue to be osmotic stress [14] and chemical toxicity [37].

Animal models provide a powerful platform to study basic biological functions, with many mechanisms and processes being found to be highly conserved across species. The free-living nematode *Caenorhabditis elegans* is one of the leading model systems in biology. In this species, eggs hatch to produce first larval stage worms (L1s) that require food to initiate subsequent development [4]. When there is food available, L1s moult through three further larval stages (L2-4) to mature as reproductive adults that are predominantly self-fertile hermaphrodites. Under standard laboratory conditions, plentiful food and a culture temperature of 20°C, this takes three days. In contrast, when no food is available, newly hatched L1s can remain at that larval stage for a prolonged period, starting development only when food becomes available [20]. *C. elegans* is now established as a standard experimental system for the investigation of a great variety of biological processes including aging, sex determination, cell lineage, cell death, myogenesis and neural development [15]. An additional benefit of *C. elegans* as a model organism is the ease with which worms can be cryopreserved, with the early larval stage (L1) best surviving freezing and thawing [5]. Here, worms are frozen slowly to -80°C in a simple medium containing glycerol and can then be transferred to liquid nitrogen for long term storage [5]. More complex protocols have been developed that allow the freezing of other stages of *C. elegans* (e.g. [13]), but as it is possible to easily obtain many thousands of worms and cryopreservation success is high, there has been little work undertaken on optimising cryopreservation in the species or on investigating the extent to which cryopreservation affects individual worms.

In the studies that have investigated the effects of cryopreservation in *C. elegans*, it has been shown that both slow-freezing and vitrification do not affect memory retention [36]. The ability to survive

freezing has also been shown to be linked to the action of the insulin/IGF-1 receptor *daf-2*, with a reduction in *daf-2* function improving survival, locomotion and muscle cell protection [17]. Here, we have examined how cryopreservation by slow freezing affects fitness in *C. elegans*, investigating lifespan, lifetime fecundity, growth development and survival. Our aim in this work is to develop a model system in which cryopreservation damage can be investigated.

2. Materials and Methods

Here we used N2 (Bristol), the standard wild type *C. elegans* strain, and a transgenic GFP-labelled *unc-54* strain [35], which results in the production of a myosin-GFP hybrid protein, UNC-54::GFP, in the body wall and vulval muscles. Worms were maintained on nematode growth media (NGM) plates [33] at 20°C, with *Escherichia coli* strain OP50 as a food source. All experiments were initiated using synchronized arrested L1s obtained by incubating eggs liberated from the sodium hypochlorite treatment of gravid adults [33] in 600µl of M9 overnight on a rotary shaker at 175rpm. All assays were run at 20°C unless otherwise noted. For cryopreservation (freeze treatment), 100µl of M9 worm suspension was mixed with 100µl of freezing solution [5,33], and tubes were then transferred to a −80°C freezer in a small Styrofoam box with slots for holding the tubes for 24 hours. For recovery, tubes were thawed at room temperature until all the ice had turned to liquid (15-20 minutes) and then the contents of the tube were transferred to a fresh NGM plate. For the controls, 100µl of M9 worm suspension was mixed with 100µl of freezing solution, left at room temperature for 20 minutes and then transferred to a fresh NGM plate. Our comparisons therefore specifically test the effects of freezing for 24 hours as all worms are exposed to freezing solution. In all experiments, treatments were blind coded and the position of plates within experimental blocks was randomised. In line with previous work [5] cryopreservation at −80°C of other lifecycle stages resulted in very limited survival, making subsequent analysis and interpretation of phenotypic effects problematic.

To determine the survival rate, freeze treated and control N2 worms were allowed to develop on food for 48 hours, after which the number of worms was counted. To test the effect of previous exposure to low temperatures, two additional treatment groups were included here in which arrested L1s were maintained in M9 for 24 hours prior to freezing at either 12 or 20°C.

To determine lifespan, freeze treated and control N2 worms were transferred *en masse* onto a seeded plate, and after 48 hours were then moved onto new seeded plates with 10 worms per plate. These assays were therefore initiated with worms that had survived the freeze treatment and resumed development. Worms were observed daily to assess survival, with individuals considered to have died if they were not moving and subsequently failed to respond to touch with a worm-pick. As fluorodeoxyuridine (FUdR), an inhibitor of DNA synthesis that prevents reproduction in *C. elegans*, was not used in these assays, worms were transferred to new plates daily until reproduction had ceased and subsequently transferred every other day to avoid food deprivation. Worms that died due to the internal hatching of progeny, a phenotype called matricidal hatching or bagging as it can result in a "bag of worms" phenotype, were censored out of the analysis of lifespan.

To determine fecundity, freeze treated and control N2 worms were individually transferred onto seeded NGM plates. During the reproductive period, worms were then transferred daily to new plates, with the progeny on the original plates allowed to develop for a further two days before they were counted. The total number of progeny produced was taken as the lifetime reproductive success (LRS) and the number of progeny produced on the first day of reproduction taken as a measure of early reproduction.

To determine body size, three groups of freeze treated and control N2 worms were imaged. To compare size directly after freezing, worms were imaged 2 hours after transfer to plates using a Dino-Lite camera (AnMo Electronics Corporation, New Taipei City, Taiwan). To look at subsequent growth,

147 separate populations of worms were imaged after 24 and 48 hours on food. For these three groups,
148 body length was determined as previously described [31], by measuring the length from the mouth to
149 the base of the tail in ImageJ [30].
150

151 Pharyngeal pumping reflects pharyngeal muscle activity, with pumping rates decreasing in old
152 worms [7], and the age-related decline in pharyngeal pumping correlating with lifespan [18]. To
153 determine if cryopreservation impacted the rate of pharyngeal pumping, the pumping rates of
154 freeze treated and control N2 worms were quantitated by counting pharyngeal contractions as
155 described by Avery and Horvitz [3]. The pumping rates were counted at days 1 and 5 of adulthood
156 for 15 seconds.

157

158 To determine the effect of cryopreservation on muscle structure, freeze treated and control UNC-
159 54::GFP worms were allowed to develop for 72 hours (day 1 of adulthood) or 168 hours (day 5 of
160 adulthood). Worms were then picked into a drop of a 1:1 mixture of 99% Ethanol and M9, covered
161 with a cover slip and imaged using an Olympus IX83 inverted fluorescence microscope (Olympus,
162 Hamburg). Overall muscle integrity was assessed qualitatively by comparing GFP fluorescence
163 between freeze treated and control worms – here we assessed worm images visually for signs of
164 damage or disruption to muscle structure – and quantitatively by counting the number of A bands
165 present in cells adjacent to the vulva. High rates of internally hatched progeny were noted in these
166 assays and the proportions of freeze treated and control worms that had progeny that had hatched
167 internally (matricidal hatching) was also determined.
168

169 All analyses were performed in R version 3.6.0 [28]. The effect of incubation prior to freezing and of
170 freezing on the number of worms was analysed by ANOVA, with *post hoc* comparisons undertaken by
171 TukeyHSD. Kaplan-Meier survival analysis was used to determine if the lifespan of freeze treated and

control worms differed. The rates of matricidal hatching in freeze treated and control worms were compared using a one-proportion Z test. All other traits were analysed by Mann-Whitney U tests.

3. Results

Survival and lifespan

Comparison of the number of worms that developed in freeze treated and control populations indicated that freezing reduced the number of worms ($F_{1,52} = 78.56$, $p < 0.001$), *i.e.* that freezing for 24 hours was directly affecting worm survival (Figure 1). These data also indicated that survival also differed between treatments (Treatment: $F_{2,52} = 7.57$, $p = 0.0013$), with *post hoc* comparisons indicating, contrary to expectations, that the incubation at 12°C for 24 hours reduced the numbers of worms (Tukey HSD $p < 0.001$ for comparison of the 20°C and 12°C incubations). Overall, this indicates that there is an approximately 40% reduction in the number of worms that develop in the frozen populations. Survival after freezing for 24 hours was however not observed to differ between treatments (Treatment x Freezing interaction: $F_{2,52} = 2.91$, $p = 0.06$), indicating that incubation at 20 or 12°C for 24 hours did not alter the response to freezing (Figure 1).

Reproduction and development

As expected, a large proportion of frozen worms survived and resumed development (Figure 1). To determine if there were long term consequences of cryopreservation, we compared freeze treated and control worms for a range of life history and phenotypic traits. Although lifespan did not differ between freeze treated and control worms (Figure 2, Kaplan Meir $X^2 = 0.6$, 1 d.f., $p = 0.4$), analysis of reproduction indicated that early reproduction was reduced in frozen worms (Figure 3A, $W = 241.5$, $p < 0.001$). As LRS did not differ between freeze treated and control worms (Figure 3B, $W = 271.5$, $p = 0.54$) this suggests that worms frozen for 24 hours may be developing more slowly than controls. Consistent with this, we observed that from a similar size at the L1 stage (Figure 3C, $W = 1001.0$, $p = 0.61$), frozen worms were smaller than controls after both 24 and 48 hours of development (Figure

198 3C, W = 2285.5, p < 0.001 and W = 2179.5, p < 0.001, respectively). These data therefore indicate that
199 frozen worms are developing more slowly, that they initiate reproduction later, but that their LRS is
200 the same as that of controls.
201
202 Muscle integrity and function
203 The *C. elegans* pharynx contains 77 cells divided between eight sections of muscle and three of
204 marginal cells and is used for pumping food into the gut [1,22] . Pharyngeal pumping is primarily a
205 consequence of pharyngeal muscle activity and normally declines with age [7]. Here, comparison of
206 pumping rates of worms that survive slow freezing and unfrozen controls indicates that the rates do
207 not differ in young adults (Figure 4, W = 14833.0, p = 0.49), but that pumping is reduced in frozen
208 worms by the fifth day of adulthood (Figure 4, W = 16477.5, p < 0.001).
209
210 Muscle structure and integrity were also assessed directly by visualising main muscle component
211 myosin. In *C. elegans*, *unc*-54 (also known as *myo-4*) codes for myosin 2 and this protein is the major
212 component of the body-wall muscle. The UNC-54::GFP therefore allows direct visualisation of the
213 parallel A bands of the body wall muscle cells. This visualisation allowed qualitative comparison of
214 muscle integrity in worms that survive slow freezing and in unfrozen controls. This revealed no large-
215 scale differences in muscle integrity or structure at either the first or the fifth day of adulthood (see
216 Figure 5A for a representative image of a worm that survived slow freezing and was subsequently
217 allowed to develop to the fifth day of adulthood). Comparison of the number of A bands in cells
218 adjacent to the vulva (Figure 5B) also revealed no effect of freezing (Figure 5D, W = 2061.5, p = 0.91
219 and W = 2121.5, p = 0.55, for day 1 and 5 respectively). This analysis did however reveal that the rate
220 of matricidal hatching (see Figure 5C for a representative image of a worm with internally hatched
221 progeny) is increased in worms that survive slow freezing in comparison to unfrozen controls (Figure
222 5E, X^2 = 9.29, df = 1, p = 0.002).
223

4. Discussion

In this study, we have focused on the biological effects of freeze-thaw stress on *C. elegans.* We have compared survival rates, lifespan, fecundity, growth, pharyngeal pumping, and muscle structure after freezing at -80˚C, a method that is widely used to store and preserve *C. elegans* [5], for 24 hours. Comparison of survival between frozen and control worms indicates that freezing for 24 hours does result in direct mortality (Figure 1), with an approximately 40% reduction in the number of worms developing in the frozen populations. Contrary to expectations, incubation of worms at low temperature prior to freezing did not increase survival. As exposure to 10°C does improve tolerance of lethal cold (0°C) in actively growing worms [23,24], our observations here are likely to be a consequence of the arrested state of the L1s used.

We next investigated lifespan in the frozen and non-frozen worms, finding no differences between the frozen and control worms (Figure 2). In this analysis of lifespan we censored worms that died from matricidal hatching, as is standard in lifespan analysis [32]. We did, however observe a very high incidence of maternal hatching among both frozen and control groups, an effect we also observed in other assays (Figure 5E).

In contrast to the lack of effect of freezing on lifespan, differences between frozen and control worms were seen for early reproduction (Figure 3A) and for body length (Figure 3B), with the frozen worms growing more slowly and laying fewer eggs on the first day of reproduction. Whilst *C. elegans* can actively modulate egg-laying depending on the environmental conditions, for instance food availability [29], the reduced growth suggests that the difference in early reproduction is a consequence of reduced size or of a delay in the onset of reproduction. As LRS is not reduced in frozen worms (Figure 3B) these data also indicate that freezing has not damaged the primordial gonadal cells of *C. elegans*, which are responsible for the formation of their reproduction system [16].

Our analysis of pharyngeal pumping did not reveal any significant differences between frozen and non-frozen worms on the first day of adulthood, but showed that pumping rates were reduced in the frozen worms by the fifth day of adulthood (Figure 4). Reduced pharyngeal pumping is associated with ageing in *C. elegans* [7] and pumping rates can be used to predict lifespan [18]. As we do not detect any differences in lifespan this may imply that there are distinct phenotypic classes, with some frozen worms pumping at a reduced rate and being at a greater risk of matricidal hatching. This would explain the reduction in pharyngeal pumping, but these worms would have been censored from the lifespan analysis. This scenario may therefore be analogous to the distinct, and separately controlled, causes of death that have been observed in *C. elegans* [38].

Analysis of muscle structure did not detect any damage in the muscles of frozen worms, either qualitatively or quantitatively (Figure 5D). As we visualized muscle structure using only UNC-54, one component of myosin 2, it is possible that there may have been damage that we could not detect, but gross changes would have been expected to alter UNC-54 localisation either directly or indirectly. These analyses did however again detect high rates of matricidal hatching in control worms; under normal circumstances rates of matricidal hatching in N2 are <5% (e.g.: [31]), and increased rates as a response to freezing (Figure 5E). This indicates that even comparatively short exposure to freezing media increases matricidal hatching and that this is exacerbated by freezing. These effects may result from damage, perhaps mimicking the intrinsic age-related degeneration of the egg-laying system [27]. Alternatively, changes in rates of matricidal hatching may be a consequence of changes in their reproductive strategy that actively increase the rate of matricidal hatching, as it has been argued that this represents a conserved stress response in *C. elegans* [6].

Overall, these results indicate that cryopreservation for 24 hours produces limited long-term effects on worms that survive freezing and thawing. We do however identify a number of phenotypes that could be used to model some aspects of the cellular damage induced by cryopreservation. Given the

growing knowledge of the environments in which *C. elegans* naturally lives (e.g. [11] and [9]) it would therefore be of interest to test to see if there is any variation between worm isolates in their freeze tolerance that might be linked to the environments from which they were isolated. We also find that there are deleterious effects of exposure to the standard freezing solution used in the cryopreservation of *C. elegans.* There is therefore scope both to improve the efficiency of this protocol and to investigate how inter-laboratory variation in cryopreservation protocols affect the worms. It would also be of interest to investigate additional priming temperatures and treatments and if the responses to cryopreservation differ between isolates.

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Competing interests:

The authors declare no competing interests.

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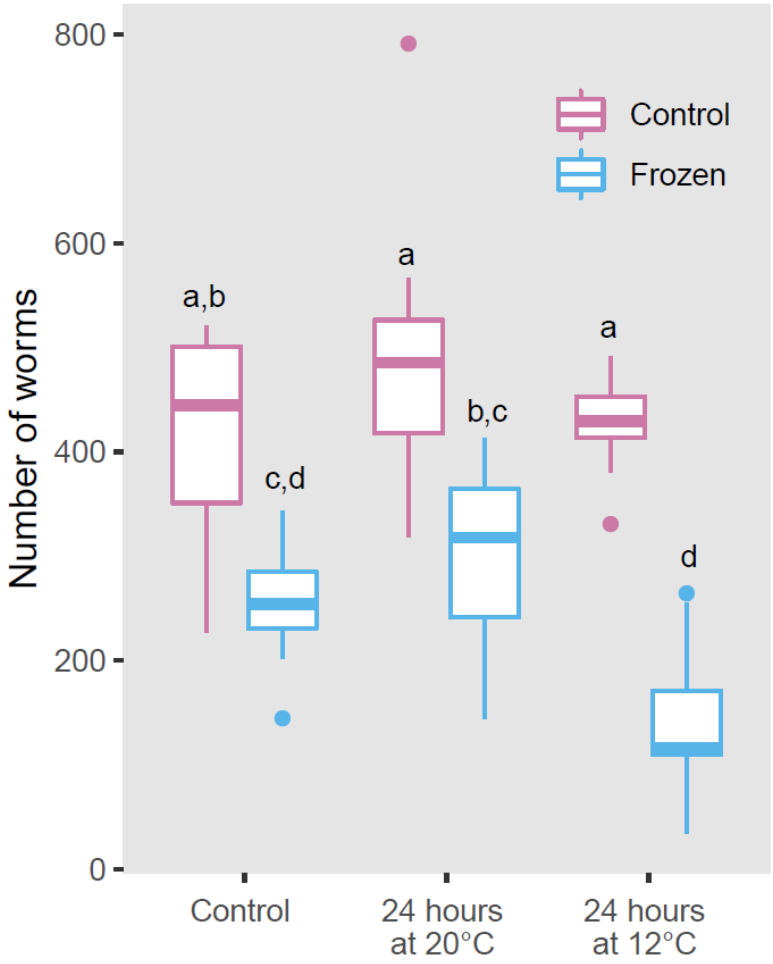
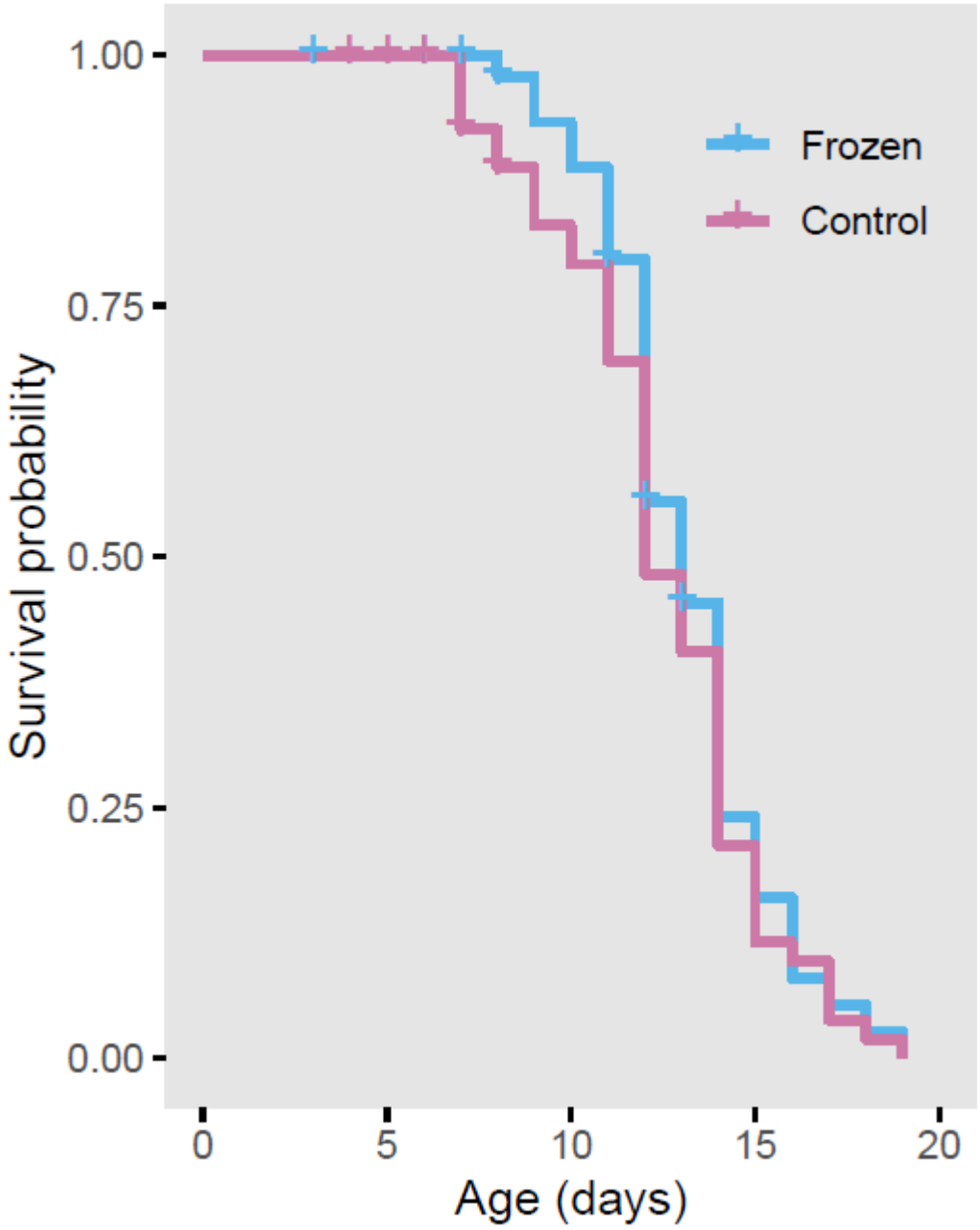


Figure 1. Slow freezing kills some worms. Box plots, with the middle bars showing the median values and any outlying points plotted individually, showing the number of worms that survive and develop to adulthood after slow freezing in comparison to unfrozen controls for worms that were incubated at either 20°C for 0 or 24 hours or at 12°C for 24 hours. Letter codes show results of *post hoc* analysis, where treatments that do not share the same letter are significantly different (Tukey HSD, $p < 0.05$).



396

397 **Figure 2. Worms that survive slow freezing have a normal lifespan.** The survival curves of worms that

398 survive slow freezing does not differ from that of unfrozen controls (Kaplan Meir X^2 = 0.6, 1 d.f., p =

399 0.4).

400

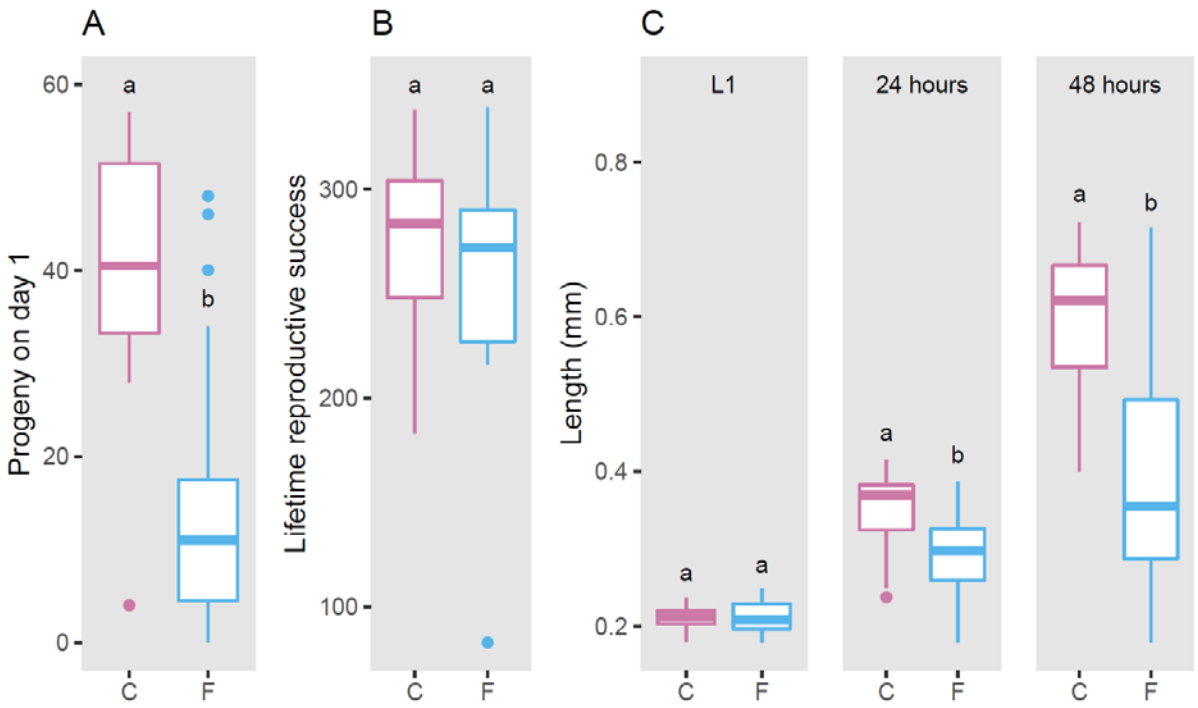


Figure 3. Early fecundity and size are reduced in worms that survive slow freezing. Box plots, with the middle bars showing the median values and any outlying points plotted individually, showing the A) number of progeny produced on the first day of reproduction, B) lifetime reproductive success, and C) body length of worms that survive slow freezing in comparison to unfrozen controls. Treatments that do not share the same letter code are significantly different (Mann-Whitney U test, $p < 0.05$).

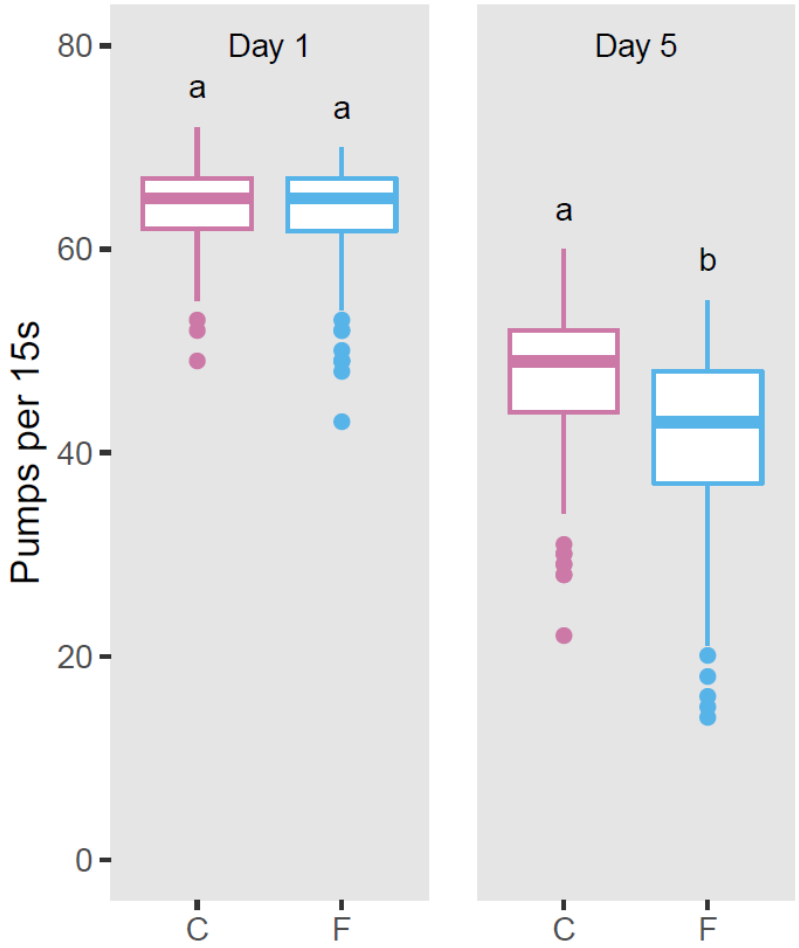


Figure 4. Pharyngeal pumping is reduced in old worms that survive slow freezing. Box plots, with the middle bars showing the median values and any outlying points plotted individually, showing the number of pharyngeal pumps in a 15s period are shown for worms on the first and fifth days of adulthood for worms that survive slow freezing in comparison to unfrozen controls. Treatments that do not share the same letter code are significantly different (Mann-Whitney U test, $p < 0.05$).

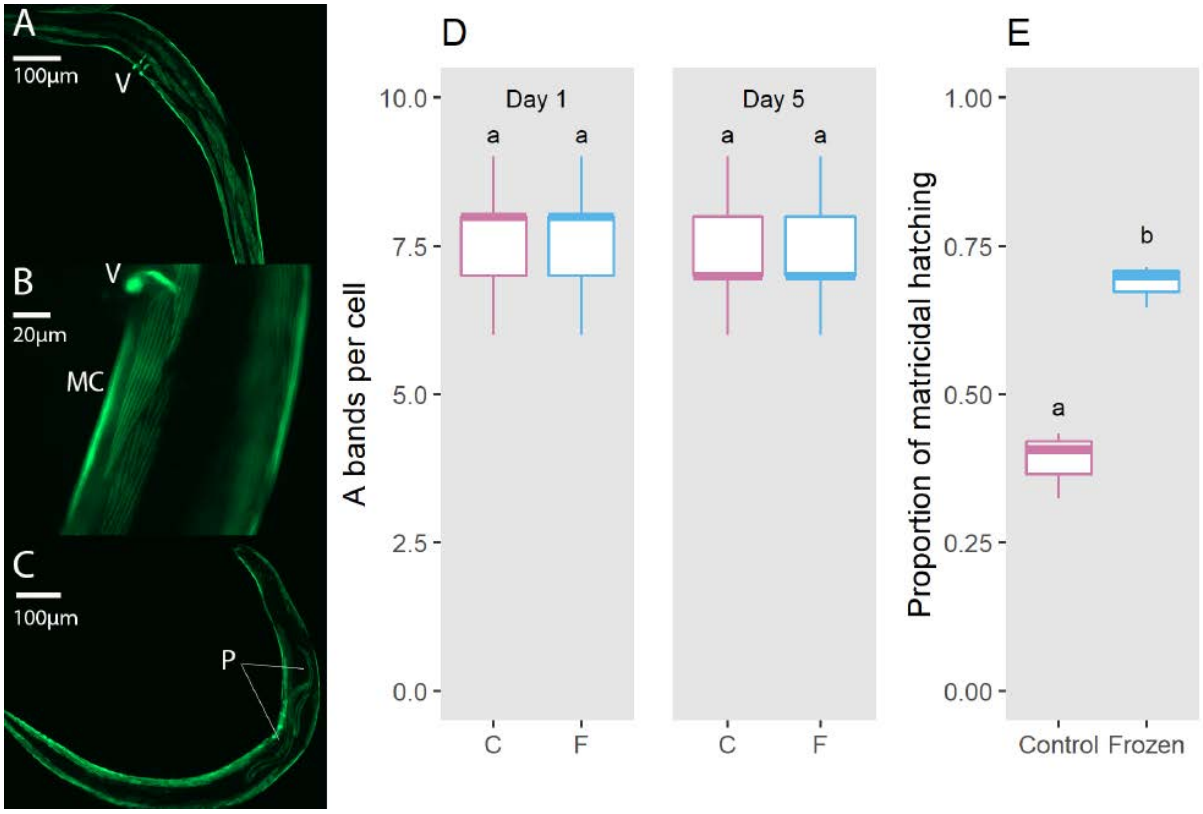


Figure 5. Worms that survive slow freezing show no signs of muscle damage, but have increased rate of matricidal hatching. Shown are representative images of A) a worm that survived slow freezing on the fifth day of adulthood as visualised by UNC-54::GFP, this shows the overall muscle structure, with the vulva (V) indicated to allow orientation, B) detail of the parallel A bands of the single spindle-shaped body-wall muscle cells (MC) directly posterior to the vulva (V), and C) a worm that survived slow freezing on the fifth day of adulthood with internally hatched progeny (arrows from P indicating the position of two internally hatched progeny). Also shown are box plots, with the middle bars showing the median values and any outlying points plotted individually, of the D) number of A bands present in cells adjacent to the vulva for worms on the first and fifth days of adulthood for worms that survived slow freezing in comparison to unfrozen controls (note that the medians here are coincident with box edges), and E) proportion of matricidal hatching for worms that survived slow freezing in comparison to unfrozen controls. Here, treatments that do not share the same letter code are significantly different (Mann-Whitney U test, $p < 0.05$).

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