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Chronic exposure to soil salinity in terrestrial species: does plasticity and underlying physiology differ among specialized ground-dwelling spiders?

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Abstract

In salt marshes, the alternation of low and high tides entails rapid shifts of submersion and aerial exposure for terrestrial communities. In these intertidal environments, terrestrial species have to deal with an osmotic loss in body water content and an increase in sodium chloride concentration when salt load increases. In salt marshes, spiders represent an abundant arthropod group, whose physiological ecology in response to variations of soil salinity must be further investigated. In this study, we compared the effect of salinity on the survival and physiology of three species of Lycosidae; two salt marsh species (*Arctosa fulvolineata* and *Pardosa purbeckensis*) and one forest species (*P. saltans*). Spiders were individually exposed at three salinity conditions (0‰, 35‰ and 70‰) and survival, changes in body water content, hemolymph ions (Na^+, Ca^{2+}, Mg^{2+}, K^+; ICP-MS technique) and metabolites (mainly amino acids, polyols, sugars; LC and GC techniques) were assessed. The survival of the forest species *P. saltans* was very quickly hampered at moderate and high salinities. In this spider, variations of hemolymph ions and metabolites revealed a quick loss of physiological homeostasis and a rapid salt-induced dehydration of the specimens. Conversely, high survival durations were measured in the two salt-marsh spiders, and more particularly in *A. fulvolineata*. In both *P. purbeckensis* and *A. fulvolineata*, the proportion of Na^+, Ca^{2+}, Mg^{2+}, K^+ remained constant at the three experimental conditions. Accumulation of hemolymph Na^+ and amino acids (mainly glutamine and proline) demonstrated stronger osmoregulatory capacities in these salt-marsh resident spiders. To conclude, even if phylogenetically close (belonging to the same, monophyletic, family), we found different physiological capacities to cope with salt load among the three tested spider species. Nevertheless, physiological responses to salinity were highly consistent with the realised ecological niches of the spiders.

Key-words: Araneae, ions, amino-acids, sugars, polyols, ICP-MS, UPLC, GC-FID

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Introduction

Geographical distribution of species correlates with their ecological needs. In this context, physiological plasticity is an important prerequisite for species experiencing varying and varied abiotic conditions (changes in of drought/wet, cold/warm seasons, oxygen and biotic conditions, pH fluctuations, etc..., Spencer & Gaston 1999). Theoretically, high flexibility should allow thriving in a wider range of habitats. This level of physiological tolerance to abiotic variations is mainly modulated by the ability of organisms to adjust their behaviour, physiology and biochemistry.

In temporary flooded habitats such as salt-marsh, hydrological characteristics (duration, intensity, frequency of drought events) and productivity (nutrient availability) impose physiological constraints to organisms that in turn rule population and species distribution. The alternation of low and high tides entails rapid shifts of submersion and aerial exposure for terrestrial communities. Salt marshes are located between land and marine systems, and their habitats are subjected to regular tides (leading to submergence approximately once per month), which, through their duration, frequency and seasonality, produce variations in soil salinity. In these intertidal environments, species adapted to seawater have to counteract problems of ionic regulation if salt load declines, whereas terrestrial species have to deal with an osmotic loss in body water content and an increase in sodium chloride concentration when salt load increases (Witteveen *et al.* 1987). Together with the duration of the submersion, all of these parameters drive community structure and diversity (Döbel *et al.* 1990) as they impact on many physiological functions of salt-marsh fauna (*e.g*. Levin & Talley 2000).

The way arthropods cope with salinity (both hypo and hyperosmotic stress) include behavioural (Pétillon *et al.* 2009, Fanini *et al.* 2012), morphological (tegument waterproofing: Beament 1961) and physiological (isoconformers: Amphipods; Calosi *et al.* 2005) responses. Two main strategies can be defined to explain the occurrence of terrestrial species within salt marshes: migrate or survive under water (see Tufova & Tuf 2005 for a study on the survival underwater of millipedes, centipedes and isopods). A spatial escape of the environmental stress can be observed, and this strategy is often used for short periods of flooding at high tide (Foster & Treherne 1975, Wyatt 1986, Hadley 1994, Pétillon *et al.* 2010a). The non-escaping species that stay underwater have evolved adaptations enabling organisms to maintain activity (either metabolic, reproductive and for insects, locomotion)

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during the unfavourable period (Foster 2000), *i.e.* elevation of soil salinity after flooding events. Indeed, in daily flooded salt marshes, a tidal rhythm of activity is less optimal (Foster 1983, Witteveen & Joose 1988). The tolerance strategy is often restricted to highly adapted species. The incurred ecological costs associated with tolerance often narrow their distributions to few niches, such as salt marshes (*i.e.* they should only be found in the habitats exhibiting those specific conditions as a result of the ability of the individual to modify their traits to maximise performance and fitness in this environment). Reduced metabolic rate, structure of the epicuticle (responsible of hygrophobic properties) and elevation of hemolymph osmolality are elements of the arsenal strategies employed by arthropods to combat osmotic stress.

Some studies on Annelids, Crustaceans and Bivalves demonstrated that variations in salinity modify body water content, ionic and osmotic regulation (Moore & Francis 1985, Sowers *et al.*, 2006). Synthesis of compatible solutes is frequently observed in species exposed to environmental constraints, including osmotic stress. Salinity causes changes in polyols (especially glycerol, sorbitol, myo-inositol), sugars (trehalose) and some amino acids (as proline or alanine) (Edwards 1982, Misra & Gupta 2005).

In the available literature, the ways terrestrial arthropods, and among them spiders, a dominant group of predators in intertidal habitats (*e.g.* Pétillon *et al.* 2005), cope with soil salinity are poorly documented (Foster & Treherne 1976, Foster 2000; but see Heydemann 1970 for *Pardosa purbeckensis*; Bethge 1973 for *Erigone longipalpis* and Foucreau *et al.* 2012 for *Arctosa fulvolineata*). In the present work, we compared the effect of soil salinity among three ground-dwelling spider species (Lycosidae), of which two are qualified as salt-marsh "resident" species, *Arctosa fulvolineata* and *Pardosa purbeckensis*, whereas the third one, *P. saltans*, is a forest species (and likely not adapted to salinity exposures). Because the realised niche of these species should be correlated with their ecophysiological capabilities (*e.g.* for ground-dwelling spiders: DeVito *et al.* 2004, Frick *et al.* 2007), we first hypothesised survival patterns depending on soil salinity (here, 0‰, 35‰ and 70‰) to differ among these three species; increasing and decreasing survivals along increasing salinities should be observed for salt-marsh and forest species, respectively. We assumed that body water loss would follow survival patterns. Lastly, we expected synthesis of inorganic and organic osmo-compatible solutes to increase with salinity and duration of experiment, but to remain qualitatively similar among species as the three species we studied are phylogenetically

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close (belonging to the same, monophyletic, family: Murphy *et al.* 2006). This last hypothesis was tested in an exploratory way by measuring changes in ions (by ICP-MS), amino-acid, sugar and polyol levels (by GC-FID) over time and along increasing soil salinities.

2. Material and Methods

2.1 Collection sites

Experiments were carried out from April to June 2008 using hand-collected spiders: adult females of *Pardosa saltans* were collected under dead wood and from the litter of a deciduous forest (Paimpont, France, 48°1'0N, 2°10'60O); adult females of *Pardosa purbeckensis* and *Arctosa fulvolineata* (Araneae: Lycosidae) were sampled in an intertidal salt marsh (Le Vivier sur Mer, Mont St Michel Bay, France, 48°40'N, 1°40'O). After collection, spiders were kept for one day under controlled conditions at constant 20 ± 1 °C before being used for experiments. Only females were used in the present study; they exhibit higher survival durations to saline conditions over males (DeVito & Formanowicz 2003, Pétillon *et al.* 2011), likely because of their higher energetic reserves (Moya-Laraño *et al.* 2008).

2.2 Experimental design

2.2.1 Salinity conditions

Female spiders were individually placed into hermetic plastic boxes (6 cm diameter, 7 cm depth, 125 mL), half filled with sand. Three distinct experimental conditions were performed: *(i)* null salinity (sand and mineral water considered as being non-salted, 0‰ salt), medium salinity (sand [*ca.* 33 cm^3] and mineral water-35‰ salt [added until saturation of sand]), and high salinity (sand [*ca.* 33 cm^3] and mineral water-70‰ salt [added until saturation of sand]). The hypersaline condition corresponds to the highest soil salinity the spiders can endure in the monthly-flooded portions of the salt marsh (Pétillon *et al.* 2010b). Commercial marine salt 'Sel de Guérande' was used for setting the desired salinity. For each species, spiders were individually held into plastic boxes at the three saline conditions (L/D: 16/8, temperature: 20 ± 1 °C, Relative humidity (RH): 78 ± 5%). Spiders were food deprived during the whole experiment to standardize the trophic status of the specimens. Soil salinity and hygrometry were monitored with a W.E.T. sensor (5cm depth; specific sand soil calibration) connected to a moisture meter HH2 (both by Delta-T Devices Ltd., Cambridge,

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UK). These parameters did not vary significantly over time till the end of the experiment (see data from Pétillon *et al.* 2011).

2.2.2 Survival experiments and body water content

Salinity tolerance of *A. fulvolineata*, *P. saltans* and *P. purbeckensis* was assessed at the three saline conditions (0, 35 or 70 ‰). Survival of spiders was monitored every day until the death of all spiders (*N* = 15 females / species / experimental condition). When assessing the survivorship, females that were not reactive to mechanical stimulation with paintbrush were considered as dead. Mortality was further confirmed by transferring immobile spiders to control conditions (null salinity and access to trophic resources); spiders were reassessed for survival 24h later.

In a second experiment, changes in body water content was monitored in the three spider species exposed at the three saline conditions for 0, 3 and 7 days (*P. saltans*) or for 0, 7 and 14 days (*A. fulvolineata* and *P. purbeckensis*). Survival data were considered for the selection of these durations of exposure (maximum mortality was about 30% for these durations, except for *P. saltans* kept at 70‰ for 7 days whose mortality reached 40-50%). For each experimental condition, water mass (mg) was measured on 15 spiders immediately after collection (fresh mass; Sartorius A200S, d = 0.1 mg). Then, females were plunged into liquid nitrogen before being freeze-dried and rapidly reweighed before being stored at - 20 °C. Water mass was calculated from these data. Samples exposed for 7 (*P. saltans*) or 14 (*P. purbeckensis* and *A. fulvolineata*) days were further used for metabolic profiling.

2.2.3 Ion concentration

Samples of hemolymph were collected from alive spiders maintained at 0, 35 or 70‰ salinities for 0 and 7 days (*Pardosa saltans*) or for 0 and 14 days (*A. fulvolineata* and *P. purbeckensis*). Four legs of each spider were cut off, and they were immediately placed into 0.2 mL PCR tubes and centrifuged at 1 000 *g* for 30 seconds. One biological replicate consisted of the hemolymph gathered from 10 individuals (N = 2 biological replicate per experimental condition). The total hemolymph volume of each biological replicate was measured with a calibrated microcapillary tube, and samples were vacuum-dried (Speedvac™) to obtain their dry masses. A 200 µL volume of nitric acid was added to each biological replicate, followed by an adding of two droplets of hydrogen peroxide (destruction

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of organic matter and stabilisation of samples for subsequent measurements). This solution was then diluted in 10 mL of ultrapure water containing 10 ppb of indium (internal standard). Concentrations of four metal ions, Na^+, K^+, Mg^{2+} and Ca^{2+} were determined by ICP-MS (Agilent Technologies HP4500), with normal plasma conditions, as described in Gaillou *et al.* (2008). Quantitative analyses were performed using a conventional external calibration procedure (Bouhnik-Le Coz *et al.* 2001). Calibration curves were calculated from intensity ratios of the internal standard and the analysed elements.

2.3 Metabolic profiles

2.3.1 Sample preparation

For each experimental condition, five replicates, each consisting of three randomly collected female spiders, were prepared. The samples were resuspended in 1000 µL of a methanol: chloroform (2:1, v:v) solution and homogenised with two tungsten beads (Ø: 3 mm) for 2 min at 30 Hz (Retsch™ MM301 Retsch GbmH, Haan, Germany). Then, 666 µL of ultrapure water was added, and the samples were vortexed, and centrifuged at 4 000 *g* for 10 min at 4 °C. One aliquot (700 µL) of the upper phase was transferred to clean microtubes and vacuum-dried (Speed Vac Concentrator, MiVac, Genevac Ltd., Ipswich, England). Finally, 500 µL of ultrapure water was added to each sample and they were stored to -80 °C until analyses.

2.3.2 Amino acid analysis

Amino acid analysis was adapted from Renault *et al.* (2010). Briefly, aliquots (10 µL) of the crude aqueous extract were used for amino acids' derivatisation according to the AccQ•Tag Ultra Derivatisation Kit protocol (Waters Corporation, Milford, MA). Amino acids were analysed using an Acquity UPLC®533 system (Waters Corporation, Milford, MA) by injecting 1 µL of the derivatisation mix onto an Acquity UPLC®534 BEH C18 1.7 µm 2.1 x 100 mm column heated at 55 °C. External standards were run and used for the drawing of calibration curves allowing the quantification of metabolites from the samples.

2.3.3 Sugar and organic acid analysis

Sugars and organic acids were quantified with the method described by Hidalgo *et al.* (2013). Briefly, a Trace GC Ultra chromatograph with flame ionization as the detector (GC-FID, Thermo Fischer Scientific Inc., Waltham, MA, USA) was used. The injector temperature was

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held at 260 °C. The oven temperature ranged from 70 to 147 °C at 9 °C min-1, from 147 to 158 °C at 0.5 °C min-1, from 158 to 300 °C at 5.0 °C min-1, and remained for 3 min at 300 °C. A 30 m fused silica column (DB-5, 30 m x 0.32 mm x 0.25 µm, 5%-Phenyl-methylpolysiloxane, 95% dimethylpolysiloxane) was used, with helium as the carrier gas at a rate of 1 mL.min^{-1}. For each sample, 100 µL of the crude aqueous extract was vacuum-dried, and dry residues were redissolved in 50 µL of methoxyamine hydrochloride solution in pyridine (20 mg/mL). Samples were agitated for 90 min at 30 °C, 50 µL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma) were added, and derivatisation was conducted at 37 °C for 30 min under agitation. One microliter of each sample was then injected to the gas chromatograph. Chromatograms were deconvoluted using ChemStation v2.0.7, and metabolite levels were quantified using arabinose as internal standard and by comparison with individual external standards.

2.4 Statistical analysis

All data are given as means ± SE. Prior to analysis, normality and homogeneity of variances were checked.

Time-mortality regression equations and hours to Lt_{50} along with both 95 and 99% confidence limits were calculated by probit analysis (Minitab Statistical Software Release 13, Minitab Inc, State College, PA). Confidence limits were used to compare spider survival among experimental conditions: lethal times were significantly different ($P < 0.05$) when the fiducial limits of the two values did not overlap.

The effect of salinity on body water content (ratio [fresh mass – dry mass] / dry mass) over the course of the experiment was investigated with two ways ANOVA, with duration of exposure and salinity treatment as the independent variables, and water content as the dependent variable. *P* values were adjusted for multiple comparisons with Bonferroni correction. Concentrations in mineral elements were the mean of two replicate samples. For each species, variations of the proportions of the four ions among the three experimental conditions was assessed with χ^2 tests.

Concentrations of all metabolites were log-transformed. Effects of species and saline conditions on the metabolic profiles of female spiders were investigated using Partial-Least Squares Discriminant Analysis (PLS-DA). Multiple correlation coefficient (R^2) and cross-validated R^2 (Q^2) were used to confirm the predictive power of the fitted model. The

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statistical significance of the PLS-DA was also assessed with permutation tests (1000 permutations, $P < 0.001$). Variable Importance in Projection (VIP) scores, which are the weighed sums of squares of the PLS loadings, were obtained from the PLS-DA. Analyses of variance were performed on all the metabolites to search for a significant effect of salinity level on their concentrations. Together with the multivariate analysis, one-way ANOVAs were performed for each detected metabolite, with experimental condition (salinity) as explanatory variables. ANOVAs were followed, when required, by Tukey HSD *post-hoc* procedures among levels of significant factors. The statistical *P*-values were adjusted using the two-stage Benjamini-Hochberg algorithm to control for false discovery rate (α=0.05). These analyses were conducted using the statistical software of 'R 2.13.0' (R Development Core 2008) and MetaboAnalyst (Xia *et al.* 2012).

3. Results

3.1 Survival

Survival of *P. saltans* was highly reduced with increased salinity levels, with Lt_{50} decreasing from *ca.* 40 to 10 to 7 days at 0, 35 and 70‰ respectively (Fig. 1). The duration of survival of *P. purbeckensis* was the lowest of the three species at 0‰ ($P < 0.05$), but these spiders highly significantly outcompete *P. saltans* for survival capacity at 35 and 70‰. Finally, females of *A. fulvolineata* were characterised by the highest duration of survival, whatever the saline condition (Fig. 1).

3.2 Water content

Body water content was significantly affected by salinity in the three spider species (Table S1, Figure 2). In both *P. saltans* and *P. purbeckensis*, water content was the lowest at the highest salinity when duration of exposure lasted 7 and 14 days, respectively. Water content was the highest at the null salinity in *A. fulvolineata* (Table S1, Figure 2).

3.3 Ions

Initial ion concentration in hemolymph, measured after the females were exposed to control condition (20 °C, null salinity) for one day, is presented in table 1. Concentration of ions in hemolymph varied with saline exposure over the course of the experiment in the three

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spider species (Fig. 3). Overall, the proportion of the four ions varied according to the saline conditions in *P. saltans* ($\chi^2 = 8.5$, $P = 0.20$) whereas it remained equivalent in *P. purbeckensis* ($\chi^2 = 92.1$, $P < 0.001$) and *A. fulvolineata* ($\chi^2 = 53.5$, $P < 0.001$). Levels of Na^+ were augmented in the three species at 35 and 70‰, as compared with 0‰-exposed spiders, with the most striking Na^+ elevation being recorded in *P. saltans* (4-fold increase of Na^+ concentration in between 0 and 35‰). In this species, concentrations of the three other ions (Mg^{2+}, K^+ and Ca^{2+}) were also increased when salt concentration rose (Fig. 3); total hemolymph ions highly rose with salinity. For *P. purbeckensis*; total hemolymph ions was similar at 0 and 35‰ and slightly (not statistically significant) decreased at 70‰ (Fig. 3). Finally, In *A. fulvolineata*, both Ca^{2+} and Mg^{2+} had nonsignificant elevated concentrations with increasing saline conditions, resulting in total hemolymph ions higher at 35 and 70‰.

3.4 Metabolic profiles

A set of 34 metabolites was identified in the spiders with our chromatographic procedure, including amino acids, carboxylic acids, cyclitols, polyamines, polyhydroxy acids, polyols and sugars. PLS-DA containing all experimental samples revealed the existence of significant differences among the groups (Permutation tests; $P < 0.001$, 0/1000). Thus, in the subsequent analyses, the effect of salinity on metabolite concentration was assessed at the species level.

The PLS-DA depicting the class separation by the 34 discriminant variables in *P. saltans* is presented in figure 4. In this species, the first (LD1) and second (LD2) Linear Discriminant axes accounted for 47.8 and 10.1 % of the total inertia (the inertia is the total variance of a dataset), respectively. The amounts of 11 metabolites, of which α-alanine, myo-inositol, proline, serine, succinic acid and tryptophane can be mentioned, varied with increased salinity level (Fig. 4, Table S2). LD1 separated females maintained at null salinity from females acclimated at 35 and 70 ‰, females from these two latest groups had overlapping metabolic profiles (Fig. 4).

For *P. purbeckensis*, LD1 and LD2 accounted for 28.7 and 46.5 % of total inertia, respectively (Fig. 5). Two main groups were observed: spiders exposed at 0 ‰ on the one hand, and spiders exposed at 35 and 70 ‰ salinities, whose metabolic profiles overlapped, on the other hand. Increasing concentrations of eight metabolites, including aspartate,

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glutamine, malate and proline, were measured from female *P. purbeckensis* exposed to augmented salinity levels (Fig. 5, Table S2). Conversely, two metabolites were characterised by decreased amounts at higher salinities (lysine and serine).

Variations of the 34 metabolites across the saline conditions are presented in Figure 6 for female *Arctosa fulvolineata*. The first (LD1) and second (LD2) Linear Discriminant axes accounted for 36.3 and 13.3% of total inertia, respectively. For this species, the three experimental groups were separated on LD1 according to increasing salinity levels. The VIP scores showed that separation on LD1 was mainly due to increasing levels of 12 metabolites. Eight metabolites, including asparagine, glutamine and proline had augmented amounts with elevated salinity amount rose, whereas four metabolites, including lysine, had decreased amounts (Fig. 6, Table S2).

4. Discussion

In the present work, we compared the physiological responses to salinity of three ground-dwelling spiders, *P. saltans*, *P. purbeckensis*, and *A. fulvolineata*, thriving in distinct habitats. Even if phylogenetically close, the three tested spider species exhibited different physiological capacities to cope with salt load, and physiological responses to salinity were highly consistent with their realised ecological niches.

Survival

Survival of the forest spider was quickly hampered at medium and high salinities, which is consistent with our previous experiment on other species from the *Pardosa* genus (Pétillon *et al.* 2011). *Pardosa saltans* is mostly restricted to the wet litter layer of deciduous forests (*e.g.* Hendrickx *et al.* 2001), where salinity is highly likely low. This finding also suggests that the ability to cope with saline exposure requires the development of specific physiological and metabolic strategies in this species (see Bradley 2008, for a synthesis on the evolution of salinity tolerance in mosquitoes). Consistently, both salt-marsh inhabiting species *(P. purbeckensis* and *A. fulvolineata*) greatly outcompeted *P. saltans* in terms of salinity tolerance. The highest capability to cope with salinity was measured in *A. fulvolineata*, with half of the individuals being able to survive prolonged exposures (up to 36 days) at salinity levels twice higher than that of the sea. This spider thrives in humid sediments, in the first four centimeters of the ground, where salt likely accumulates and

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necessitates enhanced osmoregulatory capacities. Finally, survival remained higher at 0‰ in these two salt-marsh species. This finding delineates the physiological costs required for combatting salt load and underscores that salt is not a prerequisite for the survival of *A. fulvolineata* and *P. purbeckensis*. Thus, biotic interactions, rather than salinity, likely represent an important driver of the distribution of terrestrial spiders in saline habitats (Carbonell *et al.* 2012).

Water and ion contents

Arthropods facing hypertonic environments are usually prone to dehydration. The decreased body water content of the two ground-dwelling spiders (*Pardosa* sp.) with increasing salinity is congruent with this assumption. Interestingly, the highest amount of body water lost was measured in the resident salt-marsh spider *P. purbeckensis* exposed at 70 ‰. Moloney & Nicolson (1984) showed that loss of water the middle shore living spider *Desis formidabilis* was higher than that of *Amaurobioides africanus*, which lives at higher elevations in the shore. We did not report such a difference in the spatial distribution of *A. fulvolineata* and *P. purbeckensis*, and, alternatively differential respiration and excretion losses (Walter *et al.* 2012) could explain the distinct body water patterns between these two salt-marsh spider pecies.

Inter-specific variations in the amount of body water lost have already been measured in spiders (likely explained by active secretion of epidermal cells: Davies & Edney 1952). In spiders, water may be mainly lost from hemolymph (which represents almost 32% of female fresh mass in lycosid species: Carrel 1990). This assumption is congruent with the conclusions drawn from other taxa like amphipods (Moore & Francis 1985) and isopods (Holdrich & Mayes 1976).

Differences in the ionic composition of hemolymph was observed among the three spider species. Such qualitative and quantitative differences are consistent with the primary literature, with ionic composition varying according to the feeding preferences of the specimens (Djamgoz 1987, Zeiske 1992). Body water content and, depending on spider species and experimental saline conditions, concentration of metal ions (Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}) were differentially altered among species. High variations of ion levels were observed in *P. saltans*, with maximum ionic concentrations recorded in specimens exposed at 35‰. Together with the survival results, this pattern strongly suggests a dysregulation of ion

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regulation (Yancey 2005), as soon as this species is subjected to elevated salt load. In turn, this results in a quick loss of physiological homeostasis and a progressive salt-induced dehydration of *P. saltans* females, which may have provoked DNA breaks, protein denaturation, and uncontrolled molecular interactions (Benoit *et al.* 2009, Gusev *et al.* 2010). The increased [K^+] with salinity in *P. saltans* also supports this assumption, as augmentation of [K^+] has been correlated to accumulation of injuries, and ultimately mortality, in cold-exposed insects (Koštál *et al.* 2007). In this work, we gathered evidence of the limited ability of *P. saltans* to cope with salinity, thus partially explaining its distribution restricted to forest habitats.

A different story emerges for the two salt-marsh spiders, whose proportions of the four ions were not altered by salinity treatments. The high survival duration, decreased body water content of *A. fulvolineata* from 0 to 70‰ salinities, and concomitant increase in [Na^+], probably depict osmoregulatory adjustments. Sodium has been reported as one of the most osmotic active constituents in hemolymph of insects (Ramsey 1950, Edney 1977) and spiders (Nentwig 1987, Moloney & Nicolson 1984) facing saline stress. Tracking variations in osmotic pressure of the environment requires in-time adjustments of osmolarity of the body fluids, a mechanism that is typical from osmoconformer species. Conversely, females of *P. purbeckensis* rather behave as osmoregulators, with global ionic concentrations maintained constant at salinities of 0 and 35‰. At the highest salinity, the accumulation of organic solutes may have contributed to augment hemolymph osmolality, thus necessitating a reduction [Na^+] in the supposed osmoregulator *P. purbeckensis*. Heydemann (1970) previously showed a better regulation of Na^+ levels in *P. purbeckensis* as compared to the eurytopic *P. amentata*

Metabolites

The amassing of several metabolites has been assumed to aid species to overcome saline stress (Patrick & Bradley 2000, Foucreau *et al.* 2012, Hidalgo *et al.* 2013). Among the different classes of organic compounds, amino acids are generally efficient contributors for the regulation of cell volume (Yancey 2005). In spiders, adaptations to osmotic stress is achieved through the production of osmo-protectants, or by the selection of preys containing particular amino acids and/or sugars have been reported for spiders (see Heydemann 1970 for *Pardosa purbeckensis*; Bethge 1973 for *Erigone longipalpis*). Overall,

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our data are consistent with the literature: accumulation of amino acids, which were generally measured in higher amounts in *P. purbeckensis* and *A. fulvolineata* as compared to *P. saltans*, likely played important osmoregulatory role during saline exposure. Specifically, asparagine, aspartic acid, glutamine, proline, and serine were all increased with increasing salinity in the three spider species in this study.

Asparagine and alanine can be synthesised intensively in organisms in response to salt stress (Witteveen *et al.* 1987). Alanine represents a common feature in several salt-exposed organisms (Patrick & Bradley 2000; Renault *et al.* 2014), including spiders (Foucreau *et al.* 2012). Alanine, only accumulated significantly in *P. saltans* and *P. purbeckensis*, is an important contributor of intracellular osmoregulatory processes (Holmstrup & Bayley 2013). In this line, the large concentrations of aspartic acid in the two *Pardosa* spiders exposed to saline conditions should be underscored. Indeed, this accumulation is consistent with the alanine-oriented synthesis in salt-exposed spiders, as a quick additional build-up of alanine can be achieved through intense decarboxylation of aspartic acid (Kasschau *et al.* 1984). Alternatively, in *P. saltans* particularly, it cannot be excluded that the increasing amounts of aspartic acid could result from the detrimental effects of NaCl on enzyme activities, as hypersaline conditions affect the activity aspartate transaminase (Pollard & Wyn Jones 1979)

Proline is often accumulated at high levels in halophyte plants, bacteria or insects subjected to salt load (Misra & Gupta 2005). The physicochemical characteristics of this amino acid make it an excellent osmolyte, and it acts as regulator of the osmotic pressure at the cellular level during salt stress (Stewart & Lee 1974). Interestingly, proline concentrations measured from *P. purbeckensis* exposed at salinities of 35 and 70‰ were three times higher than in the two other species. Such an elevation in the concentration of this amino acid highly likely contributed to augmenting hemolymph osmolality, which is consistent with the above-discussed variations of [Na^+] in *P. purbeckensis*.

Amounts of circulating glutamine can be high in non-stressed arthropods (Evans & Crossley 1974). This metabolite plays a prominent role in the control of cell volume, especially in shrunk cells (Fumarola *et al.* 2001), and also stimulates fluid secretion (Hazel *et al.* 2003). The increased amounts of this amino acid suggest its possible osmoregulatory role in the three spider species over the salinity gradient.

Interestingly, sugars varied little in the present work, but we cannot rule out their involvement in coping with saline exposure. Indeed, their energetic importance for fuelling

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the metabolism together with food deprivation imposed to the spiders may have masked their possible role in balancing osmotic stress

Conclusion

The two salt-marsh species *P. purbeckensis* and *A. fulvolineata* exhibited strong abilities to deal with salty environments as compared with the forest species, *P. saltans*. This finding is consistent with the realised ecological niches of these spiders. Also, we demonstrated that both *P. purbeckensis* and *A. fulvolineata* are not strict halophilic spiders. Their salt marsh restricted distribution is permitted by enhanced osmoregulatory capacities, either by osmoregulation (*P. purbeckensis*) or osmoconformation (*A. fulvolineata*). Meanwhile, these spiders were also characterised by strong abilities to live in unsalted situations. It should be evaluated in future studies if their adaptations allowing thriving in salt-marsh is associated with poorer competitiveness, explaining their habitat distribution restricted to salt marshes. Finally, the well-being of spiders exposed to saline gradients could be evaluated in future studies. For instance, the variations of HSC70 expression could be monitored, as these proteins have been suggested as a relevant molecular indicator for that purpose in a carabid beetle exposed to saline conditions (Siaussat *et al.* 2013).

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Figure captions

Figure 1. Mean survival duration (Lt_{50}: mean ± SE, days) of *Pardosa saltans*, *P. purbeckensis* and *Arctosa fulvolineata* females along the three salinity levels (probit analysis: different successive letters indicate significant differences at $\alpha = 0.05$).

Figure 2. Box plot of the body water content ([final fresh mass – final dry mass] / final dry mass) according to salinity level in female (a) *Pardosa saltans*, (b) *Pardosa purbeckensis* and (c) *Arctosa fulvolineata*. Boxes represent upper and lower quartiles; the body water content median is represented by the black line within each box. Whiskers indicate 90% percentile values, open circles represent outlier values.

Figure 3. Hemolymph concentration of Na^+, Mg^{2+}, K^+ and Ca^{2+} ions (pmol ion /mg of dry hemolymph mass) in spiders exposed at salinities of 0, 35 or 70 ‰ for one (*Pardosa saltans*) or two (*Pardosa purbeckensis* and *Arctosa fulvolineata*) weeks. Due to the small number of replicates for each experimental condition, errors are given as absolute uncertainty for each ion concentration.

Figure 4. Multivariate analysis (PLS-DA) on metabolomics data. **(A)** Sample projection (5 samples per experimental group) onto the first PLS-DA discriminant plane of female *Pardosa saltans* exposed at salinities of 0 (red dots), 35 (green dots) or 70 (bleu dots) ‰ for two weeks. Each dot represents an individual sample (one female). Ellipses represent the 95% confidence region for each experimental group, *i.e.* the region that contains 95% of all samples. Permutation tests (N = 1000) validated the model ($P < 0.01$). **(B)** Variable importance plot showing the metabolites that contributed the most to the first axis (based on their VIP scores). Variables with VIP scores > 1.3 were retained in the full model (all metabolites), which resulted in VIP scores > 0.8 in the reduced model (non-varying metabolites were removed). The colored squares, ranging from green to red, on the right represent the relative metabolite amount in the samples; green-colored squares indicate low metabolite amount, a red color indicates a high metabolite amount, and a yellow-colored square reflects an intermediate amount.

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Figure 5. Multivariate analysis (PLS-DA) on metabolomics data. **(A)** Sample projection (5 samples per experimental group) onto the first PLS-DA discriminant plane of female *Pardosa purbeckensis* exposed at salinities of 0 (red dots), 35 (green dots) or 70 (bleu dots) ‰ for two weeks. Each dot represents an individual sample (one female). Ellipses represent the 95% confidence region for each experimental group, *i.e.* the region that contains 95% of all samples. Permutation tests (N = 1000) validated the model (P < 0.01). **(B)** Variable importance plot showing the metabolites that contributed the most to the first axis (based on their VIP scores). Variables with VIP scores > 1.3 were retained in the full model, which resulted in VIP scores > 0.8 in the reduced model. The colored squares, ranging from green to red, on the right represent the relative metabolite amount in the samples; green-colored squares indicate low metabolite amount, a red color indicates a high metabolite amount, and a yellow-colored square reflects an intermediate amount.

Figure 6. Multivariate analysis (PLS-DA) on metabolomics data. **(A)** Sample projection (5 samples per experimental group) onto the first PLS-DA discriminant plane of female *Arctosa fulvolineata* exposed at salinities of 0 (red dots), 35 (green dots) or 70 (bleu dots) ‰ for two weeks. Each dot represents an individual sample (one female). Ellipses represent the 95% confidence region for each experimental group, *i.e.* the region that contains 95% of all samples. Permutation tests (N = 1000) validated the model (P < 0.01). **(B)** Variable importance plot showing the metabolites that contributed the most to the first axis (based on their VIP scores). Variables with VIP scores > 1.3 were retained in the full model, which resulted in VIP scores > 0.8 in the reduced model. The colored squares, ranging from green to red, on the right represent the relative metabolite amount in the samples; green-colored squares indicate low metabolite amount, a red color indicates a high metabolite amount, and a yellow-colored square reflects an intermediate amount.

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Table 1. Mean (± 3 % confidence limit) hemolymph concentrations (pmole ion /mg of dry hemolymph mass) of sodium; magnesium and potassium, [Na^+], [Mg^{2+}] and [K^+], respectively, in *Pardosa saltans, Pardosa purbeckensis* and *Arctosa fulvolineata*) after one day acclimation at null salinity at 20°C.

Species	[Na^+] pmole /mg	[Mg^{2+}] pmole/mg	[K^+] pmole/mg
P. saltans	984 ± 29	113 ± 3	64 ± 2
P. purbeckensis	2203 ± 66	26 ± 1	123 ± 4
A. fulvolineata	1918 ± 57	18 ± 1	104 ± 3

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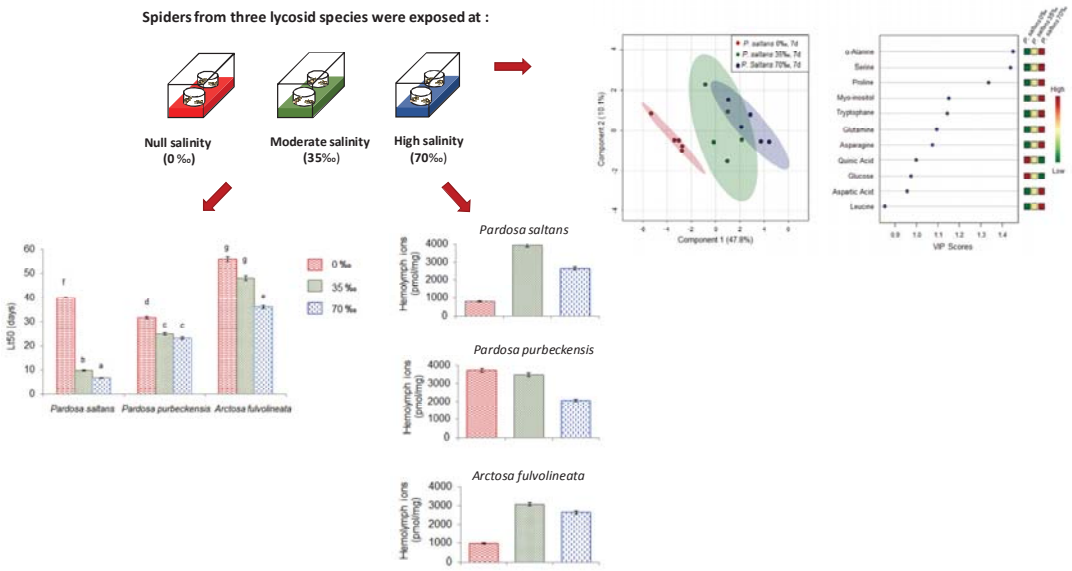

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- The effect of salinity on the survival and physiology of three lycosids was compared
- Spiders were individually exposed at three salinity conditions (0‰, 35‰ and 70‰)
- Survival, body water content, hemolymph ions and metabolites were measured
- Survival of the forest species was quickly hampered at moderate and high salinities
- Accumulation of hemolymph Na^+ and amino acids enhanced survival of salt marsh spiders

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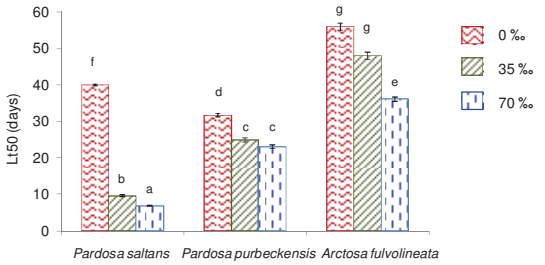

Fig. 1

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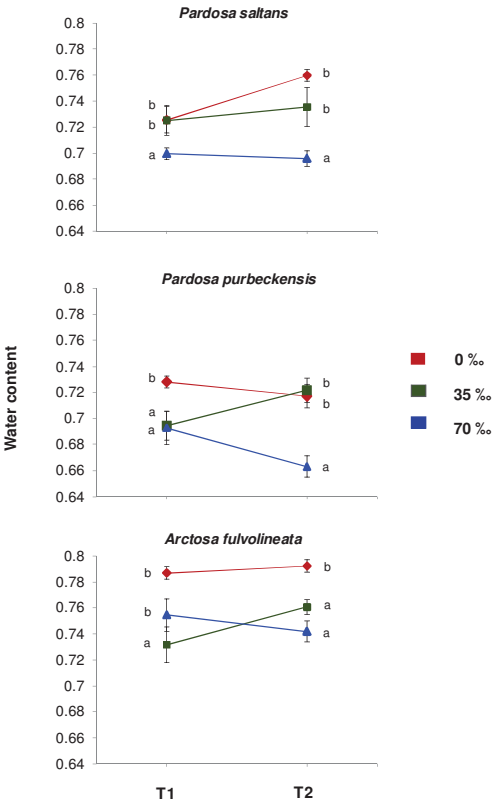

Fig. 2

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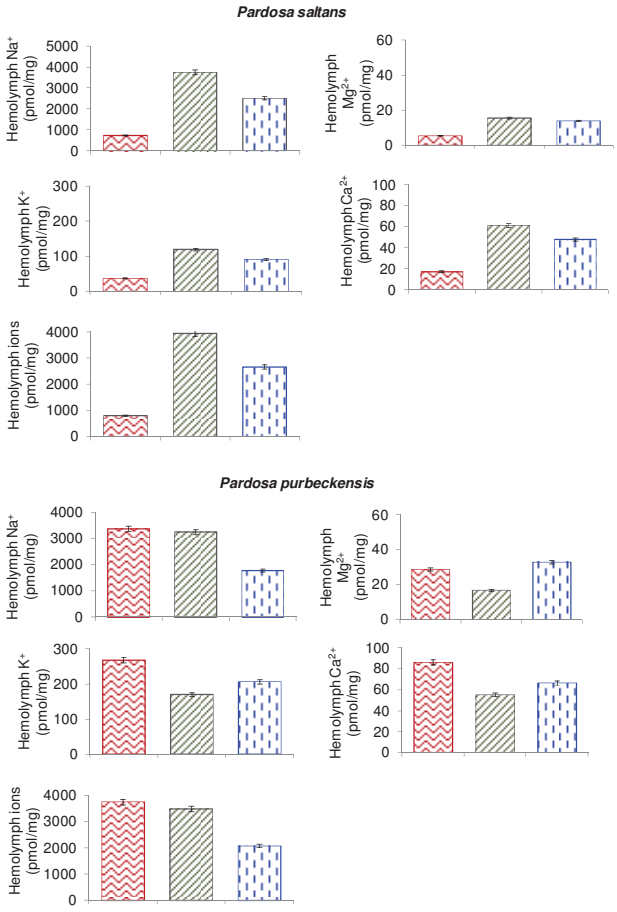

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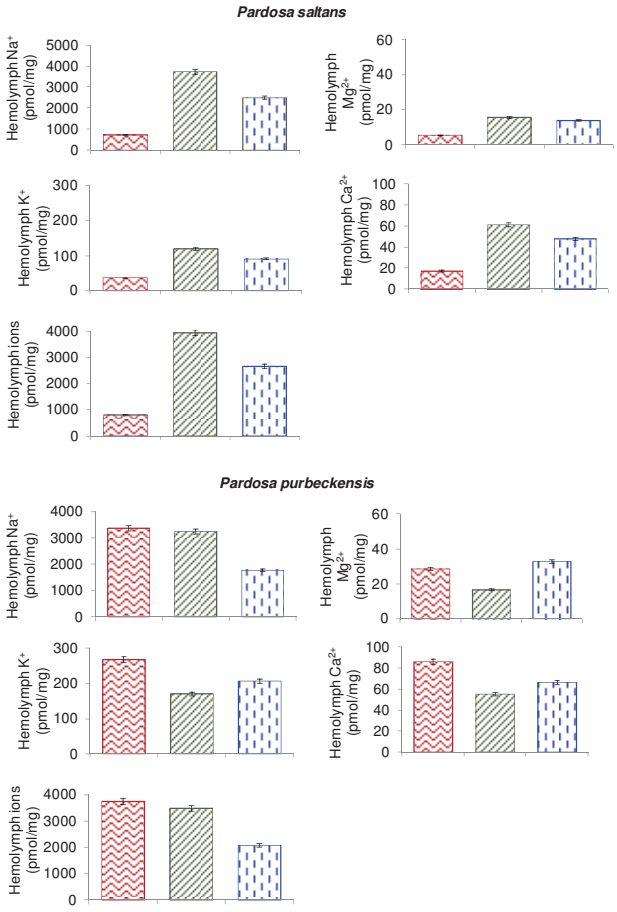

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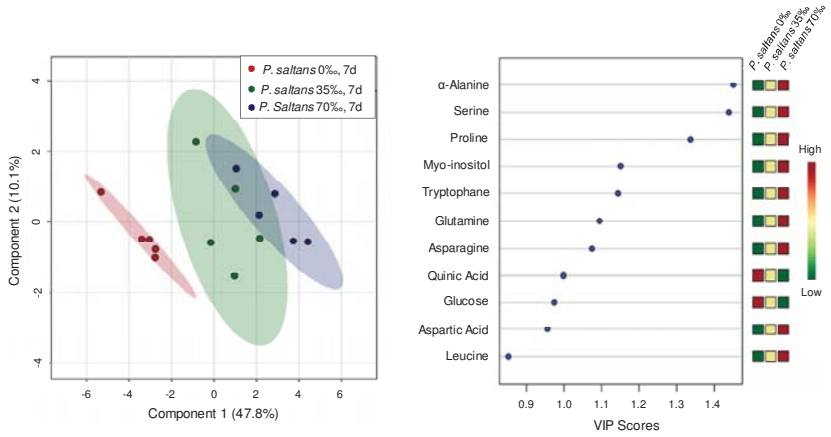

Fig. 4

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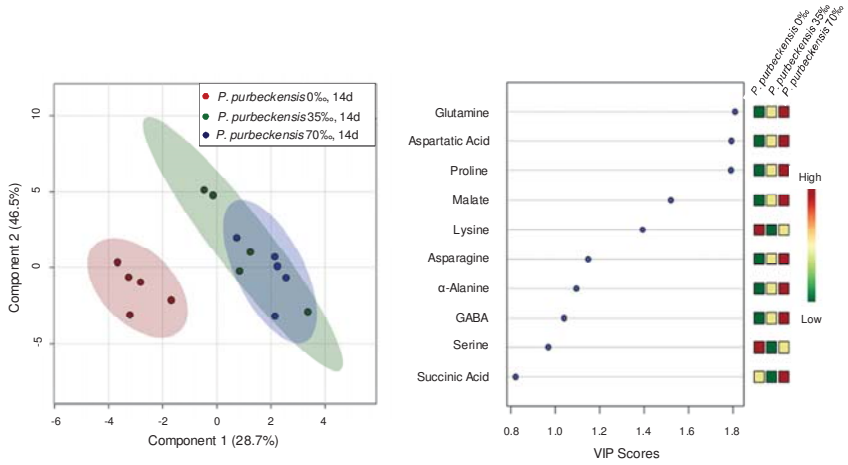

Fig. 5

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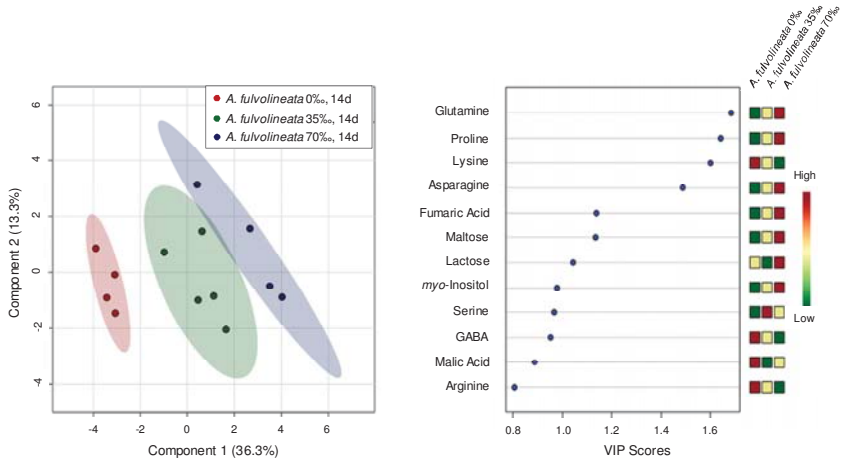

Fig. 6

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