Alanine, proline and urea are major organic osmolytes in the snail *Theodoxus fluviatilis* under hyperosmotic stress

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Abstract

Hyperosmotic stress may result in osmotic volume loss from the body to the environment in animals which cannot control the water permeability of their integument. Euryhaline (having a wide tolerance range of environmental salinities) animals have generally evolved the ability to counteract cell volume shrinkage by accumulating inorganic and organic osmolytes within their cells to balance internal and external osmolalities. Molluscs use very different combinations of amino acids and amino acid derivatives to achieve this goal. *Theodoxus fluviatilis* (Linneaus, 1758) is a neritid gastropod that is distributed not only in limnic habitats in Europe but also in brackish waters (e.g. along the shore line of the Baltic Sea). Animals from brackish sites survive better in high salinities than animals from freshwater locations. The results of this study indicate that these differences in salinity tolerance cannot be explained by differences in the general ability to accumulate amino acids as organic osmolytes. Although there may be differences in the metabolic pathways involved in osmolyte accumulation in foot muscle tissue, both groups of animals accumulate amino acid mixtures equally well when stepwise acclimated to their respective maximum tolerable salinity for extended periods. Among these amino acids, alanine, proline as well as the osmolyte urea hold a special importance for cell volume preservation in *Theodoxus* under hyperosmotic stress. It is possible that the accumulation of various amino acids during hyperosmotic stress occurs via hydrolysis of storage proteins, while alanine and proline are most likely newly synthesised under conditions of hyperosmotic stress in the animals.

Keywords: free amino acids, *Theodoxus fluviatilis,* salinity acclimation, salinity tolerance, proline, alanine, urea

Introduction

Animals facing unstable environments have evolved a range of genetic adaptations to deal with extreme environmental conditions (Rowińsky and Rogell, 2017). Within this genetic framework animals respond to rapid changes of conditions by gene regulatory or post-transcriptional mechanisms leading to altered protein expression, or by post-translational means like protein modification, changes in enzyme activities or alterations in metabolism etc. (Lockwood and Somero, 2011). The ability of individuals to modify their phenotype as a response to environmental change or stress is termed phenotypic plasticity (Woods, 2014).

Aquatic animals whose integument is not entirely water-impermeable (Oglesby, 1981) undergo osmotic water gain when exposed to external salinities lower than that of their own body fluids (hypoosmotic stress). This results in volume expansion of the internal body fluids that has to be limited by excretion of a hypotonic urine through the kidneys (Dantzler, 2016) or other excretory organs (Kirschner, 1967). Concomitantly, such animals reduce their internal osmolyte load by degrading or excreting inorganic ions or organic osmolytes to reduce the driving force for osmotic water influx (Pierce, 1982). If such animals are exposed to external salinities higher than that of their own body fluids the animals lose water to the environment and their body fluid volume decreases. To avoid detrimental shrinkages in extra- and intracellular fluid volumes, animals accumulate inorganic ions and organic osmolytes (amino acids and their derivatives, polyols, sugars, methylamines, methylsulfonium compounds or urea) in their internal fluid compartments to reduce the driving forces for osmotic water loss (Burg, 1995; Yancey et al., 1982; Yancey, 2005; Burg and Ferraris, 2008). These substances have to be taken up, be newly synthesised or mobilised from high molecular mass precursors (Wehner et al., 2003). In the case of animals that accumulate amino acids as organic osmolytes under conditions of hyperosmotic stress in their body fluids, it is still unclear

whether these small molecules are taken up from the external medium, acutely synthetised, generated by degradation of storage proteins or accumulated through combinations of such processes (Manahan et al., 1983; Deaton, 1987; Gilles and Pequeux, 1981).

Euryhaline molluscs mainly accumulate amino acids as organic osmolytes in their cells under hyperosmotic stress (Shumway et al., 1977; Pierce and Amende, 1981). While bivalves have been thoroughly investigated in terms of organic osmolyte accumulation under hyperosmotic stress, there is only a limited number of studies in oligohaline gastropods (Deaton, 2009; Taylor and Andrews, 1988; Symanowski and Hildebrandt, 2010) with highly differing results. Although *Littorina littorea* is an intertidal gastropod species experiencing harsh changes in environmental salinity, it does not utilise intracellular accumulation of free amino acids to avoid cell volume changes under hyperosmotic stress (Taylor and Andrews, 1988). In contrast, the euryhaline snail *Theodoxus fluviatilis* (Linneaus, 1758) which occurs in freshwater lakes in central Europe as well as in brackish water along the coastline of the Baltic Sea accumulates substantial amounts of free amino acids and urea (measured as the sum of ninhydrin-positive substances, NPS) in foot muscle tissue upon being transferred to higher salinities (Symanowski and Hildebrandt, 2010). The snails collected from freshwater sites (FW) and those collected from brackish water sites (BW) showed clear differences in their respective salinity tolerance (Zettler et al., 2004; Bandel, 2001; Hubendick, 1947, Wiesenthal et al., 2018). Freshwater (FW) individuals were not able to cope with salinities as high as brackish water (BW) ones. Even when given the time to acclimatise to gradually increasing salinities of their environmental medium over several days FW snails barely survived in salinities up to 21‰. Snails collected from brackish water sites, however, survived salinities up to 28‰ when gradually acclimated (Wiesenthal et al., 2018).

This study was conducted to elucidate 1) whether differences in the ability to accumulate free amino acids in the tissues explains these differences in tolerance to hyperosmotic stress, to 2) identify those amino acids that contribute the most to intracellular osmotic adjustments, and to 3) find indications whether accumulated amino acids are newly synthesised or generated by hydrolysis of storage proteins.

Methods

Animal collection and transfer experiments

Theodoxus fluviatilis were collected from 3 freshwater (FW) and 2 brackish water (BW) sites in northern Germany. The FW lakes 'Schmaler Luzin' and 'Carwitzer See' are part of the 'Feldberger Seenlandschaft' and lie about 100 km north of Berlin. The BW sites are at the coastline of the Baltic Sea near Greifswald and on the island of Hiddensee. Collections took place between May and September 2016 and snails were subject to 15 day long transfer experiments in the laboratory (Fig. 2, inserts) at room temperature (21°C). FW animals were transferred from their original medium (salinity of 0.5‰, 1 day) to their maximum salinity of 21‰ for 3 days using a step by step regime (3.7‰ for 3 days, 6.9‰ for 3 days, 10‰ for 5 days). BW animals were also transferred from their original medium (salinities of 7.5 (S5) or 9‰ (S6), 1 day) to their maximum salinity of 28‰ for 3 days using a stepwise regime (12‰ for 3 days, 18‰ for 3 days, 24‰ for 5 days). Control animals were held at their original salinities throughout the experiment. Detailed information on collection, storage and the transfer experiments have previously been provided (Wiesenthal et al. 2018).

Sample preparation

The snails were quickly cooled down to 4°C for 10-15 min before the foot muscle was dissected. The isolated foot muscle of each individual was blotted dry, weighed using a precision scale (Quintix, Sartorius, Göttingen, Germany) to the nearest 0.001 g and immediately placed in liquid nitrogen. Each of the frozen tissue samples was homogenised on ice (T-8 Ultraturrax, IKA, Staufen, Germany) in 300 µl deionised water and centrifuged at 16 000 x g for 4 min at 4 °C (HERAEUS Fresco 21, Thermo Scientific, Waltham, MA, USA). The supernatant was transferred to a new reaction tube. Proteins in the supernatant were precipitated by adding 60 µl of an ice-cold 0.575 mol/l solution of 5-sulfosalicylic acid dihydrate (Roth, Karlsruhe, Germany) to the homogenate. The mixture was vortexed (REAX 2000, Heidolph, Schwabach, Germany) and centrifuged at 16 000 x g for 4 min at 4°C,

leaving the extracted FAA, amino acid derivatives and urea from both intra- and extracellular fluids in the supernatant. The supernatant was frozen at -20°C in aliquots of 50 µl.

Quantification of the free amino acids and other ninhydrin-positive substances, including urea

For the amino acid analyses, 50 µl of the extracted osmolyte samples were diluted with 50 µl of lithium loading buffer (Laborservice Onken, Gründau, Germany) and filtered using micro-centrifuge filter tubes equipped with a 0.2 µl nylon membrane (Laborservice Onken, Gründau, Germany). Samples were filtered by centrifugation at 7 000 x g for 3 min at 4°C. The filtrates were transferred to HPLC sample vials (Macherey & Nagel, Düren, Germany). A 1:20 dilution of the standard amino acid mixture provided by the manufacturer (Laborservice Onken, Gründau, Germany) was further diluted with lithium buffer in order to obtain 100 µl of a 1:200 dilution. This standard mixture was freshly prepared for every analysis series of biological samples and run at the beginning and at the end of each series of HPLC analyses to check for differences in signal intensities potentially related to the replacement of HPLC solvents during a series.

Analyses were performed on a Biochrom 30+ amino acid analyser (Laborservice Onken, Gründau, Germany) using the original buffer kits (Laborservice Onken, Gründau, Germany). Samples and standards were automatically applied to the machine by an autosampler that kept the samples at 4°C before injection. Post-column ninhydrin-derivatised substances were detected using a SPD-20AV Prominence HPLC UV-Vis detector (Shimadzu, Columbia, MD, USA). Editing of the chromatograms was done using the OpenLAB (Agilent Technologies, Waldbronn, Germany) software. Peak areas were normalised against internal standards (hydroxylysine), and relative substance amounts in the samples (mmol/kg tissue fresh weight) were calculated using the analysis data of the amino acid standard mixtures and Microsoft Excel.

Quantification of the potential compatible osmolytes TMAO, glycine betaine, sarcosine, glycerol, myo-inositol and GPC

Only samples of snails from the collection site S5 were prepared. This was done as described above ('Sample preparation') with the addition of 43 µl of a 1 mol/l sodium bicarbonate solution (Roth, Karlsruhe, Germany) to each 50 µl aliquot in order to neutralise the pH value. These steps were carried out as preparation for the quantification of compatible osmolytes with a sample size of 3.

The potential compatible osmolytes glycerol, myo-inositol, sarcosine and glycerophosphorylcholine (GPC) were quantified in the thawed samples (n=3) using the 'Enzytec fluid Glycerol' Assay Kit (ID-N°: 5360; Thermo Fisher Scientific, Vantaa, Finland), the 'VitaFast Inositol' Assay Kit (ID-N°: 1009; Institut für Produktqualität GmbH, Berlin, Germany), the 'Sarcosine Colorimetric/Fluorometric Assay Kit' (ID-N°: K636-100; BioVision Inc., Milpitas, CA, USA) and the 'Glycerophosphorylcholine Assay Kit' (ID-N°: K433-100; BioVision Inc., Milpitas, CA, USA) respectively. The assays were carried out according to the manufacturers' instructions with a downscaling for the small volume of the sample tissue. Undiluted samples as well as a 1:100 sample dilution were measured to ensure that the standard curve covered the detected amount in the sample. The amounts of glycine betaine and trimethylamine *N*-oxide (TMAO) were determined as described in Valadez-Bustos (2016) and Wekell and Barnett (1991). The chemicals and solutions for these assays were acquired from Sigma-Aldrich Chemie GmbH (Munich, Germany) and Roth (Karlsruhe, Germany). Again, the instructions were downscaled to match the small volume of sample available and measured in the undiluted sample as well as in a 1:100 dilution.

Statistics

The statistical analysis was carried out with the free software R 3.3.2 (R Development Core Team, 2008). The result for each individual component (NPS) was tested for normal distribution with the Wilk-Shapiro test and for homogeneity of variance with the Fligner-Killeen test. When testing for potential increases in the NPS between the control groups and stressed groups per site either the Welch-t-test was used or the Kolmogorov-Smirnov test (KS-test), depending on the outcome of the Wilk-Shapiro-test (normally distributed data: Welch-t-test; non-normally distributed data: KS-test). Differences between control and stressful conditions among sites as well as differences between sites among control groups and among stressed groups were tested for with the Kruskal-Wallis-test and the post-hoc test after Dunn (Dunn, 1964) with a p-value adjustment (Benjamini and Hochberg, 1995) (R package: 'PMCMR'). A Kruskal-Wallis-test was additionally used to exclusively compare the FW sites among each other under both control and high salinity conditions. Graphs were generated with R 3.3.2 (R package: 'tiff', R package: 'raster', R package: 'lattice', R package: 'RColorBrewer').

Results

Specimens of *Theodoxus fluviatilis* were collected at different locations with different environmental salinities ranging from 0.5‰ (freshwater sites, FW) to 9‰ (brackish water sites, BW). The total amount of amino acids in the foot muscle of these animals under their original salinity conditions clearly correlated with the external salinity (Fig. 1). This shows that animals of this species use organic osmolytes not only to prevent cell volume changes during acute osmotic stress but also to balance the osmotic concentration of the body fluids with that of the external medium during normal salinity conditions.

The total amino acid accumulation upon stepwise acclimation of the animals to the maximum of their tolerable salinities (21‰ for animals from FW sites S1 - S3; 28‰ for animals from BW sites S5 and S6) was very similar in all cases (Fig. 2). The amounts of all amino acids were up to 27-fold higher in stressed animals than in control animals. Such an organic osmolyte accumulation accounts for approximately 21 - 27% (osmolality of accumulated FAA / osmolality of the environment) of all osmolytes in the foot muscle tissue under the respective conditions.

To analyse the relative contributions of individual amino acids and amino acid derivatives to the observed changes in overall organic osmolytes upon hyperosmotic stress in the animals, we measured the contents of methionine (Met), tryptophan (Trp), taurine (Tau), glycine (Gly), alanine (Ala), isoleucine (Ile), leucine (Leu), arginine (Arg), lysine (Lys), proline (Pro), threonine (Thr), serine (Ser), β-alanine (β-Ala), histidine (His), phenylalanine (Phe), glutamate (Glu), valine (Val), tyrosine (Tyr), cysteine (Cys), γ-aminobutyric acid (GABA), hydroxy-proline (OH-Pro), β-amino-isobutyric acid (BAIBA), α-amino adipic acid (AAAA), α-aminobutyric acid (AABA) and Cystathionine (Cysth) in each of the samples. The results indicated that the contributions of individual amino acids to these changes under osmotic stress in the animals were very different and ranged from 2-fold (Leu in BW animals) (Fig. 3 A) to 650-fold (proline in FW animals) (Fig. 3 B). It was very obvious that the increase in amino acid content was much more pronounced in osmotically stressed FW animals than in stressed BW animals (Fig. 3). This appears to correlate with the rate of change in external osmolality (42-fold in FW animals; 3-fold in BW animals) to which these animals were exposed to in order to impose the maximum tolerable osmotic stress.

The two amino acids that accounted for most of the total change in organic osmolyte content in the foot muscle of stressed animals were alanine and proline (Fig. 3 B), both in terms of fold change and absolute amount (Fig. 4), but urea seemed to be quantitatively important as well (Fig. 5 C). Alanine increased 10- to 20-fold in brackish water animals exposed to an external salinity of 28‰, and 80- to 130-fold in freshwater animals exposed to 21‰ reaching a mean of 47 and 88 mmol/kg fresh weight respectively. Proline accumulated 60- to 80-fold in brackish water animals exposed to 28‰

(43 mmol/kg fresh weight), and 500- to 640-fold in freshwater animals exposed to 21‰ (17 mmol/kg fresh weight). The Tryptophan content also appeared to increase in the foot muscle (44-fold), but this was due to large variations in individual measurements at very low levels (< 1 mmol/kg fresh weight) and was, therefore, not considered as relevant. Other amino acids, however, also contributed to the organic osmolyte accumulation in foot muscle tissue under hyperosmotic stress (Fig. 3 A), but to lesser degrees compared to proline or alanine (Figs. 3 B, 4 B, 5 A, 6 A). As shown in Fig. 6 B, C, amino acids like taurine or glycine were generally present at higher levels in the foot muscle of osmotically stressed animals compared to the respective controls, but their contribution to the total change in organic osmolytes was relatively small. Moreover, the patterns of changes in individual amino acids in the foot muscle in FW and BW animals differed among each other. For instance, much larger quantities of taurine were accumulated in BW animals (S5, S6) than in FW animals (S1 - S3) under hyperosmotic stress (Fig. 6 B). Of glycine, on the other hand, very similar quantities were accumulated in stressed animals irrespective of their origin (Fig. 6 C). Overall, glycine, taurine as well as the amino acid derivatives GABA, BAIBA, AAAA and AABA were present at low concentrations in the foot muscle tissue in control animals (0.001 - 3.8 mmol/kg fresh weight). During hyperosmotic stress, these substances increased by 6- to 46-fold (max. total of 10 mmol/kg fresh weight) which was not considered to be a very relevant contribution to the total organic osmolyte content. β-alanine was also detected in rather small amounts in control animals and hyperosmotically stressed FW snails, but was accumulated to 16 mmol/kg fresh weight in stressed BW ones, which corresponded to an approx. 9-fold increase (Figs. 3 B, 7 A). This means that β-alanine amounted to roughly 3% of the quantity of urea that was accumulated under hyperosmotic stress in BW snails (Fig. 5 C).

As these differences in the accumulation of individual amino acids and amino acid derived molecules in cells may be explained by different potential mechanisms (entry and exit through amino acid transporters, amino acid biosynthesis and degradation or protein biosynthesis and degradation) we tried to find markers among the amino acids and amino acid derivatives that may help to disentangle

the underlying pathways of amino acid accumulation during hyperosmotic stress. The patterns of changes in amino acid quantities in the foot muscle tissue of those amino acids that are generally considered to be essential (Met, Leu, Ile, His, Phe etc.) were very similar to those observed of the non-essential ones (Gly, Ser, Arg, Glu etc.) (Fig. 3 A). Thus, a comparisons of accumulated essential and non-essential amino acids did not allow any conclusions with respect to the accumulation mechanism. Because—in animal cells—the irreversible hydroxylation of proline only takes place when this protein is integrated in protein strands (mainly collagen; Gorres and Raines, 2010), the occurrence of free hydroxy-proline indicates that cells actively turn over their protein content. Additionally, the accumulation of OH-Pro under stress may indicate that hydrolysis of storage proteins is accelerated. Also, transamination or deamination of amino acids are frequent reactions in the synthesis and the degradation of amino acids (Bröer and Bröer, 2017). They may result in free ammonia that is partially transformed to urea in limnic snails (Haggag and Fouad, 1968; Horne and Boonkoom, 1970). Differences in the urea contents of foot muscle tissue may therefore be used as an indicator for different turnover rates in amino acid metabolism.

Based on these assumptions, we analysed the proline- and the hydroxy-proline content of each of the snail samples. As shown in Fig. 5, the proline content in the foot muscle was low in FW animals as well as in BW animals under control conditions but increased substantially in both groups under osmotic stress conditions (Fig. 5 A). The OH-Pro content in the foot muscle tissue, however, already clearly differed between the groups under control conditions (Fig. 5 B). It was very low (< 0.1 mmol/kg fresh weight) in FW animals, but substantially higher in BW animals in their natural medium (approx. 1 mmol/kg fresh weight). Upon osmotic stress, OH-Pro increased considerably in FW animals, but did not change significantly in BW animals (Fig. 5 B). While the pattern of urea quantities in the foot muscle tissue (FW animals approx. 3 mmol/kg fresh weight; BW animals approx. 300 mmol/kg fresh weight) was similar to that of OH-Pro in the control animals (Fig. 5), the data showed that there was some urea accumulation under hyperosmotic stress in the FW animals

(approx. 50 mmol/kg fresh weight), but a much more substantial increase in osmotically stressed BW animals where the amount of urea virtually doubled (to approx. 600 mmol/kg fresh weight) (Fig. 5 C).

Because large urea amounts can have denaturing effects on proteins, the quantities of TMAO, glycine betaine, sarcosine, glycerol and GPC were measured as these substances are considered to be compatible osmolytes that may alleviate negative impacts of high urea concentrations on protein conformation (Withers and Guppy, 1996; Yancey, 2005). All of these substances were below detection limits in FW as well as in BW animals and did not show noticeable increases under hyperosmotic stress. Myo-inositol, however, slightly increased in individuals exposed to hyperosmotic stress (Fig. 7 B). Under control conditions a mean amount of 23.5 (± 24.3) mmol/kg fresh weight was observed. Under hyperosmotic conditions, an increase by a factor of 1.8 to 42.5 (± 17.9) mmol/kg fresh weight was observed in BW snails. This amounted to roughly 7% of the urea amount in BW snails under hyperosmotic conditions.

Discussion

Though both groups—BW and FW—of the studied *Theodoxus fluviatilis* show the ability to accumulate organic osmolytes as a response to hyperosmotic stress equally well, they differ in the pathways of acquiring these organic osmolytes. The main constituents of the increased amounts of organic osmolytes are alanine, proline and urea that seem to be most important for an initial coping with high environmental salinity conditions.

It has been known for a long time that a number of euryhaline aquatic molluscs—including bivalves and gastropods— are hyperregulators in very dilute salinities, but are basically osmoconformers at higher osmotic concentrations of the environmental medium (Deaton, 2009). As molluscs generally cannot control the water permeability of their integument very well, they undergo rapid changes in fluid volumes in extra- and intracellular compartments when exposed to environmental media whose osmotic concentrations are either hyper- or hypoosmotic with respect to the body fluids of the

animals. Such passive responses are very similar in marine bivalves (Gainey, 1987; Hosoi et al., 2003) and limnic/brackish water gastropods (Symanowski and Hildebrandt, 2010). Euryhaline species survive such substantial changes in body fluid volume and are able to prevent extreme swelling (under hypoosmotic stress) or shrinkage (under hyperosmotic stress) in cell volume by rapidly releasing or accumulating organic osmolytes, respectively, from or in their cells to adjust the intracellular osmolality to the external conditions (Pierce, 1982; Yancey, 2005).

The European neritid *Theodoxus fluviatilis* is generally considered to be a widely distributed limnic snail, but it also occurs in BW along the shore lines of the Black Sea and the Baltic Sea (Bunje, 2005; Bunje and Lindberg, 2007). Due to this species not having any pelagic larval stages, each population is quite stationary which results in a patchy distribution of populations of this species. The adult animals within one population are very similar in terms of shell size and patterning (Wiesenthal et al., 2018). However, across several different populations the variability of these parameters seems to be high (Neumann, 1960; Kangas and Skoog, 1978; Zettler et al., 2004; Symanowski and Hildebrandt, 2010; Wiesenthal et al., 2018), although some mitochondrial marker genes have very similar sequences (Bunje, 2005; Bunje and Lindberg, 2007). Thus, to date it is unclear whether the differences in shell size and patterning may be explained by genetic variation (local adaptation) or by phenotypic or developmental plasticity (Glöer and Pešić, 2015). It has previously been reported (Symanowski and Hildebrandt, 2010) that the salinity tolerance of animals from FW locations in northern Germany is less well developed than that of BW animals. Meanwhile, we have learned that reaction norms (survival in different salinities) may shift in animals of both groups by a stepwise acclimation of the animals to increasing or decreasing salinities. And yet, the FW animals were still not able to survive salinities higher than 21‰, whilst the BW animals did well in salinities of 28‰ after such a stepwise acclimation (Wiesenthal et al., 2018). This indicates that different genetic limitations of plasticity may exist in FW and BW groups of animals pointing towards local adaptation. Our main aim in this study was to elucidate whether the ability or the mode of limiting passive cell volume changes by intracellular organic osmolyte accumulation may be one of these factors.

A general observation in euryhaline molluscs under hyperosmotic stress is that the patterns of the individual free amino acids recruited for intracellular volume adjustments are diverse (Deaton, 2009). Quantitatively relevant osmolytes in different species are taurine, glycine, alanine and proline (Yancey et al., 1982; Hosoi, 2003; Yancey, 2005). Our results in *Theodoxus* show that alanine (mainly in the FW, Fig. 6 A) and proline (mainly in the BW animals, Fig. 5 A) as well as urea (Fig. 5 C) are the most relevant organic osmolytes in foot muscle tissue after a two week transfer regime and 72 h exposure to a final hyperosmotic condition. The high levels of urea and its importance as an organic osmolyte were surprising, because despite it being known to be present at high concentrations in elasmobranch body fluids and to accumulate in terrestrial snails during aestivation and desiccation, such an accumulation has not been reported in aquatic snails (Tam et al., 2003; Hazon et al., 2003; Horne and Barnes, 1970; Arad, 2001; Rees and Hand, 1993; Hiong et al., 2005).

The accumulation of amino acids as major organic osmolytes in animals under hyperosmotic stress (Fig. 2) may occur through hydrolytic degradation of storage proteins. This conclusion is supported by the observation that even essential amino acids (Met, Ile, Leu, Lys, Thr, His, Phe, Val) were accumulated 5- to 11-fold in FW animals or 2- to 9-fold in BW animals under hyperosmotic stress (Fig. 3 A). Uneven representation of amino acid residues in storage proteins may explain the observation that the abundance of some amino acids changed more (e.g. Met) than that of others (e.g. Leu). Another potential explanation would be that some of the FAAs present in cells upon protein hydrolysis were subject to metabolic conversion whilst others were largely unaffected. The rate of the increase of organic osmolytes also points towards metabolic processes of turnover or synthesis rather than the uptake of amino acids (Bishop, 1994).

Non-essential amino acids, however, may also be newly synthetised during hyperosmotic stress. This may be an explanation for the observed accumulation of alanine (Fig. 6 A). Changes in alanine were larger than those of all other amino acids (except proline), and definitely larger than would be expected from hydrolysis of a standard protein in which alanine would account for approx. 5 % of all amino acids. From our data, we cannot draw any conclusions on the mode of alanine accumulation (accelerated synthesis, decelerated metabolic conversion) in *Theodoxus* under hyperosmotic stress.

The mode may be similar to those used in other molluscs, as alanine accumulation is a widely distributed phenomenon in euryhaline molluscs during hyperosmotic stress (Deaton, 2009; Bishop et al., 1994; Kube et al., 2006) along with the accumulation of taurine, β-alanine, and glycine. However, in contrast to these other marine mollusc species, the last three mentioned organic osmolytes are not really relevant for overall adjustments of the internal osmolality in *Theodoxus*. Proline on the other hand, accumulated to a very high degree in the foot muscle tissue of animals under hyperosmotic stress which raised the question about its source. A potential mechanism is the hydrolysis of storage proteins with high proline content, e.g. collagens (Li and Wu, 2018) and proline uptake into the cells (Bröer and Bröer, 2017). Proline-rich repeats (PRRs) of proteins may contain up to 50 % proline residues (Williamson, 1994). Assuming that such a proline-rich protein is over all composed of 17 % proline residues (as in the collagen alpha-2(IX) chain-like protein of *Biomphalaria glabrata*, UniProt A0A2C9KD15) and the other amino acid residues would be evenly represented, the quotient of amino acid representation in the fully hydrolysed protein would theoretically be approximately 3 between proline and any other amino acid. The comparison of amino acid amounts in tissues of stressed animals showed that these quotients were between 3 and 25 (proline vs. Leu - 10, Ile - 25, Arg - 3, Gly - 5, Ser - 3, Thr - 15, Val - 15) in FW or between 6 and 80 (proline vs. Leu - 20, Ile - 80, Arg - 8, Gly - 6, Ser - 8, Thr - 16, Val - 50) in BW animals. Even when considering that the molecular masses of the different amino acids are somewhat different, and that actual proteins are not ideally composed by even portions of all 20 amino acids, and under the assumption that these quotients may be secondarily affected by metabolic conversion after mobilisation from storage proteins, the data imply that proline accumulation in the foot muscle of snails under hyperosmotic stress cannot be solely explained by hydrolysis of proline-rich storage proteins.

In animal and plant cells, proline levels under osmotic stress are also controlled by proline synthesis mediated by the glutamate pathway (Szabados and Savouré, 2010). Proline is synthesised from glutamate via D-1-pyrroline-5-carboxylate (D-1-P5C) through two sequential reduction reactions catalysed by the D-P5C synthase and the P5C reductase. The genes encoding these enzymes have been identified in bivalves and were transcriptionally activated during osmotic stress (Meng et al.,

2013). We have identified transcript sequences of *Theodoxus* homologues of delta-1-pyrroline-5-carboxylate-synthase and delta-1-pyrroline-5-carboxylate-reductase in a transcriptome database of these snails (GenBank accession number: MK316364; GenBank accession number: MK316365; GenBank accession number: GenBank MK316366) which suggests that proline may indeed be rapidly synthesised from glutamate during hyperosmotic stress. The sequences associated with potential homologues of the enzyme converting proline to glutamate, proline dehydrogenase and delta-1-pyrroline-5-carboxylate-dehydrogenase, have also been identified in the *Theodoxus* transcript database (GenBank accession number: MK316367; GenBank accession number: MK316368; GenBank accession number: MK316369). Proline synthesis from glutamate may be supported by the observation that glutamate levels do not change much in BW animals under osmotic stress, whilst glutamate levels undergo similar changes as other amino acids in tissues of stressed FW animals (Fig. 3 A). Proline synthesis and accumulation in foot muscle cells may also be supported by another metabolic pathway leading from arginine to delta-1-pyrroline-5-carboxylate via ornithine (Li and Wu, 2018). This pathway has also been identified as relevant for proline accumulation in the ribbed mussel (*Geukensia demissa*) (Bishop et al., 1994). As a side product of arginine conversion to ornithine, urea is generated. Because urea accumulation under hyperosmotic stress was observed in both FW and BW animals (Fig. 5 C) to a certain extent, it is likely that this pathway of proline synthesis may be generally used for supporting proline synthesis and proline accumulation.

This does not rule out the possibility that some of the accumulated proline is derived from hydrolysis of proline-rich storage proteins. Some of the proline residues become hydroxylated by prolyl 4-hydroxylase (Gorres and Raines, 2010) while they are integral protein constituents (Li and Wu, 2018). Thus, the occurrence of free hydroxy-proline in body fluids of animals is generally considered as an indication of accelerated protein degradation (Holm and Kjaer, 2010). As shown in Fig. 5 B, hydroxy-proline accumulated to a large extent in FW animals during hyperosmotic stress, whilst no statistically significant change in hydroxy-proline abundance was observed in BW animals. This indicates that the proline accumulation observed in the foot muscle tissue of hyperosmotically challenged FW specimens of *Theodoxus fluviatilis* (Fig. 7 A) must be largely derived from storage

protein degradation, while the higher accumulation of proline in stressed BW snails cannot be explained by this process, as there was no increase of hydroxy-proline (Fig. 5 B). Thus, the proline accumulation in BW snails can be explained by synthesis or transamination of this amino acid. Accelerated turnover and transamination via the glutamate or arginine pathway will lead to increased amounts of ammonia that are largely converted to urea (Bishop et al., 1994; Bröer and Bröer, 2017; Haggag and Fouad, 1968; Horne and Boonkoom, 1970). This is in accordance with the observed urea levels in FW and BW snails (Fig. 5 C). While a moderate increase in urea levels could be explained by proline synthesis via the arginine and ornithine pathway in snails from both groups, the BW snails showed a much higher accumulation than the FW ones. Since individuals of this latter group only showed a small increase in urea, but a substantial one in hydroxy-proline they seem to rely on the hydrolysis of storage proteins. BW individuals, on the other hand, showed a great increase in urea, yet no difference in the hydroxy-proline accumulation under hyperosmotic stress compared to control animals. This indicates that urea is accumulated as a side product in the process of proline and alanine synthesis (Figs. 5 and 6 A).

Bishop et al. (1994) showed that in the ribbed mussel (*Geukensia demissa*) alanine could quickly be synthesised from pyruvate and that the accumulation of this amino acid was most likely based on protein turnover, while proline could originate from synthesis or from protein breakdown. This is in accordance with our findings for FW snails that seem to acquire their alanine and proline under hyperosmotic stress through a combination of protein hydrolysis and amino acid synthesis, while BW snails rely more on synthesis alone.

Because urea, in high concentrations of several hundred mmol/kg, has a perturbing effect on protein conformation, it is known from other organisms that certain compatible osmolytes like TMAO, glycine betaine, sarcosine, glycerol, inositol or GPC, are accumulated along with urea to counteract its negative effect (Yancey, 2005; Withers and Guppy, 1996). The textbook example for this behaviour is the group of elasmobranchs that display high TMAO concentrations in their body fluids in parallel with the high urea concentrations. It is suggested that the best counteracting function of TMAO occurs at a molar ratio of 2:1 for urea:TMAO (Tam et al., 2003, Hazon et al., 2003; Yancey,

2005). Aestivating amphibians also accumulate urea, though not quite as much as *Theodoxus fluviatilis* in this study (Withers and Guppy, 1996). Similar to aestivating desert frogs, however, *T. fluviatilis* did not accumulate any TMAO along with urea under hyperosmotic stress. In fact, none of the measured potential counteracting solutes listed above showed any noticeable increase with rising urea levels. The only exception was myo-inositol where an increase was detected (Fig. 7 B) that was similar to that observed in aestivating *Neobatrachus* (Withers and Guppy, 1996). In both cases, the ratio of inositol to urea was 1:14. Whether such a ratio is meaningful in terms of stabilising protein structure in gastropods or amphibians is not clear. Khan et al. have shown that a ratio of 1:2 (myo-inositol:urea) is needed to counteract the perturbing effect of urea on vertebrate proteins (Khan et al., 2013). In the same study, however, it became clear that urea concentrations of 500 to 600 mmol/l—as reached in stressed *Theodoxus* (Fig. 5 C)—only had minor effects on the thermal stability of these proteins, and that higher urea concentrations (> 800 mmol/l) are needed to shift the denaturing temperature to substantially lower values. Yancey and Somero (1980) stated that β-alanine also has a counteracting effect, even though it is not as strong as that of sarcosine or betaine. When β-alanine levels correspond to about 8.5% of the urea levels, a protein stabilising effect is observable (Tam et al., 2003). In this study the measured amounts of β-alanine only amounted to about 2.6% of the urea concentration. Therefore, just like for myo-inositol, the role of β-alanine as a compatible osmolyte in *Theodoxus* under hyperosmotic stress remains elusive. Even though taurine was not considered as particularly relevant for the overall osmolyte content, it might contribute to counteracting the perturbing effects of urea (Khan et al., 2013). Despite the increase not being very prominent and the total accumulated amount lying below the predicted necessary ratio of 2:1 (urea:taurine), only BW snails showed an accumulation of taurine under hyperosmotic conditions parallel to an accumulation of urea (Fig. 6B) (Khan et al., 2013). This snail species may either offset the perturbing effect of urea through a mixture of β-alanine, myo-inositol, taurine and other compatible osmolytes, which are yet unknown, or they exhibit a lower protein sensitivity towards urea, as has been described in desert frogs (Withers and Guppy, 1996). Which osmolytes besides myo-inositol, β-alanine and taurine could be involved is not yet known. The potential roles of

other polyols (mannitol, sorbitol) (Rees and Hand, 1993) remain to be tested in future studies. Also, since the measured organic osmolytes represent the load in both intra- as well as extracellular fluids, it needs to be considered, where the accumulation takes place. The FAAs, the derivatives and compatible osmolytes are mainly accumulated within the cell, while urea is most likely evenly distributed between intra- and extracellular compartments as it can diffuse through the cell membrane (Gallucci et al., 1971). Therefore, the counteracting ability of these compatible osmolytes will only be effective within the cell, while urea will also have perturbing effects on extracellular proteins. When considering the distribution of organic osmolytes and urea, the mixture of the compatible osmolytes myo-inositol, β-alanine and taurine may be the only intracellular antagonists to urea in BW snails. Nevertheless, further counteracting substances must be present in the extracellular fluids to ensure protein stability.

Taken together, the differences in the upper tolerance limits of environmental salinities that have been previously observed in FW and BW snails (Wiesenthal et al., 2018) cannot be unambiguously explained by differences in the general ability to accumulate amino acids as organic osmolytes. Although there may be differences in the metabolic pathways involved in osmolyte accumulation in foot muscle tissue, both groups of animals accumulate organic osmolyte mixtures equally well when stepwise acclimated to the maximum salinity they can tolerate over extended periods (Fig. 2). The amino acids alanine and proline as well as the secondary metabolite urea are of special quantitative importance for cell volume preservation in *Theodoxus* under hyperosmotic stress. Alanine may be somewhat more important for FW animals (Fig. 6 A), while proline and urea may have a greater significance in the BW animals (Fig. 5 A, C). It seems that basal accumulation of amino acids during hyperosmotic stress occurs via hydrolysis of storage proteins, but especially alanine and proline are most likely newly synthetised. The relative contribution of protein hydrolysis and alanine/proline synthesis may be different in animals collected at the freshwater and those collected at brackish water sites.

For future studies, it would be interesting to test for the rate of osmolyte accumulation and to follow the metabolites over the entire time of hyperosmotic stress to further elucidate the pathways of osmolyte generation and metabolism.

Data accessibility

Supplementary material attached. Relevant transcript sequences are deposited on GenBank. The corresponding accession numbers are given in the text.

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Author's contributions

A.A.W and J.-P.H. conceived the ideas and designed methodology; A.A.W. prepared and measured the tissue samples; K.H. edited the chromatograms, J.-P.H. and A.A.W. analysed the data; C.M. contributed to the transcriptome analysis; A.A.W. and J.-P.H. drafted the manuscript. All authors contributed to the preparation of the final manuscript and gave approval for publication.

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Figures

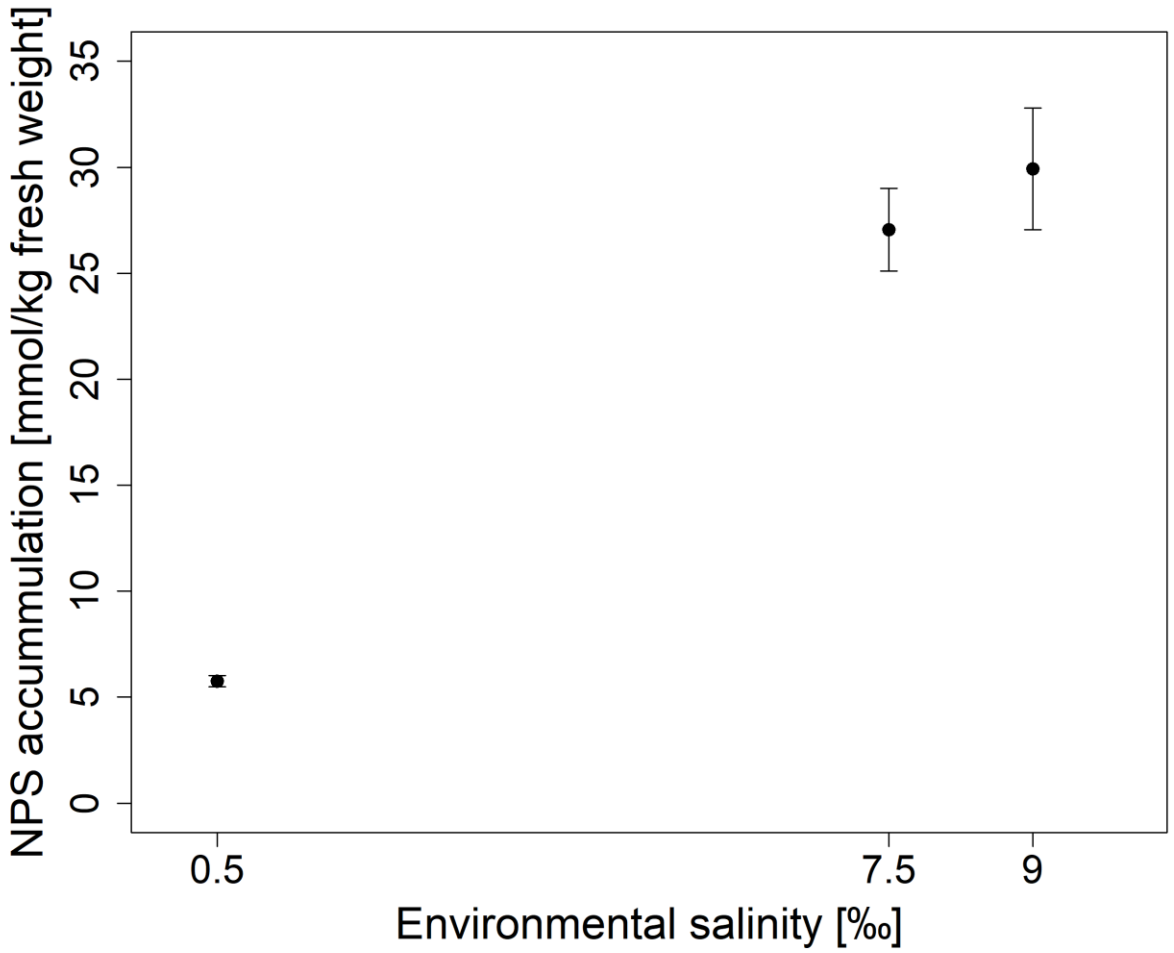

Figure 1: **Mean amounts of accumulated selected amino acids and amino acid derivatives in the foot muscle of FW and BW snails (in mmol/kg fresh weight) in relation to their basal environmental conditions** (control conditions) (FW: 0.5‰, BW S5: 7.5‰, BW S6: 9‰). Mean amounts are depicted including the standard error. Urea has been excluded from the NPS for this figure.

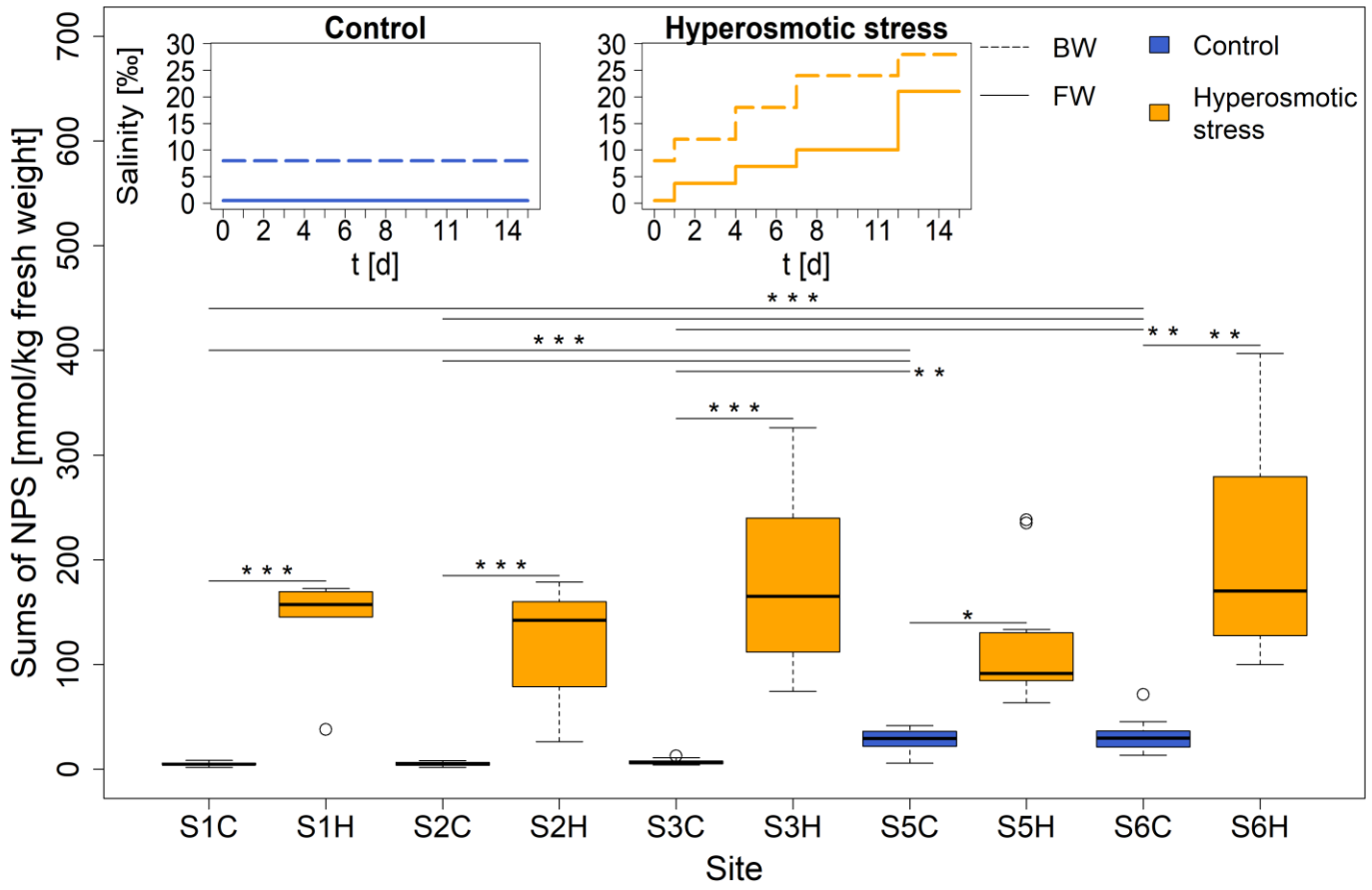

Figure 2: **The sum amounts of the selected NPS in mmol/kg fresh weight in snails from FW (S1-S3) and BW (S5-S6) under control (C, blue) and high salinity conditions (H, orange).** Urea has been excluded from the NPS for this figure. The median is represented by the middle line and the upper and lower end of the box show the 25th and 75th percentile (every site without an outlier). The whiskers either show the minimum and maximum range of the data or 1.5 times the interquartile range (approx. 2 standard deviations, every site with outliers). ° represents outliers and is defined as measurements that lie outside the whiskers, namely, more than 1.5 times the interquartile range away from the median. *p < 0.05; **p < 0.01; ***p < 0.001. The two plots at the top depict the salinity regime the snails (- - - BW and —FW) were kept in before accumulated NPS were measured. The left plot shows the control conditions and the right plot shows the high salinity conditions (FW: 21‰, BW: 28‰) over 15 days. In both plots the x-axis is the time [d] and the y-axis is the salinity [‰]. S1C: n=19, S1H: n=6, S2C: n=22, S2H: n=10, S3C: n=23, S3H: n=14, S5C: n=24, S5H: n=12, S6C: n=21, S6H: n=10

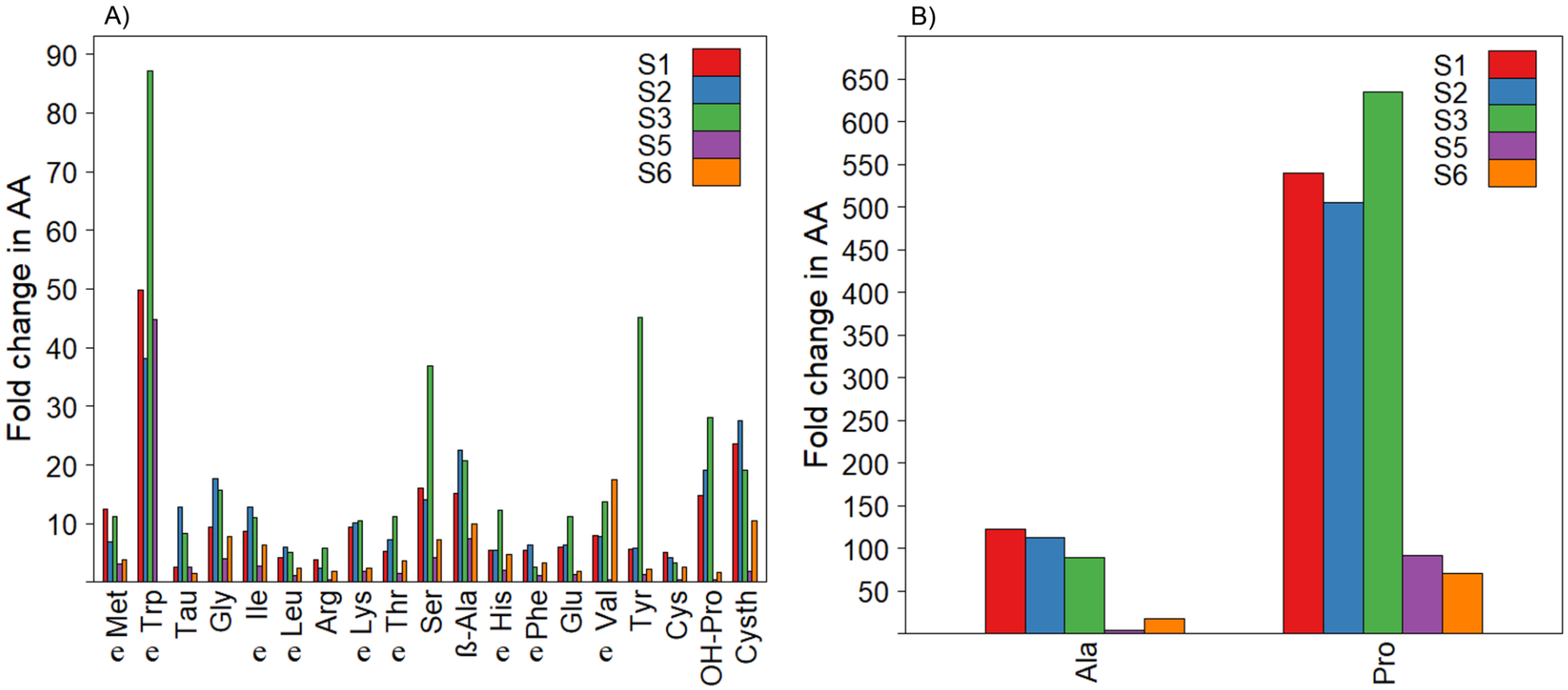

Figure 3: **The fold increase of the amino acids (AA) of the selected NPS when comparing amounts in snails from control and high salinity conditions in FW (S1-S3) and BW (S5-S6) individuals.** A): All selected amino acids without alanine and proline and without the amino acid derivatives GABA, BAIBA, AAAA and AABA. e depicts essential amino acids. B): The fold increase of alanine and proline. Note the different scaling of the y-axes.

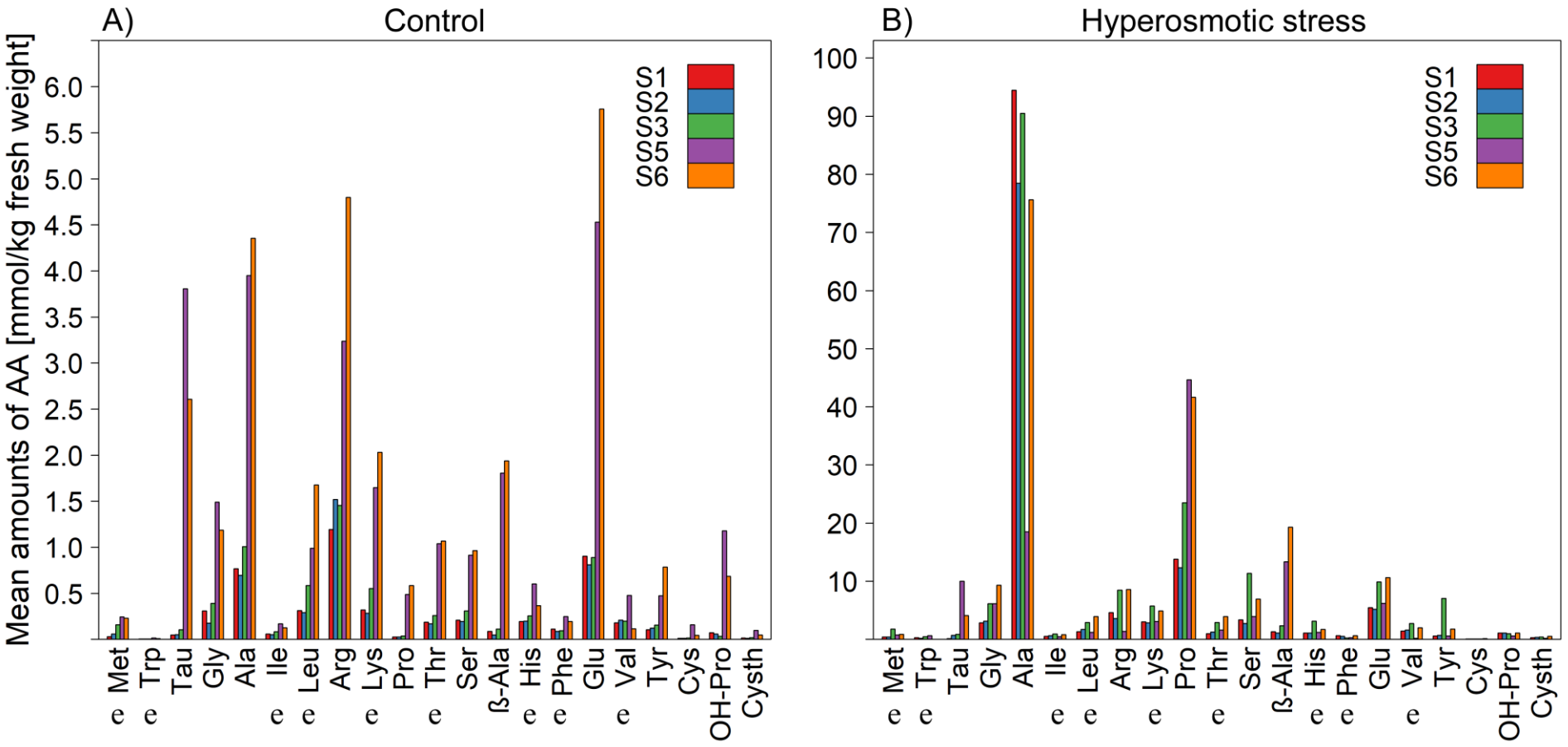

Figure 4: **The mean amounts of the amino acids (AA) of the selected NPS measured in snails from control (A) and high salinity (B) conditions in FW (S1-S3) and BW (S5-S6) individuals.** The mean accumulated amounts of all selected amino acids—excluding the amino acid derivatives GABA, BAIBA, AAAA and AABA—are depicted. A) Under control conditions B) Under hyperosmotic stress conditions. e points to amino acids that are considered to be essential in most animal species. Note the different scaling of the y-axes.

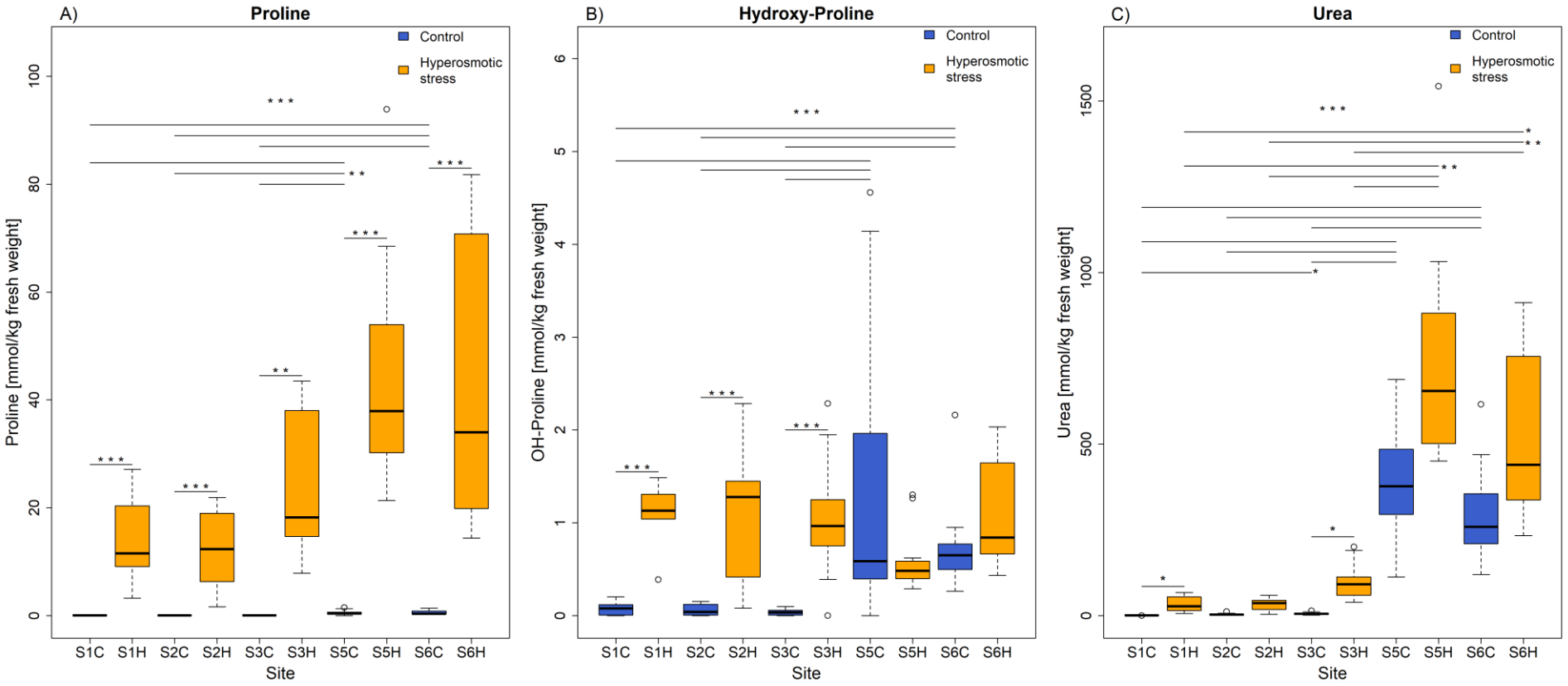

Figure 5: **Amounts of proline (A), hydroxy-proline (B) and urea (C) in mmol/kg fresh weight in snails from FW (S1-S3) and BW (S5-S6) under control (C, blue) and high salinity conditions (H, orange).** The median is represented by the middle line and the upper and lower end of the box show the 25th and 75th percentile (every site without an outlier). The whiskers either show the minimum and maximum range of the data or 1.5 times the interquartile range (approx. 2 standard deviations, every site with outliers). ° represents outliers and is defined as measurements that lie outside the whiskers, namely, more than 1.5 times the interquartile range away from the median. Note the different scaling of the y-axes. **p < 0.01; ***p < 0.001. S1C: n=19, S1H: n=6, S2C: n=22, S2H: n=10, S3C: n=23, S3H: n=14, S5C: n=24, S5H: n=12, S6C: n=21, S6H: n=10

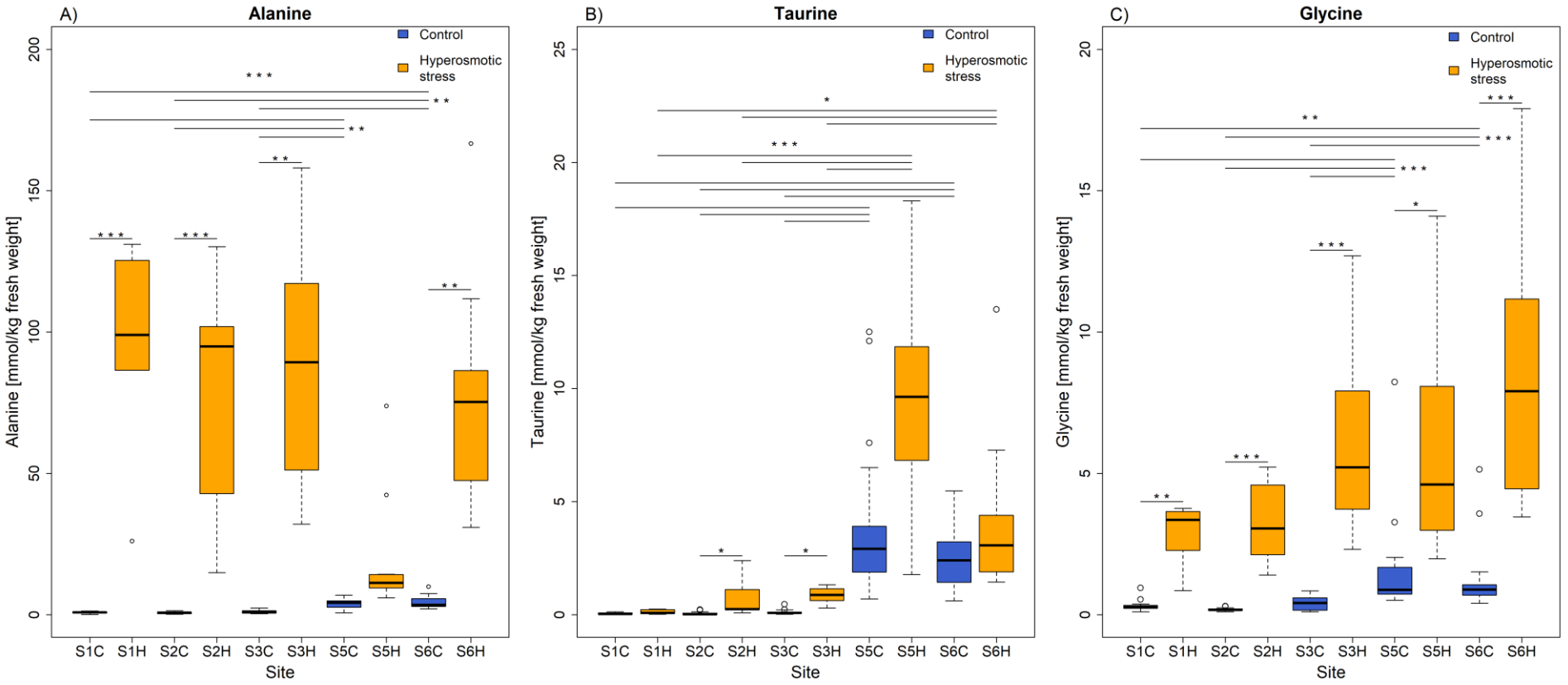

Figure 6: **Amounts of alanine (A), taurine (B) and glycine (C) in mmol/kg fresh weight in snails from FW (S1-S3) and BW (S5-S6) under control (C, blue) and high salinity conditions (H, orange).** The median is represented by the middle line and the upper and lower end of the box show the 25th and 75th percentile (every site without an outlier). The whiskers either show the minimum and maximum range of the data or 1.5 times the interquartile range (approx. 2 standard deviations, every site with outliers). ° represents outliers and is defined as measurements that lie outside the whiskers, namely, more than 1.5 times the interquartile range away from the median. Note the different scaling of the y-axes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. S1C: n=19, S1H: n=6, S2C: n=22, S2H: n=10, S3C: n=23, S3H: n=14, S5C: n=24, S5H: n=12, S6C: n=21, S6H: n=10

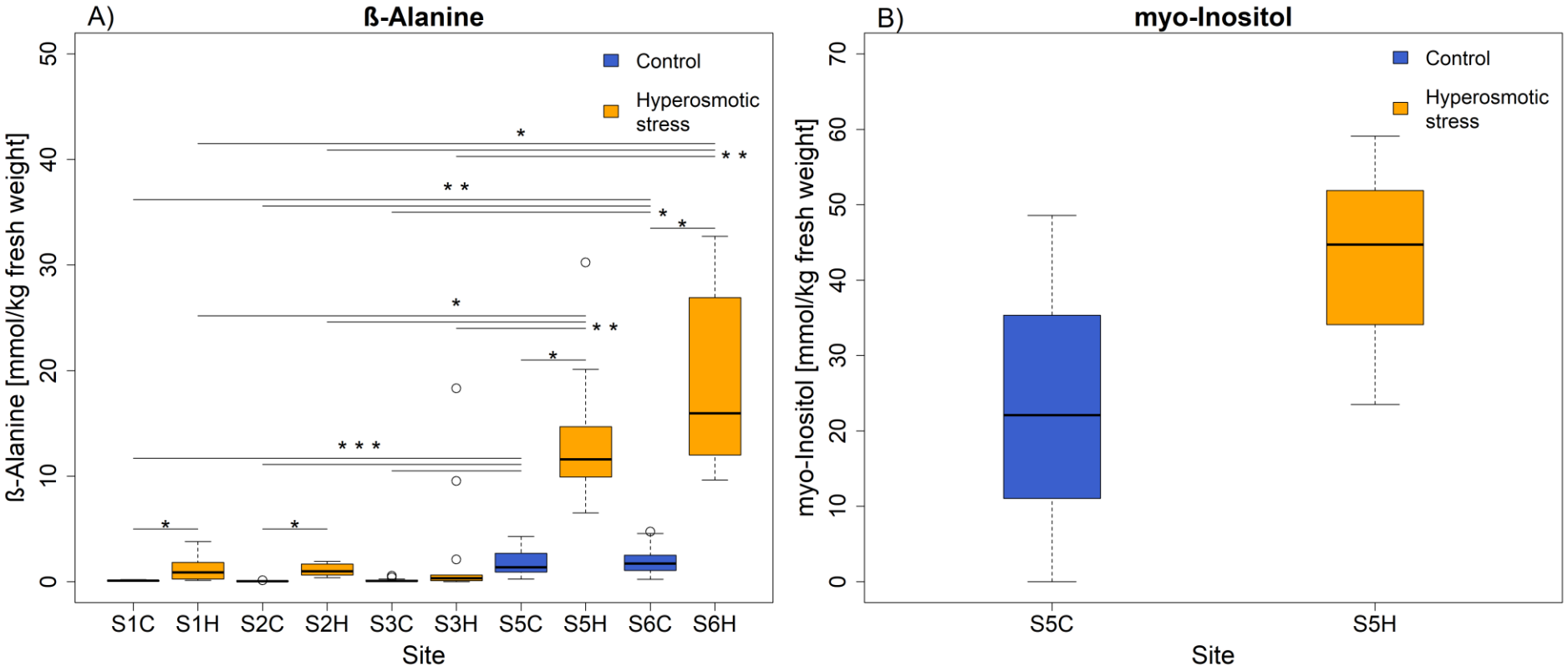

Figure 7: **Amounts of β-alanine (A) and myo-inositol (B) in mmol/kg fresh weight in snails from FW (S1-S3) and BW (S5-S6) under control (C, blue) and high salinity conditions (H, orange).** The median is represented by the middle line and the upper and lower end of the box show the 25th and 75th percentile (every site without an outlier). The whiskers either show the minimum and maximum range of the data or 1.5 times the interquartile range (approx. 2 standard deviations, every site with outliers). ° represents outliers and is defined as measurements that lie outside the whiskers, namely, more than 1.5 times the interquartile range away from the median. Note the different scaling of the y-axes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For β-alanine S1C: n=19, S1H: n=6, S2C: n=22, S2H: n=10, S3C: n=23, S3H: n=14, S5C: n=24, S5H: n=12, S6C: n=21, S6H: n=10. For myo-inositol S5C: n=3; S5H: n=3.

Supplemental Material

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