Iodine accumulation in sea urchin larvae is dependent on peroxide

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Symbols/Abbreviations: AIT- apical iodide transporter, ASW- artificial seawater, BLAST- basic local alignment search tool, CHT- choline transporter, CPM- counts per minute, CSSWV- cathodic stripping square wave voltammetry, DIDS- diisothiocyanate-2,2’-stilbenedisulfonic acid, dpi- days post-induction, DPP- differential pulse polarography, FSW- filtered synthetic seawater, H_2O_2- hydrogen peroxide, hpi- hours post-induction, I^- iodide, IO_3^- iodate, KI- potassium iodide, Na- sodium, NIS- sodium iodide symporter, NMDG- N-methyl-D-glucamine, PDD- hydrogen peroxide dependent diffusion, RCF- relative centrifugal force, ROS- reactive oxygen species, SSSF5- sodium/solute symporter family, THs- Thyroid hormones
SUMMARY:
Iodine has many important biological functions and its concentrations vary with environment. Recent research has provided novel insights into iodine uptake mechanisms in marine bacteria and kelp through hydrogen peroxide dependent diffusion (PDD). This mechanism is distinct from sodium dependent mechanisms known from vertebrates. In vertebrates, iodine accumulates in the thyroid gland by the action of the apical iodide transporter (AIT) and the sodium iodide symporter (NIS). Neither of these proteins has, thus far, been identified outside of the chordates and PDD (as an iodine uptake mechanism) has never been studied in animals. Using $^{125}$I as a marker for total iodine influx, we tested iodine uptake via sodium dependent transport versus PDD in embryos and larvae of the sea urchin *Strongylocentrotus purpuratus*. We found that iodine uptake in *S. purpuratus* is largely independent of NIS/AIT. Instead we found that uptake is dependent on the presence and production of hydrogen peroxide indicating that sea urchin larvae use PDD as a mechanism for iodine acquisition. Our data, for the first time, provide conclusive evidence for this mechanism in an animal. Furthermore, our data provide preliminary evidence that sodium dependent iodine uptake via active transporter proteins is a synapomorphy of vertebrates.

INTRODUCTION
Iodine is an important trace element with functions in many biological processes. For example, iodine is essential for the synthesis of thyroid hormones (THs), a group of developmental and metabolic hormones that are produced in the vertebrate thyroid gland (Zoeller et al., 2007). Iodine also functions independently of THs, predominantly through its action as an inorganic antioxidant (Küpper et al., 2008; Aceves et al., 2005; Berking et al., 2005; Shrivastava et al., 2006). This element occurs in several oxidative states within aqueous environments. These include di-iodine ($I_2$; oxidation state: 0), hypiodous acid ($HIO$; oxidation state: +1), iodide ($I^-$, oxidation state: -1) and iodate ($IO_3^-$, oxidation state: +5). The latter two have been shown to be most prevalent in seawater (Wong, 1991). For simplicity we use ‘iodide’ for $I^-$ and ‘iodine’ for any other form of the element.

The concentration of iodine can vary drastically between fresh and seawater ecosystems, with average values ranging from 39.4 nanomol l$^{-1}$ in surface freshwater to
453.5 nanomol l⁻¹ in surface seawater (Fuge and Johnson, 1986). Such variation in iodine availability likely affects mechanisms of iodine acquisition and its retention in aquatic organisms. The latter has been documented in teleost fish. In some species of teleost for example, iodine has been shown to enter the organism through food (Moren et al., 2008) and by diffusion across gill surfaces (Hunn and Fromm, 1966). Furthermore, data suggests that even in freshwater environments where the concentration gradient favors movement of iodine out of the organism, iodine can be actively retained within the tissue (Blanton and Specker, 2007). The same can be said for marine environments [e.g. marine copepods can contain iodine concentrations of ~94 millimol l⁻¹ (Moren et al., 2006)].

Iodine transport has been best characterized in the thyroid gland of vertebrates. Specifically, two members of the sodium solute symporter family 5 (SSSF5) coordinate the uptake of iodine through the basolateral and the apical plasma membrane of thyrocytes before it accumulates in the follicular lumen. These two SSSF5 proteins are the sodium iodide symporter (NIS; SLC5A5) and the apical iodide transporter (AIT; SLC5A8) (Eskandari et al., 1997; Lacroix et al., 2004). These transporter proteins that share 70% sequence similarity on the amino acid level, are both inhibited by potassium perchlorate, which competitively blocks their iodide transport site (Rodriguez et al., 2002). While NIS is an active transporter protein for iodine, AIT moves iodine passively along its concentration gradient (Eskandari et al., 1997; Lacroix et al., 2004).

Ecdysozoans such as the fruit fly (Drosophila melanogaster) do not appear to possess orthologs of this transporter, based on BLAST searches and sequence analysis (Campbell et al., 2004) despite the fact that the SSSF5 is widespread across both prokaryotes and eukaryotes (Wright and Turk, 2004). However, NIS orthologs have been reported in cephalochordates (Paris et al., 2008) and urochordates (Campbell et al., 2004), suggesting that this gene is restricted to Chordates.

Elemental iodine, in the form of iodide, has been shown to function as an antioxidant in Cnidarians (Aurelia aurita) and in Laminaria (Berking et al., 2005; Küpper et al., 2008) and similar functions have been hypothesized in human physiology (reviewed by Venturi and Venturi, 2009; Aceves et al., 2005; Shrivastava et al., 2006). With most reactive oxygen species (ROS) iodine is more reactive than other halogens such as bromide and chloride (Luther et al., 1995; Küpper et al., 2008). However, the
reaction of iodide with hydrogen peroxide (H$_2$O$_2$) occurs slowly in the absence of an enzyme (Luther et al., 1995) and the presence of peroxidases have been shown to greatly accelerate this reaction as iodide can act as an electron donor to scavenge H$_2$O$_2$ (Luther et al., 1995; Smyth, 2003). This reaction has also been implicated in peroxide dependent diffusion (PDD) that has been documented for marine bacteria (Amachi et al., 2007) and kelp (Küpper et al., 1998). In both taxa, iodide from seawater is oxidized extracellularly by haloperoxidases, using H$_2$O$_2$ as a substrate. The product of this reaction has been suggested to be hypoiodous acid (HIO) (Amachi et al., 2007). Due to its reactivity it is however unlikely that HOI can cross the membrane and further work is required to identify the chemical form of iodine which is transported through the membrane.

Neither NIS/AIT nor PDD have been systematically investigated as iodine uptake mechanisms in any marine invertebrate species. Here we characterize iodine acquisition in several stages of the purple sea urchin (Strongylocentrotus purpuratus). Previous work on sea urchin larvae has demonstrated iodine and TH metabolism in this group (Chino et al., 1994; Johnson and Cartwright, 1996; Saito et al., 1998; Heyland et al., 2004; Heyland and Hodin, 2004; Heyland et al., 2006). Specifically, developmental effects of THs and TH synthesis have been documented. Furthermore, the genome of the purple urchin (Sodergren et al., 2006) reveals genes orthologous to TH synthesis, metabolism and signaling genes from mammalian species (Heyland et al., 2011). Our results provide conclusive evidence for PDD in sea urchin larvae and while sodium dependent uptake cannot be ruled out as a mechanism in iodine uptake, it appears to be less important.

MATERIALS AND METHODS:

Animals

Adult S. purpuratus (Stimpson 1857) were purchased from the Cultured Abalone (Goleta, California) and were maintained in filtered synthetic seawater (Instant Ocean® Aquarium Systems Inc., Mentor, OH, USA) in conditions that mimic their natural environment – i.e., at 12°C on a 8:16 light: dark cycle- at the Hagen Aqualab, the University of Guelph and were fed rehydrated Laminaria spp. (Kombu) once a week (purchased from http://www.canadiankelp.com). Experimental larvae were reared across ten 1 male x 1 female crosses from May 2011 – July 2012. Adults were spawned by
agitation (Emlet, 1986). Eggs were collected by inversion into 0.2 micromol l\(^{-1}\) filtered Instant Ocean® seawater (FSW) and sperm was collected dry. Eggs were washed three times and fertilized at 12°C. Embryos were reared to the 6-arm larval stage and to competent stages in 1800ml of FSW at 12°C. The water was changed three times a week at which time they were fed a diet of *Isochysis galbana* (Parke 1949) (12000 cells ml\(^{-1}\)) and *Dunaliela teritolecta* (Butcher 1959) (6000 cells ml\(^{-1}\)).

To observe potential changes in iodide influx during the metamorphic transition, larvae were reared to metamorphic competence (approximately 8 weeks post-fertilization) and were exposed to biofilm to induce metamorphosis. Larvae were incubated in 8 ml of seawater in dishes that had been exposed for 6 weeks to sweater from the adult rearing tanks and contained a layer of biofilm. After an overnight incubation, juveniles were collected and any individuals that did not undergo metamorphosis were also collected and kept separately.

**Iodine uptake experiments**

Iodine influx experiments were conducted in artificial seawater (ASW: 470.57 millimol l\(^{-1}\) NaCl, 275.90 millimol l\(^{-1}\) MgSO\(_4\).7H\(_2\)O, 7.75 millimol l\(^{-1}\) CaCl\(_2\).H\(_2\)O, 26.56 millimol l\(^{-1}\) Cl\(_2\)Mg.6H\(_2\)O, 10.05 millimol l\(^{-1}\) KCl, 2.5 millimol l\(^{-1}\) NaHCO\(_3\), 0.84 millimol l\(^{-1}\) KBr, 0.01 millimol l\(^{-1}\) SrCl\(_2\).6H\(_2\)O, 0.04 millimol l\(^{-1}\) H\(_3\)BO\(_3\)) at 35ppt, pH = 8 and 14°C unless otherwise specified.

Iodine in the seawater used for the incubation experiments was measured by cathodic stripping square wave voltammetry (CSSWV) and differential pulse polarography (DPP), respectively, using a Metrohm 746VA Trace Analyzer (Herring and Liss, 1974; Campos, 1997). Trace amounts of iodine from salts used in artificial seawater added up to 100 nanomol l\(^{-1}\) based on this method. Therefore this was the concentration of iodide in ASW unless otherwise specified.

When observing iodine influx at fertilization, eggs were spawned into ASW at 12°C containing 269.25 micromol l\(^{-1}\) of sodium-ampicillin (Fisher, Fair Lawn, NJ) and washed three times in this medium. This antibiotic was used to decrease the populations of any marine bacteria that may potentially incorporate iodine and therefore confound the results. Ampicillin was removed prior to fertilization with a further wash in ASW. Dry-collected sperm was activated in ASW and fertilization success was >90%.
Larvae used for iodine incorporation experiments were starved overnight in ASW to allow the larvae to clear any live algal cells from their stomach; 269.25 micromol l⁻¹ of sodium-ampicillin was included to limit bacterial growth. Larvae were transferred into 70 micrometer cell-strainers (Vacutainer Labware Medical, Product number 32350 Franklin Lakes, NJ), the water was drained and strainers were moved into new, sterile ASW before treatment.

**Iodine influx experiment protocol**

Pharmacology experiments were conducted on fertilized eggs, 6-arm stage larvae (approximately 3-weeks post-fertilization) and metamorphically competent larvae and juveniles. The large majority of experiments were performed on 6-arm stage larvae as our preliminary work determined that later stages are easier to handle than embryos in the uptake assays. In these experiments, 50 larvae were used per replicate and ~350 eggs per replicate.

Eggs and larvae were pulse centrifuged to a maximum speed of 3000 relative centrifugal force (rcf; Eppendorf Benchtop Centrifuge, Model 5430) as this was found to be effective in concentrating larvae without damaging them. The ASW supernatant was replaced with the treatment media for pre-incubation, if necessary. 37kBq ml⁻¹ of Na-¹²⁵I (Perkin Elmer, Woodbridge ON) in ASW (¹²⁵ASW) (Klebanoff et al., 1979) was used per exposure and each replicate was incubated in 0.5 ml of ¹²⁵ASW. After ¹²⁵ASW incubation, eggs/larvae were pelleted by centrifugation (3000 rcf) and washed with ice-cold ASW containing 1 millimol l⁻¹ potassium iodide (Sigma, Oakville ON) until the supernatant activity was at background levels (<30 counts per minute (cpm)). Samples were kept on ice during the washes. Activity per replicate (represented as cpm after a 60 second measurement time) was measured using Perkin Elmer Wizard2 Automatic Gamma counter (Model #2470).

**Kinetics of iodine influx**

To assess the kinetics of iodine influx, influx rate was measured as a function of time and iodine concentration. For time, we measured influx at 2.5, 5, 10, 20, 40, 80, and 160 minutes in ASW containing 2000 nanomol l⁻¹ potassium iodide (KI). For iodine concentration we measured influx for potassium iodide ranging from 100 nanomol l⁻¹ to 1800 nanomol l⁻¹. The initial influx rate was determined from the initial slopes (occurring
between 2.5 and 10 minutes) of the transport kinetic curves. The initial influx rate from
the time experiment revealed an optimal influx rate at 5 minutes of exposure in $^{125}\text{ASW}$,
though a 2.5-minute incubation did provide a detectable signal. We also tested the effects
of temperature and pH on larval iodide influx. For temperature, larvae were exposed to
4°C, 14°C and 21°C $^{125}\text{ASW}$ and the initial influx rate was measured. For assaying the
effect of pH, larvae were exposed to one of four treatments, pH 8, pH 7.5, pH 7, and pH
6.5 for 2.5 minutes in $^{125}\text{ASW}$.

To determine the effect of metamorphosis on iodine uptake, competent larvae
were induced to undergo metamorphosis using naturally grown biofilm and the iodine
influx rate was measured on these stages. Competent larvae and induced individuals (i.e.
those exposed to biofilm) were incubated for two hours in ASW containing 269.25
micromol l$^{-1}$ of sodium-ampicillin prior to the iodine uptake experiment. The experiment
had four developmental groups with three replicates per group and 10 individuals per
replicate: competent larvae (uninduced), pre-competent (induced but did not
metamorphose), juveniles 8-hours post induction (hpi), juveniles 10 days post induction
dpi. Replicates were incubated for 30 minutes in $^{125}\text{ASW}$ and were washed and
measured as above.

**Sodium independent iodide uptake mechanisms**

To test the involvement of PDD on iodine influx, H$_2$O$_2$, reducing agents,
peroxidase inhibitors, a metabolic inhibitor and anion channel inhibitors were tested. This
addressed several components of the PDD model. Firstly the effect of H$_2$O$_2$ in larval
iodine influx is assessed through addition of this chemical. Secondly, specific
involvement of H$_2$O$_2$ produced by the larvae was tested through the use of reducing
agents that scavenge this molecule. In the PDD model proposed by Amachi et al.
(2007) a peroxidase is needed to react hydrogen peroxide with environmental iodide, as
this reaction does not occur readily abiotically. Therefore, if this model applies to sea
urchin larvae, iodide and H$_2$O$_2$ must react with a peroxidase, which was tested through
the use of peroxidase inhibitors. The final part of this model is that iodine must diffuse
into the organism through a channel and appropriate chemical inhibitors would block this.

Eggs of *S. purpuratus* release ~32 micromol l$^{-1}$ of H$_2$O$_2$ during fertilization
(Foerder et al., 1978) and we tested several H$_2$O$_2$ (Fisher) concentrations within this
The effects of ascorbate (Sigma), catalase (Sigma), aminotriazole (Sigma) and thiourea (Sigma) on iodide influx were examined. We also tested the effects of 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid (DIDS) and the metabolic inhibitor, cyanide, which were both obtained from Sigma.

To further test the role of H₂O₂ in larval iodine influx, we induced oxidative stress using the insecticide paraquat (Ultra Scientific, Kingston, RI). Larvae were pre-incubated for 30 minutes in ASW containing one of five treatments, 0 millimol l⁻¹ paraquat (control), .1 millimol l⁻¹ paraquat, 1 millimol l⁻¹ paraquat, 10 millimol l⁻¹ paraquat and 10 millimol l⁻¹ paraquat + 100 micromol l⁻¹ ascorbate (rescue). They were then exposed to ¹²⁵I-containing ASW for 5 minutes to observe paraquat’s effect on initial influx rate.

**Sodium dependent iodide uptake mechanisms**

The effect of potassium perchlorate (KClO₄) was tested at three concentrations: 1 micromol l⁻¹, 10 micromol l⁻¹ and 100 micromol l⁻¹. Larvae were pre-incubated in ASW+KClO₄ (Sigma St. Louis, USA) for 10 minutes prior to the addition of ¹²⁵ASW. Furthermore we assessed the effect of decreasing sodium availability. Sodium was replaced with the osmolyte N-methyl-d-glucamine (NMDG, Acros Organics, New Jersey, USA), but osmolality was maintained at 1000 milliOsmols (as measured by the Vapro© vapor pressure osmometer). The sea water consisted of: 486 micromol l⁻¹ NaCl and/or NMDG+HCl (pH=8), 10 micromol l⁻¹ KCl, 30 micromol l⁻¹ MgSO₄, 26 micromol l⁻¹ MgCl, 2.5 micromol l⁻¹ KHCO₃, 10 micromol l⁻¹ CaCl₂, 10 micromol l⁻¹, HEPES, and 1 micromol l⁻¹ EDTA (Beltran et al., 1996). Five sodium concentrations were used: 467 micromol l⁻¹ (control), 431.98 micromol l⁻¹, 396.95 micromol l⁻¹, 361.93 micromol l⁻¹ and 326.9 micromol l⁻¹. Larvae were incubated in ¹²⁵Treatment-ASW for 2.5 minutes, without pre-incubation. A second experiment was conducted in ASW without radioactive iodine to determine the effect of low sodium environments on survivorship. The effect of the media on mortality was assessed through observation of mobility, muscle contraction, and ciliary beating under a dissecting scope.

**Statistical analysis**

Influx rate was calculated by Eqn 1 from the modified equation from (Chang et al., 1997) and is expressed as femtomoles larvae⁻¹ hour⁻¹:

\[
J_{in} = \frac{Q_{in}}{X_{out} \cdot t_{inc}}
\]
\( J_{in} \) is the net influx of iodine (femtomoles individual\(^{-1}\) hour\(^{-1}\)), \( Q_{larvae} \) is the radioactivity of larvae or eggs (cpm individual\(^{-1}\)), \( X_{out} \) is the specific activity of the incubation water (cpm femtomole\(^{-1}\) I\(^{-1}\)) and \( t_{inc} \) is the incubation time (hours).

Pharmacological results were analyzed using SPSS (IBM). The effects of various inhibitors on iodine influx were analyzed using ANOVA with a Fisher’s LSD post hoc after an initial test for normal distribution using Shapiro-Wilk test for normality. This is with the exception of DIDS, where a t-test was used. Regression analysis was conducted on data testing the effect of KI concentration on influx rate.

**Phylogenetic analysis of the Sodium/Solute symporter family**

Human protein sequences were used in a basic local alignment search tool (BLAST) search for putative orthologs for members of SSSF5, specifically a tblastn with an e-value cut off at 1 e-04. These BLAST searches were carried out on the NCBI site (www.ncbi.nlm.nih.gov/) for orthologs of the human sodium glucose transporter 2 (SLC5A2), NIS (SLC5A5), choline transporter (CHT; SLC5A7), AIT (SLC5A8) and the sodium monocarboxylate transporter 2 (SMCT; SLC5A12). Representative sequences were obtained for: *Homo sapiens* (Hs), *Danio rerio* (Dr), *Branchiostoma florididae* (Bf), *Ciona intestinalis* (Ci), *Strongylocentrotus purpuratus* (Sp), *Saccoglossus kowalevski* (Sk), *Drosophila melanogaster* (Dm) and *Nematostella vectensis* (Nv), *Pan troglodytes* (Pt), *Canis lupus* (Cl), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Bos taurus* (Bt), *Gallus gallus* (Gg). For the full list of the 109 sequences used see Appendix A. These sequences were then aligned in SeaView and a PhylMyl tree was created using an LG matrix (Gouy et al., 2010).

**RESULTS**

**Kinetics of iodide uptake in S. purpuratus**

Incubation in \(^{125}\)I-containing ASW demonstrated that larvae of *S. purpuratus* acquire iodide directly from the environment in the absence of food (Fig. 1A). When larvae were incubated in ASW containing 2000 nanomol l\(^{-1}\) potassium iodide (KI), the iodide accumulation plateaus after 40 minutes and the initial influx rate (where the accumulation is linear) occurs within the first 10 minutes at this iodine concentration. We also found that with increasing iodine concentration (100 nanomol l\(^{-1}\) to 1800 nanomol l\(^{-1}\)
KI) the influx rate does not decrease (adj $R^2 = 0.709$, $p<0.001$, Fig. 1B). Furthermore, this influx follows a linear pattern within the environmentally relevant range (100 nanomol l$^{-1}$ to 800 nanomol l$^{-1}$ KI).

The effect of pH and temperature were tested in order to assess additional base parameters of the uptake process. For example, temperature dependence indicates the involvement of a metabolic process. Influx that is dependent on pH suggests that a specific oxidation form of iodide is translocated across the membrane as this abiotic variable has been known to influence marine iodine chemistry. Temperature had a significant impact on iodide influx ($F_{3,3} = 5.79$, $p<0.05$) where influx at 21°C was significantly higher than at 4°C (Fig. 1C). Across the range tested, pH had a non-significant effect on iodide influx ($F_{3,3} = 3.46$, $p>0.05$).

There appears to be a significant effect of metamorphosis on iodine influx in sea urchins ($F_{3,2} = 10.67$, $p<0.05$; Fig. 1D). There does not appear to be an effect of biofilm exposure on uptake as individuals induced with biofilm but did not metamorphose exhibited the same influx rates as competent un-induced individuals. The effect on iodide influx appears to be a result in change in life stage and not solely a result of exposure to biofilm as juveniles 8 hour post-induction have statistically similar iodide influx rates as juveniles 10 days post-induction (Fig. 1D).

**Sodium-independent iodide uptake mechanisms**

We found that the initial iodine influx rates of 6-arm stage larvae increase linearly with the addition of exogenous hydrogen peroxide (H$_2$O$_2$) from 0 micromol l$^{-1}$ to 30 micromol l$^{-1}$ with a non-significant decrease at 40 micromol l$^{-1}$ in 100 nanomol l$^{-1}$ iodine environment ($F_{4,3} = 7.22$, $p<0.005$; Fig 2A). Similarly, we were able to demonstrate a significant increase in iodide influx associated with fertilization in sea urchin eggs ($F_{3,3} = 31.26$, $p<0.001$, Fisher’s LSD, $p<0.001$; Fig. 2B).

Iodine incorporation at fertilization is inhibited by the addition of 100 micromol l$^{-1}$ ascorbate, a reducing agent that scavenges available hydrogen peroxide ($F_{3,3} = 31.26$, Fisher’s LSD, $p<0.001$; Table 1). When 6-arm stage larvae were exposed to an identical treatment, there was also a decrease in initial iodine influx rates, though not as pronounced as in fertilized eggs ($F_{4,3} = 6.575$, $p<0.05$, Fisher’s LSD; Table 1). We found that exposure to catalase, another consumer of hydrogen peroxide, also leads to a
reduction of iodine influx rates in larvae (F_{4,3}=6.575, p<0.05, Fisher’s LSD, p<0.05), but
not in eggs (F_{3,3}= 31.26, Fisher’s LSD, p>0.05; Table 1).

Paraquat induces oxidative stress through the production of superoxide by redox
cycling \textit{in vivo} (Bus and Gibson, 1984). This induced oxidative stress in larvae resulted in
a significant increase in the initial influx rate in 6-arm stage larvae (F_{4,3}=6.06, p<0.01; Fig. 3B). However, when 100 micromol l^{-1} ascorbate is added to the paraquat treatment,
the influx rate is not significantly different to the control (Fisher’s LSD, p >0.05; Fig. 3B).

As previously mentioned, the reaction between iodide and H_{2}O_{2} generally
requires a peroxidase to catalyze this reaction (Luther et al., 1995). We examined the
effects of thiourea and aminotriazole, two peroxidase inhibitors (Wood and Legg, 1970;
Davidson et al., 1979), on iodide accumulation. We found a statistically significant
decrease in larval iodine influx with increasing thiourea concentration (from 1-1000
nanomol l^{-1}) during a 30-minute incubation (F_{4,2} = 6.16, p<0.05; Table 1). Fertilized eggs
exposed to thiourea experience a dose dependent decrease in iodine influx as was
observed in larvae (F_{3,2} = 147.65, p<0.001; Table 1). Aminotriazole significantly disrupts
the initial iodide influx in 6-arm stage larvae (F_{3,3} = 6.88, p<0.01; Table 1).

In order to test the energy dependence of iodine uptake we exposed larvae at the
6-arm stage to cyanide and found that cyanide is a strong inhibitor of iodine influx with
increasing concentrations (F_{3,3}=13.226, p<0.01; Table 1). To test the involvement of
anion channels in the uptake process we exposed 6-arm larvae to DIDS and found a
significant decrease in iodine influx (t_{3}= 5.2, p<0.01, Table 1).

\textbf{Sodium-dependent iodide uptake mechanisms}

Decreasing sodium availability in the environment had no impact on iodine influx
in 6-arm stage larvae (F_{4,3}=2.12, p>0.05). Furthermore, larvae exposed to potassium
perchlorate, an inhibitor of NIS/AIT transport, did not experience any inhibition of iodine
influx rate (F_{3,3}=1.163 p>0.05, Table 1). There was no permanent effect of exposure to
reduced sodium seawater on larval motility and survivorship (results not shown).

\textbf{Phylogenetic analysis of the sodium/solute symporter family 5}

Our phylogeny based on an Amino LG matrix determined that for the 109
proteins included in this analysis, there are three major clades, each supported with a
Bootstrap value of 100% (Fig. 3A). There was one Nematostella sequence outside of these three major clades (Nv-XP_001621852.1) and it was not included in Fig. 3A, but is visible in Appendix B. Clade I, the Na-Anion transporter-like proteins, are a group of vertebrate and invertebrate proteins related to the vertebrate NIS, AIT and SMCT. Clade II, Na-glucose transporter-like proteins, cluster around SLC5A2, the vertebrate sodium glucose transporter 2. Clade III, the Na-Choline Transporter (CHT)-like proteins, contain several invertebrate representatives that group near the vertebrate CHT. Furthermore, Clade I contains Clade I.1- NIS/AIT/SMCT–like proteins. The vertebrate NIS, AIT and SMCT proteins are found within this group and the rest of this clade consists of only deuterostome representatives of the SSSF5 (Fig. 3B).

Outside of Clade I.1, in the Na-Anion transporter-like proteins group, the non-vertebrate proteins are clustered into two groups. The first Clade I.2 which consists of representatives from all of the taxa under examination, with the exception of vertebrates, supported by 96% Bootstrap. Sister to this is Dm II, a clade containing ten sequences from Drosophila (Appendix B).

**DISCUSSION**
Iodine uptake has not been studied in detail for non-chordate animals despite the fact that iodine uptake, function and metabolism have been described across a wide range of taxa (reviewed by Eales, 1997; Heyland et al., 2005; reviewed by Miller and Heyland, 2010). In contrast to the relatively well-described sodium-transporter dependent iodine uptake mechanisms in chordates, recent studies suggest that sodium-transporter independent mechanisms such as peroxide dependent diffusion (PDD) are critically important for iodine uptake and regulation in macroalgae, bacteria and microalgae (Küpper et al., 1998; Amachi et al., 2007). Here we investigated iodine uptake mechanisms in larvae of the purple sea urchin *Strongylocentrotus purpuratus* and tested whether iodine uptake in planktonic larvae of this species is dependent on a transporter system such as the sodium/iodide symporter (NIS) or diffusion based and dependent on peroxide.

**Initial iodide influx rates in sea urchin larvae suggest diffusion-based mechanism for iodine accumulation.**

Iodine concentration and its chemical composition (iodide and iodate being the most common) in seawater vary significantly between habitats and geographic regions. For example while average total inorganic iodine is 400-500 nanomol l⁻¹ (Fuge and Johnson, 1986), iodide in marine surface waters range from <62 nanomol l⁻¹ near the poles, to 230 nanomol l⁻¹ in tropical and subtropical environments (Wong, 1991). Kinetic data from our radioactive iodide accumulation assays strongly indicate that iodide influx is not likely transporter dependent, as the initial iodide influx rate does not saturate with increasing iodide concentrations within the range of 100 – 1800 nanomol l⁻¹ of iodide. Furthermore sea urchin larvae accumulate iodide at concentrations well above natural levels in what is presumably their habitat as planktotrophic larvae (i.e. surface waters of the Pacific) before they become competent and settle into their adult habitat. In surface water iodide occurs at approximately 78 nanomol l⁻¹ (Tsunogai, 1971). Since the larval uptake capacity greatly exceeds the availability of unbound iodide, sea urchin larvae are able to efficiently accumulate iodine regardless of its environmental availability.

This is similar to what was observed in Atlantic Halibut (*Hippoglossus hippoglossus* L.). Here, the accumulation of iodide is uninhibited by increasing environmental concentrations, such that even in media containing excessive iodine (much
higher than would be present in the natural environment), the larval influx does not plateau (Moren et al., 2008). NIS seems to be involved in this process, however as only half of the iodide uptake is inhibited by perchlorate, perchlorate-independent uptake through ingestion was proposed for early stages lacking functional gills (Moren et al., 2008). For these larvae, dietary sources of iodine seem to be critical based on the following evidence: the high iodine concentrations in their copepod prey (estimated to be an average of 94 mM; Moren et al., 2006), their extensive absorption capacity to take up iodide from seawater (Moren et al., 2008), and the positive effect dietary iodine has on their development and survivorship (Hamre et al., 2002).

This information raises the general question about how important food sources are for providing iodine to aquatic organisms. Planktonic invertebrate larvae, including feeding larvae of the purple sea urchin, spend a considerable amount of time in the water column feeding on microalgae. Previous work including recent data from our lab has demonstrated that microalgae species that larvae feed on have the capacity to accumulate iodine and potentially metabolize it to other organic forms such as thyroid hormones (Chino et al., 1994; Heyland et al., 2006; van Bergjik pers comm.). By feeding on algae, sea urchin larvae may receive iodine in a much more concentrated form. Furthermore, if larvae receive TH precursors or the active hormones themselves, these can affect their metabolism during development and metamorphosis (Chino et al., 1994; Heyland, 2004; Heyland et al., 2004; Heyland and Moroz, 2005; Heyland and Moroz, 2006; Heyland et al., 2006).

Cyanide acts by interrupting the electron transport chain (by blocking cytochrome oxidase activity) and by limiting the availability of ATP (Gensemer, 2006). Unlike the uptake system observed in marine bacteria, the metabolic inhibitor cyanide significantly disrupts sea urchin iodide influx. In bacteria, a 1 millimol l⁻¹ treatment results in a ~30% decrease in iodide influx (Amachi et al., 2007) while in sea urchin larvae this cyanide concentration results in a >90% reduction in iodide uptake. While this is not an ultimate test of whether this process is active or passive, it is a good indicator of energy dependence. Therefore, we conclude that although iodide uptake in sea urchin larvae is largely through a transporter-independent mechanism it does appear to require ATP.

It is apparent that iodide influx is significantly higher post-metamorphosis
regardless of whether or not the larvae had been treated with biofilm and regardless of
how long the individual has lived as a juvenile (8 hours post-induction vs. 10 days post-
induction). This indicates that juveniles have a significantly higher iodide uptake capacity
than late-stage larvae of comparable size. Changes in transport kinetics as a result of
developmental transitions have been documented before in *S. purpuratus* with regards to
integumental uptake of dissolved organic carbon (Allemand et al., 1984; Davis et al.,
1985; reviewed in Wright and Manahan, 1989). However, how the increase in iodine
uptake rate with metamorphosis relates to life history transitions and the subsequent
changes in environment (i.e. movement from the pelagic to the benthic environment) in *S.
purpuratus* is unclear as iodine concentrations is lower in marine surface water (0-200m)
and increases with depth (>200 m) (Truesdale, 1978). Future studies tracing iodine in
larval and metamorphic stages may be useful in determining whether changes in iodine
metabolism can explain the differences in uptake rate.

**Hydrogen peroxide affects iodide influx in sea urchin larvae**

While NIS/AIT-dependent iodide uptake has been best characterized in the
vertebrate thyroid gland, it is not the only known mechanism responsible for iodine
uptake. Peroxide dependent diffusion (PDD) has recently been described in some detail
for both bacteria and macroalgae. In a marine *Flavobacteriaceae* bacteria strain (Amachi
et al., 2007) and *Laminaria* kelp (Küpper et al., 1998) peroxide oxidizes iodine via a yet
unidentified haloperoxidase; hypoiodous acid (HIO) is speculated to be the form of
iodine produced by this system (Amachi et al. 2007). For *Flavobacteriaceae* (bacteria) it
has been further shown that the peroxide released into the extracellular environment
originates from glucose oxidase activity in the cell membrane (Amachi et al., 2007).

Our data strongly suggest that a peroxide-dependent mechanism is at least
partially involved in iodine uptake in sea urchin larvae. This conclusion is substantiated
by several experimental findings from our study. When larvae are exposed to peroxide
and paraquat (a producer of intracellular peroxide), iodine accumulation increased
significantly. We were also able to demonstrate that iodine accumulation levels decrease
in response to treatment of larvae with hydrogen peroxide scavengers (ascorbate and
catalase) and peroxidase inhibitors (thiourea and aminotriazole). Furthermore, while we
examined cyanide activity in the context of metabolism, this chemical does also have
inhibitory effects on some peroxidases (e.g. Klebanoff et al., 1979). These pharmacology
data indicate that hydrogen peroxide is produced by larval tissue and is critical for iodine
uptake. Furthermore, in normal development, sea urchin embryos experience an oxidative
burst that is associated with a sudden release of hydrogen peroxide at fertilization. This
ultimately leads to the formation of the fertilization envelope, through ovoperoxidase
mediated tyrosine cross-linking and acts as a slow block to polyspermy (Foerder et al.,
1978; Wong et al., 2004; Wong and Wessel, 2005). Our data confirm that this release of
H$_2$O$_2$ is also associated with a substantial increase of iodine accumulation.

The enzyme responsible for the oxidative burst at fertilization is Udx1, a member
of the NADPH oxidases which are characterized by a broad set of functions including
ROS metabolism and TH synthesis (Wong et al., 2004); these enzymes are
diphenyleneiodonium-sensitive (Cross and Jones, 1986) but cyanide-insensitive (Babior
et al., 1976). In sea urchins, Udx1 protein is expressed in the ectodermal domain of the
embryo and has also been shown to be expressed in later embryonic stages (Wong and
Wessel, 2005). Pharmacological and functional-blocking experiments of sea urchin Udx1
have demonstrated that hydrogen peroxide produced by Udx1 appears to be necessary for
the early cell-cycle (Wong and Wessel, 2005). Our results show that iodine uptake
increased dramatically at fertilization. These data therefore provide a strong natural test
of the hypothesis that peroxide is facilitating the uptake of iodine. Based on this evidence
we propose a new model for iodine uptake in sea urchin embryos and larvae (Fig. 4)
suggesting that Udx1 may produce the peroxide essential for iodine accumulation.

In our model, the oxidation of iodide is essential to its ability to be taken up by
cells. While there we have no information regarding the specific form of iodine taken up
by sea urchins, we hypothesize that such a compound could be hypoiodous acid (HIO) or
some oxidation product of it. When molecular iodine (I$_2$) is formed in seawater through
oxidation, a major part of it disproportionates into HIO (Truesdale, 1993). It was also
proposed that HIO is the form that can enter the cells in Flavobacteriaceae (Amachi et
al., 2007). Based on this evidence it would be important in future studies to further
investigate the chemical properties of iodide oxidation products that are involved in the
uptake process.
Note however that the oxidative burst in sea urchin fertilization is different to the oxidative burst that occurs in Laminaria, where it functions in microbial defense (Küpper et al., 2001; Küpper et al., 2002; Küpper et al., 2006). There, iodide stored in the apoplast acts as an extracellular antioxidant since natural antioxidants, such as ascorbate and glutathione, used by Laminaria are found strictly intracellularly (Küpper et al., 2008). These studies also showed that in contrast to the sea urchin oxidative burst, oxidative stress (e.g. photooxidative ROS production) or an oxidative burst in Laminaria results in an efflux of iodide (Küpper et al., 2008). Within the apoplast [where H$_2$O$_2$ concentrations can reach an excess of 1 millimol l$^{-1}$ (Küpper et al., 2001)] and the thallus surface released iodide acts as an antioxidant and scavenges ROS (Küpper et al., 2008).

While the system described by (Küpper et al., 2008) characterizes a novel mechanism for iodine storage and use, a model for iodine antioxidant function proposed for the moon jelly (Aurelia aurita) is more relevant to the system we propose for sea urchin larvae (Berking et al., 2005). For A. aurita, Berking et al. (2005) hypothesized that iodide and tyrosine synergistically function as a non-enzymatic ROS defense system. They propose that iodide enters A. aurita tissue (through a yet uncharacterized mechanism) where it reacts with endogenous ROS to form iodine. Iodine then reacts with tyrosine and produces iodotyrosines that form a waste product that can diffuse out of the tissue or, as the authors speculate, become co-opted as a signaling molecule (Berking et al., 2005). This proposed mechanism is equivalent to the biochemical process occurring during the oxidative burst at fertilization where hydrogen peroxide catalyzes the cross-linking of tyrosine to harden the fertilization membrane (Foerder et al., 1978; Wong et al., 2004; Wong and Wessel, 2005). Since sea urchins possess the necessary cellular machinery for this process, it is plausible that this mechanism is also employed in antioxidant function. Furthermore, it is conceivable that these enzymes (e.g. Udx1) are also used during later development for iodine uptake or in iodine metabolism such as the iodination of biological molecules.

**Sea urchin iodide influx is not dependent on the sodium/iodide symporter or apical-iodide transporter**

Based on current evidence, understanding of sodium-dependent iodide uptake mechanisms is restricted to the sodium/iodide symporter and the apical-iodide transporter
that both occur in the vertebrate thyroid gland. Of these mechanisms NIS is an active
transporter, whereas AIT is involved in the passive diffusion of iodide down its
concentration gradient (Eskandari et al., 1997; Lacroix et al., 2004). Beyond this, no other
sodium-dependent iodine uptake mechanism has been characterized in plants or animals.

In order to test whether iodine accumulation in sea urchin larvae is NIS/AIT-dependent
we tested the effect of perchlorate, a competitive inhibitor of NIS and AIT on iodide
accumulation in sea urchin larvae. We also tested the effect of decreased sodium
concentrations in the environment on iodide accumulation. Finally we searched the sea
urchin genome (Sodergren et al., 2006) for NIS/AIT candidate genes.

Perchlorate is a potent inhibitor of iodide transport into the thyroid gland in
vertebrates (Carrasco, 1993). This inhibition is established by a competition between
iodide and perchlorate at a specific site of the NIS molecule (reviewed by Dohan and
Carrasco, 2003). Our data show that perchlorate has no effect on iodide influx in sea
urchin larvae, which is a very distinct result from what one would expect to see in
vertebrates. The results therefore show that iodine accumulation is NIS/AIT-independent
and is also unaffected by exogenous sodium concentration. When we examined, the
effect of reduced sodium on survivorship, we found that larvae stopped swimming after
2.5 minutes in the lowest sodium concentration, however, when they were transferred
back into normal-sodium seawater, they recovered. This indicates that while sodium
limitation does have an effect on larval physiology it was not lethal for the duration of the
pharmacology experiment (i.e. 2.5 minutes)

There are a few other examples of organisms that accumulate iodine in a way that
is unaffected by perchlorate. For example, bryozoans (Bugula neretina and Shizoporella
errata) accumulate radioiodide from seawater in an ouabain and dinitriphenol-sensitive
uptake that is unaffected by perchlorate, but also thiourea (reviewed in Eales, 1997). In
scyphozoan jellyfish (Cnidarians), where iodine is required for signaling developmental
transitions (Spangenberg, 1974), iodine uptake is also unaffected by perchlorate
(Silverstone et al., 1978). Considering the vast phylogenetic distance between bryozoans,
cnidarians and echinoderms, it appears that perchlorate-inhibited iodide uptake
mechanisms, namely NIS/AIT, are more the exception than the rule among animals as a
whole, and may be even vertebrate-specific.
Evolutionary Implications

NIS and AIT proteins are part of the sodium/solute symporter family 5 (SSSF5). This family consists of at least 220 members ranging from bacteria to metazoa and, with a few exceptions, these proteins largely transport substrates (such as glucose, myoinositol and iodide) in a sodium-dependent manner (reviewed in Wright and Turk, 2004). Within humans there are currently 12 identified members expressed in a variety of tissues (Kanai et al., 1994; Jung, 2002). These proteins are highly conserved with up to 70% amino acid identity to SLC5A1: sodium/glucose transporter 1 (Wright and Turk, 2004). It is because of this conservation that we analyzed representatives of this family across several vertebrate and invertebrate taxa in an attempt to identify putative S. purpuratus NIS/AIT orthologs.

As was previously described by Wright and Turk (2004), CHT-like proteins (Clade III), Na-Glucose transporter-like proteins (Clade II) and Na-Anion transporter-like proteins (containing NIS/AIT/SMCT; Clade I) are distinct phylogenetic clades. However, our analysis revealed that Clade I and Clade II group together and separately from Clade III (CHT-like proteins). These results are markedly different from what was found using only human protein sequences. Here CHT grouped with NIS/AIT/SMCT as a separate cluster from the Na-glucose and Na-myoinositol transporters (Wright and Turk, 2004). Our results, using a wider selection of taxonomic groups including vertebrate and invertebrate sequences indicate that the diversification of Na-glucose transporter-like proteins and Na-Anion transporter-like proteins from a putative ancestral transporter occurred early in animal evolution.

With respect to the specific question addressed in this study, Clade I provides the most useful information. Specifically we found that all NIS/AIT/SMCT relatives from invertebrate species are found only in deuterostomes (Clade I.1) and no protein sequences related to NIS can be found outside of the vertebrates (Clade I.1, Clade I.2 and Dm II). Therefore we conclude that the NIS pathway, used for iodine uptake, is likely a vertebrate synapomorphy. These results contrast with the conclusions drawn by Paris et al. (2008) that Branchiostoma contains homologs of vertebrate NIS. While this group did conduct a thorough phylogenetic analysis, their phylogeny lacked sequences from Ciona (a
urochordate) and Saccoglossus (a hemichordate), which may have contributed to the

different conclusion.

In contrast to NIS/AIT mechanisms, involvement of peroxidase activity in iodide
accumulation is widespread, having been observed in macroalgae, (Küpper et al., 1998),
marine bacteria (Amachi et al., 2007) and microalgae species (van Bergjik, personal
communication). Furthermore, iodine and peroxidases were present in primitive
Cyanobacteria about 3 ½ billion years ago (Obinger et al., 1997) indicating that the
elements necessary for PDD as a mechanism for iodide uptake are very ancient. Whether
this is the ancestral state for all organisms or whether it evolved many times
independently in different lineages will require a more detailed mechanistic
understanding of this process in a variety of organisms from different kingdoms.

Finally, it is worth noting that diffusion-based iodine uptake mechanisms may
also function in aquatic vertebrates, e.g. teleosts. As mentioned previously, Moren et al.
(2008) found that when halibut larvae were exposed to perchlorate, only half of the iodine
influx was inhibited. Although PDD was never explicitly tested, it is conceivable that
NIS/AIT-independent mechanisms of iodine acquisition may be more prevalent in
vertebrates than originally assumed.

CONCLUSIONS

Our data show that sea urchins, a representative non-chordate deuterostome utilize
hydrogen peroxide dependent diffusion for iodine acquisition. Putative orthologs of
NIS/AIT transporters have been found in genomes of basal chordates but
pharmacological evidence elucidating mechanisms of iodine uptake is largely missing
from these taxa. Future work should focus on these mechanisms in such taxa as this will
assist in our understanding of whether sodium dependent iodine uptake is a vertebrate or
chordate synapomorphy. Our preliminary analysis, based only on phylogenetic
comparisons, suggests that this uptake mechanism is restricted to vertebrates.

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FIGURE LEGENDS

Figure 1: Iodine influx rate (femtomoles larvae$^{-1}$ hour$^{-1}$) for 6-arm stage larvae of *S. purpuratus* in artificial seawater (n=3). (A) The initial influx at 2000 nanomol l$^{-1}$ is apparent by approximately 2.5 minutes and reaches a plateau at approximately 40 minutes. (B) Increasing iodine concentration does not decrease iodine influx rate (femtomoles larvae$^{-1}$ hour$^{-1}$) during a 2.5-minute incubation. (C) There is a positive correlation between temperature and iodide influx rate. (D) Influx rate during a 30-minute incubation is affected by the developmental progression of metamorphosis. Late-stage competent (not induced to metamorphose) and pre-competent (induced to metamorphose) larvae have significantly lower iodide influx rates than metamorphosed individuals (induced to metamorphose) (n=3) (the horizontal lines indicate significant difference at a p<0.05 level between the two data points lying below the endpoints of each line).

Figure 2: (A) Exogenous iodine uptake rate is enhanced significantly with the addition of exogenous hydrogen peroxide in 6-arm stage larvae (n=4). (B) The oxidative burst at fertilization is associated with an increase in iodide influx. (C) Paraquat was found to increase iodine influx in 6-arm larvae at 0.1, 1 and 10 millimol l$^{-1}$ concentrations. This effect is rescued by addition of 100 micromol l$^{-1}$ ascorbate. (* indicates significant difference at a p<0.05 level from the control)

Figure 3: (A) Schematic topology of the SSSF5 using several vertebrate and invertebrate taxa. Based on an Amino LG matrix, there are three major clades (supported with a Bootstrap level of 100%): **Clade I**- Na-Anion transporter-like proteins, **Clade II**- Na-glucose transporter-like proteins, and **Clade III**-Na-Choline Transporter-like proteins. Clade I contains **Clade I.1**- NIS/AIT/SMCT –like proteins, **Clade I.2**- Invertebrate Na-Anion transporter-like proteins, **Dm II**- *Drosophila* Na-Anion Transporter-like proteins (B) Clade I.1 contains the Vertebrate NIS, AIT and SMCT proteins and deuterostome representatives of the SSSF5. All vertebrate sequences cluster together and the sister group to the vertebrate clade is Ci I, which contains eight *Ciona* representatives. The remaining four clusters are unresolved at the 60% Bootstrap level: Bf I, Sk/Sp I, Bf/Sk/Sp
and Ci II/Sk/Sp II. Bf I, a cluster of eight *Branchiostoma* proteins supported by 100% bootstrap. Sp I contains five proteins from the sea urchin. Sp II contains six proteins and Ci II contains seven proteins.

Figure 4: Hypothesized mechanism of iodine influx in sea urchin larvae based on the peroxide dependent diffusion model. In this model, iodine reacts with hydrogen peroxide through the action of a peroxidase and is translocated into the larvae through a channel as an oxidized form.
Table 1: Effects of various inhibitors on initial iodine influx rates in 6-arm stage larvae and in eggs. The influx rate is represented as the percentage of the control.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
<th>Uptake activity (% of control)</th>
<th>6-arm Stage Larvae</th>
<th>Fertilized Eggs</th>
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</thead>
<tbody>
<tr>
<td>Perchlorate</td>
<td>1 micromol l⁻¹</td>
<td>95.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 micromol l⁻¹</td>
<td>90.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 micromol l⁻¹</td>
<td>74.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminotriazole</td>
<td>10 micromol l⁻¹</td>
<td>56.9*</td>
<td>43.8*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 micromol l⁻¹</td>
<td>56.0*</td>
<td>33.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 micromol l⁻¹</td>
<td>49.7*</td>
<td>30.5*</td>
<td></td>
</tr>
<tr>
<td>Thiourea</td>
<td>1 nanomol l⁻¹</td>
<td>76.6</td>
<td>67.7*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 nanomol l⁻¹</td>
<td>45.9*</td>
<td>26.9*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 nanomol l⁻¹</td>
<td>9.7*</td>
<td>0.77*</td>
<td></td>
</tr>
<tr>
<td>Cyanide</td>
<td>1 micromol l⁻¹</td>
<td>100.0</td>
<td>26ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 micromol l⁻¹</td>
<td>49.5*</td>
<td>5ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 micromol l⁻¹</td>
<td>9.5*</td>
<td>0ᵃ</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>10 units ml⁻¹</td>
<td>79.9</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 units ml⁻¹</td>
<td>49.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 units ml⁻¹</td>
<td>36.9*</td>
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<tr>
<td>Ascorbate</td>
<td>100 micromol l⁻¹</td>
<td>61.6*</td>
<td>1.94*</td>
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<tr>
<td>DIDS</td>
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<td>39.5*</td>
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</tr>
</tbody>
</table>

*: statistically different from the control at the p<0.05 confidence level

ᵃ: data taken from Kelbanoff et al. (1979)
APPENDIX

Appendix A

The following is a list of the accession numbers of the 109 sequences analyzed in phylogenetic analysis to determine the organization of the SSSF5 across taxa. This list is in alphabetical order:

Appendix B

PhyML tree of amino acid sequences found for members and orthologs of the Sodium Solute Symporter Family 5 (SSSF5), based on an Amino LG matrix. Bootstrap values were cut-off at 60%. 109 sequences were examined for Homo sapiens (Hs), Pan troglodytes (Pt), Canis lupus (Cl), Bos taurus (Bt), Mus musculus (Mm), Rattus norvegicus (Rn), Gallus gallus (Gg), Danio rerio (Dr), Branchiostoma florididae (Bf), Ciona intestinalis (Ci), Strongylocentrotus purpuratus (Sp), Saccoglossus kowalevski (Sk), Drosophila melanogaster (Dm) and Nematostella vectensis (Nv).

Appendix C

Alignment produced by the program CLC using Clustal X. Species used: Homo sapiens (Hs), Danio rerio (Dr), Strongylocentrotus purpuratus (Sp), and Saccoglossus kowalevski (Sk). Three representatives of Strongylocentrotus and one representative of Saccoglossus are aligned against two vertebrate NIS proteins.
REFERENCES


Fig. S1. Alignment produced by the program CLC using Clustal X. Sequences used: *Homo sapiens* sodium iodide symporter (H.s. NIS), *Mus musculus* NIS (M.m. NIS), *Danio rerio* NIS (D.r. NIS), *Homo sapiens* apical iodide transporter (H.s. AIT), and three *Strongylocentrotus purpuratus* proteins detected by BLAST with H.s. NIS. (S.p. 001184672, S.p. 785969, S.p. 001202585).