

# Living with high concentrations of urea: They can!

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## ABSTRACT

Marine elasmobranchs maintain their body fluid isoosmotic or slightly hyperosmotic to the external medium by the retention of large urea concentrations. This review focuses on the strategies adopted by these fishes to maintain a large outwardly directed concentration gradient of this osmolyte minimizing the loss across the main interfaces between body fluid and the external medium such as the gills, the kidney and the rectal gland, thus reducing the cost of making urea. The high plasma osmolarity, mainly maintained by urea retention, is a challenge to volume homeostasis when fish move from seawater to water with a low salinity, since the high water permeability of branchial epithelium would cause a net flux of water into the animal. Since the renal regulation of urea retention in habitat with different salinities is crucial for the osmotic homeostasis of these species, the regulation of the activity and/or the expression of urea transporters in renal tubules will be also discussed. In addition attention will be paid on the urea-methylamine system involved in maintaining the stability and functioning of many proteins since it is known that the high urea concentration found in marine elasmobranch fish, similar only to that found in mammalian kidney, has a destabilizing effect on many macromolecules and inhibits functions such as ligand binding.

**Keywords:** Elasmobranch; Urea; TMA; Gills; Kidney; Rectal Gland

## 1. INTRODUCTION

It is known that the strategy adopted by the elasmobranch fish to live in the seawater, having an electrolyte content higher than plasma and tissue, is different from the strategy adopted by marine teleosts. They avoid dehydration and maintain plasma osmolarity lower than the surrounding medium by drinking sea water and absorb-

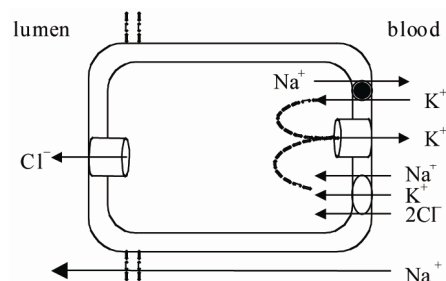
ing NaCl and hence water by the intestinal fluid desalinated at esophageal level while excess of NaCl is excreted across the gills. Elasmobranch fish achieve plasma ionic homeostasis and maintain a plasma osmolarity equal or slightly higher than sea water by actively excreting excess NaCl by rectal gland (**Figure 1**) and retaining large amounts of urea. Many works have been performed to study the osmoregulatory strategies adopted by elasmobranch fish and the results obtained have been reviewed by many authors [1-8].

This review will focus on studies performed in order to answer to the questions raised by the strategy of retaining urea, such as:

- 1) Which are the mechanisms allowing urea retention?
- 2) Since urea retention in fresh water is maladaptive, in which way does the whole picture of urea system change in euryhaline elasmobranchs?
- 3) How the destabilizing effects of urea are counteracted?

## 2. WHICH ARE THE MECHANISMS ALLOWING UREA RETENTION?

In most elasmobranch urea is the end product of ni-



**Figure 1.** NaCl excretion in a rectal gland cell.  $\text{Cl}^-$  diffuse across the apical membrane into the lumen of rectal gland driven by its electrochemical gradient. The  $\text{Na}^+ \text{K}^+$  ATPase provides the driving force for the operation of the basolateral  $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$  cotransport and hence for the active accumulation of  $\text{Cl}^-$ . The mucosal negative transepithelial potential generated by  $\text{Cl}^-$  secretion favours  $\text{Na}^+$  efflux through the cation selective tight junction (see ref 3).

trogen metabolism and it is mainly produced in the liver in the ornithine-urea cycle through carbamoylphosphate synthetase III [9-11]. Marine elasmobranchs retain large amount of urea in order to maintain body fluids isoosmotic or slightly iperosmotic to the surrounding seawater [12,13]. They accumulate urea to concentrations as high as 350 - 500 mM [14-16]. In order to maintain a large outwardly directed concentration gradient they must minimize the loss across the main interfaces between body fluid and the external medium such as the gills, the kidney and the rectal gland, thus reducing the cost of making urea.

Noteworthy a role of gut in urea conservation has been recently suggested, but the mechanisms involved are not well known [11].

## 2.1. Gills

Fish gills have a large surface area and a short diffusion distance necessary to allow an efficient exchange of respiratory gases between the blood and the extracellular medium. This arrangement is also responsible of a large diffusive movement of solute and water between the organism and sea-water. In the elasmobranchs the maintenance of high plasma concentration of urea implies that the loss of this osmolyte across the gills must be low. Indeed the pioneering work of Boylan [17] suggested that the gill epithelium of *Squalus acanthias* is exceptionally tight to urea. In order to explain this impermeability Boylan [17] and Part *et al.* [18] evaluated two hypotheses:

1) gill epithelium (particularly the apical membrane of the cells) is impermeable to urea due to the peculiar composition of the membrane, since it had been showed that in artificial membranes a selective impermeability to urea can be obtained by modifying lipid composition, *i.e.* by increasing sphingomyelin content [19].

2) Gill epithelium forms an intermediary compartment between blood and the external medium in which the intracellular urea concentration is kept lower than in the body fluid by a basolateral urea transporter that actively transports into the blood urea that enters the cell from the blood or that is synthesized in the cell. In such a way the diffusive loss across the apical membrane into the sea-water would be reduced.

Part *et al.* [18], by a study with a perfused gill preparation from dogfish, concluded that the apical membrane is extremely tight to urea, having an urea permeability coefficient of  $3 \times 2 \times 10^{-8} \text{ cm} \cdot \text{sec}^{-1}$  that is lower than one of teleost epithelium ( $2 \times 6 \times 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$ ). A low urea permeability of apical membrane was suggested also by Hill *et al.* [20] but it was concluded that it cannot account for low urea loss measured in elasmobranch.

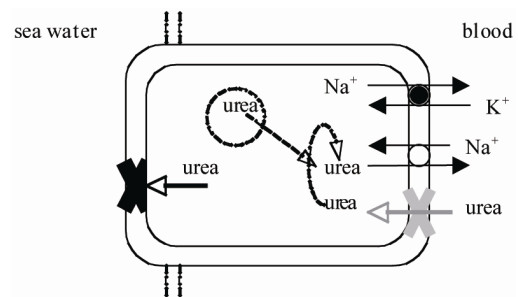
Part *et al.* [18] showed that the overall impermeability

of gill epithelium is also due to a putative back-transport of urea in the basolateral membrane, since phloretin, a non competitive inhibitor of urea transport, increases urea efflux across the gills. Following studies confirmed the presence of urea transporters in elasmobranch gills. The low-stringency Northern analysis revealed that an homologue of the renal urea transport is present in the gill of *S. acanthias* [21]. Analyses of urea uptake by vesicles prepared from enriched basolateral membrane of *S. acanthias* [22], showing a saturation kinetics at low urea concentration, suggested the presence of a carrier-mediated urea transport having high affinity for urea, it is a  $\text{Na}^+$ -coupled secondary transport functioning in an antiport function (**Figure 2**). The antiporter is responsible of a significant metabolic saving since the metabolic cost of synthesize one urea molecule (5 ATP equivalents) is much higher than the cost for transporting urea to the blood via a  $\text{Na}^+\text{-K}^+$  dependent urea transport (about 0.2 ATP equivalent for molecule of urea returned to the blood). However, Fines *et al.* [22] suggested that the low urea permeability of spiny dogfish gills is accomplished not only by the active transport of the osmolyte but also by an unusual composition of basolateral membrane that presents an elevated cholesterol to phospholipids molar ratio.

## 2.2. Rectal Gland

Another striking feature of elasmobranch is the low urea permeability of the rectal gland. This organ, having the important role to excrete excess  $\text{NaCl}$  and water, secretes a fluid isotonic with plasma. This implies that water follows the diffusive flux of the electrolytes and hence the permeability of the rectal gland must be high.

Studies performed with liposomes having different lipid composition showed a parallelism between permeability to water and to urea [20,23], so rectal gland would



**Figure 2.** Possible mechanisms explaining the impermeability of gill epithelium of elasmobranch fish: a very low permeability of the apical membrane impeding the diffusive loss to seawater; a  $\text{Na}^+$ -coupled secondary antiport that transports into the blood urea that is synthesized in the cell or that enters the cell from basolateral membrane having a low urea permeability (see text for the references).

have an high urea permeability. This conclusion contrasts with the observation that the rectal gland fluid has a high level of NaCl (500 mM vs. 340 - 350 mM in the plasma) but a low level of urea (18 mM vs. 330 mM in the plasma) [24,25] and strongly suggests a selective impermeability to urea of the rectal gland. Zeidel *et al.* [26] advanced two hypotheses to explain this unusual permeability characteristic of the rectal gland membranes:

- 1) The membranes possess specific water channel, aquaporins.
- 2) Apical or serosal membranes have a tight barrier to urea.

By using highly purified vesicles of apical or basolateral membranes of rectal gland of *S. acanthias* they measured their permeability to urea and to water. They showed that the water permeability was too low and the activation energy for water was too high to suppose the presence of aquaporins in both the apical and the serosal membranes and suggested that water crosses the membranes through the lipidic component. Comparison of water and urea permeability measured in rectal gland with that measured in artificial liposomes and in other biological membranes let the authors to conclude that the basolateral membranes have the role of selective barrier to urea in shark rectal gland. They suggested that this barrier could be due to the membrane lipidic components, as found in gill membranes [22], or to a protein component, as observed in the medullary thick ascending limb of Henle loop, where the paracellin-1, a member of claudin family, prevent the paracellular flux of water allowing the flux of  $Mg^{2+}$  and  $Ca^{2+}$  down their electrochemical gradient [27].

### 2.3. Kidney

Urea is freely filtered by the glomerulus but the urea excretion by the renal route is as low as 5% of the total body urea excretion [28]. Indeed it was calculated that 90% - 96% of the filtered urea is reabsorbed in the renal tubules [29-31]. In order to explain the efficient urea reabsorption three different mechanisms, even if they were supported by poor experimental evidences, were initially proposed:

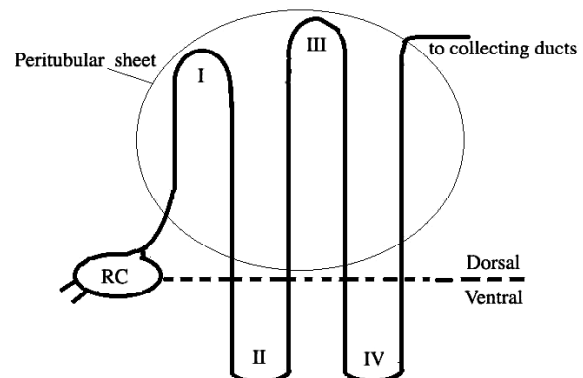
- 1) A carrier mediated passive process [12,32].
- 2) A passive reabsorption down a localized concentration gradient [33].
- 3) An active transport, possibly  $Na^+$ -dependent [34, 35].

More recently Smith and Wright [21] isolated a 2.2 kb cDNA from *S. acanthias* kidney encoding a 380 amino-acid protein with a 60% aminoacidic identity to the facilitated urea transport protein found in rat (UT-A2). They suggested that the shark urea transporter (Sh-UT), the first urea transporter isolated in a marine fish, consti-

tutes a phylogenetic ancestral form of UT-A2 and that other urea transporter homologues may be present in elasmobranch species. A cDNA encoding a urea transporter homologue to Sh-UT was isolated from another dogfish species, *Triakis scyllia* [36]. It was abundantly expressed in the kidney but low levels were found in the brain and in the liver. Immunocytochemistry, using specific antibodies raised against the cloned UT, showed that the transport is localized in the collecting duct on both the apical and the serosal membranes of the epithelial cells. This localization supported the hypothesis that urea transport is performed by carrier mediated passive permeation, the other segments of the nephron acting as a countercurrent multiplier system that produces the driving force for facilitated diffusion of urea.

The peculiar arrangement of elasmobranch nephron (see **Figure 3**) leading to a countercurrent system was described by Lacy and Reale [37,38] and by Hentschel *et al* [39]; it is typical of all marine elasmobranchs. The nephron consists of four loops beginning at renal corpuscle located between the dorsal bundle zone (where the tubule segments are tightly packed) and the ventral sinus zone (where the tubule segments are loosely packed and segregated from each other by large blood sinuses). The I loop is in the bundle zone, the tubule then extends in the sinus zone and turns back forming the II loop, from which the III loop originates; it descends in the sinus zone forming the IV loop. This last loop returning to the bundle zone joins the collecting ducts. They fuse to form a collecting tube. The loop I and III together with the distal tubule belonging to the same nephron together with a network of anastomosing capillaries form a bundle enclosed by connective tissue called "peritubular sheet", an impermeable peritubular sheet. This unit forms the countercurrent system of the nephron of elasmobranch.

Two different urea transporters were identified in the ventral and in the dorsal zone of the little skate, *Raja*



**Figure 3.** Schematic drawing of an elasmobranch nephron as described by Lacey and Reale [37]. RC = renal corpuscle; I, II, III and IV indicate the loop number.

*erinacea*, by using brush border membrane vesicles from the two different sections of the kidney [40]. The authors showed that in the dorsal zone urea transport was not saturable, was sensitive to the non competitive inhibitors phloretin and HgCl<sub>2</sub> and was significantly reduced in the presence of an urea analogue. By these results they suggested the presence of one or more facilitated urea transporters possibly coded by skUT (skate urea transporter) which expression had been already evidenced in both the dorsal and ventral section [41]. In the ventral tubules the presence of an additional transporter was proposed; it has the characteristic of a Na<sup>+</sup>-linked urea transporter, since it was stimulated by an inward Na<sup>+</sup> gradient [40].

A close homologue of shUT, strUT-2, was cloned from the kidney of the Atlantic stingray, *Dasyatis Sabina* [42]. It was suggested that the presence of homologues in two species that have diverged in the elasmobranch evolution [43] speaks in favour of a conservation of this isoform. However in the Atlantic stingray another phloretin transporter, strUT-2, was also identified [44]. It has a high degree of sequence identity with shUT but has a unique extension of the hydrophilic COOH-terminal region of about 51 aminoacids. In addition strUT-2 has a protein kinase regulatory site lacking in the shark urea transporter. The authors proposed that protein kinase may alter the function of the stingray transporter thus regulating urea reabsorption during the movement of the animal between habitats of different salinities. Indeed Atlantic stingray is an elasmobranch that, unlike the spiny dogfish, can move to habitats covering a large range of salinities.

### 3. SINCE UREA RETENTION IN FRESH WATER IS MALADAPTIVE, IN WHICH WAY DOES THE WHOLE PICTURE OF UREA SYSTEM CHANGE IN EURYHALINE ELASMOBRANCH?

Most elasmobranches live in seawater and are stenohaline while the stingrays of the genus *Potamotrygon* are the only elasmobranch permanently adapted to freshwater [45,46]. However a number of species are marginally euryhaline since they can enter in the brackish environment of river mouths [47] and a small number can move from seawater to freshwater, generally on a seasonal basis [13,46,48,49].

The high plasma osmolarity, mainly maintained by urea retention, is a challenge to volume homeostasis when fish move from seawater to water with a low salinity, since the high water permeability of branchial epithelium would cause a net flux of water into the animal.

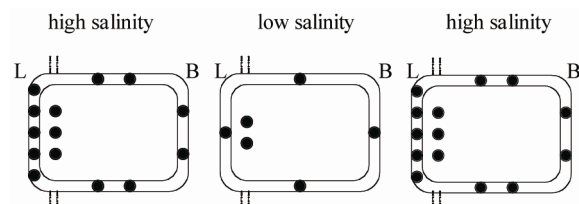
Since the kidney is the primary organ responsible of urea retention and euryhaline elasmobranchs remain ureotelic in environment with a low salinity [30,34,46,50-52]

the renal regulation of urea retention in habitat with different salinities is crucial for the osmotic homeostasis of these species. This observation suggests that the activity and/or the expression of urea transporters in renal tubules have to be regulated. Indeed an increase in urea excretion relative to urea absorption in a diluted environment was described in the euryhaline skates [30,31].

Morgan *et al.* [41], using Northern analysis of mRNA from the kidney of the little skate *Raja erinacea*, revealed three bands of 3.1, 2.8, and 1.6 kb belonging to three isoforms of an urea transporter family. When the skates were exposed to diluted environment (50% seawater) the levels of all three skUT transcripts were reduced by 1.8 - 3.5 folds. Since kidney osmolality and urea concentration decreased while water content increased, the authors suggested that the downregulation of skUT plays an important role in lowering tissue urea levels in response to the osmolality of the external medium.

A recent study of Yamaguchi *et al.* [53], by using semiquantitative fluorescent immunochemistry and real-time PCR, examined the localization and the mRNA levels of UT transporters in the collecting duct of the kidney of groups of *T. scyllium* held at different salinities. It was suggested that the transfer of this fish to a low salinity did not change the expression of UTs, as observed in *R. erinacea* [41], but modified the cellular distribution of the transporters. It was found that in the control *T. scyllium* (100% seawater) UTs localized in both the apical and the basolateral membranes, with a higher expression in the apical membrane. In fish transferred to a diluted medium (30% seawater) the immunoreactivity significantly decreased but the most pronounced change was in the apical membrane where the immunoreactivity almost disappeared, while a less pronounced immunoreactivity reduction was detected in the cytoplasm and in the serosal membrane. On the return to full seawater the apical distribution recovered its initial level (**Figure 4**).

These observations led the authors to conclude that UT distribution is regulated by its production and degrada-



**Figure 4.** Redistribuition of UT transporters (filled circles) in a cell lining the collecting duct of *T. scyllium* adapted to different salinities. In fish transferred to a diluted medium they significantly decrease but the most pronounced change is in the apical membrane. On the return to full seawater the distribution recovers its initial level (see text for the references). L = lumen; B = blood (see ref 53).

tion as well as by trafficking between apical membrane and cytoplasm and suggested that the abundance of UT in the apical membrane functions as rate limiting factor for urea reabsorption; this trafficking should be controlled to regulate urea reabsorption. It was suggested that vasotocin can act as possible regulator in *T. scyllum*, similarly to vasopressin in mammals [54,55]. This hypothesis was based on two observations:

1) Plasma levels of vasotocin increase following transfer to concentrated seawater and decrease following transfer to dilute seawater [53,56].

2) Plasma levels of the hormone correlate with the abundance of UTs in the luminal membrane and with urea plasma levels [53,56].

However it is also possible that plasma and tissue levels of urea change as a consequence of a change of hepatic urea production. A decrease of urea production during the acclimation to fresh water was suggested for the little skate *R. erinacea* [30] for the dogfish *Scyliorhinus canicula* [57] and for the bullshark, *Carcharinus leucas* [58,59]. Tam *et al.* [58] showed that the acclimation of the freshwater stingray *Himantura signifer* to diluted seawater led to an increase of urea synthesis due to an upregulation of the enzymes of the ornithine-urea cycle.

The ability to modify urea production is different in the various elasmobranch species and it is probably related to their ability to survive in habitats with different salinities. *H. signifer* that is a species that can travel along the river and hence may experience brackish water maintains the ability to synthesize urea and has a reduced capacity to retain urea [58]. The stenohaline fresh-water rays Potamotrygon spp, that permanently dwell in fresh water, being confined in the Amazonian river basin away from ocean for million years [60,61] are ammoniotelic and are not able to produce urea neither to counteract an experimental salinity stress [45,62,63] nor as strategy to detoxify ammonia. It was observed that Potamotrygon motoro is not able to upregulated urea synthesis via the ornithine-urea cycle when exposed to environmental ammonia [64].

#### 4. HOW THE DESTABILIZING EFFECTS OF UREA ARE COUNTERACTED?

It is known that the high urea concentration found in marine elasmobranch fish, similar only to that found in mammalian kidney [65], has a destabilizing effect on many macromolecules. In order to explain the mechanisms responsible of the chemical denaturation of urea two mechanisms have been proposed: the indirect and the direct mechanism. According to the indirect mechanism urea denatures protein by destroying water structure that in turn weakens the hydrophobic interaction responsible of the globular structure of the protein [66,67]. Ac-

ording to the direct mechanism urea unfolds protein through a direct interaction mediated by either electrostatic interaction or van der Waals attraction [68-70].

The loss of structure influences enzyme kinetic properties [71,72], alters the melting point transition temperature of protein [73,74], inhibits functions as ligand binding [75].

To counteract the destabilizing effect of urea, elasmobranchs accumulate methylamine organic osmolytes [14, 71,76-78], since they are stabilizer of protein structures and activator of many functional properties of proteins [71,72]. Trimethyl amine oxide (TMAO) and betaine are the most effective [15] and the predominant methylamines found in the muscle of elasmobranchs [79,80]. The level of TMAO, which primary role is that of an osmolyte, varies in the euryhaline species adapted to different salinities [76,81]. However in seawater elasmobranchs TMAO is the major component while in the transition from euryhaline to freshwater elasmobranchs betaine becomes the major methylamine [82].

A 2:1 concentration ratio of urea to these counteracting compounds is optimal for preserving proper protein function [15,71,72].

In addition urea could increase the fluidity of membranes by a destructive effect on the hydrophobic core of the membrane or by indirect effects related to changes of integral membrane proteins. Barton *et al.* [4] showed that TMAO may act as counteracting solute in the red blood cell membrane of *R. erinacea* and suggested that the counteraction is at the level of integral proteins rather than on the membrane phospholipids.

Regarding the synthesis of the predominant methylamines it was suggested that betaine synthetic pathways are probably present in most or in all elasmobranchs, like in the other vertebrates, since they play an important role in the catabolism of choline introduced by the food [83]. Betaine is synthesized from choline in metabolic pathways involving choline dehydrogenase (ChoDH) and aldehyde dehydrogenase (BADH). Measurable levels of these enzymes were found in various elasmobranch species [82].

In vertebrates TMAO derives from the oxidation of trimethylamine (TMA) through a reaction catalyzed by the trimethylamine oxidase (TMAox). This enzyme is sporadically present in elasmobranchs [82,84-86] and when present its activity is high in the liver that should be the major site of TMAO synthesis [84,86,87]. It was suggested that the ability to maintain TMAO plasma concentration in species lacking of TMAox without an exogenous supply of TMAO for a long period depends on a release from muscle [85] as well as on a very low loss from the animal [81,87,88]. This can be due to a sustained reabsorption of the filtered TMAO [30,89]. In addition, in order to maintain elevated methylamine lev-

els to counteract the destabilizing effect of urea in species lacking of TMAox, an activation of the enzyme involved in betaine synthesis during a period of exogenous supply of TMAO can occur [82].

## 5. DISCUSSION

In summary, studies hitherto performed suggest that, in order to maintain a large outwardly direct concentration gradient, both gills and rectal gland of elasmobranchs have an unusual permeability allowing the movement of water but not of urea. However in the gills an homologue of a renal urea transporter was also detected, suggesting a back-transport of the osmolyte in the basolateral membrane that contributes to avoid urea loss from the body. Various facilitated urea transporters have been identified in the elasmobranch renal tubules allowing the reabsorption of urea freely filtered by the glomerulus. The expression, the activity and/or the cellular localization of these transporters are probably regulated in the euryhaline species when they move to habitats with different salinities, in order to modify urea retention in response to osmotic challenges. The adaptation to different salinities involves also a variation of TMAO concentrations, the second osmolyte in elasmobranchs, that plays also the important role to counteract not only the destabilizing effect of urea on the structure and hence on the function of many proteins but also the urea effect on membrane fluidity.

However a multidisciplinary approach ranging from organism to molecular techniques is still necessary to complete the picture of the strategies adopted by different elasmobranch species for living with high urea concentrations. This approach will be necessary also to better understand how the urea levels are modulated to allow euryhaline species to live in habitats with different salinities.

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