Table 1 presents the results of our determinations of the effect of the removal of external Na^+ and K^+ on the efflux of acid and ammonia. It is clear that, although the ammonia efflux was unaffected, the rate of acidification of the medium was significantly reduced and actually reversed to alkalization in some experiments. Replacement of the animals into normal seawater resulted in a reestablishment of net acidification with an overshoot which was obscured by experimental variability (6 or 7 animals showed a greater acid efflux in SW_2 than in SW_1). One might propose that the overshoot was secondary to a fall in blood pH during the period in Na^+- and K^+-free seawater but data on blood pH are lacking. Only two determinations of the TEP were made during these experiments but it is apparent that changes in the TEP cannot account for the substantial alterations of the acid efflux secondary to changes in the external levels of Na^+ and K^+. The animals were not cannulated but Cohen (J. Cell Comp. Physiol. 53:204-213, 1959) found that *Squalus acanthias* maintained a renal acid excretion of the order of 4 $\mu M.100g^{-1}.hr^{-1}$, which is only 10% of the total acid excretion of the skates in Table 1. In addition, Pierce and Kent (Bull. MDIBL 8:49-53, 1968) have shown that the *S. acanthias* branchial epithelium excreted acid at a rate of approximately 100 $\mu M.100g^{-1}.hr^{-1}$ when perfused with seawater in equilibrium with 2-5% CO_2 in air. It, therefore, appears that the present experiments have determined mainly branchial acid excretion.

Kirschner et al. (Am. J. Physiol. 224:832-837, 1973) have shown that external amiloride inhibited Na^+ influx and both acid and ammonia efflux from the branchially-irrigated freshwater trout so we have tested the amiloride sensitivity of the acid and ammonia excretion from *R. erinacea*. Table 2 shows clearly that 10^{-4} M amiloride significantly inhibited acidification but had no affect on the efflux of ammonia or the TEP. The cause for the much higher control acid efflux in these experiments is unknown.

These experiments indicate clearly that, in *R. erinacea*, ammonia efflux is not coupled to external Na^+ or K^+ and is therefore not via Na^+/NH_4^+ exchange which has been described for *Scyliohinus canicula* and a wide variety of teleost species (see refs. in introduction). However, it is equally clear that acidification of the medium by this species is sensitive to external Na^+ (and/or K^+) and is, therefore, presumably via Na^+/H^+ (or possibly K^+/H^+) exchange. Since the few determinations of the unidirectional Na^+ fluxes across marine elasmobranchs indicate a rate of from 33-100 $\mu M.100g^{-1}.hr^{-1}$ (Evans, in "Osmotic and Ionic Regulation in Animals," G.M.O. Maloiy editor, Academic Press, 1979) it appears that this ionic exchange system (assuming a 1:1 stoichiometry) may represent a significant Na^+ load to the skate which must be excreted via branchial and rectal gland extrusion mechanisms. Research supported by NSF PCM77-09915.

PRELIMINARY STUDIES OF OSMOREGULATION BY THE PREMATURE "Pup" OF THE DOGFISH, *Squalus acanthias*. AND THE UTERINE LINING OF THE FEMALE

Gregg A. Kormanik and David H. Evans, Department of Biology, University of Miami, Coral Gables, Florida

The dogfish, *Squalus acanthias*, is ovoviviparous with developing young maintained *intra utero* for periods up to two years (Woodhead, Bull. MDIBL 16:103-106, 1976). During the later periods of gestation, the yolk sac bearing "pups" are exposed to seawater secondary to the maternal flushing of the uteri with sea water (Burger, Sharks, Skates and Rays, p. 178, 1967). In addition, we (and other workers) have found that late-term "pups" removed in the summer months during surgery of the female are able to survive in sea water (15°-17°C) for periods up to two weeks as long as care is taken to avoid rupture of the yolk sac membrane.

Since the highly vascularized uterine walls of the female are also exposed to seawater late in development it is also of interest to investigate the ability of the uterine lining to maintain ionic

gradients between the maternal blood stream and seawater. We therefore have investigated the blood Na^+, K^+ and Cl^- levels of the *intra utero* "pups" as well as the rates of whole-body Na^+ efflux and cloacal Na^+, K^+ and Cl^- efflux from premature "pups." In addition, we shall report on preliminary experiments performed to determine the relative ionic permeabilities of premature "pups" and early- and late-term uteri.

To determine the blood Na^+, K^+ and Cl^- concentrations of maternal and "pup" blood, samples were withdrawn from the hepatic portal vein of spinal-transected pregnant females and from tail severance of some of the resident "pups" from the uteri of the female. All samples were centrifuged immediately, frozen, shipped to Miami under dry ice and analyzed for Na^+ and K^+ (via flame photometry) and Cl^- (via amperometric titration). In addition, two samples of lumen fluids from late-term uteri were also taken, frozen and analyzed. To determine the rate of unidirectional Na^+ efflux, 4 µCi of ^{22}Na was intraperitoneally injected into premature "pups" (at least 48 hours after removal from the female) and the "pup" was placed into 200 ml of seawater in a plastic container maintained at 15°-17°C via external running seawater. One hour was allowed for isotopic equilibration within the animal and then samples of the flux bath were removed at various intervals for periods up to 20 hours post injection. At the end of the efflux period, the "pups" were sacrificed by spinal transection, a sample of blood removed, and a measured aliquot of plasma analyzed for radioactivity in order to determine the Na^+ space within the animal. All radioactive samples were analyzed on the Autogamma system. Effluxes were calculated as described by Evans and Kormanik (this bulletin). Cloacal Na^+, K^+ and Cl^- efflux rates were measured by cannulation with surgical tubing about 12 cm in length, flared at the proximal end and tied with the cloaca with a purse-string suture. The distal end was left open, the trapped air space providing sufficient pressure to prevent back flow of seawater into the cannula. Preliminary experiments using India Ink had determined that this cannulation system did not allow any leakage of cloacal fluids around the site of cannulation. At various times post-cannulation, samples of fluid were aspirated by gentle suction down to the cloacal opening, measured for volume and stored frozen for subsequent ionic analyses as described previously.

To measure the transepithelial electrical potentials (TEPs) and to approximate relative ionic permeabilities of premature "pups" and early and late uteri (early uteri had 4 to 6 eggs per uteri contained in a "candle" of very fragile material which is presumably the homologue of the skate egg case, each egg with a small-about 15 mm-embryo), we examined some electrical characteristics of the "pups" and isolated uterine epithelium. The TEP (and its alteration by ionic substitutions) was measured via PE 10 bridges (filled with 3 M KCl in 2% Agar) connected to matched calomel electrodes via 3 M KCl filled test tubes. The calomel electrodes fed into a Radiometer Model PHM 62 digital pH meter. Measurement of the TEP across the intact, premature "pup" was carried out as described for teleost fish (Evans et al., J. Exp. Biol. 61:277-283, 1974). In order to measure the TEP across the isolated uterine lining, a sac was prepared by taking a 3-6 cm length of the uterus near the distal end, tying off one end, inverting and tying the other end around a flared piece of surgical tubing. The sac was then filled with Forster's elasmobranch Ringer's solution (ERS) (Forster et al., Comp. Biochem. Physiol. 42A:3-13, 1972) and suspended in various experimental solutions. One bridge was placed in the inside of the sac (serosal or blood side) and the other in the external solutions (mucosal or uterine lumen side). All TEPs were recorded as serosa (or blood) relative to mucosa. In order to determine relative ionic permeabilities, "pups" or uterine sacs were transferred from either seawater or ERS to either Na^+ and K^+ free (choline as the impermeant cation) or Cl^- and HCO_3^- free (benzenesulphonate as the impermeant anion) artificial seawater solutions. In all cases the experimental "pup" or sac preparation was first "washed" in tap water for 30 seconds (to remove external ions) before transfer to ion-substituted solutions. To check for alteration of the permeability characteristics, the fish or sacs were transferred back into seawater between each ion-substituted

solution. TEPs were recorded for at least 5 minutes in each solution but were found to be stable (± 1 mV) for up to 20 minutes in other experiments.

TABLE 1

Ion Concentrations in the Plasma of the "Pup" and the Female, S. acanthias, and the Uterine Fluids

	$[Na^+]$	K^+	Cl^-
Female (4)	234 ±7 *	3.28±0.2	221 ±9
"Pup" (16)	224 ±7	7.95±0.68	221 ±12
Late Uterine Fluids	461,425	11.6,12.0	503,511

*$\bar{X}$ ± S.E. (N), $mM.l^{-1}$

TABLE 2

Transepithelial Electrical Potentials Across Squalus acanthias "pups" in Sea Water and Ion-substituted Sea Water Solutions.

SW	$-K^+, -Na^+$ ASW*	SW	$-Cl^-, -HCO_3^-$ ASW	SW
-4.4±0.6**	-7.0±0.6	-4.5±0.7	+9.1±0.7	-2.6±0.5
(14)	(14)	(13)	(13)	(12)

*ASW = Artificial Sea Water

**TEP in mV, $\bar{X}$ ± S.E. (No. of animals)

Table 1 shows that *intra-utero* "pups" maintain blood Na^+ and Cl^- concentrations equivalent to that of the mother; embryonic blood K^+ is, however, twice that of the female. Both the mother and the embryo maintain blood Na^+, K^+ and Cl^- much below that of the uterine fluids (Table 1). It is, therefore, clear that late-term addition of seawater to the uterine fluids represents a potential ionic imbalance to both the embryo and the mother.

The rate of unidirectional Na^+ efflux from "pups" was the same whether we used animals with intact or ruptured yolk sacs. In addition, analysis of the amount of radioactivity in the yolk sac at the termination of some efflux experiments showed that only 3.5 ± 0.7% (6) of the total ^{22}Na activity in the fish was in the yolk sac. It, therefore, appears that despite an extensive vascularization, the yolk sac and its contents do not represent either a high Na^+-permeability pathway or a deposit for Na^+ during development. The rate constant of Na^+ efflux from 11 pups was 2.9 ± 0.9 x $10^{-3}.hr^{-1}$($\bar{x}$ ± S.E.) and the Na^+ space was 63.0 ± 8.5 $ml.100\ g^{-1}$. Since the blood Na^+ concentration of these animals was 237.7 ± 7.0 $mM.l^{-1}$ (not significantly different from those in Table 1) the unidirectional Na^+ efflux can be calculated (as the product of these three parameters) to be 41.2 $\mu M.100\ g^{-1}.hr^{-1}$, somewhat below, but of the same order as, that described for the adult (68 $\mu M.100\ g^{-1}.hr^{-1}$, Horowicz and Burger, Am. J. Physiol. 214:635-642, 1968). Thus, the premature "pup" of *S. acanthias* is apparently able to maintain the extremely low Na^+ permeability which characterizes marine elasmobranchs (Evans, in "Osmotic and Ionic Regulations in Animals," G.M.O. Maloiy editor, Academic Press, 1979).

Table 2 demonstrates that the TEP across the premature "pup" is inside negative to seawater, similar to that described for other elasmobranchs (Evans, Am. J. Physiol., in press; Evans and Kormanik, this bulletin). Since both $[Na^+]$ and $[Cl^-]$ are maintained distinctly below that of the surrounding seawater (or late uterine fluids) (see Table 1), it is evident that *S. acanthias* "pups" maintain both ions out of electrochemical equilibrium with the external environment. Removal of external Na^+ and K^+ affected the TEP to a much lesser extent than removal of Cl^- and HCO_3^-. Since the ionic gradients (between seawater and blood) for Na^+ and Cl^- are similar this indicates that this species is relatively more permeable to Cl^- than Na^+. This is to be directly contrasted with most teleost species which show a decidedly greater cation than anion permeability (Evans, in "Osmotic and Ionic Regulations in Animals," G.M.O. Maloiy, editor, Academic Press, 1979). In addition, the relatively small changes in the TEP elicited by removal of either Na^+ or Cl^- from the seawater (the TEP across a typical teleost may depolarize by 10 to 50 mV when external Na is removed (Evans, *ibid*) demonstrates again the relative ion-impermeability of this elasmobranch (see flux data above).

TABLE 3

Cloacal Fluid Flow, Ion Concentrations and Ionic Loss from S. acanthias "Pups"

Time Period	Flow ($\mu l.100g.h^{-1}$)	$[Na^+]$ ($mM.l^{-1}$)	$[Cl^-]$ ($mM.l^{-1}$)	Na Flux* ($\mu M.100g.h^{-1}$)	Cl Flux ($\mu M.100g^{-1}.h^{-1}$)
10:00-16:00 (Max. flow)	117.±17. (11)	397±40 (10)	468±17 (9)	42.5±6.4 (10)	57.6±11.0 (9)
22:00-8:00 (Min. flow)	52.9±9.0 (12)	378±28 (11)	484±34 (8)	20.5±4.4 (11)	22.6±4.0 (8)
Significance between Max. & Min. Flow Periods	p<0.01	n.s.	n.s.	p<0.01	p<0.01

*calculated for individual fish

The cloacal flow rate displayed significant diurnal variation so Table 3 presents data for the two time periods which represent the maximum and minimum cloacal flow rates. It is obvious that the cloacal fluids from the premature "pups" contain significantly more Na^+ and Cl^- than the blood, indicating a net excretion of both ions via the cloacal fluids. It is interesting to note that, despite the diurnal variation of flow rate, the cloacal fluid ionic concentration remained relatively constant so that the cloacal efflux rate of both Na^+ and Cl^- varied with the flow rate. The flow rates and concentrations of Table 2 are of the same order as published data on the adult elasmobranch rectal gland (Evans, *ibid*)

TABLE 4

Transepithelial Electrical Potentials across Isolated Early and Late Term S. acanthias Uteri in various Electrolyte Solutions

	Ringers	SW	$-K^+,-Na^+$ ASW	SW	$-Cl^-,-HCO_3^-$ ASW	SW	Ringers
Early term	+0.7±0.8 (3)	+0.8±1.0 (5)	-1.6±0.6 (5)	+0.3±1.2 (5)	+8.7±2.3 (5)	+1.4±0.7 (3)	+1.2±1.5 (3)
Late term	+8.7±2.4 (5)	+7.8±1.6 (7)	+2.1±1.2 (7)	+7.8±1.6 (7)	+17.5±3.5 (7)	+6.5±1.4 (5)	+11.8±2.8 (7)

All data are expressed as X ± S.E. (N).

Each everted uterine "sac" was transferred through the series of solutions (mucosal side out) as indicated. The serosal side was always filled with elasmobranch Ringer's solution.

which indicates that either renal fluid and ionic production is rather limited in the "pup" or else both renal and rectal function in the "pup" is far below that found in adults. Comparison of the cloacal Na^+ efflux with the total Na^+ efflux determined isotopically indicates that from 50-100% (depending on time of day) of the total is via cloacal loss. Unfortunately isotopic efflux data were not gathered in a way which could distinguish diurnal variation. The rate constant of 2.9×10^{-3} was derived from overnight flux determinations. Clearly, a substantial portion of the Na^+ efflux from the "pup" is extracloacal and presumably represents the sum of diffusional and active efflux components since the rectal gland is not a vital organ (Burger, Physiol. Zool. 38:191-196, 1965, Chan et al., Comp. Biochem. Physiol. 23:185-198, 1967). Maetz and Lahlou (J. Physiol. Paris 58:249, 1966) have also found that an appreciable amount of the Na^+ efflux from the adult, *Scyliorhinus canicula*, is extra-rectal.

Table 4 indicates that the electrical parameters of the early uterus are distinctly different from those of the later uterus. While the early uterus is unable to develop a TEP with ERS on both

sides it is clear that the later uterus is able to sustain a serosal positive potential which presumably indicates either net uptake of cations from the uterine fluids or net secretion of anions into the lumen contents. These alternatives cannot be distinguished until tracer flux studies are performed. Further, the TEP across the young uterus is rather refractory to substantial alteration of the ionic content of the mucosal solutions except that removal of external Cl^- did hyperpolarize the membrane by 8 mV, indicating some sort of finite, but low, Cl^- permeability. In contrast, the later uterus displays more substantial changes in the TEP when the dominant cations (Na^+ and K^+) or the dominant anion (Cl^-) are removed from the mucosal solutions. Thus, the relative ionic permeabilities have increased during development of the uterus, and, as in the case with the "pup" (see above), Cl^- appears to be the most permeant.

In summary, our preliminary investigations of some aspects of osmoregulation by developing *S. acanthias* embryos and juxtaposed uteri indicate that the pups are able to maintain blood Na^+ and Cl^- levels below uterine fluids (and seawater) because of low ionic permeability and cloacal (presumably rectal gland) extrusion of these ions (the typical adult pattern). In addition, the uterus maintains a rather low ionic permeability, despite extensive vascularization, with Cl^- being more permeant than Na^+. Most interestingly, the older uteri are able to develop measurable TEPs in elasmobranch Ringer's solutions, which indicates that some sort of electrogenic ionic transport must be taking place across this epithelium. Further experiments on the ontogeny of osmoregulation by elasmobranchs are certainly warranted. Research supported by NSF PCM 77-09915.

EPITHELIAL CELL PROLIFERATION IN FLOUNDER INTESTINE: A PRELIMINARY REPORT

Jerry S. Trier,* Pamela C. Moxey* and Michael Field,** Departments of Medicine, Harvard Medical School (Peter Bent Brigham Hospital)* and The University of Chicago**

It has been suggested that cholera toxin-induced secretion in mammalian small intestine occurs primarily at the level of the immature proliferating undifferentiated cells in the crypts. Unlike mammalian small intestine, the intestine of the winter flounder, *Pseudopleuronectes americanus* does not respond to cyclic AMP with fluid and electrolyte secretion (Field and Smith, MDIBL Bull. Vol. 15). Moreover, unlike mammalian small intestine, morphologically defined crypts are not present in the mucosa of flounder small intestine or in other fish studied to date (Field et al., J. Memb. Biol. 41:265).

To determine whether immature proliferating epithelial cells are present in the small intestine of the winter flounder and to determine their distribution in the mucosa, the DNA precursor, [^{3}H]-thymidine (2 μCi/g body wt) was injected intravenously into fasted (at least 72 hr) and fed flounder. Fasted and fed fish were compared because the rate of active chloride absorption was found to be appreciably greater in the fasted fish (M. Field, D. Clayton and R.A. Frizzell, unpublished observations). Fish were killed 2 to 48 hours after thymidine injection and samples of proximal, middle and distal intestine were fixed in Bouin's solution and embedded in paraffin. Tissues were serially sectioned, dipped in photographic emulsion, incubated, developed and stained with hematoxylin and eosin. Adjacent samples of proximal, middle and distal intestine were fixed with glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy resin.

To date we have completed preparation and initial examination of radioautographs of the proximal middle and distal intestine of fasted flounder. The general morphology of flounder intestine was the same at all levels. Whereas flounder intestine is not characterized by crypts and villi as is mammalian small intestine, it is covered by mucosal folds which, when cross-sectioned, give an appearance similar to that of villi. Within 2 hours after exposure to [^{3}H]-thymidine, labeled epithelial cells in fasted