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Cold tolerance mechanisms of two arthropods from the Andean Range of Central Chile: *Agathemera crassa* (Insecta: Agathemeridae) and *Euathlus condorito* (Arachnida: Theraphosidae)

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ABSTRACT

Two strategies have been described for cold tolerance in arthropods: (1) freeze-tolerant organisms, which can survive the formation of ice crystals and (2) freeze-avoidant organisms, which prevent the ice crystal formation by super cooling their internal fluids. We studied two arthropods from the Andean Range in central Chile (2400 m a.s.l.), the stick insect *Agathemera crassa* commonly named as "Chinchemolle", and the tarantula spider *Euathlus condorito* commonly named as "Araña pollito", in order to evaluate how they respond to low temperatures at the physiological and molecular levels. We sampled the soil temperature during one year to track the temperature changes that these organisms must overcome. We found minimum temperatures around −6 °C in autumn, while the temperature were stable at 0 °C in winter due to the snow. The average field-cooling rate was 0.01 ± 0.006 °C min^{-1}. For both arthropods we determined the super cooling point (SCP) at a cooling rate of 1 °C min^{-1} and its subsequent survival, finding that *A. crassa* is a freezing tolerant organism with a SCP of −3.8 ± 1.8 °C and 100% survival, while *E. condorito* is a freezing avoidant organism with a SCP of −3.0 ± 1.3 °C and 0% survival. The SCP and survival were not affected by the season in which individuals were collected, the SCP was significantly affected by the cooling rate of the experiment. Both species had low molecular weight cryoprotective in their hemolymph that could explain their cold-tolerance behavior. Glucose, glycerol, and trehalose were found in *A. crassa's* hemolymph, only glucose and glycerol were found in *E. condorito's*. We analyzed the hemolymph proteins and found no seasonal differences in composition for either species and also we detected protein antifreeze activity in the hemolymph from both arthropods.

1. Introduction

Arthropods have developed different strategies to survive in temperate, polar regions and high mountains, using several physiological, behavioral and biochemical mechanisms to deal with temperatures that fall below 0 °C (Lee, 2010). Cold can produce damage due to changes in either metabolism, phase transition in membranes, and protein denaturation (Ramløv, 2000). Moreover, ice formation can produce osmotic stress on cells, causing massive water outflow and cell death associated with shrinking (Storey and Storey, 2013).

Cold tolerance refers to the capacity of an organism to survive low temperatures, even lower than the melting point of its body fluids (Lee, 1991). Traditionally, there have been recognized two cold tolerance strategies by which arthropods survive low temperatures, freeze-avoidance and freeze-tolerance. Some species avoid ice formation, by supercooling their corporal fluids below melting point, hence they are commonly recognized as freezing-avoidant animals (Zachariassen, 1985; Catley, 1992; Bale, 1993; Sformo et al., 2011). In contrast, organisms that can survive freezing of their body fluids are known as freezing-tolerant (Salt, 1936; Raml∅v et al., 1992; Bale, 1993; Block et al., 1998; Toxopeus et al., 2016).

The molecular mechanisms that underlie cold tolerance strategies in arthropods have been previously studied (Salt, 1959, 1961; Rudolph and Crowe, 1985; Lee, 1991; Storey, 1997; Duman, 2001; Graether and Sykes, 2004; Storey and Storey, 2013). Two types of cryoprotectant substances have been found in the hemolymph of cold tolerant arthropods: (1) Sugars, polyols and aminoacids (also known as low molecular weight cryoprotectants), which act in a colligative manner by

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lowering the freezing point of the hemolymph or by stabilizing supercooled fluids (Salt, 1959; Ramløv, 1999, 2000). In fact, the multifunctional nonreducing disaccharide trehalose can bind to phospholipids polar groups (Villarreal et al., 2004), stabilizing membranes and protecting them from phase transition or freezing dehydration (Crowe et al., 1984; Rudolph and Crowe, 1985; Storey, 1997). And (2) ice binding-proteins (IBPs), which bind to the ice crystal surface and affect its growth (Davies, 2014). Two groups of IBPs with opposite functions have been described, antifreeze proteins (AFPs) and ice nucleating proteins (INPs) (Zachariassen and Hammel, 1976; Davies, 2014; Duman, 2015). AFPs are capable of inhibiting both ice formation and ice recrystallization by the Kelvin effect while INPs initiate ice crystal growth at a relatively high subzero temperature (Davies, 2014; Duman, 2015).

The cryoprotectants described above can be present in both freeze-avoidant and freeze-tolerant species; nevertheless, their biological function can be different in each case. In freezing avoidant species, low molecular weight cryoprotectants can stabilize membranes and supercooled fluids and AFPs can enhance the supercooling capacity of the body fluid or mask the activity of ice nucleating agents (Duman, 2015). On one hand, in freeze-tolerant species, the ice nucleating agents may start ice nucleation at high subzero temperatures and then AFPs can control the growth and distribution of ice crystals, by means of ice recrystallization inhibition (IRI) activity (Wilson and Ramløv, 1995; Sinclair et al., 1999; Wharton, 2011; Duman, 2015). On the other hand low molecular weight cryoprotectants can stabilize supercooled intracellular fluids and protect membranes from freezing dehydration (Storey and Storey, 2013).

In spite of the knowledge on cold tolerant arthropods, how Andean arthropods survive the low temperatures during winter is still unknown (Sinclair et al., 2003b; Sinclair and Chown, 2005; Wharton, 2011; Dennis et al., 2014). Previously, on winter field observations at Andean Range of central Chile, we found frozen *Agathemera crassa* (Insecta: Agathemeridae) individuals, while in the same location *Euathlus condorito* (Arachnida: Theraphosidae) spiders remained unfrozen. This observation called our attention, and suggested that they have different adaptations to survive the same environmental conditions. For these reasons, we explored the cold tolerance mechanisms of the stick insect *A. crassa* and the tarantula spider *E. condorito*, terrestrial arthropods that inhabit the Andes Range in Central Chile. They are commonly found under rocks between 1900 and 3000 m a.s.l. In this habitat, the climate is dry and warm during summer, with some snow events being possible. During winter, the climate is cold/snowy, with a snow cover of two or three meters depth between June and October (Hoffman et al., 1998). Both species present nocturnal activity and body size dimorphism, with adult females having between 8 and 12 g of body mass and males around 4 g (Camousseight, 1995; Vera et al., 2012; Thienel et al., 2015; Veloso et al., 2012; Perafán and Pérez-Miles, 2014). Since there are few studies relating physiological aspects with the ecology of cold tolerant arthropods (Ramløv, 1999; Sinclair, 1997) we studied the cold tolerance responses for these two organisms and correlated it with the presence of low and high molecular weight cryoprotectant substances in their hemolymphs.

2. Materials and methods

2.1. Specimen collection and habitat characterization

Forty-eight individuals of *A. crassa* and 40 of *E. condorito* were caught under rocks in Farellones (Andean Range of central Chile: 33° 21′ 3″ S; 70° 18′ 50″ W, 2.400 m a.s.l.). Summer specimens were collected in March while winter specimens were collected in late May or late September due to zone inaccessibility during the winter. Animals were divided into two groups, one group of 15 specimens of *A. crassa* (6 from summer and 9 from winter) and 14 of *E. condorito* (5 from summer and 9 from winter) were used for hemolymph extraction. The other 32 specimens of *A. crassa* (9 from summer and 23 from winter) and 26 of *E. condorito* (6 from summer and 20 from winter) were transferred to the laboratory in individual boxes and used for SCP determination. After the experiments animals were released at the same location where they were captured.

The microhabitat temperature from December 2009 to January 2011 was measured in the same area where animals were collected. The temperature was recorded every hour using a data logger ACR Smart Button (± 0.5 °C), which was placed on the soil substrate. All data were downloaded every two months. The field cooling rate was calculated every day that the temperature was lower than 0 °C. We calculated the field cooling rate in the intervals between 3 h before the temperature reached 0 °C and the time when the temperature reached a minimum and began to rise again (Sinclair, 2001).

2.2. Cold tolerance strategy determination

Protocols for cold tolerance strategy determination were designed after reviewing the work of Sinclair et al. (2015). Specifically, the supercooling point (SCP) and freezing survival of the animals were determined. For the SCP determination, animals were fasted for one week and kept at 4 °C. Body mass was measured just before the experiment. We pinned down the animals over a wooden tablet and attached a thermocouple type K to the dorsal thorax. We placed the animals in an in-house designed closed system, with an acrylic tube and two hoses (Fig. 1). The whole system was introduced in a temperature-regulated bath. The system was cooled from 4 °C to −8 °C at rates of 1 °C min^{-1} or 0.5 °C min^{-1} (Sinclair, 2015). The animal temperature was recorded every second for 4.5 h. Subsequently, animals were thawed at room temperature. Additionally, 4 *E. condorito*, spiders were cooled at 0.5 °C min^{-1} taking care that they do not reach the SCP while being cooled, reaching temperatures of −3, −4.4, −5.2 and −5.6 °C, and maintaining these temperatures during one hour. We evaluated the animal survival rate three days after the cooling experiment by observing their feeding and coordinated movement capacity.

Covariate analysis (ANCOVA) of these data was made with the statistic package STATISTICA, Soft Stat 8.0. We adjusted the data to a normal distribution using the Johnson model (Johnson, 1949) with

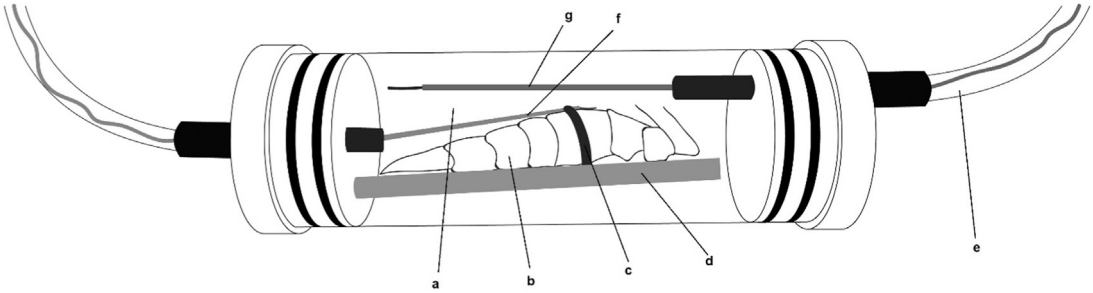

Fig. 1. Design of a closed system to measure the SCP. a) environment; b) animals body; c) elastic band; d) wooden tablet; e) hose; f) animal body thermocouple; g) environmental thermocouple.

statistic package Minitab 17. We used body mass as the covariable. We analyzed seasonal effect and cooling rate as categorical variables over the SCP of each species.

2.3. Hemolymph cryoprotectants characterization

Hemolymph samples were collected immediately after animals were caught. Hemolymph was extracted from each spider's heart (50–200 μL) by cardiac puncture and from each insect trochanters (10–100 μL) by puncture. Samples were centrifuged for 15 min at 4 °C at 14,000 × *g*, and supernatants were stored at −20 °C until use.

Low molecular weight cryoprotectants were detected in the hemolymph. Glucose and trehalose concentrations were determined spectrophotometrically using the Trehalose K-TREH kit by Megazyme International (Bray, Ireland) and glycerol concentrations using the GK (Glucokinase format) K-GCROLGK kit by Megazyme International (Bray, Ireland) following the manufacturers' instructions.

The protein content of the samples was determined using the Bradford assay (Bio-Rad) (Bradford, 1976) with BSA as a standard. Hemolymph proteins were separated by 15% SDS-PAGE and visualized by Coomassie blue staining. We compared the hemolymph SDS-PAGE migration pattern from winter and summer specimens.

Thermal hysteresis (TH) was measured for crude hemolymph samples from both arthropods using an Otago nanolitre osmometer, as described by Wharton et al. (2009) and Ramløv (2010). The melting and freezing points were measured through observing the melting and freezing temperature of single ice crystals using a Zeiss stereomicroscope Stemi 2000-C. The thermal hysteresis value was calculated as the difference between the melting and freezing points. We also considered the ice crystal shape, given that the hexagonal shapes are indicative of binding between AFPs and the ice crystal (Davies, 2014).

The IRI activity of different samples was measured using the gold nanoparticles (AuNPs) method as described by Park et al. (2013) with BSA as the negative control. Briefly, freezing produces AuNPs aggregation (Albert et al., 2009), which could be followed by the change in the solution color from red to blue; however, AFPs inhibits AuNPs aggregation, preventing the color change. Therefore, it is possible to quantify IRI activity (Mitchell et al., 2015) of proteins spectrophotometrically by measuring the extinction spectrum of the protein-AuNPs solution after a freezing-thawing cycle, and calculating the 520/650 nm ratio. To remove low molecular weight cryoprotectants from hemolymph proteins we used gel filtration chromatography (HiTrap Desalting 5 mL column), with nanopure water as the mobile phase at room temperature in an automated chromatography Akta Prime plus system (GE Healthcare biosciences). Fractions containing proteins were collected and pooled. In the case of *E. condorito*, we also prepared a sample enriched in the main protein component (≈70 kDa) by size exclusion chromatography: the hemolymph supernatant was applied to a Tricorn 10/600 column packed with Sephacryl S-300 (GE Healthcare Biosciences) following the fabricant instructions. The column was equilibrated and eluted in buffer 0.1 M Tris HCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.3 M NaCl, pH 7.5 at a flow rate of 0.8 mL/min. Fractions were evaluated by Coomassie Blue Stained SDS-PAGE (Supplementary Information) and the pool of selected fractions was concentrated using an Amicon Ultra-4 centrifugal filter device.

3. Results

3.1. Soil temperature

Soil temperature was recorded for one year. The spring and summer monthly temperature range varied between close to 0 °C to close to 60 °C. In the autumn the temperature began to decrease progressively to reach the lowest observed temperature (around −6 °C), which was recorded in April and May before the winter snow fall. In winter, the soil temperature was constantly close to 0 °C due to the snow cover. (Fig. 2). The average field-cooling rate was 0.01 ± 0.006 °C min^{-1}.

3.2. Cold tolerance strategy

Cold tolerance strategy was determined by measuring the SCP of the spiders and insects collected in summer and winter. The significance level of the comparison among results was calculated using ANCOVA, incorporating the body mass as the covariable. All stick insects survived the freezing protocol, which indicates that *A. crassa* is a freeze-tolerant insect. Conversely, all *E. condorito* individuals died after the freezing protocol. However, when we avoided reaching the SCP in the cooling experiment, we observed that all spiders survived, indicating that the SCP was, in fact, the low lethal temperature. We concluded that *E. condorito* is a freeze-avoidant species. The SCP does not show significant differences between the species, and neither shows seasonal changes between individuals of the same specie (Fig. 3a; Table 1a). The mean SCP was −3.9 °C for *A. crassa* and −3 °C for *E. condorito*. Noteworthy, decreasing the cooling rate significantly affected the SCP, lowering it to −6.1 °C for *E. condorito*, and to −5 °C for *A. crassa* (Fig. 3b; Table 1b). However, when comparing the marginal means corrected for body mass, the cooling rate effect is explained only for *E. condorito*.

3.3. Characterization of hemolymph cryoprotectants

Three potential low molecular weight cryoprotectant substances were quantified: trehalose, glucose, and glycerol. Trehalose could be detected only in the *A. crassa* hemolymph, and it was at a high concentration. Glucose and glycerol were found in both *E. condorito* and *A. crassa* hemolymphs (Table 2).

We also characterized the pattern of protein content of the hemolymph from animals collected in every season. The protein concentration in the hemolymph was 75 ± 33 mg mL^{-1} for *E. condorito* and 51 ± 17 mg mL^{-1} for *A. crassa*, without important changes between seasons. In addition we did not find any seasonal difference for either organism in the SDS-PAGE pattern of hemolymph proteins. In the case of the spiders, we detected various ~70 kDa bands (Fig. 4b), which probably correspond to the subunits of the hetero multimeric hemocyanin as already observed for other tarantula spiders (Trabalon et al., 2010; Burmester, 2013). In the case of the insects, the main protein component is also around 70 kDa, but additional bands are also conspicuous at low molecular weights, which could correspond to AFPs as molecular weights between 3.2 and 32 kDa have been reported for AFPs (Ramløv, 2000; Bar Dolev et al., 2016) (Fig. 4a). In the case of the spider hemolymph, proteins with low molecular weight were not detected.

Protein antifreeze activity was analyzed for both species by using an Otago nanoliter osmometer (TH activity) and by a colorimetric assay based on gold nanoparticles (IRI activity). A low level of thermal hysteresis was observed in both species. The TH value for the hemolymph of *A. crassa* was 0.20 ± 0.05 °C (n = 3) while the value for *E. condorito* was 0.06 ± 0.01 °C (n = 4). Furthermore, we observed a hexagonal shape of ice crystals in both species, which could be indicative of AFPs binding to the ice surface (Fig. 5).

In the case of the colorimetric assay, for samples obtained from both organisms, the solution of AuNPs in the presence of hemolymph proteins kept its red characteristic color before and after the freeze/thaw cycle (Fig. 6a), suggesting that the hemolymph inhibits ice crystallization (Mitchell et al., 2015). The IRI activity of these samples was always higher than that observed for the BSA control. Moreover, the assay was performed with three different protein concentrations (Fig. 6b), showing a dose effect on the antifreeze activity for *A. crassa* hemolymph proteins, but not for *E. condorito*. To determine if the behavior observed for the hemolymph proteins of *E. condorito* could be attributed to its ~70 kDa components, we partially purified them and assayed (Supplementary information), finding the same performance and absence of concentration effect.

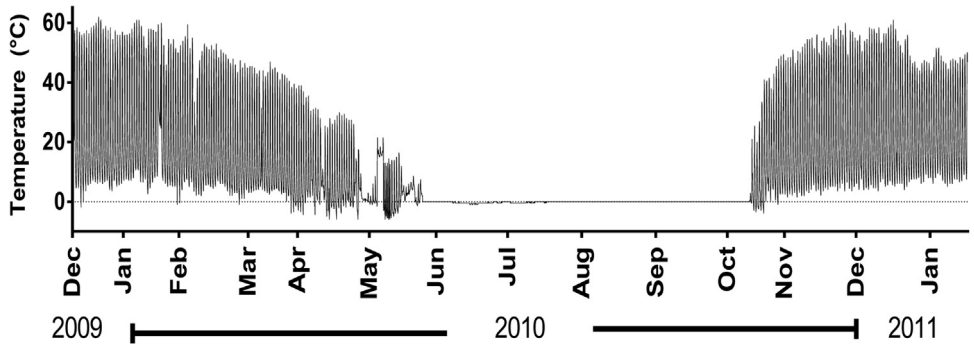

Fig. 2. Temperature recorded on Farellones soil for fourteen months. The coldest days are between April and October and the annual minimum temperatures are −6 °C in April and May.

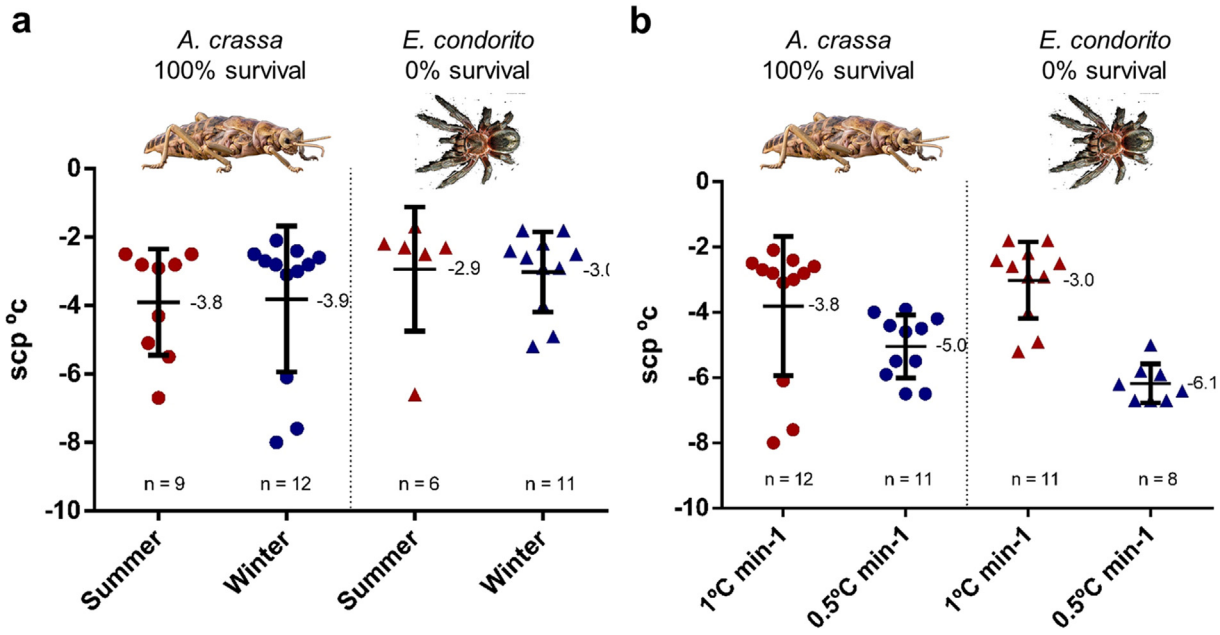

Fig. 3. Supercooling point determination. SCP was determined for both *A. crassa* and *E. condorito*, bars represent the mean and SD. a) The seasonal effect over the SCP at 1 °C min^{-1} of both species. b) Cooling rate effect over the SCP of both species.

Table 1
Two way ANCOVA using organism weight as the covariable. "Model a" corresponds to ANCOVA using species and season as factors and "model b" using species and cooling rate.

Effect	DF	F	P
Model a			
Intercept	1	12.89	0.001*
Species	1	0.83	0.369
Season	1	0.25	0.614
Body mass	1	20.95	< 0.001*
Species x Season	1	0.01	0.891
Error	30		
Model b			
Intercept	1	17.21	< 0.001*
Species	1	1.55	0.21
Cooling rate	1	23.64	< 0.001*
Body mass	1	16.29	< 0.001*
Species x Cooling rate	1	10.57	0.002*
Error	52		

* Significant *p* values < 0.05.

Table 2
Low molecular weight cryoprotective substances in hemolymph samples of *A. crassa* and *E. condorito*. Concentrations were determined for different hemolymph samples from different organisms with 3 technical replicas.

Species	Season	Trehalose (mM)	n	Glucose (mM)	n	Glycerol (mM)	n
A. crassa	Winter	200 ± 65	7*	39 ± 3	7	1.0 ± 0.6	5
	Summer	93 ± 16	8*	41 ± 8	8	1.4 ± 0.8	5
E. condorito	Winter	nd	5	9 ± 1	5*	4.0 ± 1.3	6
	Summer	nd	5	12 ± 1	5*	2.4 ± 1.6	6

The values correspond to concentration ± SD. nd = not detected, i.e. out of the minimum range of detection for this method.

* Means differs significantly by nonparametric Mann-Whitney test.

4. Discussion

Farellones is a representative zone of the Andes Range at 2400 m a.s.l. Soil temperature varies widely over a month, getting to maximum temperatures of around 60 °C and minimum temperatures as cold as 0 °C during the summer season. In winter, heavy snow covers the soil and acts as a temperature buffer, with the temperature being close to 0 °C. These temperature conditions are very similar to those observed in the New Zealand alpine environment, where other cold tolerant phasmid insects have been described (Dennis et al., 2014, 2015). More importantly, in the autumn before the snowfall, the minimum temperatures on the soil can reach −6 °C. This temperature is more than low enough to freeze animal fluids. Therefore, poikilothermic animals that inhabit this region must be able to respond to extremes

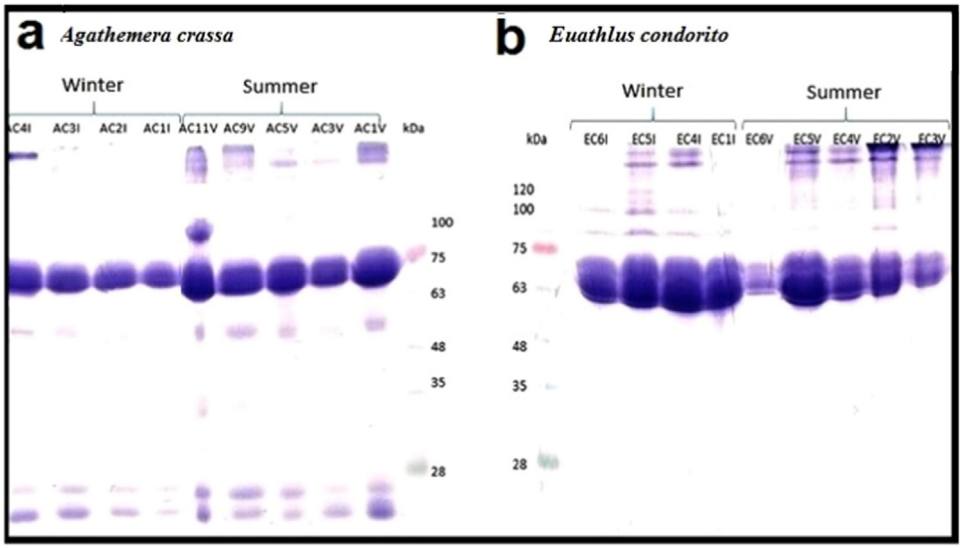

Fig. 4. 15% SDS-PAGE of hemolymph samples from different organisms of a)*A. crassa*and; b)*E. condorito* collected during summer or winter. 20 μg of total protein were loaded in each lane.

climatological conditions in both winter and summer.

We measured freezing temperatures, close to the SCP of both species, at several times in May and June. Both species use two potential alternative strategies to survive low temperatures. *Euathlus condorito* could be considered as a freeze-avoidant organism, because all frozen spiders died and spiders which were cooled tenths of degrees over their SCP survived. In comparison, *A. crassa* can survive moderate freezing (i.e. not less than −10 °C) (Sinclair, 1999). Both species show no seasonal SCP differences, which is expected because the animals must survive subzero temperatures even in summer (Sinclair et al., 2003a). In the animal freezing experiments, our data showed that the cooling rate is a considerable factor for survival, principally for *E. condorito*, which reached lower SCP temperatures when the cooling rate was slower. In the case of *A. crassa* SCP is slightly affected by the cooling rate change. This is reasonable considering the cold tolerance strategies of both species. The field cooling-rate was considerably lower than the experimental rates. This situation can enhance the animal's survival in the field, especially in the spiders case, as maintaining their body fluids in liquid state is critical for survival. Thus, cooling rate is an important factor to consider for understanding the arthropods survival in an ecologically relevant context (Sinclair, 2001).

The high levels of trehalose and glucose detected in *A. crassa* hemolymphs could contribute to explain its cold hardening behavior. With respect to trehalose, concentrations in the stick insect hemolymph varied significantly between winter and summer (Table 2). However, as we did not observe seasonal changes in the SCP, we suspect that trehalose could be associated to membrane protection (Crowe et al., 1984; Rudolph and Crowe, 1985; Storey, 1997). Summer concentrations of trehalose are enough to resist low temperature events that could occur in this season but the concentration rise during autumn when the lowest temperatures are reached and occur more frequently. This is similar to the trehalose concentration rise observed in winter for the cockroach *Celatoblatta quinquemaculata* (Wharton et al., 2009), which tolerates cold over the whole year. This high levels of trehalose can be a threat for the organism survival due to the risk of precipitation, this risk can be overcome by the presence of AFPs which have been reported to inhibit trehalose precipitation in the hemolympm (Wen et al., 2016); however such a role remains to be assessed for the potential AFPs that we are reporting. On the other hand, glucose was detected at concentrations slightly higher to those in *Hemideina thoracica* (Leader and Bedford, 1978), a moderately freeze-tolerant organism (Sinclair et al., 1999). However, the concentration of glycerol found in *A. crassa* hemolymph was lower than that found in other freezing tolerant insects (Wharton, 2011), suggesting that glycerol is not a major factor in cold tolerance in *A. crassa*.

With respect to *E. condorito*, we could not detect trehalose in its hemolymph, however, the concentration of glucose (~12 mM) is considerably higher than the concentration detected in other lowland theraphoside spiders, *e.g. Eurypelma californicum*, at 0.7 mM (Schartau and Leidescher, 1983); *Grammostola rosea*, at 0.97 mM and *Theraphosa*

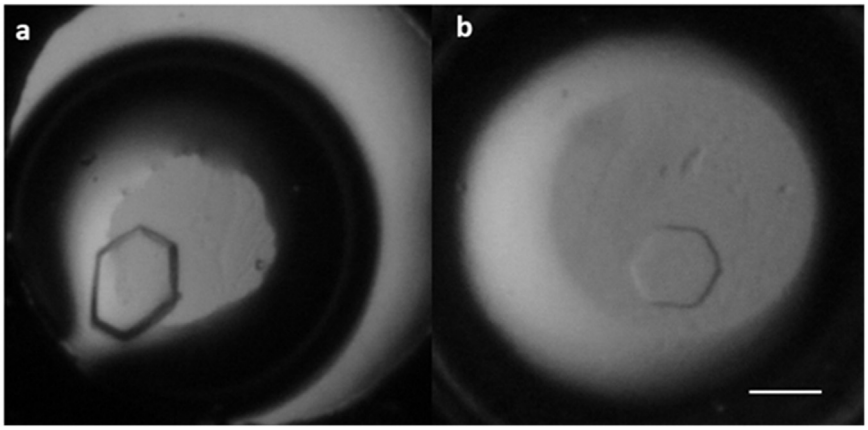

Fig. 5. Hemolymph AFPs. Ice crystal hexagonal shape could indicate presence of hemolymph AFPs binding to ice crystals. a) *E. condorito*. b) *A. crassa*. Scale bar = 100 μm.

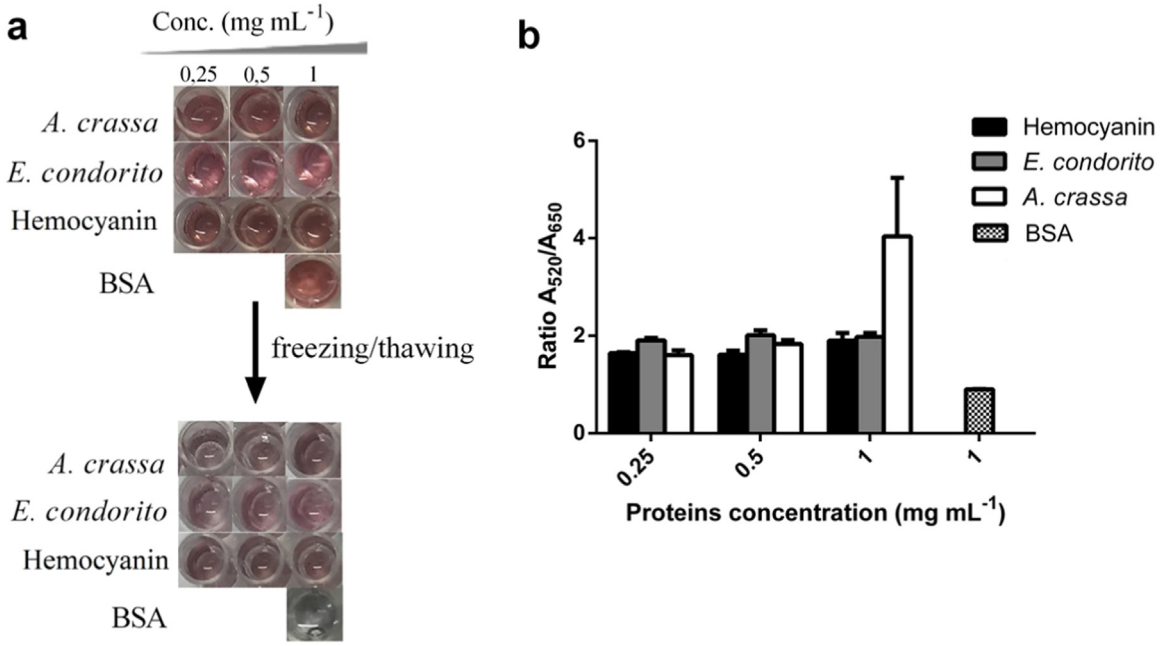

Fig. 6. AuNPs-based colorimetric assay of hemolymph proteins from *A. crassa* and *E. condorito* and *E. condorito* hemocyanin and a) AuNPs assay for hemolymph proteins and hemocyanin before (upper columns) and after (bottom columns) a freeze/thaw cycle; b) Effect of protein concentration on the IRI activity quantified by the extinction ratio (A_{520}/A_{650}). The error bars represent the standard deviation for three independent experiments.

blonda, at 1 mM (Zachariahd et al., 2007). This could, in part explain the supercooling ability of *E. condorito*, unfortunately, cold tolerance has not been studied for any of these spiders, and so a proper comparison cannot be made.

Hemolymph glucose concentrations were different between winter and summer (Table 2); however, this difference cannot be related to the cold exposure response because the cold tolerance strategy and SCP remains the same for the whole year. Thus, an explanation for these changes could be related to a metabolic response to the energetic requirements during summer. Glycerol concentrations were detected at lower levels compared to other freezing avoidant spiders (Duman, 1979), hence it is not probable that the supercooling capacity of this spiders can be explained only by this low molecular weight cryoprotectant.

There are several methods to detect AFP activity, despite each one has advantages and disadvantages (Ramløv, 2010), the most common technique used in the last decades has been to measure TH using a nanoliter osmometer. Also, when TH is too low, AFPs can inhibit normal crystal growth by adsorption to a particular ice planes or sites (Griffith and Yaish, 2004; Ramløv, 2010). If planes remain unprotected, crystals continue growing on these planes and exhibit different ice shapes (Bar-Dolev et al., 2012). Compared with these techniques the colorimetric assay based on gold nanoparticles aggregation by freezing (Park et al., 2013) does not require specialized equipment and it is compatible with 96 well plates for high-throughput studies (Mitchell et al. 2015). Nevertheless, special care must be taken to interpret these results, because the surface functionality of the AuNPs could produce aggregation (Park et al., 2013).

We report here potentials AFPs based in different approaches, we observed hexagonal ice crystal shapes (Fig. 5) probably produced by AFPs binding to the ice crystal surfaces. In addition, we detected low levels of TH activity in crude hemolymph and IRI activity in hemolymph proteins (Fig. 6) from both arthropods. These potential hemolymph AFPs could play important roles in the cold tolerance strategies of both species (Duman, 2001, 2015; Duman et al., 2004; Bar Dolev et al., 2016). For *E. condorito*, hemolymph extracts enriched in 70 kDa proteins showed IRI activity (Fig. 5), suggesting that these proteins could be responsible for the total activity observed. Although the TH observed is low, we reasoned that it in conjunction with glucose and glycerol, it might be sufficient to protect the animal from freezing events, i.e., stabilizing the supercooled state. In *A. crassa*, proteins with IRI activity could control both growth and distribution of ice crystals in its hemolymph (Duman, 2001, 2015; Ramløv et al., 1996; Wharton et al., 2009; Storey and Storey, 2013; Davies, 2014), with the effect of trehalose and glucose explaining the 100% survival during the freeze/thaw cycles.

A hypothesis about the evolution and geographical distribution of the arthropod's cold tolerance strategies has been proposed (Sinclair et al., 2003b). From an evolutionary point of view, the freeze avoidance would be an ancestral adaptation within the arthropod lineage, and the freezing tolerance would have evolved many times for different insect taxa (Vernon and Vannier, 2002; Sinclair et al., 2003b). While the geographic distribution of the cold tolerance strategy is different between organisms in the Northern and Southern hemispheres, the freezing tolerance is more common in the Southern hemisphere (Sinclair et al., 2003b; Sinclair and Chown, 2005; Chawn and Sinclair, 2010). Nevertheless, mortality in Southern hemisphere freeze-tolerant species generally occur below −10 °C, while Northern hemisphere species present mortalities several degrees below −10 °C (Chown and Nicolson, 2004). Our results are consistent with this hypothesis, although we propose that this hypothesis should be reviewed after the incorporation of new data from more South American species. Our findings here contribute to the knowledge of Southern cold tolerant animals, with members of two taxa with different strategies of cold tolerance at both the eco-physiological and molecular levels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtherbio.2018.03.018.

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