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RESEARCH ARTICLE

MAPK phosphorylation is implicated in the adaptation to desiccation stress in nematodes

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SUMMARY

Some nematodes can survive almost complete desiccation by entering an ametabolic state called anhydrobiosis requiring the accumulation of protective molecules such as trehalose and LEA proteins. However, it is not known how anhydrobiotic organisms sense and regulate the response to water loss. Mitogen-activated protein kinases (MAPKs) are highly conserved signalling proteins that regulate adaptation to various stresses. Here, we first compared the anhydrobiotic potential of three nematode species, *Caenorhabditis elegans*, *Aphelenchus avenae* and *Panagrolaimus superbus*, and then determined the phosphorylation status of the MAPKs p38, JNK and ERK during desiccation and rehydration. *Caenorhabditis elegans* was unable to undergo anhydrobiosis even after an initial phase of slow drying (preconditioning), while *A. avenae* did survive desiccation after preconditioning. In contrast, *P. superbus* withstood desiccation under rapid drying conditions, although survival rates improved with preconditioning. These results characterise *C. elegans* as desiccation sensitive, *A. avenae* as a slow desiccation strategist anhydrobiote and *P. superbus* as a fast desiccation strategist anhydrobiote. Both *C. elegans* and *A. avenae* showed increased MAPK phosphorylation during drying, consistent with an attempt to mount protection systems against desiccation stress. In *P. superbus*, however, MAPK phosphorylation was apparent prior to water loss and then decreased on dehydration, suggesting that signal transduction pathways are constitutively active in this nematode. Inhibition of p38 and JNK in *P. superbus* decreased its desiccation tolerance. This is consistent with the designation of *P. superbus* as a fast desiccation strategist and its high level of preparedness for anhydrobiosis in the hydrated state. These findings show that MAPKs play an important role in the survival of organisms during anhydrobiosis.

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INTRODUCTION

Anhydrobiosis is the ability of some organisms to survive extreme water loss through evaporation by entering a state of suspended animation without measurable metabolism (Barrett, 1982). Upon rehydration, anhydrobiotic organisms recover and resume normal behaviour as if no time has elapsed (Ricci and Caprioli, 2005). This ability to survive almost complete desiccation is a property of many organisms including bacteria, yeasts, plant seeds and metazoans like nematodes, rotifers, brine shrimps and tardigrades (Crowe et al., 1992).

Nematodes are the most abundant metazoans on the planet, comprising four out of five animals (Platt, 1994). They have colonised all known habitats and have evolved mechanisms to survive almost every stress, including desiccation through evaporative water loss. Nematodes can remain in the anhydrobiotic state for long periods, the longest recorded being 32 years by *Anguina tritici* (Norton, 1978). Various protective molecules are thought to be required for nematodes to survive anhydrobiosis including non-reducing sugars such as trehalose. One proposed function of trehalose is the stabilisation of proteins and membrane structures by formation of an organic glass, which ‘freezes’ the cell and its contents in time and space (reviewed in Crowe, 2002). Other molecules that accumulate and are thought to be important in anhydrobiotic nematodes include heat shock proteins (HSPs), anti-oxidants and late embryogenesis abundant (LEA) proteins (Browne et al., 2004; Reardon et al., 2010).

Anhydrobiotic nematodes have been divided into slow and fast desiccation strategists (Womersley, 1987). Slow desiccation strategists, like *Aphelenchus avenae*, require a period of slow drying, sometimes referred to as preconditioning, before they can successfully survive exposure to very dry environments (Crowe and Madin, 1975). Preconditioning allows *A. avenae* time to produce anhydrobiotic adaptations, e.g. trehalose (Madin and Crowe, 1975), antioxidants like glutaredoxin and glutathione peroxidase (Browne et al., 2004; Reardon et al., 2010), and an LEA protein with molecular shield activity (Browne et al., 2004; Chakrabortee et al., 2007). In contrast, fast desiccation strategists such as *Panagrolaimus superbus* do not require preconditioning and can enter an anhydrobiotic state immediately on exposure to very dry conditions. It is thought that these organisms constitutively produce protective molecules; for example, *P. superbus* contains high levels of trehalose, up to 10% of dry weight, even when fully hydrated (Shannon et al., 2005; Tyson et al., 2012). Alternatively, slow desiccation strategists have also been described as external dehydration strategists because their rate of water loss is related to the relative humidity of their environment, while fast desiccation

strategists have been termed innate dehydration strategists as it is thought they can control their rate of water loss to allow them more time to induce biochemical adaptations (Perry and Moens, 2011). While *A. avenae* and *P. superbus* can undergo anhydrobiosis at all life stages, other organisms only do so during specific developmental stages; for example, the dauer larvae of the popular model nematode *Caenorhabditis elegans* were recently shown to survive extreme water loss (Erkut et al., 2011).

Although our understanding of the effector molecules in anhydrobiosis is improving, little is known about how their production is controlled. One group of proteins involved in the transduction of sensor signals to stress response genes are the mitogen-activated protein kinases (MAPKs). Once activated by phosphorylation, MAPKs either enter the nucleus and regulate gene expression or remain in the cytoplasm and phosphorylate substrates (reviewed in Manning et al., 2002). All three types of MAPKs (ERK, p38 and JNK) play a role in *C. elegans* stress responses (Lant and Storey, 2010; Sakaguchi et al., 2004). For example, during oxidative stress, the *C. elegans* p38 MAPK, PMK-1, is required for nuclear translocation of the transcription factor SKN-1 that upregulates protective genes (An and Blackwell, 2003; Inoue et al., 2005). In mammalian cells, studies have shown that all three MAPKs are phosphorylated during desiccation stress and that chemical inhibition of ERK reduces the expression of desiccation-responsive genes (Huang et al., 2010). The involvement of MAPKs in various stress responses across a wide range of organisms suggests they might play a role in transducing the desiccation stress signal during evaporative water loss in nematodes.

Therefore, in this study, the phosphorylation status of p38, JNK and ERK was examined in three nematode species with varying tolerance to water stress. We show that MAPKs are phosphorylated during entry into and recovery from anhydrobiosis in nematodes that require upregulation of genes necessary to survive desiccation (slow/external desiccation strategists). Conversely, MAPKs in a nematode that constitutively expresses these genes (a fast/innate desiccation strategist) are phosphorylated in the hydrated state and become dephosphorylated during desiccation. As far as we are aware, this is the most comprehensive study to date to examine MAPK activation during desiccation stress in anhydrobiotic organisms, and shows that MAPKs play an important role in anhydrobiosis.

MATERIALS AND METHODS

Culture and harvesting of nematodes

Caenorhabditis elegans N2 Maupas 1900 and *P. superbus* Fuchs 1930 were grown at 25°C on a lawn of tetracycline-resistant *Escherichia coli* HT115 containing the carbenicillin- and tetracycline-resistant plasmid L4440 (Timmons et al., 2001) on nematode growth medium (NGM) plates supplemented with 50 μmol l^{-1} carbenicillin and 12.5 μmol l^{-1} tetracycline. Nematodes were harvested by rinsing the NGM plates with M9 buffer and cleaned by centrifugation thrice at 1000 ***g*** for 3 min. Synchronised young adult populations of *C. elegans* were obtained by bleaching gravid worms (0.5 mol l^{-1} NaOH, 1% NaOCl) for 5 min with vigorous shaking before washing thrice in M9 buffer for 3 min at 1000 ***g***. *Caenorhabditis elegans* eggs were hatched overnight in M9 buffer with gentle agitation, deposited onto *E. coli* HT115-covered NGM plates and grown for 38 h at 25°C. *Aphelenchus avenae* Bastian 1865 were grown for 2–3 weeks at 22°C on the fungus *Botrytis cinerea*, which was maintained on autoclaved wheat in 500 ml Schott bottles, and then harvested by rinsing the bottles with tap water. Large particles of debris were removed from the worm suspension by passing it through a 300 μm sieve and cleaning by centrifugation thrice at 1000 ***g*** for 3 min.

Nematode desiccation and rehydration

Two-thousand adult *C. elegans*, 20,000 mixed-stage *A. avenae* or 5000 mixed-stage *P. superbus* individuals were placed onto a filter membrane (25 mm Supour-450, Pall Life Sciences, Portsmouth, UK). Filter membranes on small plastic Petri dishes were placed inside sealed plastic boxes containing a saturated salt solution to achieve a specific relative humidity (RH) at 25°C. The following salt solutions were used: 100% ultra high purity (UHP) water, 97% potassium sulphate, 90% barium chloride, 75% sodium chloride and 10% freshly baked silica gel. After desiccation, worms were rehydrated by the addition of 1.6 ml of M9 buffer (*C. elegans* and *P. superbus*) or tap water (*A. avenae*) and agitated on a horizontal shaker at 80 r.p.m. After 1 h of rehydration, filters were removed and rinsed with a further 0.8 ml buffer/water. Worms were left to rehydrate for a total of 2 h (*C. elegans*) or 16–24 h (*A. avenae* and *P. superbus*) at 80 r.p.m. unless otherwise stated. The motility of 80 worms from three replicates per condition was used to determine survival.

Nematode treatment with kinase inhibitors

Stock solutions of JNK, p38 and PI3K inhibitors [SP600125 (cat. no. 420119), SB203580 (cat. no. 559389) and LY294002 (cat. no. 440202), respectively; all from Calbiochem-Novabiochem, Nottingham, UK] dissolved in DMSO (Sigma-Aldrich, Poole, UK), were diluted in tap water (*A. avenae*) or M9 buffer (*P. superbus*) containing 20,000 mixed-stage *A. avenae* per ml or 5000 mixed-stage *P. superbus* per ml, respectively. The final concentration of DMSO in the inhibitor solution was adjusted to 1%. Nematodes were incubated in the inhibitor solution for 3 h in the dark at 115 r.p.m. Untreated nematodes were also incubated in 1% DMSO under the same conditions. Nematodes were subsequently desiccated as described above and their survival determined by counting the motility of 80 worms from three replicates per condition.

Determination of water content

Nematode water content was determined using a Q500 thermogravimetric analyzer (TA Instruments, Elstree, UK) by heating nematodes to 150°C at a rate of 10°C min^{-1} and calculating their change in mass. Three replicates were used per condition.

Protein extraction

After desiccation, nematodes were either immediately frozen in liquid nitrogen and stored at −80°C or rehydrated for the appropriate time, collected by centrifuging at 1000 ***g*** for 3 min, then frozen in liquid nitrogen and stored at −80°C. Frozen worms were thawed on ice, washed with ice-cold UHP water and pelleted by centrifugation. Worm pellets were resuspended in lysis buffer [50 mmol l^{-1} Tris HCl pH 7.5, 5% SDS, 5% 2-mercaptoethanol, one complete mini protease inhibitor tablet (Roche Products Ltd, Welwyn Garden City, UK) per 10 ml], boiled for 25 min and centrifuged twice at 13,000 ***g*** for 20 min to remove debris.

SDS-PAGE and western blotting

Proteins were resolved on a 10% acrylamide gel and transferred onto a PVDF membrane (GE Healthcare, Chalfont St Giles, UK). The PVDF membrane was blocked for 1 h in 5% non-fat milk powder in PBS supplemented with 0.1% TWEEN 20 (NFPBST) then incubated with primary antibody for 1 h in 1% NFPBST, washed 6×5 min in 1% NFPBST, incubated with secondary antibody

for 1 h in 1% NFPBST, washed again and incubated with ECL plus (GE Healthcare). Antibodies used for western blotting included phospho-p38 (cat. no. 9216; Cell Signaling Technology, Hitchin, UK), phospho-ERK (cat. no. M8159; Sigma-Aldrich), phospho-JNK (cat. no. 4688; Cell Signaling Technology), total-ERK (cat. no. Sc-153; Santa Cruz Technology, CA, USA), total-JNK (cat. no. Sc-571; Santa Cruz Technology), anti-PMK-1 (recognises total p38 in *C. elegans*; gift of Prof. Kunihiro Matsumoto, Nagoya University, Nagoya, Japan), actin (cat. no. A 2066; Sigma-Aldrich), anti-mouse IgG, horseradish peroxidase linked (cat. no. NA931; GE Healthcare) and anti-rabbit IgG, horseradish peroxidase linked (cat. no. NA934; GE Healthcare). Western blots were stripped by incubation for 45 min in stripping buffer (625 mmol l^{-1} Tris HCl pH 6.8, 2% SDS, 0.8% 2-mercaptoethanol) at 55°C then re-blocked, washed 6×5 min in PBS and re-probed as described above. Western blot band intensity was quantified using FIJI (v.1.45).

Statistics

Significant difference between data values was determined using ANOVA (analysis of variance) with confidence intervals at 95%. The number of moving worms was used for statistical analysis. Actual significance values were determined using a Tukey *post hoc* test where $P>0.05$ was considered not significant (n.s.), $0.01<P\leq0.05$ was considered significant (*), $0.001<P\leq0.01$ was considered highly significant (**) and $P\leq0.001$ was considered extremely significant (***).

RESULTS

Anhydrobiotic potential of three nematode species

Desiccation tolerance of the three nematode species was assessed after incubation at various relative humidities. Synchronised populations of young *C. elegans* adults were placed at 100%, 97%, 90%, 75% or 10% RH for 6 and 24 h (Fig. 1A). No appreciable mortality was observed when *C. elegans* was incubated at 100% or 97% RH for up to 24 h, or at 90% RH for 6 h, but only 30% of worms were still moving after 24 h at 90% RH. There was no survival at 75% or 10% RH. To test whether preconditioning (a period of slow dehydration before complete water loss) might allow *C. elegans* adults to survive anhydrobiosis, they were incubated at 97% RH (Fig. 1A) for 24, 48 and 72 h before transfer to low (10%) RH for 24 h. However, even after preconditioning, *C. elegans* adults were unable to survive exposure to 10% RH and therefore were judged to be incapable of anhydrobiosis.

Aphelenchus avenae were preconditioned at either 100% RH or 97% RH for various times (24, 36, 48 or 72 h) and either immediately rehydrated or subjected to complete desiccation by incubation at 10% RH for 24 h (Fig. 1B). Exposure of nematodes to 97% RH for up to 72 h did not decrease nematode survival compared with non-desiccated samples at 100% RH. However, preconditioning at 97% RH significantly increased anhydrobiotic survival (survival after complete desiccation at 10% RH) and the longer the preconditioning time (up to 72 h) the better the results. In comparison, a harsher preconditioning regime, i.e. 90% RH, itself had a detrimental effect

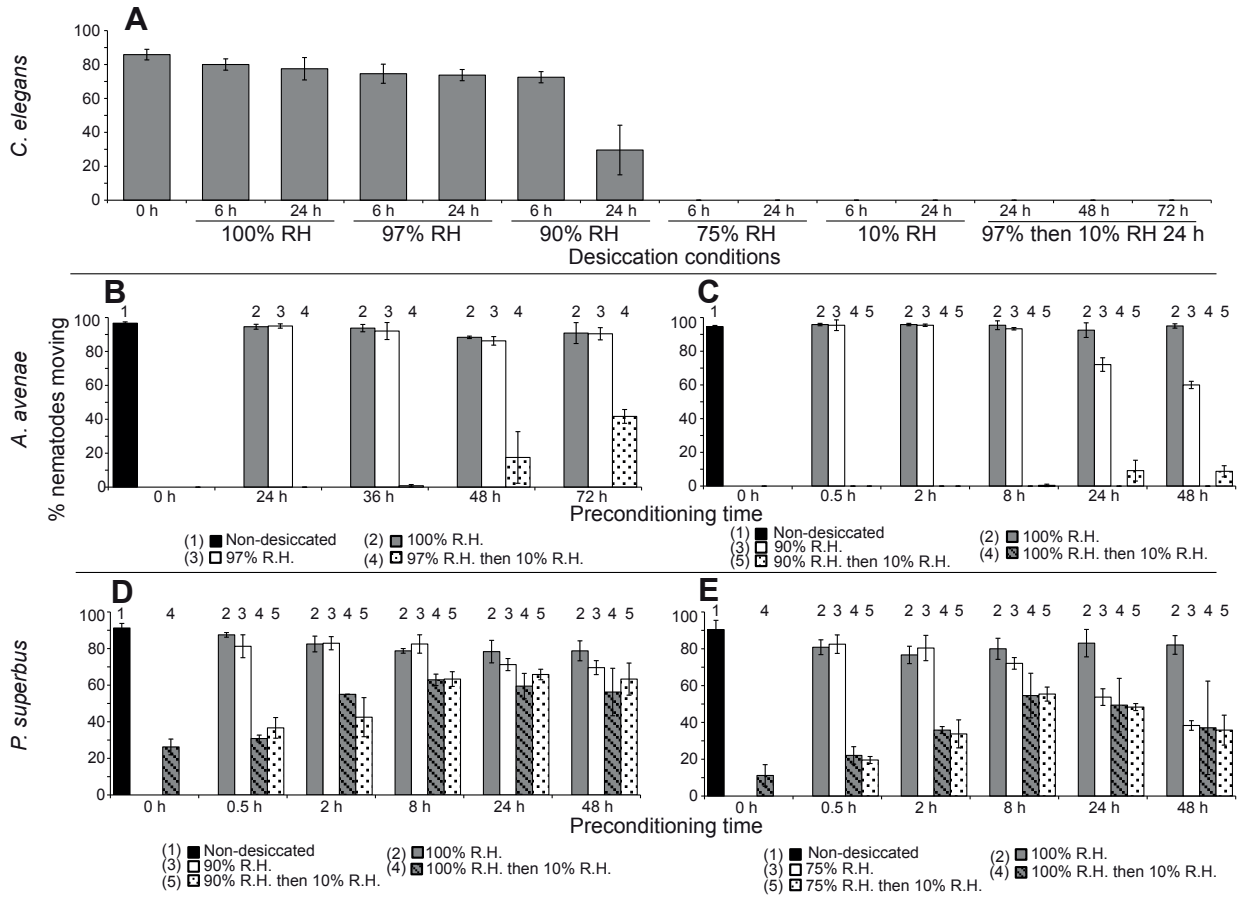

Fig. 1. Desiccation survival of three nematode species with varying tolerance to water loss. (A) Synchronised young *Caenorhabditis elegans* adults desiccated under different conditions. (B) Mixed-stage *Aphelenchus avenae* preconditioned at 100% and 97% relative humidity (RH) for various times. (C) Mixed-stage *A. avenae* preconditioned at 100% and 90% RH for various times. (D) Mixed-stage *Panagrolaimus superbus* preconditioned at 100% and 90% RH for various times. (E) Mixed-stage *P. superbus* preconditioned at 100% and 75% RH for various times. N=3. Error bars show ±1 s.d.

on viability, as survival was only 70% and 50% after 24 and 48 h, respectively (Fig. 1C). However, preconditioning *A. avenae* at 90% RH accelerated the onset of desiccation tolerance as nematodes survived anhydrobiosis after only 24 h, compared with 48 h after being preconditioned at 97% RH (Fig. 1B). Nevertheless, the maximum anhydrobiotic survival rate after preconditioning at 90% RH was only 10%, compared with 40% survival after preconditioning at 97% RH. *Aphelenchus avenae* preconditioned at 100% RH were unable to survive anhydrobiosis at all (Fig. 1C). These data confirm *A. avenae* as a slow desiccation strategist anhydrobiote.

No effect of preconditioning itself on the viability of *P. superbus* was observed after incubation at 100% or 90% RH for up to 48 h (Fig. 1D) or at 75% RH at time points up to 8 h, although longer incubation times at 75% RH led to decreased survival (Fig. 1E). Approximately 11–26% of the *P. superbus* population was able to survive immediate exposure to 10% RH, but preconditioning (up to 8 h) at either 90% (Fig. 1D) or 75% RH (Fig. 1E) increased survival. Interestingly, preconditioning the worms at 100% RH (for up to 8 h) before complete desiccation, when theoretically no water loss should occur, also increased anhydrobiotic survival (Fig. 1D,E). These results show *P. superbus* to be a fast desiccation strategist, but also indicate that its anhydrobiotic potential can be increased by preconditioning at a range of relative humidities (75–100%).

The rate of recovery from the dry state was investigated next to ask whether differences occur between slow and fast desiccation strategists. *Aphelenchus avenae* incubated at 10% RH for 24 h after preconditioning at 100% or 97% RH for 96 h was rehydrated and the number of motile nematodes assessed over time. As shown previously (Fig. 1B,C), none of the nematodes preconditioned at 100% RH survived anhydrobiosis, while preconditioning at 97% RH ensured up to 55% viability (Fig. 2A). Only 10% of worms were moving after 0.5 h and it took 24 h for all live nematodes to fully recover, after which there was no further increase in viability: survival was the same at 48 h. In contrast, the recovery of *P. superbus* preconditioned at either 100% or 90% RH for 24 h before complete desiccation at 10% RH was considerably quicker, with all worms regaining full motility within 0.5 h (Fig. 2B). No difference in recovery rate was observed between *P. superbus* preconditioned at either 100% or 90% RH.

To confirm that nematodes desiccated at 10% RH for 24 h were fully dehydrated, and to uncover any differences in water loss between fast and slow desiccation strategists, the residual water content of both *A. avenae* and *P. superbus* after various desiccation regimes was determined using thermogravimetry. The two species showed comparable water content when freshly harvested or desiccated at 10% RH (around 70% and 8% water content, respectively; Fig. 2C). This confirms that both nematodes undergo anhydrobiosis and that there are no differences in final water content. The water content of *A. avenae* at optimum preconditioning conditions (97% RH for 72 h) was around 40%, while the water content of *P. superbus* at optimal preconditioning (90% RH for 24 h) was around 30%. This shows that water is lost during preconditioning consistent with the RH of the desiccation chamber. Interestingly, while *P. superbus* control worms incubated at 100% RH did not lose water but could survive complete desiccation at 10% RH, *A. avenae* lost about 29% of their water content (Fig. 2C) and yet were unable to survive anhydrobiosis (Fig. 1C,D).

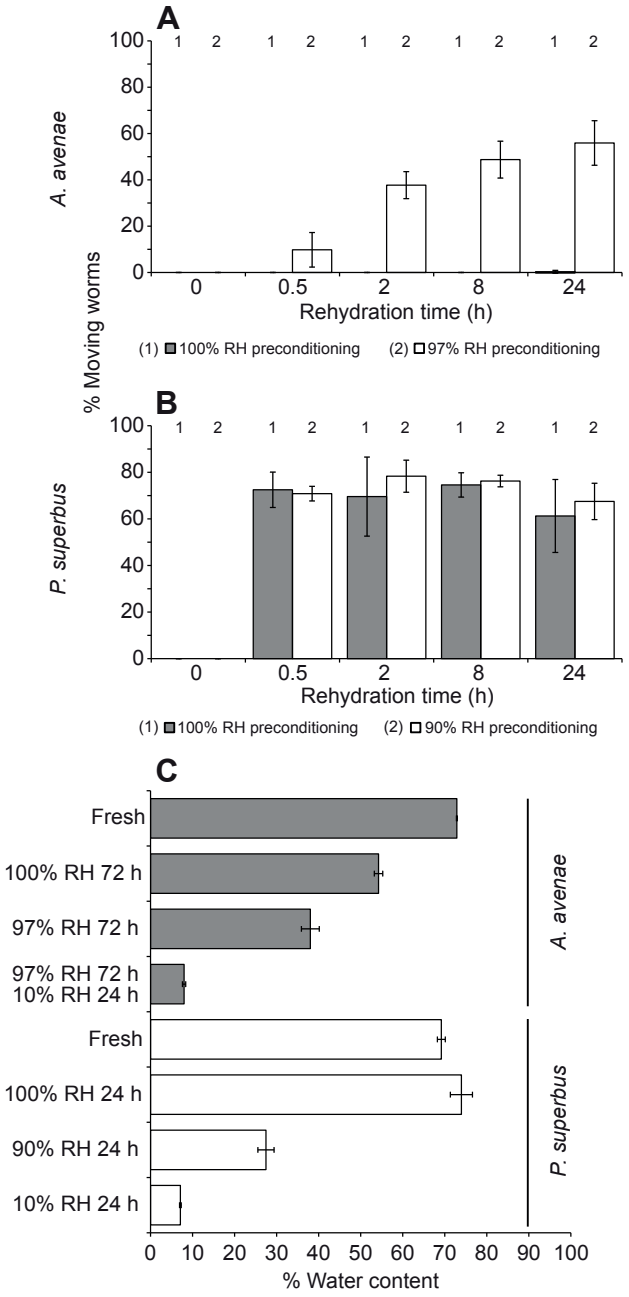

Fig. 2. Recovery from, and water content during, anhydrobiosis in *A. avenae* and *P. superbus*. (A) Mixed-stage *A. avenae* were preconditioned for 96 h at either 100% or 97% RH and then desiccated for 24 h at 10% RH. Recovery of activity was monitored for various times. (B) Mixed-stage *P. superbus* were preconditioned for 24 h at either 100% or 90% RH and then desiccated for 24 h at 10% RH. Recovery of activity was measured for various times. (C) Water content of *A. avenae* and *P. superbus* after various desiccation regimes. *N*=3. Error bars show ±1 s.d.

MAPK phosphorylation during entry into anhydrobiosis

If MAPKs are involved in signal transduction leading to a desiccation stress response, then their phosphorylation status should change with water loss. Therefore, the phosphorylation of p38, JNK and ERK was examined during desiccation in the three nematode species characterised above. The adult stage of *C. elegans* was incubated under non-desiccating (100% RH) and desiccating conditions (90% RH) over time (0, 0.5, 2, 8 and 24 h) and the activation status of the MAPKs determined by western blotting using antibodies specific to their phosphorylated forms (Fig. 3A). All three MAPKs became more phosphorylated after 24 h of desiccation at

90% RH while phosphorylation remained unchanged during incubation at 100% RH. Re-probing the blot with antibodies that recognised both phosphorylated and non-phosphorylated forms of the MAPKs confirmed no change in the total amount of each MAPK.

Desiccation of the anhydrobiotic nematode *A. avenae* at 90% RH caused all three MAPKs to become phosphorylated at 24 and 48 h, while little change in phosphorylation occurred during incubation at 100% RH (Fig. 3B). Total ERK and JNK remained constant during the experiment. The total amount of p38 in *A. avenae* could not be determined because no suitable antibody was available. As *A. avenae* is a slow desiccation strategist, the anhydrobiotic survival of *A. avenae* after preconditioning at 90% RH is low, but can be improved by preconditioning at a higher RH (Fig. 1B,C). At 97% RH, only a temporary increase in p38 phosphorylation at 36 h was observed, while JNK phosphorylation increased at 48 h and 72 h and ERK phosphorylation remained unchanged (Fig. 3C). A slight increase in JNK and p38 phosphorylation at 0.5 h was seen under both desiccating and non-desiccating conditions and therefore could not be attributed to dehydration.

The MAPK profile of the anhydrobiotic nematode *P. superbus* was noticeably different from those of *A. avenae* and *C. elegans*, perhaps reflecting its status as a fast desiccation strategist. After 24 h at 90% RH, p38 and ERK, but not JNK, became dephosphorylated (Fig. 3D). The total amount of ERK and JNK also increased slightly after 8 h, which cannot be explained by unequal protein loading as demonstrated by blotting for actin. Following incubation of *P. superbus* at 100% RH, there was a slight increase in phosphorylation of all three MAPKs at 24 and 48 h, but this probably reflects some variation in protein loading. When *P. superbus* was preconditioned at 75% RH, ERK and p38 phosphorylation decreased earlier in comparison with preconditioning at 90% RH (compare Fig. 3E and 3D), after just 8 h, while JNK phosphorylation also apparently decreased at later time points (Fig. 3E).

MAPK phosphorylation during recovery from anhydrobiosis

Successful entry into anhydrobiosis might not be sufficient for nematode survival as cellular damage can also occur in the desiccated state (e.g. membrane disruption, protein aggregation and DNA damage). This damage will need to be repaired once the nematode is rehydrated for it to be viable. It is plausible that MAPKs might activate cellular repair pathways in response to a rehydration signal, ensuring successful recovery from anhydrobiosis. To examine this, the phosphorylation status of p38, ERK and JNK was compared after recovery from anhydrobiosis in *A. avenae* and *P. superbus*. *Aphelenchus avenae* was preconditioned at 97% RH for 96 h, then desiccated with silica gel for 24 h and rehydrated for 0.5–24 h. As a comparison, non-desiccated (not exposed to silica gel) but preconditioned worms were investigated concurrently. In Fig. 3C, it was shown that p38 and ERK phosphorylation returned to pre-desiccated levels within 72 h of preconditioning at 97% RH while JNK phosphorylation remained elevated. This was also true in non-desiccated worms after 96 h of preconditioning (Fig. 4A). During rehydration, phosphorylation of all three MAPKs increased transiently after 0.5 h; ERK was the first MAPK to return to pre-desiccated phosphorylation levels after 8 h, followed by p38 after 24 h and finally JNK after 48 h. Total ERK and JNK levels remained unchanged.

Panagrolaimus superbus was preconditioned for 24 h at either 100% or 90% RH, then desiccated with silica gel for 24 h and rehydrated for 0.5–24 h (Fig. 4B). As seen in Fig. 3D, both p38 and ERK phosphorylation decreased in non-desiccated nematodes preconditioned at 90% RH (Fig. 4B). However, after 0.5 h of rehydration the phosphorylation levels of these MAPKs returned to pre-desiccation levels. JNK phosphorylation increased slightly between 0.5 and 2 h of rehydration independent of whether preconditioning occurred at 100% or 90% RH. Interestingly, after 24 h of rehydration, total JNK increased considerably in both 100% and 90% RH preconditioned worms. It was also observed that after 8 h of rehydration the phosphorylation of all three MAPKs decreased, perhaps in response to a prolonged time in solution without food.

MAPK inhibitors reduce *P. superbus* recovery from anhydrobiosis

To confirm the importance of MAPK phosphorylation in anhydrobiotic survival, *A. avenae* and *P. superbus* were treated for 3 h prior to harvesting with kinase inhibitors (SB203580, SP600125 and LY294002) against p38, JNK and phosphatidylinositol 3-kinase (PI3K), respectively. A PI3K inhibitor was used because it is the only kinase inhibitor reported to increase stress tolerance in a nematode (Babar et al., 1999), but it appears to have no effect on desiccation tolerance (Fig. 5). Inhibitor-treated *A. avenae* were preconditioned at 97% RH for 48 or 72 h then desiccated using silica gel for 24 h. However, no significant effect of the inhibitors on the anhydrobiotic survival of *A. avenae* was observed compared with worms treated with DMSO (Fig. 5A). *Aphelenchus avenae* treated with the inhibitors but preconditioned at 100% RH only was used as a control to examine the toxic effects of the inhibitors on the nematodes. No toxic effects on *A. avenae* were observed using this control. A second control experiment where *A. avenae* was soaked for up to 100 h in solutions containing the inhibitors also showed no toxicity (data not shown).

Inhibitor-treated *P. superbus* was immediately desiccated with silica gel for 24 h or preconditioned at 90% RH for 24 h first before complete desiccation for 24 h. None of the inhibitors affected the survival of preconditioned *P. superbus*, but the p38 and JNK inhibitors did significantly reduce the anhydrobiotic survival of non-preconditioned worms compared with DMSO-treated worms (P=0.000, P=0.013, respectively), while the PI3K inhibitor had no effect (P=1.000) (Fig. 5B). This reduction in survival was not due to toxicity of the inhibitors as control *P. superbus* preconditioned at 100% RH or soaked in the inhibitors showed no difference in survival (Fig. 5B and data not shown). Increasing the inhibitor concentrations resulted in a further reduction in anhydrobiotic survival in worms treated with the JNK and p38 inhibitors but not the PI3K inhibitor (P=0.000, P=0.000, P=1.000, respectively, for the highest inhibitor concentration compared with DMSO-treated worms), while no decrease in the viability of control worms occurred (Fig. 5C and data not shown).

DISCUSSION

The anhydrobiotic survival strategy employed by different nematodes is probably related to the ecological niche they inhabit. The fast desiccation strategist *P. superbus* was isolated from a gull's nest on Surtsy Island, Iceland, a harsh and changeable environment (Shannon et al., 2005). *Panagrolaimus superbus* can enter anhydrobiosis without preconditioning and recover rapidly within 0.5 h, important attributes in a habitat that regularly freezes and thaws. In contrast, the slow desiccation strategist *A. avenae* lives in soil, a fairly stable environment that dries slowly and infrequently, allowing time to upregulate adaptations in preparation for the dry state and consistent with a need for slow entry into, and recovery from, anhydrobiosis. The non-tolerant *C. elegans* adults inhabit rotting fruit, a more stable environment, making it unnecessary to be particularly tolerant to

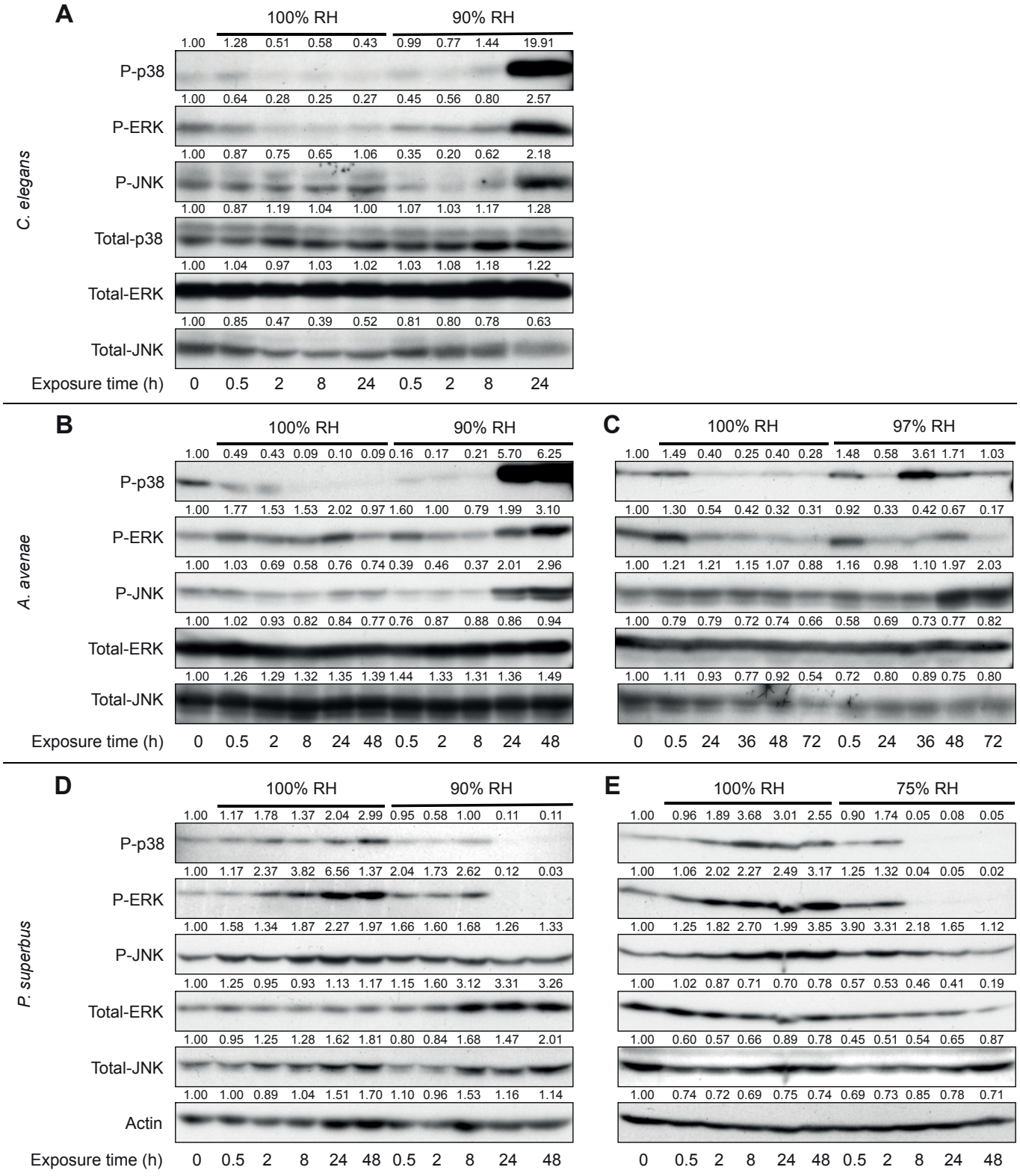

Fig. 3. Mitogen-activated protein kinase (MAPK) phosphorylation during preconditioning in three nematode species with varying tolerance to desiccation stress. (A) Synchronised young *C. elegans* adults preconditioned at 100% and 90% RH for various times. (B) Mixed-stage *A. avenae* preconditioned at 100% and 90% RH for various times. (C) Mixed-stage *A. avenae* preconditioned at 100% and 97% RH for various times. (D) Mixed-stage *P. superbus* preconditioned at 100% and 90% RH for various times. (E) Mixed-stage *P. superbus* preconditioned at 100% and 75% RH for various times. Western blots were first probed with antibodies against phosphorylated MAPKs before being stripped and re-probed with the corresponding total MAPK antibody or, in the case of the phosphorylated p38 blot, with an antibody against actin. Relative quantification of each band is shown. The ERK phosphorylation state during preconditioning in *C. elegans* and *A. avenae* has been presented previously (Huang et al., 2010) but is shown here to provide a complete picture of MAPK activation.

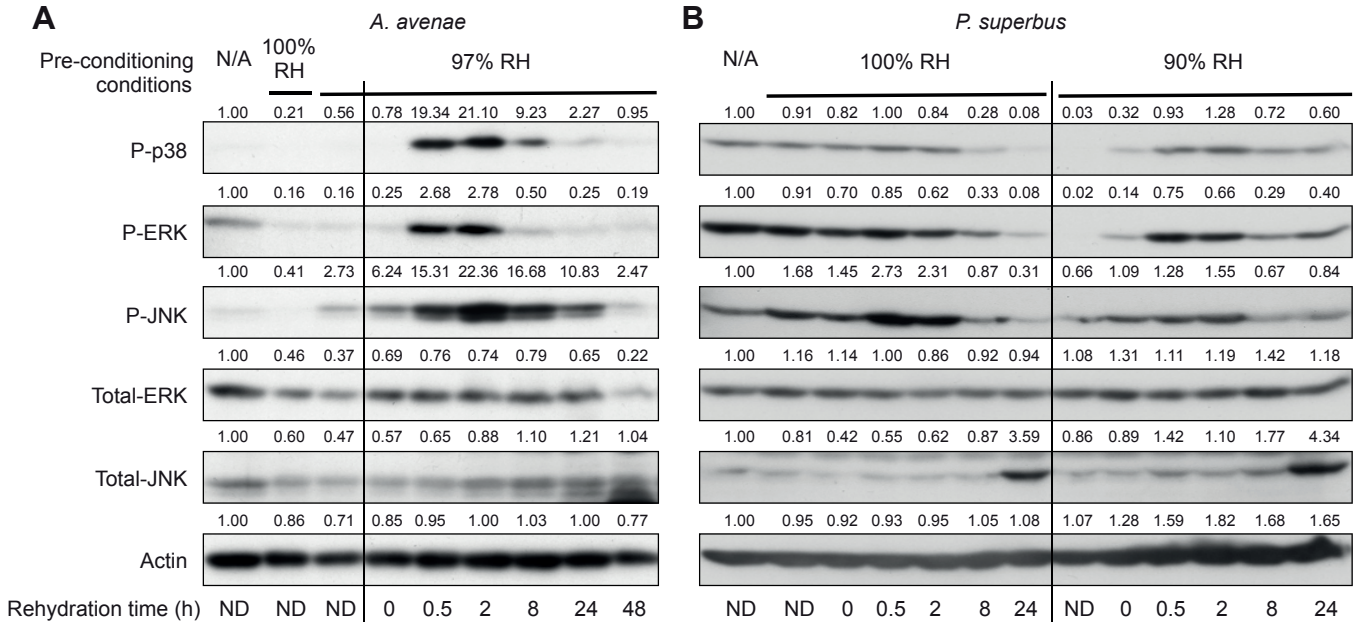

Fig. 4. MAPK phosphorylation in *A. avenae* and *P. superbus* during recovery from anhydrobiosis. (A) Mixed-stage *A. avenae* were preconditioned at 100% or 97% RH for 96 h, desiccated at 10% RH for 24 h and subsequently rehydrated for various times. (B) Mixed-stage *P. superbus* were preconditioned at 100% or 90% RH for 24 h, desiccated at 10% RH for 24 h and subsequently rehydrated for various times. In both cases, non-desiccated (ND) or preconditioned-only worms were also examined as a comparison. Western blots were first probed with antibodies against phosphorylated MAPKs before being stripped and re-probed with the corresponding total MAPK antibody or, in the case of the phosphorylated p38 blot, with an antibody against actin. Relative quantification of each band is shown. Full blots are shown in supplementary material Fig. S1.

water stress. In fact, *C. elegans* adults cannot enter anhydrobiosis even with preconditioning, but this species does possess an anhydrobiotically capable dauer stage, should its habitat become less hospitable (Erkut et al., 2011; Gal et al., 2004). Dauer larvae express several stress-tolerant genes including an LEA protein gene, and it has been shown that knockdown of this LEA protein transcript through RNAi reduces dauer desiccation tolerance (Gal et al., 2004). The different desiccation tolerance shown by the three nematode species is also reflected in their tolerance to osmotic stress, with *P. superbus* able to survive high hypertonicity, *A. avenae* being somewhat less osmotolerant, and *C. elegans* surviving osmotic stress poorly (Banton, 2009).

In previous studies, hydrated (non-dried) controls are usually represented by nematodes in water, but we attempted a more appropriate control where non-desiccated nematodes are treated in the same way as desiccated nematodes but with incubation at 100% RH instead of lower humidities. Interestingly, *A. avenae* did lose water at 100% RH, while *P. superbus* did not (although over a shorter time course), suggesting that some loss can occur even in a completely saturated environment. However, more remarkable is that without any measurable loss of water, preconditioning of *P. superbus* at both 100% and 90% RH increased its anhydrobiotic survival. A recent study where over 9000 expressed sequence tags (ESTs) from *P. superbus* were sequenced found that while many desiccation tolerance genes were constitutively expressed, some of them were also upregulated in response to dehydration (Tyson et al., 2012). It would be interesting to test whether such genes also respond to incubation of *P. superbus* at 100% RH. In contrast, despite significant water loss in *A. avenae* incubated at 100% RH for 72 h, these nematodes were unable to enter and survive anhydrobiosis, in agreement with previous data showing that preconditioned *A. avenae* require a water content of between 40% and 12% to successfully survive anhydrobiosis (Higa and Womersley, 1993). The increased water loss by *A. avenae* at 100% RH compared with *P. superbus* is in agreement with *P. superbus* being an innate (or fast) desiccation strategist that can control its rate of water loss during preconditioning. The fact that preconditioning *P. superbus* also increased its anhydrobiotic survival agrees with the findings of Perry and Moens (Perry and Moens, 2011), who state that biochemical adaptations occur in innate desiccation strategists during preconditioning.

Nematodes with different survival strategies have distinct patterns of MAPK activation during entry into and exit from anhydrobiosis (Fig. 6 summarises the relative variation in phosphorylation of each MAP kinase). Where upregulation of protective molecules is attempted (*C. elegans* and *A. avenae*), p38, ERK and JNK phosphorylation is elevated during preconditioning and rehydration. Therefore, in these worms MAPKs might be acting as downstream signal transducers, activating transcription factors in response to upstream ‘dehydration’ signals. It is noteworthy that this occurs in *C. elegans*, even though its adults do not survive desiccation, suggesting that it is able to respond to the stress, but not activate the effector molecules involved in survival. Presumably, however, such effectors can be produced by *C. elegans* dauer larvae (Erkut et al., 2011).

In *P. superbus*, p38, ERK and possibly JNK phosphorylation decreased during preconditioning at 90% and 75% RH, but not at 100% RH. However, preconditioning did increase anhydrobiotic survival and *P. superbus* incubated at 100% and 90% RH survived equally well. Therefore, it is unlikely that MAPK dephosphorylation is required for anhydrobiosis.

Once an anhydrobiotic nematode is rehydrated, it can take time to regain motility. It is thought that, during this lag phase, any cellular damage that has accumulated during anhydrobiosis is repaired

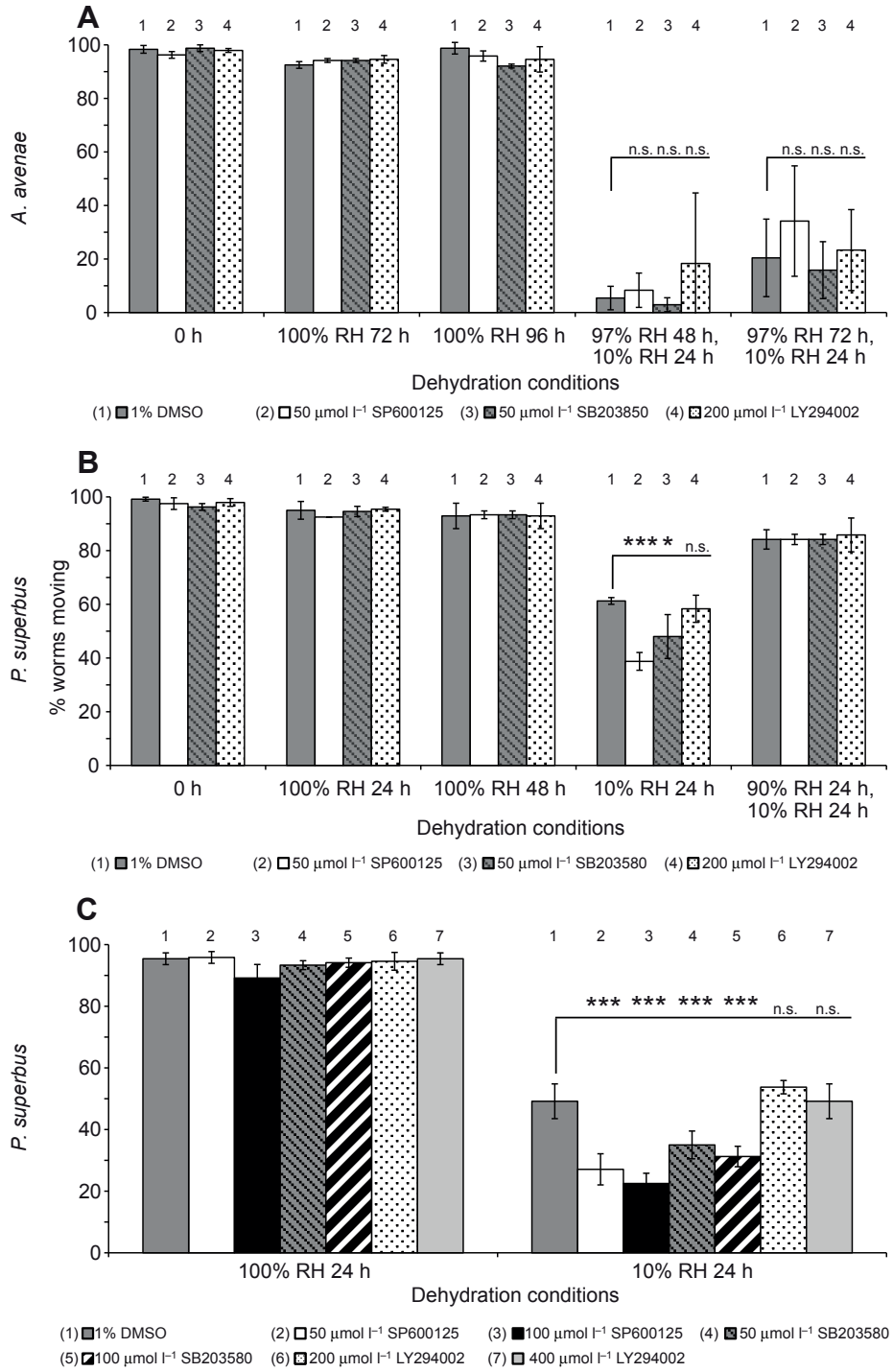

Fig. 5. The effect of kinase inhibitors (SB203580, SP600125 and LY294002) against p38, JNK and phosphatidylinositol 3-kinase (PI3K), respectively, on the anhydrobiotic survival of *A. avenae* and *P. superbus*. Nematodes were treated with kinase inhibitors for 3 h before preconditioning and subsequent desiccation at 10% RH for 24 h. Control worms were preconditioned for the equivalent time at 100% RH without desiccation at 10% RH. 0 h worms were pre-treated with the inhibitors only. (A) Mixed-stage *A. avenae* preconditioned at 97% RH. (B) Mixed-stage *P. superbus* with and without preconditioning at 90% RH. (C) Mixed-stage *P. superbus* desiccated at 10% RH after treatment with increasing concentrations of inhibitors. N=3. Error bars show ±1 s.d. Statistical significance between DMSO only and inhibitor-treated worms is indicated as follows: P>0.05 was considered not significant (n.s.), 0.01<P≤0.05 was considered significant (*), 0.001<P≤0.01 was considered highly significant (**) and P≤0.001 was considered extremely significant (***).

(Wharton and Barrett, 1985). We found that all three MAPKs became phosphorylated during the lag phase in *A. avenae*, suggesting that MAPKs might activate pathways involved in cellular repair. In contrast to *A. avenae*, which takes 2 h for motility to be restored after anhydrobiosis, *P. superbus* requires only 0.5 h. This quick recovery was reflected in the *P. superbus* MAPK phosphorylation pattern, which also returned to pre-stress levels within 0.5 h, suggesting that little damage occurs in these nematodes during desiccation.

Under certain conditions, each MAPK responded differently to the stress; for example, when *A. avenae* was preconditioned at 97% RH and during its recovery from anhydrobiosis (Fig. 3C, Fig. 4A). This differential phosphorylation pattern could be evidence that each MAPK responds to different stimuli and/or regulates alternative processes. Differential MAPK activation could also occur in different tissues, as reported in frog organs during freezing and thawing (Greenway and Storey, 2000).

In this study it was found that the temporal activation of each MAPK could be different. It is conceivable that each MAPK responds to different stimuli during preconditioning in order to regulate specific survival adaptations at different stages of desiccation. For example, transient p38 activation observed during

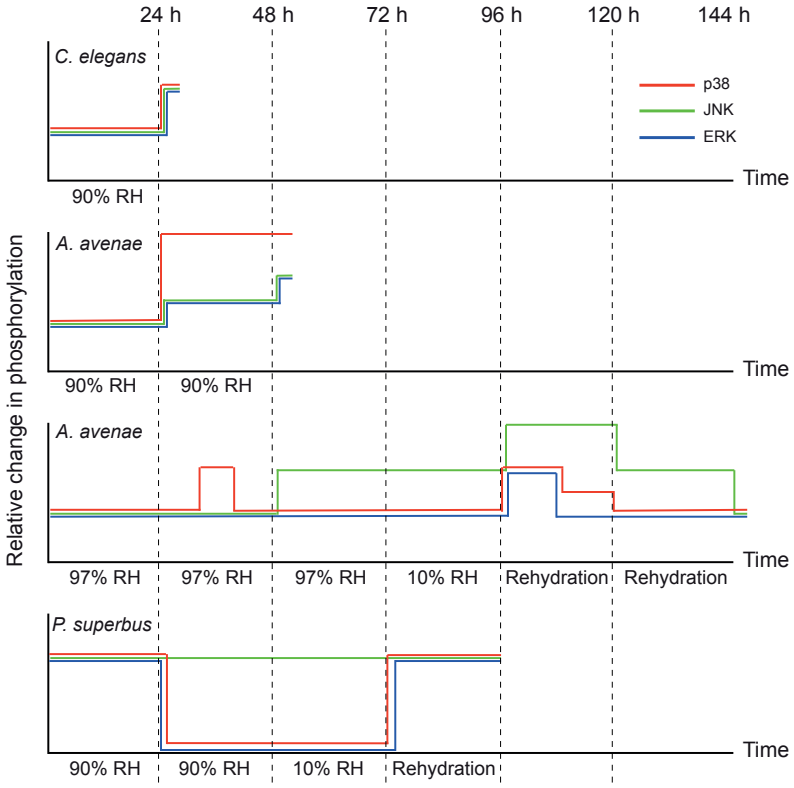

Fig. 6. Overview of the variation in phosphorylation of p38, JNK and ERK during preconditioning, anhydrobiosis and rehydration in *C. elegans*, *A. avenae* and *P. superbus*. The graphs represent the relative changes in MAPK phosphorylation (*y*-axis) over time (*x*-axis; time points as described in Results), as inferred from the western blotting data presented in this report. The changes in phosphorylation are not quantitative but represent the relative increase or decrease in phosphorylation from the initial state. In *C. elegans*, all three MAPKs become phosphorylated after 24 h of preconditioning at 90% RH. In *A. avenae*, all three MAPKs become phosphorylated after 24 h of preconditioning at 90% RH, with p38 phosphorylation increasing more than that of either ERK or JNK. ERK and JNK phosphorylation continues to increases after a further 24 h at 90% RH. When *A. avenae* is preconditioned at 97% RH, a transient increase in p38 phosphorylation after 36 h is observed, while after 48 h a sustained increase in JNK phosphorylation occurs. During anhydrobiosis (10% RH), the phosphorylation state of all three MAPKs remains unchanged but is increased within 0.5 h of rehydration. ERK is the first MAPK whose phosphorylation returns to its pre-desiccated state after 2 h followed by p38 and JNK after 24 and 48 h, respectively. Preconditioning *P. superbus* at 90% RH causes a dramatic decrease in p38 and ERK phosphorylation after 24 h and no variation in JNK; in the desiccated state, MAPK phosphorylation levels remain unchanged but quickly return to their original pre-desiccated levels within 0.5 h of rehydration.

A. avenae preconditioning could occur in response to cellular perturbations such as changes in intracellular tonicity, which would lead to the activation of early response genes such as trehalose synthase, while sustained JNK activation was found later during preconditioning, which could be in response to more severe stresses such as protein denaturation and could upregulate later response genes encoding LEA proteins, HSPs and antioxidants. Interestingly, Lamitina and colleagues (Lamitina et al., 2006) found that protein damage might trigger the expression of survival genes in *C. elegans* during osmotic stress; therefore, protein damage could also be an important inducer of survival genes in anhydrobiotic organisms.

All three MAPKs investigated here (p38, JNK and ERK) are known to be involved in stress tolerance in other organisms. For example, the role of p38 in osmotolerance in yeast and mammalian cells has been intensely studied (reviewed in Burg et al., 2007). Sensors upstream of p38 have been hypothesised to detect changes in intracellular tonicity, perhaps through mechanical effects on the plasma membrane (Reiser et al., 2003). These sensors indirectly activate the p38 MAPK cascade, leading to p38 phosphorylation, which then either enters the nucleus to drive the expression of protective genes or phosphorylates intracellular targets such as other kinases. Desiccation and hypertonic osmotic stress share similar characteristics as both result in the loss of internal water, causing macromolecular crowding and increases in intracellular pH, viscosity and ion concentration, leading to DNA damage, protein denaturation and disruption of transmembrane potentials. In response to water loss, the two stresses induce similar adaptations, including the production of compatible solutes (e.g. trehalose), HSPs, antioxidants and LEA proteins (Burg et al., 2007; Kikawada et al., 2006). It is plausible that as osmotic and desiccation stresses are similar, the signalling mechanisms are also conserved. Indeed, in human cells it has been shown that p38 is activated in response to both osmotic and desiccation stress and that osmotic stress can precondition the slow desiccation strategist *A. avenae* so that it survives anhydrobiosis (Huang and Tunnacliffe, 2004; Otsubo et al., 2006). In *C. elegans*, p38 is activated in response to oxidative stress, whereupon it phosphorylates the transcription factor SKN-1, causing it to translocate into the nucleus and drive gene expression. The p38 cascade in *C. elegans* is also necessary for survival of *Pseudomonas aeruginosa* infection (Inoue et al., 2005; Kim et al., 2002). Therefore, the p38 phosphorylation observed in this study is unlikely to be specific to desiccation stress alone in the nematodes studied.

The JNK cascade plays a role in a variety of stresses in different organisms, including heavy metal and heat stresses in *C. elegans*. During heat stress, JNK activates the *C. elegans* DAF-16 pathway, which is constitutively upregulated in the stress-tolerant and anhydrobiotically capable *C. elegans* dauer larvae (Oh et al., 2005; Wolf et al., 2008). It would be interesting to investigate whether JNK also functions through the DAF-16 pathway in anhydrobiotic nematodes.

We found that JNK either became (*A. avenae*) or remained (*P. superbus*) phosphorylated for prolonged periods during preconditioning compared with p38, which is transiently activated during slow *A. avenae* preconditioning or dephosphorylated during *P. superbus* preconditioning. In mammalian cells, prolonged JNK activation can be induced by stresses such as UV radiation, resulting in apoptosis (Shaulian et al., 2000). Apoptosis would not be beneficial in anhydrobiotic organisms such as nematodes as cells do not replicate in adults and therefore essentially all cells would need to survive desiccation for the whole organism to recover from anhydrobiosis. Therefore, apoptosis would need to be suppressed in anhydrobiotic organisms.

In this study, it has been shown that ERK phosphorylation state is affected by desiccation stress and that it often (but not exclusively) follows p38 MAPK activity. ERK is also involved in a number of other stresses in nematodes, including pathogen attack and starvation (Nicholas and Hodgkin, 2004; You et al., 2006). This is in contrast to other studies in *Drosophila*, mammalian cells and amphibians,

which demonstrate that ERK is not normally phosphorylated during stress and that it is predominantly involved in controlling cell proliferation and differentiation (Cowan and Storey, 2003). Therefore, the involvement of ERK during stress in nematodes might be evolutionarily conserved and its function in stress could have been acquired after nematodes diverged from the insect and vertebrate lineages around 600 million years ago (Manning et al., 2002). Alternatively, the involvement of the ERK cascade in the response to stress in both mammals and insects might not have been fully appreciated yet as ERK is phosphorylated in response to desiccation, osmotic and oxidative stresses in mammalian cells (Garg and Chang, 2003; Huang et al., 2010; Yang et al., 2000). Therefore, further work is needed to precisely define the role of ERK in stress in nematodes and other organisms.

We attempted to use inhibitors to reduce MAPK activity before nematodes were desiccated using concentrations reported in other studies on nematodes (Babar et al., 1999; Berman et al., 2001; Orsborn et al., 2007). Treatment of *A. avenae* with these inhibitors had no apparent effect on survival, which might suggest that these kinases are not involved in desiccation tolerance. However, it is possible that during preconditioning the effects of the inhibitors wore off once nematodes were removed from the inhibitor solution. Therefore, any effects of the inhibitors could have dissipated within a few hours of preconditioning, before the first MAPKs become activated, which takes up to 36 h (Fig. 3C). This could also explain why the inhibitors had no effect on the anhydrobiotic survival of preconditioned *P. superbus* but did decrease its survival if the worms were desiccated at 10% RH immediately after treatment and is consistent with phosphorylation of p38 and ERK being required for the constitutive expression of anhydrobiotic survival genes. Similarly, perhaps, MAPKs might be dephosphorylated when *P. superbus* is desiccated as expression of these genes is no longer necessary during the dry state. Finally, although these results are consistent with a role for MAPKs in anhydrobiosis, it is important to appreciate that kinase inhibitors are known to have non-specific effects and might also inhibit other kinases not examined. For example, the SB203580 inhibitor is quite specific for p38 but it has been shown to partially inhibit protein kinase B (Davies et al., 2000; Lali et al., 2000), while the JNK inhibitor SP600125 can also reduce the activities of p38 and ATF-2 (Bennett et al., 2001; Davies et al., 2000).

This study represents the first investigation into the role of MAPKs in anhydrobiosis, showing that MAPK phosphorylation patterns change during desiccation and rehydration in nematodes, and that inhibition of p38 and JNK phosphorylation is detrimental for survival during anhydrobiosis in at least one species (*P. superbus*).

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