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# Sodium uptake in different life stages of crustaceans: the water flea *Daphnia magna* Strauss

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## SUMMARY

**The concentration-dependent kinetics and main mechanisms of whole-body $Na^+$ uptake were assessed in neonate and adult water flea *Daphnia magna* Strauss acclimated to moderately hard water (0.6 mmol $l^{-1}$ NaCl, 1.0 mmol $l^{-1}$ $CaCO_3$ and 0.15 mmol $l^{-1}$ $MgSO_4 \cdot 7H_2O$; pH 8.2). Whole-body $Na^+$ uptake is independent of the presence of $Cl^-$ in the external medium and kinetic parameters are dependent on the life stage. Adults have a lower maximum capacity of $Na^+$ transport on a mass-specific basis but a higher affinity for $Na^+$ when compared to neonates. Based on pharmacological analyses, mechanisms involved in whole-body $Na^+$ uptake differ according to the life stage considered. In neonates, a proton pump-coupled $Na^+$ channel appears to play an important role in the whole-body $Na^+$ uptake at the apical membrane. However, they do not appear to contribute to whole-body $Na^+$ uptake in adults, where only the $Na^+$ channel seems to be present, associated with the $Na^+/H^+$ exchanger. In both cases, carbonic anhydrase contributes by providing $H^+$ for the transporters. At the basolateral membrane of the salt-transporting epithelia of neonates, $Na^+$ is pumped from the cells to the extracellular fluid by a $Na^+,K^+$-ATPase and a $Na^+/Cl^-$ exchanger whereas $K^+$ and $Cl^-$ move through specific channels. In adults, a $Na^+/K^+/2Cl^-$ cotransporter replaces the $Na^+/Cl^-$ exchanger. Differential sensitivity of neonates and adults to iono- and osmoregulatory toxicants, such as metals, are discussed with respect to differences in whole-body $Na^+$ uptake kinetics, as well as in the mechanisms of $Na^+$ transport involved in the whole-body $Na^+$ uptake in the two life stages.**

Key words: crustacean, *Daphnia magna*, ion transport, life stage, $Na^+$ uptake.

## INTRODUCTION

Most aquatic crustaceans are marine species and only a few of them are able to hyper-osmoregulate their haemolymph in diluted seawater, i.e. to maintain a haemolymph osmotic concentration higher than that of the surrounding medium in diluted seawater. Furthermore, among those capable of performing haemolymph hyper-ionoregulation several of them are only weak regulators, being able to live for only a short time in freshwater. Most crab and shrimp species are in this group. Among the strong regulators, we can find some stenohaline freshwater crab and crayfish species.

In contrast to other invertebrates and fish (Kirschner, 2004), crustaceans living in freshwater present a somewhat different ionoregulatory picture (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). For freshwater fish, data available in the literature are sufficient to allow researchers to model the main routes of $Na^+$ and $Cl^-$ transport and the mechanisms involved in such processes (Kirschner, 2004; Evans et al., 2005). However, much less is known about the ion transport mechanisms in invertebrates. For example, ion-transporting epithelia (midgut and Malpighian tubules) in insects appears to be energized through a proton-motive force generated by a vacuolar-type proton ATPase (V-$H^+$-ATPase proton pump). However, secondary transport mechanisms that are coupled to the V-$H^+$-ATPase activity are not fully elucidated (Pullikuth et al., 2003). In crustaceans, models of ion transport in salt-transporting epithelia, especially gills, have also been generated over the last decade (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). However, ionoregulatory studies in freshwater crustaceans have been mainly limited to a few crayfish, crab and shrimp species, which because of their large size are convenient for *in vivo* experiments. In this regard, crayfish species are by far the most studied (Kirschner, 2004). However, very small cladocerans have proved suitable for radiotracer studies.

*Daphnia magna* Strauss is a small hyper-regulating freshwater cladoceran showing a vigorous, active NaCl uptake (Holm-Jensen, 1948; Stobbart et al., 1977; Potts and Fryer, 1979; Bianchini and Wood, 2002; Bianchini and Wood, 2003; Glover and Wood, 2005; Glover et al., 2005). This NaCl uptake is essential to counteract the continuous ion loss to the hypo-osmotic medium. This situation is aggravated in daphnids because they show a high surface to volume ratio. In fact, we have previously demonstrated that the sodium uptake rate is dependent on the body size and clearly determines the sensitivity of freshwater animals to ionoregulatory toxicants such as metals (Bianchini et al., 2002a; Grosell et al., 2002). Daphnids have been shown to be the most sensitive aquatic organisms to both waterborne copper and silver after either acute or chronic exposure (Ratte, 1999; Bianchini et al., 2002b; Grosell et al., 2002). Furthermore, the mechanisms of acute and chronic toxicity of these metals are associated with an alteration of the whole-body $Na^+$ concentration as a consequence of a metal-induced inhibition of the whole-body $Na^+,K^+$-ATPase activity (Bianchini and Wood, 2002; Grosell et al., 2002; Bianchini and Wood, 2003). The inhibition of the $Na^+$ uptake by $Ag^+$ is clearly competitive and $Ag^+$ does not affect whole-body $Cl^-$ levels (Bianchini and Wood, 2003), which suggests that different ionoregulatory effects occur in

freshwater crustaceans than in freshwater fish (Hogstrand and Wood, 1998; Wood, 2001; Evans et al., 2005). Taken together, these findings clearly indicate that the knowledge of the mechanisms of ion transport involved in the NaCl uptake in *D. magna*, the most metal-sensitive aquatic species, is essential to better interpret toxicity data and to have a good understanding of the mode of action of contaminant metals in the aquatic environment.

Toxicological data have also shown that the early-life stages of aquatic animals are the most sensitive to ionoregulatory toxicants such as metals (Ratte, 1999; Bianchini et al., 2002b; Grosell et al., 2002). In turn, physiological studies have clearly demonstrated that significant changes in the ability to cope with hypo-osmotic environments occur during the ontogenesis of the osmoregulatory organs in various crustacean groups, including cladocerans (daphnids), isopods, amphipods and decapods (crabs, lobsters, shrimps and crayfish) (Charmantier, 1998; Charmantier and Charmantier-Daures, 2001; Charmantier et al., 2002; Cieluch et al., 2004; Khodabandeh et al., 2005a; Khodabandeh et al., 2005b). The ontogenesis of the osmoregulation can occur at either the embryonic or the postembryonic phase (Charmantier and Charmantier-Daures, 2001).

According to Charmantier (Charmantier, 1998), crustaceans show three different patterns of ontogeny of osmoregulation. The first pattern is shown by weak osmoregulators, in which only small changes in the ability to osmoregulate during the course of development are seen. The true marine osmoconformers belong to this category. The second pattern is shown by crustaceans, in which the first postembryonic stage possesses the same osmoregulatory pattern as the adults. This category is represented by the freshwater-invading species. The third pattern is shown by species in which changes in the osmoregulatory pattern occur during their development, usually at or after metamorphosis. In this case, they shift from an osmoconforming to an osmoregulating response. This category is composed of the transitional species between the true marine osmoconformers and the very strongly regulating species (Charmantier, 1998).

Osmoregulation in daphnids has been extensively reviewed (Aladin and Potts, 1995). Briefly, eggs are incubated in brood chambers, which are closed in several species such as *D. magna*. Some species, such as *D. magna*, inhabit fresh or slightly saline waters. However, most of them live in marine and coastal waters. In both cases, osmolality of the embryonic fluid is isosmotic to the osmolality of the brood chamber fluid, which in turn is close to the osmolality of the haemolymph. This feature protects embryos until late in their development. Shortly before hatching, the brood chamber opens, and the emerging larvae are able to osmoregulate, since they have their osmoregulatory organs already developed. The osmoregulatory organs in daphnids include the neck organ, which in some cases is later replaced by epipodites, which have developed during the embryonic stages (Aladin and Potts, 1995).

It is clear from the toxicological and physiological findings described above that structural, biochemical and physiological changes could occur at the different life stages of crustaceans to cope with the challenges imposed by environmental salinity variations. Therefore, it is important to understand the possible ontogenetic changes in the mechanisms involved in ion-transporting mechanisms in daphnids, especially those associated with the $Na^+$ uptake, and link them to the differential sensitivity of the life stages to ionoregulatory toxicants, such as copper and silver.

Although it has long been known that whole-body $Na^+$ uptake is both active and occurs in a concentration-dependent, saturable manner in daphnids (Holm-Jensen, 1948; Stobbart et al., 1977; Potts and Fryer, 1979), there has been only one recent investigation on the actual mechanism(s) involved (Glover and Wood, 2005). This study used 7–8-day-old specimens of *D. magna* and provided evidence that the electrogenic $2Na^+/1H^+$ exchanger played an important role (Glover and Wood, 2005). In light of the above, the objectives of the present study were to use radiotracer and pharmacological techniques to characterize the mechanism(s) of transport involved in whole-body $Na^+$ uptake in *D. magna* of two size and age classes. Neonate animals (<24 h old) were compared with adults (7–8 days old), which were approximately sevenfold greater in body mass. Our results indicate that significant ontogenetic differences occur between the two age classes, and provide new information on the mechanisms of $Na^+$ transport in freshwater cladocerans.

## MATERIALS AND METHODS

### Daphnid maintenance

The water fleas *Daphnia magna* Strauss (ARO strain, Aquatic Research Organisms, Hampton, NH, USA) employed in the present study were obtained from an established laboratory culture. They were reared as previously described (Bianchini and Wood, 2002; Bowles et al., 2002; Bianchini and Wood, 2003; Bianchini et al., 2002a; Bianchini et al., 2002b; Bianchini et al., 2005). Briefly, the *Daphnia* colony was maintained in moderately hard synthetic freshwater, of which the ionic composition (0.6 mmol $l^{-1}$ NaCl, 1.0 mmol $l^{-1}$ $CaCO_3$ and 0.15 mmol $l^{-1}$ $MgSO_4 \cdot 7H_2O$; pH 8.2) was similar to the water from the Lake Ontario (Canada). Synthetic water used for all tests was prepared as a single batch employing 1000 l of reverse-osmosis-purified water in a food-grade polyethylene tank.

During the maintenance period, as well as during experiments, *D. magna* were fed algae (*Ankistrodesmus convolutus*; $1.82 \times 10^8$ cells $l^{-1}$, which is equal to 33 mg dry mass $l^{-1}$) and a mixture of a yeast–Cerophyll–trout chow slurry (YCT; 18.5 mg dry mass $l^{-1}$) on a daily basis.

Water was not aerated, but the experimental medium, including food, was renewed each day. Temperature and photoperiod were fixed at 20°C and 16 h:8 h L:D, respectively.

### Sodium uptake

To assess the possible influence of body mass on $Na^+$ uptake, neonate (<24 h) and adult (1 week) daphnids ($N$=48) were collected from the culture using plastic pipettes. They were randomly divided in three groups (16 daphnids per chamber) and tested in 50 ml glass beakers, as described below. Wet mass of all tested daphnids ranged from 0.050 to 3.700 mg. For the remaining experiments, daphnid size was selected in order to avoid the effect of body mass on sodium fluxes. Mean wet mass (± s.e.m.) of neonate (<24 h; $N$=48) and adult (1 week; $N$=48) daphnids used to determine the kinetic parameters of the whole-body $Na^+$ uptake was 0.181±0.014 and 3.279±0.197 mg, respectively. Mean wet mass (± s.e.m.) of neonate (<24 h; $N$=78) and adult (1 week; $N$=78) daphnids used to characterize the different mechanisms involved in the $Na^+$ uptake was 0.056±0.008 and 3.087±0.206 mg, respectively. In all cases, whole-body $Na^+$ uptake was measured using $^{22}Na$ (0.37 mBq $l^{-1}$, Amersham, specific activity 11.2 TBq $g^{-1}$ $Na^+$) as a radiotracer and measurements were done at 20°C.

Whole-body $Na^+$ uptake was determined as previously described (Bianchini and Wood, 2002; Bianchini and Wood, 2003; Glover and Wood, 2005). Briefly, neonate and adult daphnids were collected from the culture medium, quickly rinsed in deionized

water and transferred to a new glass beaker containing 50 ml of a simplified synthetic freshwater (0.6 mmol $l^{-1}$ NaCl and 0.5 mmol $l^{-1}$ $CaCl_2$; pH adjusted to 8.2 with KOH), which served as the basic test solution in all trials. Potassium concentration after adjusting pH was within the range found in the Lake Ontario [0.049–0.069 mmol $l^{-1}$ (NRCC, 1977)] and the tap water in Ontario province [0.001–0.512 mmol $l^{-1}$ (Health Canada, 2007)]. $^{22}Na$ was then added to this new experimental medium at a final specific activity of 1.85 kBq/μEq $Na^+$. Water samples for measurement of $^{22}Na$ radioactivity and total sodium were taken at 0 and 1 h, after which the test was ended. These samples were used for $^{22}Na$ radioactivity measurement using a Canberra-Packard MINAXI gamma counter, and for total sodium measurement using a Varian AA-1275 atomic absorption unit operated in flame emission mode. After the 1 h flux period, daphnids were collected using plastic pipettes, washed for 15 s in a concentrated (600 mmol $l^{-1}$) NaCl solution to displace loosely bound $^{22}Na$, blotted dry on filter paper, weighed on an electronic microscale (Mettler UMT2; 0.001 mg accuracy; Mettler-Toledo, Columbus, OH, USA), and transferred to plastic vials. The $^{22}Na$ radioactivity in the whole body was then measured as described for the water samples. $Na^+$ uptake rate was calculated based on the incorporation of $^{22}Na$ in the whole body during the 1 h flux period, the mean measured specific activity of the $^{22}Na$ in the water, the body mass of the animal, and the elapsed time, as previously described (Bianchini and Wood, 2002; Bianchini et al., 2002a; Bianchini and Wood, 2003; Grosell et al., 2002).

To determine the kinetic parameters of the whole-body $Na^+$ uptake, groups of daphnids ($N$=6 in each group) were exposed (1 h) to different NaCl levels (0.05 to 4.80 mmol $l^{-1}$) in the test water. Media were prepared adding NaCl to a 0.5 mmol $l^{-1}$ $CaCl_2$ solution to reach the desired $Na^+$ concentration and the pH of the solution was adjusted to 8.2 with KOH. The $Na^+$ uptake rates at the different $Na^+$ concentrations were calculated as described above. Kinetic parameters [Michaelis constant ($K_m$) and maximal velocity ($V_{max}$)] for sodium uptake in neonate and adult daphnids under control conditions were determined by means of nonlinear regression analyses (one-site binding), as previously described (Bianchini and Wood, 2003).

Several pharmacological tools were used to characterize the different mechanisms involved in the $Na^+$ uptake in daphnids. Considering that *D. magna* can tolerate and live in freshwater and brackish waters (Schuytema, 1997), the drugs tested were selected based on the mechanisms described to operate in salt-transporting epithelia of weak and strong hyperosmoregulator crustaceans. These mechanisms have been recently reviewed and modelled (Kirschner, 2004; Freire et al., 2007). In turn, concentrations of drugs tested in the present study were selected to fall within the range of concentrations showed to significantly inhibit the desired target mechanisms in weak and strong hyperosmoregulator crustaceans. Original studies reporting these concentrations have been extensively reviewed (Péqueux, 1995; Onken and Riestenpatt, 1998; Kirschner, 2004; Freire et al., 2007). Based on these considerations, the following enzyme inhibitors and ion channel and transporter blockers were added to the external water in separate tests: acetazolamide ($10^{-3}$ mol $l^{-1}$; carbonic anhydrase inhibitor); bafilomycin $A_1$ ($5\times10^{-7}$ mol $l^{-1}$; V-$H^+$-ATPase inhibitor); phenamil ($10^{-4}$ mol $l^{-1}$; $Na^+$ channel blocker); diphenylamine-2-carboxylate (DPC; $10^{-3}$ mol $l^{-1}$; $Cl^-$ channel blocker); amiloride ($10^{-3}$ mol $l^{-1}$; $Na^+/H^+$ exchanger); bumetanide ($10^{-3}$ mol $l^{-1}$; $Na^+/Cl^-$ and $Na^+/K^+/2Cl^-$ cotransporters blocker); furosemide ($10^{-3}$ mol $l^{-1}$; $Na^+/K^+/2Cl^-$ cotransporter blocker); thiazide ($10^{-3}$ mol $l^{-1}$; $Na^+/Cl^-$ cotransporter blocker); di-isothiocyanostilbene-2,2′ disulfonic acid (DIDS; $10^{-3}$ mol $l^{-1}$; $Cl^-/HCO_3^-$ exchanger blocker); and 2,4,6-triaminopyrimidine (TAP; $10^{-3}$ mol $l^{-1}$; paracellular pathway blocker).

All drugs used were purchased from Sigma (St Louis, MI, USA), except the bafilomycin $A_1$, which was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). They were previously dissolved in dimethylsulfoxide (DMSO) such that the final DMSO concentration in the test solution was 1%. All drugs were added to the external water in separate tests. For each test, daphnids were pre-exposed for 15 min to the specific blocker or inhibitor, prior to the addition of $^{22}Na$ to start the flux measurement. Pre-exposure was carried out in the same test solution and the drug remained present during the 1 h flux measurement. In control treatments, whole-body $Na^+$ uptake was similarly measured in the presence of 1% DMSO to rule out a possible effect of this chemical on the whole-body $Na^+$ influx. Also, some tests were carried out in the absence of $Cl^-$ (replaced by gluconate) in the test solution to check for possible $Cl^-$ dependence of the whole-body $Na^+$ uptake.

Data from pharmacological studies were expressed as mean ± s.e.m. ($N$=6). Significant differences between treatments were assessed by analysis of variance (ANOVA) followed by the *a posteriori* Tukey's test. The significance level adopted was 95%.

## RESULTS

### Kinetics of whole-body $Na^+$ uptake

The whole-body $Na^+$ uptake rate was inversely related to the body mass over a wide range of body mass (0.050–3.700 mg). The slope of this relationship on a log–log plot was –0.305 (Fig. 1).

The kinetics of whole-body $Na^+$ uptake rate followed a typical Michaelis–Menten relationship for both neonates and adults. However, a marked difference was observed between the two life stages. The maximum capacity for sodium transport ($J_{max}$) on a mass-specific basis was twofold higher in neonates (Fig. 2A) than in adults (Fig. 2B). Also, the $K_m$ constant for $Na^+$ was 2.5-fold higher (i.e. transport affinity was lower) in neonates (Fig. 2A) than in adults (Fig. 2B).

### Effect of ion channel and transporter blockers on whole-body $Na^+$ uptake

In both neonate and adult daphnids, exposure to 1% DMSO did not induce significant changes in the whole-body $Na^+$ uptake. Also, no

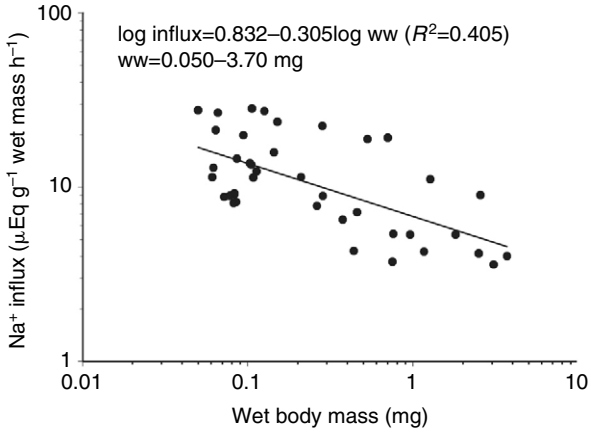


Fig. 1. Influence of wet body mass (ww) on mass-specific $Na^+$ uptake rate (influx) in the water flea *Daphnia magna*. Note that both axes are logarithmic.

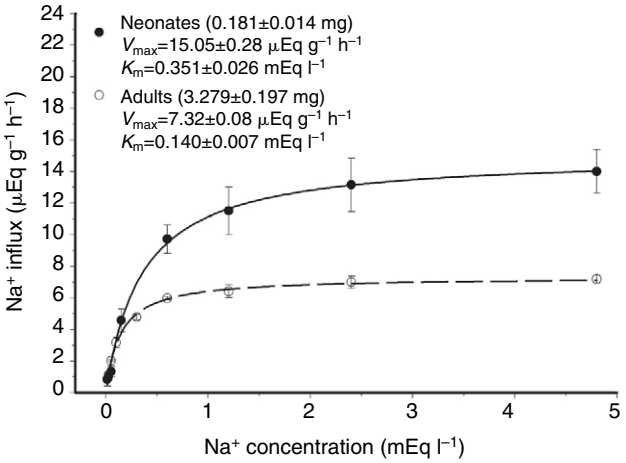


Fig. 2. Kinetics of mass-specific $Na^+$ uptake rate in neonate and adult water flea *Daphnia magna* as a function of sodium concentration. Data are expressed as mean ± 1 s.e.m. ($N$=6). Kinetic parameters (Michaelis constant [$K_m$] and maximal velocity [$V_{max}$]) for sodium uptake in neonate and adult daphnids were determined by means of nonlinear regression analyses (one-site binding), as previously described (Bianchini and Wood, 2003).

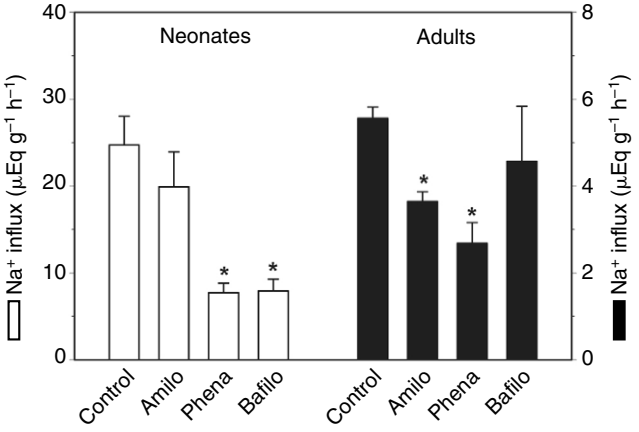


Fig. 4. Whole-body $Na^+$ uptake rate in neonate and adult water flea *Daphnia magna* after pre-exposure to amiloride (Amilo, $10^{-3}$ mol l$^{-1}$), phenamil (Phena, $10^{-4}$ mol l$^{-1}$) or bafilomycin $A_1$ (Bafilo, $5\times10^{-7}$ mol l$^{-1}$). Data are expressed as mean ± 1 s.e.m. ($N$=6). Note the different axis scale for neonate (left) and adult (right) daphnids.

significant changes in the whole-body $Na^+$ uptake were observed either in the absence of $Cl^-$ (replaced by gluconate; Fig. 3). However, several drugs had a significant inhibitory effect on the $Na^+$ uptake in both neonate and adult daphnids, but the life stages differed significantly from each other in their profile of sensitivity to various inhibitors. Results from the pharmacological studies and the ontogenetic differences observed will be outlined below, in light of the current models of ion transport in weak and strong hyperosmoregulator crustaceans. These models have been recently proposed based on structural, biochemical, pharmacological and physiological studies (Kirschner, 2004; Freire et al., 2007).

In weak hyperosmoregulators, both transcellular and paracellular transepithelial transport of $Na^+$ have been reported. It has been shown that $Na^+$ absorption occurs *via* the paracellular pathway, and that an apical $Na^+/H^+$ exchanger and a $Na^+/K^+/2Cl^-$ cotransporter

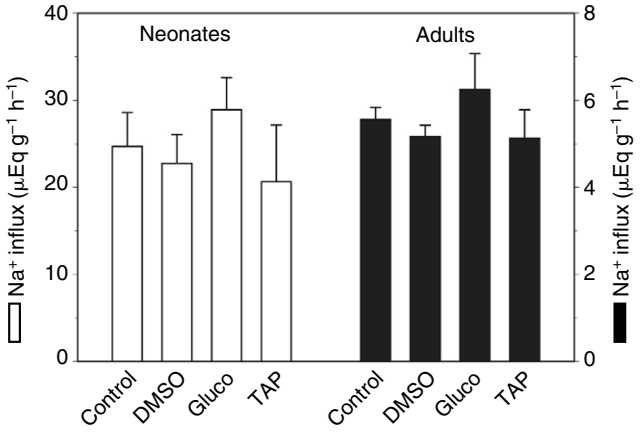


Fig. 3. Whole-body $Na^+$ uptake rate in neonate and adult water flea *Daphnia magna* after pre-exposure to 1% dimethylsulfoxide (DMSO), in the absence of $Cl^-$ (replaced with equimolar gluconate) in the external medium (Gluco) or after pre-exposure to 2,4,6-triaminopyrimidine (TAP, $10^{-3}$ mol l$^{-1}$). Data are expressed as mean ± 1 s.e.m. ($N$=6). Note the different axis scale for neonate (left) and adult (right) daphnids.

are implicated in transcellular $Na^+$ uptake from the external medium by the salt-transporting epithelia. On the basolateral side of these epithelia, a $Na^+/K^+$ pump uses the energy resulting from the $Na^+/K^+$-ATPase activity and pumps the intracellular $Na^+$ into the haemolymph. In the present study, we did not test the effect of ouabain, a well-known inhibitor of the $Na^+/K^+$-ATPase activity, since high specific whole-body $Na^+/K^+$-ATPase activities have been already reported in both neonate and adult *D. magna* (Bianchini and Wood, 2002; Bianchini and Wood, 2003). However, TAP ($10^{-3}$ mol l$^{-1}$) was employed to block a possible paracellular entry of $Na^+$, whereas amiloride ($10^{-3}$ mol l$^{-1}$) was used to target the $Na^+/H^+$ exchanger. In the presence of TAP, no significant change in whole-body $Na^+$ uptake was observed in either neonate or adult daphnids (Fig. 3). By contrast, a significant decrease (34%) in whole-body $Na^+$ uptake was observed after exposure to amiloride in adult daphnids, but not neonates (Fig. 4).

Regarding the $Na^+/K^+/2Cl^-$ cotransporter, both furosemide ($10^{-3}$ mol l$^{-1}$) and bumetanide ($10^{-3}$ mol l$^{-1}$) were used in the present study to target this mechanism. The use of furosemide is more common in pharmacological studies, but bumetanide was also employed because of its higher effectiveness, at least in mammalian tissues (O'Grady et al., 1987). However, bumetanide at the concentration used can also block the $Na^+/Cl^-$ cotransporter. Therefore, thiazide ($10^{-3}$ mol l$^{-1}$) was tested to identify a possible inhibition of both $Na^+/K^+/2Cl^-$ and $Na^+/Cl^-$ cotransporters by bumetanide. It is important to note that thiazide has been shown to block the $Na^+/Cl^-$ cotransporter, but not the $Na^+/K^+/2Cl^-$ exchanger. In the present study, furosemide was without significant effect on whole-body $Na^+$ uptake in either neonate or adult daphnids (Fig. 5). However, whole-body $Na^+$ uptake significantly decreased after exposure of neonates (61%) or adults (53%) to bumetanide. Furthermore, a significant decrease (53%) in whole-body $Na^+$ uptake was observed in neonates exposed to thiazide but no significant effect of this drug was observed in adult daphnids (Fig. 5).

In strong hyperosmoregulators, $Na^+$ uptake from the external medium occurs *via* apical $Na^+$ channels associated with a V-$H^+$-ATPase. As observed in weak hyperosmoregulators, $Na^+$ is also

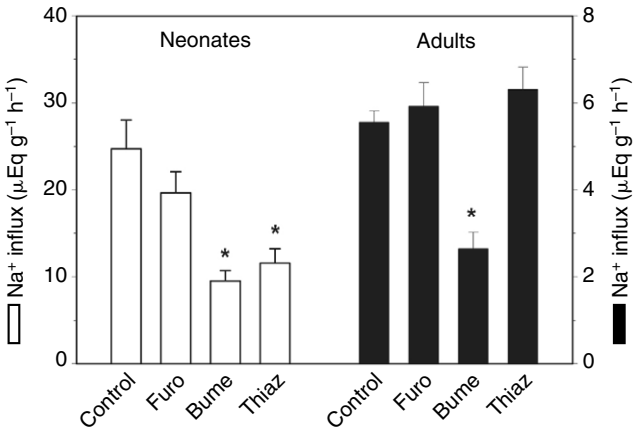


Fig. 5. Whole-body $Na^+$ uptake rate in neonate and adult water flea *Daphnia magna* after pre-exposure to furosemide (furo, $10^{-3}$ mol $l^{-1}$), bumetanide (bume, $10^{-3}$ mol $l^{-1}$) or thiazide (thiaz, $10^{-3}$ mol $l^{-1}$). Data are expressed as mean ± 1 s.e.m. (*N*=6). Note the different axis scale for neonate (left) and adult (right) daphnids.

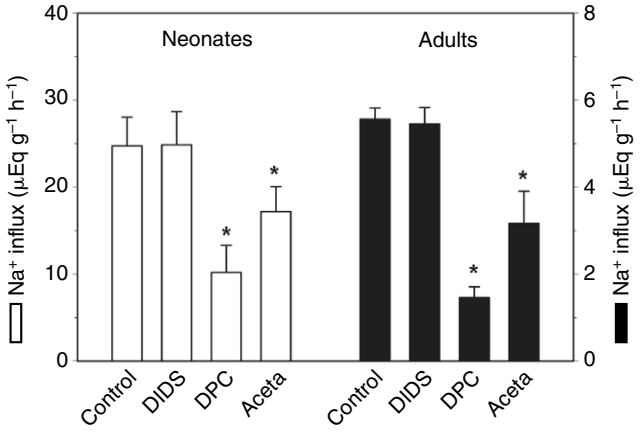


Fig. 6. Whole-body $Na^+$ uptake rate in neonate and adult water flea *Daphnia magna* after pre-exposure to 4,4′ diisothiocyanostilbene-2,2′ disulfonic acid (DIDS, $10^{-3}$ mol $l^{-1}$), diphenylamine-2-carboxylate (DPC, $10^{-3}$ mol $l^{-1}$) or acetazolamide (Aceta, $10^{-3}$ mol $l^{-1}$). Data are expressed as mean ± 1 s.e.m. (*N*=6). Note the different axis scale for neonate (left) and adult (right) daphnids.

pumped from the cell into the haemolymph through a basolateral $Na^+/K^+$ pump (Kirschner, 2004; Freire et al., 2007). As explained above, the effect of ouabain on the whole-body $Na^+$ uptake was not tested in the present study. However, phenamil ($10^{-4}$ mol $l^{-1}$) and bafilomycin $A_1$ ($5\times10^{-7}$ mol $l^{-1}$) were used to block the apical $Na^+$ channels and inhibit the V-$H^+$-ATPase activity, respectively. In neonate daphnids, a significant decrease in the whole-body $Na^+$ uptake was observed after exposure to either phenamil (69%) or bafilomycin $A_1$ (68%; Fig. 4). In adult daphnids, a significant decrease (52%) in whole-body $Na^+$ uptake was also observed, but only for phenamil (Fig. 4).

Regarding $Cl^-$ transepithelial transport, the $Cl^-/HCO_3^-$ exchanger and $Cl^-$ channels seem to be involved in this process at the apical and basolateral membrane, respectively. This picture is similar in both weak and strong hyperosmoregulators (Kirschner, 2004; Freire et al., 2007). In the present study, DIDS ($10^{-3}$ mol $l^{-1}$) and DPC ($10^{-3}$ mol $l^{-1}$) were employed to target the $Cl^-/HCO_3^-$ exchanger and $Cl^-$ channels, respectively. DIDS was used instead of SITS (stilbene isothiosulphonic acid) because the whole-body $Na^+$ uptake in *D. magna* was previously reported to be independent of the $Cl^-$ concentration in the external medium (Glover and Wood, 2005). DPC exposure induced significant decreases in whole-body $Na^+$ uptake in both neonate (59%) and adult (74%) daphnids. However, DIDS was without significant effect in both life stages (Fig. 6).

Finally, gill carbonic anhydrase has been implicated in the $H_2CO_3$ formation through $CO_2$ hydration, generating both $H^+$ and $HCO_3^-$, which serve as counterions for the exchange of $Na^+$ and $Cl^-$, respectively (Kirschner, 2004; Freire et al., 2007). In the present study, acetazolamide ($10^{-3}$ mol $l^{-1}$), a specific inhibitor of carbonic anhydrase, was employed to inhibit the enzyme activity. This drug significantly inhibited the whole-body $Na^+$ uptake in both neonate (30%) and adult (43%) daphnids (Fig. 6).

## DISCUSSION

In the present study, we used radiolabelling techniques combined with pharmacological tools to assess the kinetic characteristics and mechanisms of transport involved in the $Na^+$ uptake in free-swimming *Daphnia magna*. We considered only measurements of whole-body $Na^+$ uptake due to the very small size of daphnids, and therefore cannot partition transport to the various structures that might be involved.

$Na^+$ uptake rate, and therefore probably $Na^+$ turnover, is inversely related to the body mass in *D. magna* over a wide range (0.050–3.700 mg; Fig. 1). This relationship can be explained by the fact that, in freshwater fish and crustaceans, the body mass-specific area of the salt-transporting organs, especially the gills, is also inversely related to body mass (Hughes and Morgan, 1973; Santos et al., 1987). Note that the slope (–0.305) obtained for the log–log relationship between the whole-body $Na^+$ uptake and body mass is very close to that (–0.328) observed when several aquatic species were considered (Grosell et al., 2002). Furthermore, this slope is similar to that expected for other surface-dependent physiological processes in aquatic invertebrates, such as respiration (Barnes et al., 1993). Thus, these findings clearly indicate that small daphnids would be more sensitive than larger ones to environmental toxicants with ionoregulatory actions. In fact, a significant and highly negative correlation was observed between the body mass and metal (Cu and Ag) sensitivity in several aquatic species (Grosell et al., 2002), including *D. magna* at different life stages (Bianchini et al., 2002a). In addition to the dependence on size, sensitivity to metals within daphnids could be also associated with structural, biochemical, and/or physiological ontogenetic differences, as discussed below.

Transport kinetics data indicate that in both neonate and adult daphnids whole-body $Na^+$ uptake follows a typical Michaelis–Menten saturation curve (Fig. 2). This feature is similar to that described for other freshwater crustaceans, such as the crayfish *Astacus pallipes* (Shaw, 1959). However, a marked difference was observed between the two life stages. Both the maximum capacity for sodium transport ($J_{max}$) and the affinity constant for sodium transport ($K_m$) were much higher in neonates than in adults. Based on the mean sizes of the two groups of daphnids used to determine the kinetic parameters of the whole-body $Na^+$ uptake and the relationship shown in Fig. 1, the whole-body $Na^+$ uptake rates are 11.44 and 4.73 μEq $g^{-1}$ $h^{-1}$ for the neonates (0.181 mg) and adults (3.279 mg) used, respectively. These data show that the lower affinity (higher $K_m$) for $Na^+$ in neonates is compensated by a higher

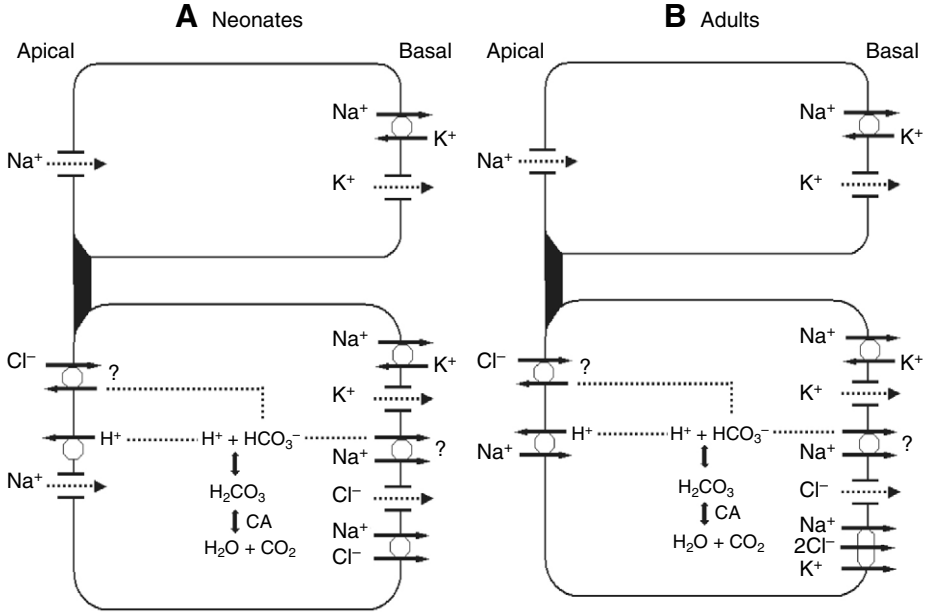


Fig. 7. Functional models of ion transport mechanisms involved in whole-body $Na^+$ uptake in neonate (A) and adult (B) water flea *Daphnia magna*. CA, carbonic anhydrase. Solid and broken lines indicate transport and diffusion, respectively. ? indicates the need for more data to confirm or not the presence of the mechanism of transport.

maximum capacity of $Na^+$ uptake. It is possible that these differences occur because the same mechanisms of $Na^+$ uptake are present in neonates and adults, but with biochemically mediated (e.g. intracellular modulators) or structurally mediated differences (e.g. diffusion access distances), or that the mechanism(s) of $Na^+$ uptake is (are) fundamentally different in neonates and adults. Notably, ontogenetic changes seen in hyper-regulating crustaceans are generally associated with anatomical and/or structural changes in the ion-transporting cells and organs (Charmantier and Charmantier-Daures, 2001; Charmantier et al., 2002; Cieluch et al., 2004; Khodabandeh et al., 2005a; Khodabandeh et al., 2005b).

Our experimental approach compared the $Na^+$ uptake kinetics in neonate and adult daphnids, as well as employed pharmacological tools to evaluate whether there were differences in the fundamental mechanisms of whole-body $Na^+$ uptake between these two life stages. At this point, it is important to note that the experimental approach generally used for crayfish, the most studied stenohaline freshwater crustacean with regard to the mechanisms of ion transport, almost exclusively involves intact animals, whereas in crabs, the gill perfusion and the split-gill lamella mounted in Ussing chamber are the techniques most commonly used (Kirschner, 2004; Freire et al., 2007). Therefore, results obtained in the present study for daphnids are more directly comparable to those described for crayfish than for crabs.

Data from kinetic experiments performed in the present study show that $Na^+$ uptake is saturable in both neonate and adult *D. magna* (Fig. 2). A similar saturable $Na^+$ uptake has been previously reported in *D. magna*, but only for adults (Stobbart et al., 1977; Potts and Fryer, 1979; Glover and Wood, 2005). This finding indicates that a specific transport mechanism or mechanisms is/are involved in $Na^+$ uptake in both life stages. In this context, it is important to note that $Na^+$ uptake in *D. magna* is dependent on pH, reducing as pH decreases (Potts and Fryer, 1979; Glover and Wood, 2005). Therefore, it seems that sodium uptake may be linked to proton excretion in *D. magna*. Furthermore, in agreement with a previous report for adult daphnids (Glover and Wood, 2005), no significant effect on whole-body $Na^+$ uptake was observed in both neonate and adult daphnids when $Cl^-$ was completely replaced by gluconate in the external medium (Fig. 3). This finding indicates that the whole-body $Na^+$ uptake in both neonate and adult daphnids is not dependent on $Cl^-$ concentration in the external medium, at least at $Na^+$ levels typical of those occurring in natural freshwater. This finding is similar to that reported for other crustaceans adapted to freshwater, such as the Chinese mitten crab *Eriocheir sinensis* (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). Furthermore, the $Na^+$ uptake characteristics shown by daphnids are quite similar to those reported for the freshwater crayfish *Astacus pallipes*, i.e. whole-body $Na^+$ influx in this species is also independent of the ambient $Cl^-$ concentrations, but dependent on the external $Na^+$ concentration, following typical Michaelis–Menten kinetics (Shaw, 1959).

The fact that TAP, a well-known blocker of paracellular permeability in salt-transporting epithelia, did not affect the whole-body $Na^+$ influx (Fig. 3) clearly indicates that the $Na^+$ uptake in both neonate and adult daphnids is mainly occurring through the transcellular pathway. In fact, transcellular $Na^+$ influx has been widely reported to occur in gills, i.e. the main organ involved in ionic and osmotic regulation in freshwater and euryhaline crustaceans such as shrimp, crab and crayfish (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007).

Regarding a possible involvement of a $Na^+/H^+$ exchanger in the whole-body $Na^+$ uptake in *D. magna*, a significant amiloride-sensitive whole-body $Na^+$ uptake was observed in adult daphnids (Fig. 4). This result is in complete agreement with those previously reported for adult daphnids (Glover and Wood, 2005). Regarding the putative elements of a $Na^+/H^+$ (or $NH_4^+$) exchanger in crustaceans, they have been reported for freshwater and euryhaline crabs, as well as for freshwater crayfish (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). As performed here, most studies have employed pharmacological tools to demonstrate the presence of this mechanism in aquatic crustaceans. For example, amiloride, at the same concentration tested in daphnids in the present study ($10^{-3}$ mol $l^{-1}$), also inhibited the whole-body influxes of both $Na^+$ and $H^+$ without significant effects on the whole-body $Na^+$ efflux in salt-depleted crayfish (Kirschner et al., 1973; Ehrenfeld, 1974). Furthermore, several lines of evidence from studies on whole-body $Na^+$ and $H^+$ fluxes, as well as the effect of external $NH_4^+$ concentration on these fluxes, also suggested that a $Na^+/H^+$ exchanger may play an important role in whole-body $Na^+$ uptake in freshwater crayfish (Shaw, 1960a; Shaw, 1960b; Kirschner, 2002).

Based on their findings, Glover and Wood (Glover and Wood, 2005) suggested that a putative electrogenic $2Na^+/1H^+$ exchanger would be involved in the whole-body $Na^+$ uptake in adult *D. magna*. In fact, invertebrate epithelial cells from different tissues, including crustacean gills, posses an electrogenic brush-border $2Na^+/1H^+$ antiporter protein that is analogous to the vertebrate electroneutral $1Na^+/1H^+$ exchanger. This electrogenic antiporter is also sensitive to

amiloride, but performs an extensive array of transport functions because of its electrogenic nature and wide substrate range, involving both monovalent and divalent cations (Ahearn and Franco, 1990; Ahearn et al., 1994; Zhuang et al., 1995; Ahearn, 1996; Ahearn et al., 1999; Ahearn et al., 2001; Mandal et al., 2003; Pullikuth et al., 2003). In this case, a $2Na^+/1H^+$ system would be expected to yield a sigmoidal relationship of $Na^+$ uptake rate with water $Na^+$ concentration because of co-operativity effects. However, a typical hyperbolic Michaelis–Menten kinetic relationship was observed in both neonates and adults in the present study, as well as for adults in the study by Glover and Wood (Glover and Wood, 2005). This finding suggests that at low $Na^+$ concentrations, as observed in most freshwaters, a $1Na^+/1H^+$ exchanger would be operating at the apical surface of the salt-transporting epithelia in daphnids. This electroneutral exchange is also reported to operate in different invertebrate salt-transporting epithelia, such as the Malpighian tubules of mosquitoes (Weng et al., 2003) and gills of freshwater and euryhaline crabs (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). In turn, the electrogenic exchange has been implicated in maintaining the alkaline lepidopteran or mosquito midgut lumen or the acidic lumen of crustaceans (Pullikuth et al., 2003). In fact, the prevalence of electrogenic cation–proton exchange in invertebrates has been viewed as an ancestral mechanism, whereas the electroneutral exchange occurring in mammals is considered an evolutionary adaptation (Grinstein and Wieczoreck, 1994; Ahearn et al., 2001).

Despite the clear inhibitory effect of amiloride on the $Na^+$ uptake in adult daphnids, no significant effect was observed in neonates (Fig. 4). This differential response of neonate and adult daphnids to amiloride could be associated with different cuticle properties, which hinder the drug from approaching the apical membrane of ion-transporting epithelia. It has been demonstrated that the crustacean cuticle has ion-selective properties (Avenet and Lignon, 1985; Lignon and Lenoir, 1985), which would certainly affect the local water chemistry next to the salt-transporting epithelia, thereby also affecting the kinetics (e.g. $K_m$) of whole-body ion uptake. Otherwise, we need to consider that fundamentally different mechanisms of ion transport are operating in the different life stages. This second hypothesis is clearly supported by the data from our pharmacological studies. Whole-body $Na^+$ uptake was inhibited by bafilomycin $A_1$ (Fig. 4) and thiazide (Fig. 5) only in neonates, whereas amiloride had a significant inhibitory effect only in adults (Fig. 4).

If we assume that a $Na^+/H^+$ exchanger is not operating in the whole-body $Na^+$ uptake in neonate daphnids, based on the lack of effect of amiloride, another possibility is that the $Na^+$ influx could be driven across the apical membrane by the activity of a V-$H^+$-ATPase proton pump. Arguing in favour of this hypothesis, a clear inhibition (~50%) of the whole-body $Na^+$ uptake was observed in the present study in neonate daphnids (Fig. 4) after pre-exposure to either phenamil, a well-known inhibitor of epithelial $Na^+$ channels, or bafilomycin $A_1$, a potent and specific inhibitor of the V-$H^+$-ATPase. Taken together, these findings indicate that a V-$H^+$-ATPase associated with an epithelial $Na^+$ channel is involved in the whole-body $Na^+$ uptake in neonate daphnids. This enzyme has been shown to be also present in gills of both freshwater and euryhaline crabs (Onken and Putzenlechner, 1995; Tresguerres et al., 2003; Weihrauch et al., 2004), as well as in freshwater crayfish (Putzenlechner, 1994; Zare and Greenaway, 1998; Zetino et al., 2001). In insects, the V-$H^+$-ATPase proton pump also plays a pivotal role in generating a proton-motive force that energizes the ion-transporting epithelia (midgut and Malpighian tubules) (Pullikuth et al., 2003).

The fact that a similar effect of phenamil, but not of bafilomycin $A_1$, was seen in adult daphnids (Fig. 4) suggests that they are probably not expressing V-$H^+$-ATPase or else are expressing this enzyme only at low levels, but the epithelial $Na^+$ channel is still present and operating. In this context, an ATP-driven $Na^+$ influx in neonates would be in agreement with the higher transport capacity (higher $J_{max}$) for $Na^+$ showed by neonate daphnids. Furthermore, as observed in other aquatic species, neonate daphnids exhibit higher $Na^+$ turnover rates than adults because of their higher mass-specific area for exchange with the environmental medium (Bianchini et al., 2002a), thus requiring a better efficiency for $Na^+$ uptake. In addition, a consequently higher metabolism would be occurring (Baillieul et al., 2005), requiring a higher capacity of $H^+$ extrusion across the apical membrane of salt-transporting epithelia in neonates.

In summary, the $Na^+$ influx across the salt-transporting epithelia of neonate daphnids seems to occur through an epithelial $Na^+$ channel, powered by an active proton extrusion *via* a V-type $H^+$-ATPase, which would provide the required electrical gradient for $Na^+$ uptake across the apical membrane against a concentration gradient, as observed in the ion-transporting epithelia of insects (Pullikuth et al., 2003) and gills of freshwater-adapted crabs (Kirschner, 2004; Freire et al., 2007). By contrast, the $Na^+$ influx across the salt-transporting epithelia of adult daphnids seems to involve the $Na^+/H^+$ exchanger, as observed in freshwater crayfish and brackish water-acclimated crabs (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). In both cases, carbonic anhydrase would be providing the $H^+$ needed for the ion transport operation, as observed in freshwater crayfish (Ehrenfeld, 1974; Kirschner, 2004; Freire et al., 2007). This statement is based on the fact that acetazolamide, a well-known inhibitor of carbonic anhydrase (CA), induced a significant decrease in the $Na^+$ influx in both neonate and adult daphnids (Fig. 6). In fact, CA is the enzyme responsible for $H_2CO_3$ formation from $CO_2$ hydration, generating both $H^+$ and $HCO_3^-$ in gills of aquatic invertebrates (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007).

Regarding the ion movements across the basolateral membrane of the salt-transporting epithelia in daphnids, $Na^+$ would be driven by an ATP-dependent sodium–potassium pump ($Na^+/K^+$-ATPase), as observed in salt-transporting epithelia of insects (Pullikuth et al., 2003) and several freshwater and brackish crustaceans (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007). This statement is based on the fact that high specific whole-body $Na^+/K^+$-ATPase activities have been reported in both neonate and adult *D. magna* (Bianchini and Wood, 2002; Bianchini and Wood, 2003). Thus, a putative pump-and-leaky system would transport $Na^+$ from the intracellular to the extracellular fluid across the basolateral membrane with $K^+$ being transported in the opposite direction at the expense of ATP. In turn, intracellular $K^+$ would diffuse from the intracellular to the extracellular fluid through specific $K^+$ channels, following an electrochemical gradient generated by the $Na^+/K^+$-ATPase pump. The diffusive movement of $K^+$ would generate a local electric gradient enough to drive the $Cl^-$ from the intracellular to the extracellular fluid through specific $Cl^-$ channels across the basolateral membrane of the salt-transporting epithelia. This hypothesis is supported by the fact that a marked and significant inhibition of the whole $Na^+$ uptake was observed after exposure of both neonate and adult daphnids to DPC (Fig. 6), a known specific inhibitor of $Cl^-$ channels. Furthermore, evidence for the existence of epithelial $Cl^-$ channels in intact crustaceans has already been reported in the literature (Zetino and Kirschner, 1993). Notably, the absence of an effect of DIDS in either life stage suggests that a basolateral $Na^+/HCO_3^-$ cotransport system is not involved in $Na^+$ uptake, in contrast to recent models in insects (Pullikuth et al., 2003) and freshwater fish (Perry et al., 2003; Scott et al., 2005). However, further studies should be performed to better elucidate this point,

since a DIDS-insensitive $Na^+/HCO_3^-$ cotransport system has been reported to occur in the basolateral membrane of different mammalian tissues (Aickin, 1994; Odgaard et al., 2003). Despite the absence of and effect of DIDS on whole-body $Na^+$ uptake in neonate and adult daphnids, it is also possible that the $HCO_3^-$ generated by the carbonic anhydrase would diffuse from the intracellular fluid to the surrounding medium through a $Cl^-/HCO_3^-$ exchanger. This idea is based on two facts: an apical $Cl^-/HCO_3^-$ exchanger has been widely reported in ion-transporting epithelia of both freshwater and euryhaline crustaceans (Péqueux, 1995; Kirschner, 2004; Freire et al., 2007); and a $Na^+$-independent $Cl^-/HCO_3^-$ exchanger resistant to inhibition by DIDS has been identified and cloned in the apical membranes of mammalian ion-transporting epithelia (Alper, 1991; Godinich and Jennings, 1995; Romero and Boron, 1999; Soleimani and Burnham, 2000; Royaux et al., 2001; Tsuganezawa et al., 2001; Barmeyer et al., 2007). Therefore, future studies should be performed to verify the possible expression and localization of this kind of $Cl^-/HCO_3^-$ exchanger in daphnids.

In addition to the ATP-dependent $Na^+$ transport driven by the $Na^+/K^+$ pump ($Na^+/K^+$-ATPase) at the basolateral membrane, other mechanisms of $Na^+$ transport seem to be involved as well. The significant inhibition of the whole-body $Na^+$ uptake induced by bumetanide in both neonate and adult daphnids (Fig. 5) strongly suggest that, at least part of the $Na^+$ influx could be associated with the action of a $Na^+/K^+/2Cl^-$ cotransporter in both life stages. However, a significant inhibition of the whole-body $Na^+$ uptake after exposure to thiazide was only observed in neonate daphnids (Fig. 5). The combination of these results indicate that in neonates a $Na^+/Cl^-$ cotransporter would play an important role in the whole-body $Na^+$ uptake whereas in adults the mechanism probably involves the $Na^+/K^+/2Cl^-$ cotransporter. The clear lack of effect of the absence of $Cl^-$ in the external medium on the whole-body $Na^+$ uptake in both neonate and adult daphnids (Fig. 3) strongly suggests that these mechanisms are in fact located at the basolateral membrane and are involved in extrusion of $Na^+$ from the intracellular fluid to the extracellular fluid. In turn, the lack of effect of furosemide on $Na^+$ uptake in both life stages of daphnids (Fig. 5) could be explained by a possible lower sensitivity of the salt transporting epithelia to furosemide than to bumetanide, as observed in some salt-transporting epithelia in mammals (O'Grady et al., 1987). A bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter has been also suggested to operate in the basolateral membrane of Malpighian tubules of insects (Pullikuth et al., 2003).

Functional models of the schemes outlined above for neonate and adult daphnids are depicted in Fig. 7, based on the findings described in the present study. These models do not necessarily mean that all $Na^+$ transport is occurring at only one kind of salt-transporting epithelial cell. In fact, it has been reported that the epithelial cells of the epipodites are those involved in the osmoregulation in *D. magna* (Goldmann et al., 1999), and that two kinds of epithelial cells, dark and light types, are alternately arranged in this 'gill' of *D. magna* (Kikuchi, 1983). Thus, it could be possible that the different mechanisms of ion transport involved in the whole-body $Na^+$ uptake in *D. magna*, identified in the present study, are partitioned into the two different kinds of cells (dark and light cells). This picture would be very similar to that described for the amphibian skin, where αMR cells are associated with principal cells (Kirschner, 2004).

Taken together, the findings reported here clearly suggest that differences in whole-body $Na^+$ uptake kinetics, as well as in the mechanisms of $Na^+$ transport involved in the whole-body $Na^+$ uptake in neonate and adult *D. magna* could be the physiological basis for the differential sensitivity of these two life stages to iono- and osmoregulatory toxicants, such as metals (Bianchini et al., 2002a; Grosell et al., 2002).

The higher sensitivity of neonates could be explained by the lower affinity of the $Na^+$ uptake mechanisms in this life stage. This lower affinity for $Na^+$ would favour the ionoregulatory toxicants, such as $Cu^+$ and $Ag^+$, in the competition for the binding sites for $Na^+$ at the ion-transporting epithelia of daphnids. In fact, we found that the inhibition of whole-body $Na^+$ uptake by $Ag^+$ is clearly competitive in *D. magna* (Bianchini and Wood, 2003). This higher competition associated with the higher maximum capacity for sodium transport ($J_{max}$) observed in neonates would lead to a higher whole-body accumulation of the toxicant in individuals of this life stage. In fact, we have reported a highly significant negative correlation (slope around 0.4) between body mass of *D. magna* and whole-body silver accumulation, which was directly related to the whole-body $Na^+/K^+$-ATPase inhibition induced by $Ag^+$ (Bianchini and Wood, 2003). At this point, its important to stress that inhibition of the whole-body $Na^+$ uptake induced by $Ag^+$ is directly associated with the metal-induced inhibition of the whole-body $Na^+/K^+$-ATPase activity, constituting the physiological basis of both $Cu^+$ and $Ag^+$ toxicity in *D. magna* (Bianchini and Wood, 2002; Grosell et al., 2002; Bianchini and Wood, 2003).

In addition to the increased metal accumulation associated with the features described above, the major mechanism controlling $Na^+$ uptake in neonate daphnids, i.e. the epithelial $Na^+$ channel associated with the V-type $H^+$-ATPase, could be also favouring the metal accumulation in this life stage. This statement is based on the fact that silver, probably as $Ag^+$, has been demonstrated to enter the branchial epithelial cells *via* the $Na^+$ channel coupled to the proton ATPase in the apical membrane of freshwater fish (Bury and Wood, 1999). On the other hand, the absence of a V-type $H^+$-ATPase and the expression of a $Na^+/H^+$ exchanger in the apical membrane of adult daphnids seem to be at the basis of a lower metal accumulation rate and a consequently lower sensitivity of adult daphnids to metal exposure.

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