Pablo Sabat · Karin Maldonado
Antonio Rivera-Hutinel · Gonzalo Farfan

Coping with salt without salt glands: osmoregulatory plasticity in three species of coastal songbirds (ovenbirds) of the genus *Cinclodes* (Passeriformes: Furnariidae)

Abstract We investigated the phenotypic plasticity of renal function in three South American coastal passerine *Cinclodes* (ovenbirds) differing in the proportion of marine prey they consume. Individuals were acclimated to two regimes of salinity for 15 days, and then the maximal urine-concentrating ability (U_{max}), hematological parameters and kidney morphology of each species were determined. The proportion of kidney mass occupied by medullary tissue, the number of medullary cones in the kidneys, plasma osmolality and U_{max} differed among the three species, supporting the hypothesis of an adaptation for excretion of the high salt load in the strictly marine *C. nigrofumosus*. Our results indicate that species of *Cinclodes* are able to modify the proportion of medullary tissue and the U_{max}. In addition, we found interspecific differences in the magnitude to which these osmoregulatory parameters can be modified. The greater ability to modify the osmoregulatory features in the migrant species *C. oustaleti* may enable it to cope with seasonal changes in salt load imposed by the winter consumption of hypertonic prey.

P. Sabat (✉) · K. Maldonado · G. Farfan
Departamento de Ciencias Ecológicas, Facultad de Ciencias,
Universidad de Chile, Casilla 653, Santiago, Chile
E-mail: psabat@uchile.cl

A. Rivera-Hutinel
Center for Advanced Studies in Ecology & Biodiversity
and Departamento de Ecología, Facultad de Ciencias Biológicas,
Pontificia Universidad Católica de Chile, Santiago, Chile

Introduction

Phenotypic plasticity, the ability to change and modify the phenotype in response to environmental cues, is crucial for the maintenance of homeostasis under changing environments (Stearns 1989; Pigliucci 2001). Many birds experience seasonal shifts in their diet in response to changing environmental resource availability or variations in nutritional requirements (Karasov 1996; Sabat et al. 1998). Dietary shifts are often coupled with changes in the chemical composition of food resources (Bell 1990). In order to maintain biological homeostasis, animals respond to these changes by modifying their physiology.

In sea birds, consumption of marine invertebrates is accompanied by significant osmotic load (e.g. Sabat et al. 2003). When birds switch from terrestrial to marine prey or consume food with high salinity, salts are absorbed by the small intestine, and their concentrations in the body fluids increase (Holmes et al. 1961; Hughes 2003). Unless birds can excrete fluids that are more concentrated than ingested water, the body becomes dehydrated. Most marine groups eliminate highly concentrated NaCl solutions through the nasal salt glands. In birds that lack salt glands, renal function is particularly important, becoming the only way to eliminate the salts ingested with food. Many studies have shown that the structure and function of the kidney varies with the feeding habitats and water availability of species (Goldstein and Braun 1989; Casotti et al. 1998). For example, kidney mass and percentage of renal medulla seem to be greater in birds inhabiting environments where fresh water is scarce or unavailable, whereas the cortex is larger in birds that ingest greater amounts of water with their food. As a short-term response, renal function may be modified by conditions of hydration (Braun 1978; Goldstein and Zahedi 1990). However, plasticity of renal function has received little attention (Goldstein et al. 2001).

Although there is an increase in the use of phylogenetically based statistical methods to inferring adaptation

(see Garland and Adolph 1994; Garland et al. 1999), comparative studies of closely related species minimize the effects of potentially confounding variables associated with phylogeny, and give insight into how differences in organismal physiology evolved (e.g. Tieleman et al. 2003). The aim of this study was to characterize the ability of three passerine species in the genus *Cinclodes* (Furnariidae) to adapt to different conditions of salt ingestion. Species of *Cinclodes* are well-suited models for the study of phenotypic plasticity of the renal function because they are conspicuous members of marine coastal and freshwater stream habitats. *Cinclodes nigrofumosus* is probably the most marine passerine species, with more than 80% of prey consumed being of marine origin, whereas *C. patagonicus* and *C. oustaleti* incorporate more terrestrial prey in coastal habitats (Sabat and Martinez del Río 2002; Sabat and Gonzalez 2003; Sabat et al. 2003). Also, because some populations of *C. patagonicus* and *C. oustaleti* move seasonally from the coast to inland streams during the dry austral summer (Sielfeld et al. 1996; Jorge et al. 1998), they probably shift their diet between salt water and fresh water prey, possibly experiencing temporal variations in electrolyte and fresh water intake, causing osmoregulatory demands to change during their life span. Phenotypic plasticity in renal function is probably a critical function allowing these species to have wider food niches. Also, based on dietary preferences and the osmoregulatory features exhibited in the field, we expected marked differences in the maximal urine-concentrating ability among these species of *Cinclodes*.

Materials and methods

C. nigrofumosus (11 individuals) and *C. oustaleti* (13 individuals) were captured with mist nests in the coastal locality of El Quisco (33°34′S, 71°37′W), whereas 10 *C. patagonicus* were captured in an inland fresh-water stream at El Manzano (33°39′S, 70°22′W, approximately 120 km from the coast) in central Chile. Birds were transported to the laboratory and maintained at 22 ± 2 °C, L:D 12:12 in separate cages of 50×50×50 cm with mealworm (*Tenebrio mollitor*) larvae and water ad libitum. After 24 h, animals were weighed with an electronic balance (± 0.01 g) and then randomly assigned to one of two treatments: one group had access to tap water and the other to an 800 mOsmol kg^{-1} NaCl solution ad libitum for 2 weeks. The concentration of 800 mOsmol kg^{-1} was used as the best estimation of the concentration of food and drinking water (Sabat and Martinez del Rio 2002) because it is near the maximum concentration of stomach fluids of *Cinclodes* species in the field.

After the 2-week acclimation period, blood samples were collected from the humeral vein of conscious birds into heparinized hematocrit tubes. Blood samples were centrifuged at 9,000 *g* for 5 min, the hematocrit was recorded, and the plasma was stored frozen (−40 °C) for no more than 1 week before osmometry analysis. Animals were then salt loaded with 0.02 ml g^{-1} bird of a solution of 600 mmol l^{-1} NaCl. Poulson and Bartholomew (1962) showed that a maximum concentration of urine was elicited in sparrows when this solution was supplied. During loading, birds were gently held in a standing position and a rubber tube was inserted into the oesophagus to deliver the salt solution. Birds were placed in individual 25×25×30 cm plastic cages and were continuously observed, and urine samples were taken every 15 min after salt-loading administration for at least 2 h. Ureteral urine was obtained by inserting a small closed-ended cannula into the birds' cloaca. Urine drained into the cannula via a window placed dorsally over the ureteral orifices, and the closed end avoided contamination by intestinal fluids (Goldstein and Braun 1989). Although this procedure generally prevents contamination with intestinal fluids, samples with evidence of brown coloration were discarded. Urine samples were centrifuged, and the supernatant was saved for later analysis to obtain the maximal urine concentration after salt loading (U_{max}). Osmolality of the blood plasma and supernatant of urine were determined by vapour pressure osmometry (Wescor 5130B).

After the experiments were completed, birds were weighed, sacrificed by decapitation, dissected and the organs were removed. Kidneys were weighed (± 0.001 g) and preserved in paraformaldehyde-glutaraldehyde. The area of medulla was estimated by point counting using the Cavalieri principle on the right kidney, which was processed for routine light microscopy (Gundersen et al. 1988; Wauri 1989). Medullary cones were dissected from the left kidney using a dissecting microscope, and the entire collection was counted. The effects of species and salt concentration were tested using a two-way ANOVA (analysis of variance), using species and acclimation condition as factors. To test for differences in morphological data, a two-way ANCOVA (analysis of covariance) was performed, using body mass as the covariate. In addition, to assess how much of the trait variation is due to taxonomic level (i.e. phylogenetic proximity), a nested analysis of variance with two variables as random factors was performed. The first factor grouped the sister species in one category (*C. nigrofumosus* and *C. patagonicus*)) and the third species in another category, reflecting the hierarchical phylogeny (T. Chesser, unpublished results). The second factor, nested in the former, has one category for each species. Hence, any effect due to phylogenetic proximity would appear as a significant effect of the first factor (see Harvey and Pagel 1991; Gittleman and Luh 1992). All statistical comparisons were conducted using Statistica for the Windows 95 operating system (StatSoft Inc., Tulsa, Oklahoma, 1997).

Results

Renal morphology

Before acclimation, average body mass did not differ between groups in any of the species. When we

combined all three species in a repeated-measures ANOVA using body mass after and before the acclimation period as dependent variables, and species and acclimation as independent factors, we found strong interspecific differences ($F_{(2,28)} = 216.6$, $P < 0.001$) but no significant effect of treatment ($F_{(1,28)} = 1.3$, $P = 0.26$, Table 1) nor any interaction among factors ($F_{(2,28)} = 0.04$, $P = 0.95$). We concluded that the three species are able to maintain mass on the 800-mOsmol kg^{-1} NaCl drinking regime. On average, the mass of both kidneys was $2.0 \pm 0.6\%$ of the body mass in *C. nigrofumosus*, which was greater (ANCOVA $F_{(2, 28)} = 19.87$, $P < 0.001$) than the $1.7 \pm 0.4\%$ in *C. oustaleti* and $1.2 \pm 0.2\%$ in *C. patagonicus*. In addition, there was a significant effect of acclimation on kidney mass ($F_{(1, 28)} = 5.43$, $P = 0.037$), but no significant interaction between factors ($F_{(2, 28)} = 1.40$, $P = 0.26$, Table 1).

The mass of kidneys was higher in the salt water (SW) than in the tap water (TW) treatment. Nevertheless, a posteriori Tukey tests did not detect any differences between treatments in any species. The proportion of kidney mass consisting of medullary tissue was higher in *C. nigrofumosus* than in *C. oustaleti* and *C. fuscus* ($F_{(2,28)} = 21.49$, $P < 0.001$, Fig. 1). The effect of acclimation on the proportion of medullary tissue approached significance ($F_{(1,28)} = 4.06$, $P = 0.053$), as did the interaction between species and acclimation ($F_{(2,28)} = 0.76$, $P = 0.5$), the SW acclimated birds tending to have more medullary tissue (Fig. 1). However, the a posteriori Tukey test revealed that the proportion of kidney mass consisting of medullary tissue in *C. oustaleti* was higher in SW birds than in TW birds ($P = 0.048$).

The total number of medullary cones differed among species (ANCOVA $F_{(2,28)} = 6.22$, $P = 0.007$, Fig. 1) but was not affected by acclimation ($F_{(2,28)} = 1.25$, $P = 0.275$) nor by the species × acclimation interaction ($F_{(2,28)} = 1.68$, $P = 0.20$). The number of medullary cones per gram of kidney of the marine species *C. nigrofumosus* was almost twice that of the two congeners. In addition, there was a significant and positive correlation ($r^2 = 0.50$; $P < 0.001$) between the proportion of medullary tissue and number of medullary cones per gram of
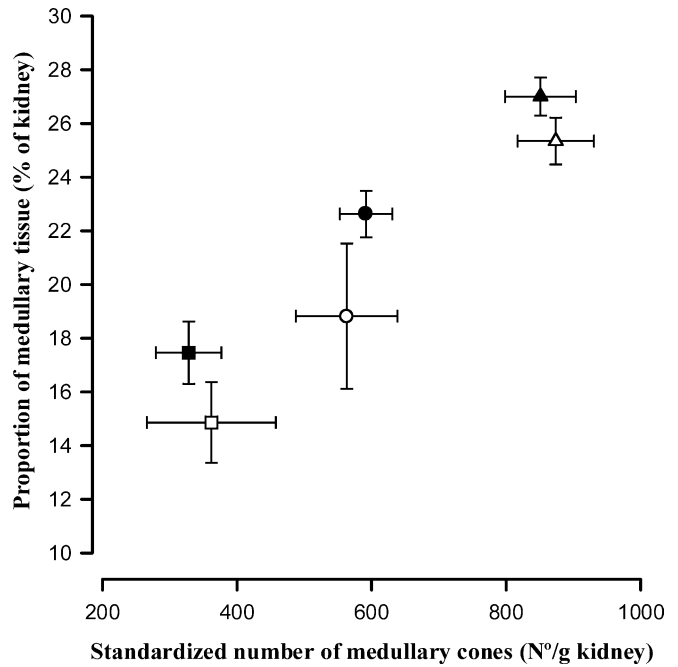

Fig. 1 Proportion of kidney mass composed of medullary tissue and number of medullary cones per gram of kidney in *Cinclodes patagonicus* (*squares*), *Cinclodes oustaleti* (*circles*) and *Cinclodes nigrofumosus* (*triangles*) acclimated for 15 days to tap water (*open symbols*) and 800 mOsmol kg^{-1} NaCl solution (*closed symbols*). Data are mean ± SE

kidney (Fig. 1). Table 3 shows the results of the hierarchically nested ANOVA. Results agree with those of conventional ANOVA and, without exception, all variables were unaffected by the phylogenetic effect, expressed as the effect of the random variable grouped by phylogenetic proximity.

Physiological parameters

The hematocrit of *Cinclodes* species did not differ significantly among species ($F_{(2,28)} = 1.42$, $P = 0.26$). After 4 weeks of acclimation, the hematocrit was affected by

Table 1 Osmoregulatory features of *Cinclodes nigrofumosus* (*Cn*), *Cinclodes patagonicus* (*Cp*) and *Cinclodes oustaleti* (*Co*) acclimated to salt water (*SW*) or tap water (*TW*). Summary of results of two-way ANOVA (and repeated-measures ANOVA for body mass) using SW = 800 mOsmol kg^{-1} of NaCl solution and TW as factors are shown. Values are presented as mean ± 1 SD

	SW ($n = 6$)	TW ($n = 6$)	SW ($n = 5$)	TW ($n = 5$)	SW[b] ($n = 8$)	TW ($n = 5$)	Spp	Acc	Spp×acc
	C. nigrofumosus		*C. patagonicus*		*C. oustaleti*		Effect[a]		
Body mass (g)	72.1 ± 11.6	66.5 ± 7.8	37.4 ± 1.9	40.4 ± 1.7	27.1 ± 1.7	23.8 ± 5.6	s[c]	ns	ns
Kidney mass (g)	1.38 ± 0.16	1.25 ± 0.24	0.48 ± 0.06	0.45 ± 0.03	0.47 ± 0.06	0.45 ± 0.09	s[d]	s[e]	ns
Hematocrit	48.8 ± 10.3	50.7 ± 0.5	49.8 ± 3.5	46.2 ± 4.8	48.4 ± 8.8	42.4 ± 8.9	ns	ns	ns
Urine (mOsmol kg^{-1})	793 ± 110	738 ± 216	538 ± 102	516 ± 141	796 ± 202*	591 ± 186	s[d]	ns	ns
Plasma osmolality (mOsmol kg^{-1})	387 ± 25	379 ± 12	357 ± 12	360 ± 14	378 ± 12	373 ± 32	s[d]	ns	ns

[a] *Spp* Species; *acc* acclimation; *s* significant effect; *ns* non-significant effect
[b] *Asterisk* denotes significant within-species differences between treatments, $P < 0.05$ (Tukey-type a posteriori tests)

[c] $Cn > Cp > Co$
[d] $Cn > Cp = Co$
[e] $SW > TW$

Table 2 Urine osmolalities after the ingestion of 0.02 ml g^{-1} bird of a solution of 600 mmol l^{-1} NaCl of *Cinclodes nigrofumosus, Cinclodes patagonicus* and *Cinclodes oustaleti* acclimated to salt water (*SW*) or tap water (*TW*). Note that means of urine osmolalities in most cases differ from U_{max} because each bird reached U_{max} at different times

		Time after salt load (min)							
		15	30	45	60	75	90	105	120
C. nigrofumosus	SW	831 ± 106	861 ± 125	938 ± 180	1036 ± 166	909 ± 97	990 ± 14	986 ± 19	853 ± 128
C. nigrofumosus	TW	888 ± 63	935 ± 78	943 ± 75	9535 ± 40	926 ± 85	955 ± 63	–	900 ± 115
C. patagonicus	SW	592 ± 84	740 ± 69	804 ± 53	793 ± 11	805 ± 42	–	705 ± 164	–
C. patagonicus	TW	543 ± 113	688 ± 77	752 ± 103	753 ± 102	820 ± 60	–	760 ± 57	–
C. oustaleti	SW	812 ± 159	893 ± 79	937 ± 104	912 ± 94	946 ± 28	–	850 ± 70	–
C. oustaleti	TW	680 ± 325	692 ± 194	822 ± 159	845 ± 190	–	830 ± 183	–	–

neither the experimental condition ($F_{(1,28)}=1.39$, $P=0.24$) nor the species × acclimation interaction ($F_{(2,28)}=0.11$, $P=0.37$, Table 1). Plasma osmolality was higher in *C. nigrofumosus* ($F_{(2, 28)}=3.41$, $P=0.048$) but was not affected by experimental condition ($F_{(1,28)}=0.11$, $P=0.73$) or by the interaction between species and experimental condition ($F_{(2, 28)}=0.14$, $P=0.86$). After the acclimation period, urine osmolality was higher in *C. nigrofumosus* ($F_{(2, 28)}=6.23$, $P=0.006$), but there were no effects of treatment or interaction ($F_{(1,28)}=0.6$, $P=0.43$ and $F_{(2, 28)}=1.6$, $P=0.21$, respectively, Table 1).

Temporal profiles of urine osmolalities are shown in Table 2. Maximal ability to concentrate urine was greater in *C. nigrofumosus* than in *C. oustaleti* and *C. patagonicus* ($F_{(2, 28)}=19.39$, $P<0.001$), with no difference between *C. oustaleti* and *C. patagonicus* (Fig. 2). In addition, maximal urine-concentrating ability was higher in birds acclimated to SW ($F_{(1,28)}=5.29$, $P=0.003$), but the interaction between species and experimental condition was not significant ($F_{(2, 28)}=0.61$, $P=0.54$). *Cinclodes oustaleti* acclimated to SW had a significant increase of 14% in U_{max}, whereas *C. nigrofumosus* and *C. patagonicus* did not (Fig. 2). In spite of the great variability in U_{max}, none of the *C. patagonicus* and *C. oustaleti* reached urine-concentration values higher than seawater (1,000 mOsmol kg^{-1}), whereas *C. nigrofumosus* did. To test whether morphological and physiological variables can explain the variability of U_{max}, a multiple forward stepwise regression analysis was performed using standardized kidney mass, plasma osmolality, proportion of medullary tissue, and the standardized number of medullary cones as independent variables. Results of this analysis showed that medullary tissue and plasma osmolality best explained the variability in U_{max} [multiple $r^2=0.76$, $P<0.001$; $U_{max}=8.87$ (medullary tissue) + 2.16 (plasma osmolality) − 114].

Discussion

Comparative kidney morphology and physiology

Several studies have attempted to link the morphology of avian kidneys with the ability to concentrate urine (Goldstein and Brown 1989; Casotti et al. 1998). For example, Casotti and Braun (2000) reported that differences in renal anatomy in three species of sparrows could be correlated with the ability to produce concentrated urine. Our results are consistent with those reported in sparrows, and also with those previously reported in species of *Cinclodes* in the field (Sabat and Martinez del Río 2002). Also, we found that the number of medullary cones of kidneys in the marine species is almost twice than in migratory species (Fig. 1). The number of medullary cones per gram of kidney in *C. nigrofumosus* is similar to that recorded in the salt-marsh savannah sparrow *P. sandwichensis beldingi* (Johnson and Mugaas 1970) but higher than that reported for non-marine bird species examined to date (see Johnson and Mugaas 1970; Johnson and Ohmart 1973; Goldstein and Braun 1989; Goldstein and Ellis 1991).
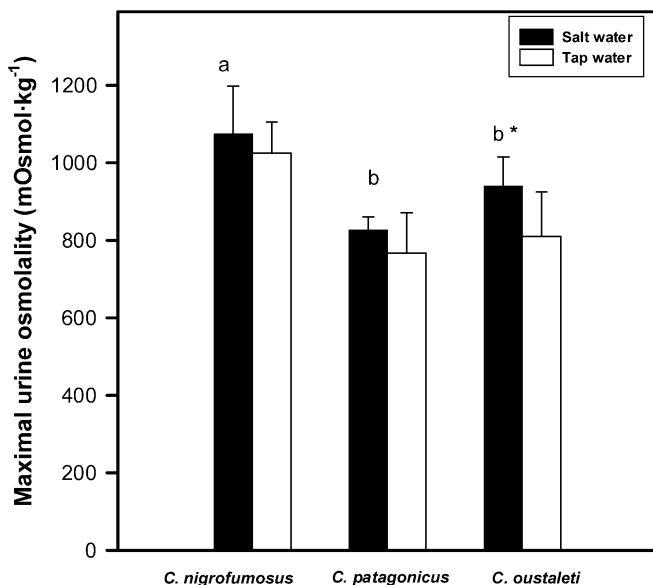
Fig. 2 Maximal urine-concentrating ability in three species of *Cinclodes* acclimated to different salt loads for 15 days. *Different letters* indicate significant differences among species, and *asterisks* denote significant differences between groups, after a Tukey test. Data are mean ± SD

Table 3 Results of the hierarchical nested ANOVA for some features of *Cinclodes nigrofumosus, Cinclodes patagonicus* and *Cinclodes oustaleti*

	First random factor (phylogenetic proximity)		Second random factor (species)	
	F	P	F	P
Body mass (g)	1.57	0.43	44.64	< 0.0001
Kidney mass (g)	0.006	0.95	8.58	0.008
Medullary cones	0.001	0.98	68.30	< 0.0001
Medullary tissue	0.16	0.76	63.38	< 0.0001
Hematocrit	42.08	0.31	0.31	0.58
U_{max} (mOsmol kg^{-1})	0.04	0.87	23.74	< 0.0001
Plasma osmolality	0.17	0.76	2.13	0.15

Cinclodes nigrofumosus has a greater ability to concentrate urine, producing on average urine with osmolalities slightly higher than that of seawater (i.e. 1,000 mOsmol kg^{-1}). In contrast, none of the individuals of *C. patagonicus* and *C. oustaleti* had urine concentrations higher than seawater. The U_{max} of *C. nigrofumosus* is significantly higher than those produced by any other species of passerine birds (see McNab 2002; Fleming and Nicolson 2003), with the exception of salt-loaded salt-marsh savannah sparrows in which values near 2,000 mOsmol kg^{-1} have been recorded (Poulson and Bartholomew 1962, but see Goldstein et al. 1990 for data on field-caught birds). Thus although we are unable to present maximal concentrating abilities as urine:plasma osmolality ratios (plasma osmolality was not measured after salt loading), it seems clear that the ability to concentrate urine is unusually high in *C. nigrofumosus*. In addition, the high plasma osmolality of *C. nigrofumosus* on both acclimation treatments can be viewed as an adaptation to cope with prey of high salt concentration year-round. Taking into account the significant positive relationship between the morphological and physiological variables in *Cinclodes* species, and the interspecific differences in kidney morphology and physiological parameters, our data strongly support the hypothesis that the ability to incorporate marine prey is linked to a higher urine-concentrating ability which, in turn, is coupled with a more developed medulla and number of medullary cones in the kidney (Fig. 1).

Our concern about the possible effect of phylogenetic inertia affecting our analysis was put aside by the nested ANOVA results (Table 3). In addition, a molecular phylogenetic hypothesis of the relationships of *Cinclodes* suggests that the marine specialist condition appears to have been derived twice in *Cinclodes*—once in the *C. nigrofumosus/C. taczanowskii* group and once in *C. antarcticus* from a non-marine ancestor (T. Chesser, unpublished data). The marine condition in *Cinclodes* appears to be an apomorphic condition, whereas the life in limnic habitats is the plesiomorphic condition. In this sense, the fact that osmoregulatory features in *Cinclodes nigrofumosus* are clearly different from those in the other species reinforces the idea of a physiological adaptation to cope with salt load.

Flexibility of renal function

The few studies documenting phenotypic plasticity of renal function in birds show results and conclusions that are contradictory (e.g. Holmes et al. 1968; Johnson and Ohmart 1973; Bankir and Rouffignac 1985; Goldstein and Zahedi 1990; Goldstein et al. 1990; Casotti 2001). Our results indicate that *Cinclodes* species have the ability to produce urine with higher concentration if birds are previously acclimated to salt water. However, neither *C. oustaleti* nor *C. patagonicus* from both treatments approached the values of U_{max} measured in *C. nigrofumosus*. The results of within-species effects suggest that this ability is more pronounced in *C. oustaleti* (Fig. 2). The same is true for kidney morphology (Table 1). The ability to change U_{max} exhibited by *Cinclodes oustaleti* was coupled with a more developed medulla in salt-acclimated birds. In spite of the correlation between the number of medullary cones and the proportion of medullary tissue, the absence of any change in the number of medullary cones following acclimation suggests that salt-acclimated *C. oustaleti* probably enhance medullary tissue by hypertrophy. Likewise, Goldstein and Ellis (1991) found that medullary tissue mass (but not kidney mass) was enhanced in dehydrated adult quails, and that this increase did not involve any increase in number of medullary cones. In addition, Goldstein et al. (2001) and Sabat et al. (2004) have documented changes in renal structure associated with experimental changes in dietary protein concentration. It seems that the need for elimination of great amounts of any electrolyte (salt or nitrogen) elicits a similar response in kidney morphology, producing a hypertrophy of medullary tissue.

Our results indicate that *Cinclodes* species are probably able to change the proportion of medulla by medullary tissue hypertrophy and also U_{max}. However, it seems that *C. oustaleti* has a greater phenotypic plasticity in renal function, which is coupled with structural changes in the kidneys. *Cinclodes patagonicus* seems to be less flexible in both physiology and morphology, and *C. nigrofumosus* virtually does not change in any variable. What could explain the interspecific differences in the extent to which these osmoregulatory parameters are modified in *Cinclodes*? Plasticity of physiological and

morphological traits may be constrained in specialised individuals, and might have an adaptive value in those that experience great variation in the physical and biotic environment (Stearns 1989; Padilla and Adolph 1996). *Cinclodes nigrofumosus* is exclusively marine, and thus would be expected to have a more stable year-round diet and salt loads. In contrast, *C. oustaleti* and *C. patagonicus* migrate between freshwater and coastal habitats. Hence, variation in the salt loads that individuals experience is expected through time. The extent to which the ability to modify renal morphology as a function of osmotic load depends on the ecological habits of species or populations is a question that needs to be further assessed.

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