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Costs of osmoregulation by Black-winged Stilt chicks (*Himantopus himantopus*) raised in high saline environments.

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Table of contents

Resumo

As zonas húmidas naturais estão ameaçadas em todo o mundo devido ao aumento da população humana e às consequências adversas associadas à sua atividade. Devido à rápida regressão das zonas húmidas costeiras e interiores nas quais as aves se alimentam e reproduzem, estas têm vindo a ocupar habitats artificiais que são geridos pelo Homem. Devido à sua elevada produtividade e proximidade das zonas naturais afetadas, as salinas são um importante habitat artificial alternativo para as aves costeiras migradoras e invernantes como local de refúgio durante a maré cheia, local de alimentação e nidificação e podem atenuar o impacto da perda das áreas húmidas naturais. No entanto as aves são obrigadas a lidar com as elevadas concentrações de sal nas salinas, principalmente as crias que não têm capacidade de procurar locais menos salinos para se alimentar. Para manter o equilíbrio osmótico, as aves têm que eliminar o excesso de sal ingerido nas presas e na água. Uma vez que o sistema renal das aves têm uma reduzida capacidade de concentração, elas usam as glândulas de sal que excretam soluções altamente concentradas para eliminar o excesso de sal da corrente sanguínea. No entanto, experiências com aves costeiras têm demonstrado que manter um sistema osmorregulatório é energeticamente dispendioso. De facto, há escassez de informação acerca dos custos da osmorregulação e do funcionamento das glândulas de sal nas aves costeiras e pouco se sabe acerca dos efeitos da ingestão de elevados conteúdos de sal pelas aves que habitam as salinas. Pensa-se que as crias são mais afetadas pelo sal e manifestam os seus efeitos de forma mais evidente do que os adultos uma vez que as suas glândulas de sal podem estar subdesenvolvidas. O Pernilongo *Himantopus himantopus* é uma ave costeira que pode usar uma vasta gama de habitats com diferentes salinidades como barragens, zonas húmidas interiores de água doce, estuários e está fortemente associada a habitats hipersalinos como as salinas. O presente estudo pretende avaliar de que forma as crias de Pernilongo estão aptas a osmorregular em condições salinas e verificar se elas apresentam custos energéticos devido à osmorregulção e ao sal ingerido. Para levar a cabo o nosso objetivo, mantivemos em cativeiro crias de Pernilongo submetidas a quatro tratamentos salinos: 0g/l, 20g/l, 60g/l e 120g/l e, para cada tratamento, analisámos variáveis fisiológicas (taxa de crescimento, assimetria flutuante, concentração de iões Na^+ e Cl^- no plasma, hematócritos, taxa metabólica basal) e comportamentais (frequência com que abanam a cabeça para eliminar o sal das presas, antes e após a sua ingestão).

Os nossos resultados demonstram que os níveis de salinidade apenas tiveram efeito no comportamento das crias enquanto estas se alimentavam através do aumento dos movimentos da cabeça antes e após a ingestão das presas. Isto sugere que o principal mecanismo para prevenir as consequências do sal é expelir o excesso de sal da presa e do bico e evitar ingeri-lo. Portanto, as glândulas de sal das crias parecem funcionar de forma semelhante em diferentes salinidades e deverão estar aptas a excretar o inevitável sal ingerido sem provocar um aumento nas taxas metabólicas basais devido ao seu comportamento lhes permite manter as concentrações internas de solutos e o seu normal desenvolvimento.

Este estudo contribui para salientar que as salinas são um habitat de nidificação importante para o Pernilongo, dado que esta espécie apresenta características comportamentais que lhe permite lidar com a dieta salina sem sofrer desvantagens em termos energéticos, permitindo assim o desenvolvimento normal das suas crias.

Palavras-chave: salinas, osmorregulação, taxa metabólica basal, glândulas de sal, *Himantopus himantopus*.

Abstract

Natural wetlands are under threat all over the world owing to the increase of the human population and adverse consequences of human activities. Due to the degradation and alteration of coastal and inland wetlands in which birds feed and reproduce, they have occupied man-made habitats. Due to their high productivity and proximity to natural altered areas salinas are an important alternative and artificial habitat for migrating and wintering shorebirds, as an area for roosting during high-tide, feeding and breeding and can mitigate the impact of natural wetlands loss. However birds are obligate to deal with high salt concentrations in salinas, particularly chicks which cannot search for less saline areas to feed. In order to maintain the osmotic equilibrium, birds have to eliminate the excess of salt ingested with prey and water. Birds are known to have poor renal concentrating capacity, and use salt glands to excrete highly concentrated solutions and thus to eliminate the excess of salt from the bloodstream. But experiments on shorebirds have demonstrated that maintaining active osmoregulatory machinery is energetically expensive. Indeed, there is a lack of information about osmoregulation costs and salt gland function in shorebirds and little is known about the effects of high salt contents ingested by birds that inhabit salinas. It is thought that chicks are more affected by salt loads and manifest its effects more clearly than the adults, because the salt glands of chicks may be under-developed. The Black-winged Stilt *Himantopus himantopus* is a shorebird species that can inhabit a wide range of habitats with different salinities such as dams, inland freshwater marshes and estuaries, and is strongly associated with hypersaline environments such salinas. The present study aims to evaluate in which extent Black-winged Stilt chicks are able to osmoregulate in saline conditions and assess whether they experience energetic costs due to osmoregulation and salt loads ingested. To achieve this we maintained Black-winged Stilt chicks in captivity in four saline treatments: 0g/l, 20g/l, 60g/l and 120g/l and measured for each treatment physiological (chick growth rate, fluctuating asymmetry, Na^+ and Cl^- ions concentration in plasma, hematocrit, basal metabolic rate) and behavioural (head-shake movements before and after prey ingestion) variables.

Our results show that salinity levels only had an effect on chick's behaviour while they were feeding by increasing head-shake movements before and after the ingestion of prey. The main mechanism to prevent physiological salt consequences is therefore to expel excess salt from the prey and the bill, thus avoiding its ingestion. Hence, chick’s salt glands seem to functioning similarly for different salinities and should be able to excrete the inevitable salt ingested without lead to an increase in basal metabolic rates, because the behaviour of chicks should allows to keep their internal concentrations of solutes and the normal development of chicks.

This study contributes to conclude that salinas are an important breeding habitat for Black-winged Stilt, because this species possesses behavioural characteristics enabling it to deal with a high salt diet without suffering energetic disadvantages, which enables the normal development of their chicks.

Key words: salinas, osmoregulation, basal metabolic rate, salt glands, *Himantopus himantopus*.

Chapter 1 – Introduction

1.1. Salinas as wetlands for waders

Wetlands are among the most productive and valuable ecosystems in the world (Bildstein et al 1991), and are the main habitats for waders that are dependent on inland and coastal wetlands for survival and reproduction (Bellio et al 2009). Due to human activities wetlands have been reduced and altered, resulting in adverse consequences for waterbird populations (Ma et al 2010). Man-made and agricultural habitats, such as fish-ponds, rice fields and salinas emerged as alternative or complementary habitats (Masero 2003). Salinas are relatively stable habitats and can act as a buffer area against the impact of natural habitat loss (Weber et al 1999). Due to its close location to remaining natural wetlands (Magno Toral & Figuerola 2012), high biological richness, low levels of disturbance, spatial heterogeneity and productive microenvironments (Lopez et al 2010), salinas are important habitats for roosting during high-tide (Dias 2009) and feeding by migrating (Masero 2003) and wintering shorebirds (Masero et al 2000), and constitute also an important habitat for some shorebird breeding species (Lopez et al 2010). For instance, In Portugal, it is estimated that 68.9% of the Black-winged Stilt *Himantopus himantopus* breeding pairs occur in salinas (Rufino & Neves 1991).

1.2. The cost of living and breeding in salinas

Although salinas constitute a fundamental alternative habitat for waterbirds in all life stages (e.g., Weber & Haig 1996, Elphick & Oring 1998, Connor & Gabor 2006), and may be crucial for the conservation of waterbird populations (Masero 2003), little is known about the effect of salinity on breeding birds that feed in hypersaline conditions, where they have only saline water to drink and almost all available prey (especially invertebrates) are in osmotic equilibrium with this water (Sabat 2000). Because salinization may result in reduced reproduction through chick mortality, and reduced vigor associated dehydration may increase mortality at all life stages (Rubega & Robinson 1996), a key question is to understand in what extent breeding in salinas can affect chick development and, therefore, breeding success and ultimately the conservation of species, when compared with natural habitats with lower saline conditions such as freshwater or brackish water areas.

It is known that waterbirds may suffer adverse effects when living in highly saline environments without access to freshwater (Hannam et al 2003), and inhabiting salinas entails osmotic and ionic costs for waders due to salt ingestion. Therefore salinity may be an important abiotic factor that affects the occurrence of birds in salinas (Ma et al 2010), influencing their distribution, reproductive success and physiological performance (Lei & Poulin 2011). However there are morphological, physiological and behavioural adaptations that enable animals living in saline environments to successfully maintain an osmotic balance (Peaker & Linzell 1975, Skadhauge 1981, Schmidt-Nielsen 1997, Bentley 2002). For instance, Hannam et al (2003) reported that preening was higher in saline-raised chicks of American Avocets *Recurvirostra americana* than in chicks kept in freshwater and brackish water. This suggests that chicks growing under salt conditions try to maintain normal feather structure consistent with thermoregulatory ability, possibly to compensate for salt stress and minimize their energetic costs. Furthermore, to deal with osmotic stress induced by high salinity, shorebirds may maintain a low body mass in order to reduce maintenance costs during energy-costly activities, thus enabling to save energy in order to cope with costs related with hyper-saline environments (Gutierrez et al 2011). Peña-Villalobos et al (2013) investigated how physiological adjustments can lead to kidney morphological alterations in Rufous-collared Sparrow *Zonotrichia capensis* after 20 days of acclimation to fresh and salt water (200 mM NaCl): renal mass was higher in the salt water group to enhance the ability of hyperosmotic urine production to cope with the high salt load diet. Therefore some bird species are capable of modifying their kidney morphology in response to salt acclimation.

Despite these capacities, when drinking salty water, birds cannot maintain water balance through renal excretion alone (Willoughby & Peaked 1979) because avian kidneys are known to have poor concentrating ability (Sturkie 1976, Dantzler & Braun 1980, Braun 1981, Goldstein & Braun 1989). The ingestion of high levels of salt obligate birds to excrete the excess of salt, because salts ingested are absorbed by the small intestine and the concentration in the body fluids increases (Purdue & Haines 1977, Simon 1982, Holmes & Phillips 1985), by a extrarenal excretory system: the supraorbital or nasal salt glands (Sabat 2000) that allow to successfully maintain internal salt and water balance (Gutierrez et al 2013). Nasal salt glands allow marine and coastal birds to osmoregulate in a hyperosmotic environment by excreting excess sodium chloride (NaCl) ingested with food and water from de bloodstream (Dosch 1997), via the nostrils (reviewed in Schmidt-Nielsen 1960, Peaker & Linzell 1975, Skadhauge 1981, Holmes & Phillips 1985, Goldstein & Skadhauge 2000, Sabat 2000, Goldstein 2002). Salt gland secretion is a sodium chloride solution more concentrated than seawater that generates osmotically free water that sustains other physiological processes of birds (Hughes 2003). The salt loading of an individual bird may vary with habitat (saline water vs. fresh or brackish water), salt concentration of its prey, energy intake (i.e. volume of water ingested) and internal hydration level (which may be affected by evaporative cooling demands) (Gutierrez et al 2012b).

It is believed that physiological stress associated with life in marine and other saline habitats does not result from de absence of water but from the necessity of eliminating excess electrolytes (Sabat 2000). Maintaining a functional salt-gland and excreting excess salt should impose significant physiological costs and are energetically expensive processes for birds (Burger & Gochfeld 1984, Nyström & Pehrsson 1988) due to the increased size and metabolic intensity of the organs and tissues involved in salt excretion (Gutierrez et al 2011); i.e. to excrete the excess of salt involves active transport of Na^+ and $C1^-$ ions across cell membranes (Rubega & Robinson 1996) and considerable ATP is required (Sabat 2000). In extreme cases, high salt loading can create such excessive osmoregulatory demands that may lead to the death of birds (Fletcher & Holmes 1968, Poulson & Bartholomew 1962).

Although little is known about the development and function of salt glands in juvenile birds, it is believed that such costs and the effect of excess salinity are unequal in adults and chicks. Salt tolerance appears to be lower in hatchlings than in adults, even in some species whose adult life stages are largely or wholly marine (Nyström & Pehrsson 1988, Johnston & Bildstein 1990, De Santo 1992, Bildstein 1993), because the salt glands of adults and young may function at different levels (Dosch 1997): young waterbirds may have incomplete developed osmoregulatory organs (Barnes & Nudds 1991) and have a higher dependence on freshwater availability at breeding sites, since they cannot fly to freshwater areas. Consequently, it appears that the osmoregulatory system of chicks may be insufficient to impact the dehydration caused by high salinity, which may increase chick mortality rates (Barnes & Nudds 1991, Stolley et al 1999ab).

Responses to adverse environmental conditions are manifested as physiological, morphological and behavioural modifications. Birds raised in high saline environments can present behavioural changes to cope with high salinity. Modification of food by regurgitation when parent feed their chicks and prey selection can be used as behavioural mechanisms in order to diminish osmotic load imposed by high saline foods (Sabat 2000). For instance, Purdue & Haines (1977) suggested that Kentish Plover *Charadrius alexandrinus* at hypersaline lakes in the western United States maintain water balance by not consuming salt water and eating insects with high freshwater contents.

The ability to maintain a favorable energy balance is one of the major factors that determines survival or mortality of free-living birds (Kersten & Piersma 1987). Basal metabolic rate (BMR) is a highly flexible physiological trait which measurement provides important information on the physiological performance of an organism in a particular environment (Gutierrez et al 2012a). BMR is a function of several basic physiological variables (Piersma et al 2004) including body temperature (Reinertsen 1983), metabolic hormone levels (Stokkan 1994, Kersten et al 1998) and body composition including organ sizes (Daan et al 1990, Piersma 2002). In terms of the oxygen consumption, BMR is the minimum rate of energy expenditure that the organism must have to keep itself alive, representing the ‘minimum cost of life’ (Gutierrez 2012c). In shorebirds, BMR may reflect the effects and interactions of salinity with the organism itself in terms of the minimal energetic requirements. Gutierrez et al (2012a) carried out interspecific analyses and showed that, BMR was significantly lower in inland adult Dunlin *Calidris alpina* than in coastal shorebirds after the effects of potentially confounding climatic (latitude, temperature, solar radiation, and wind conditions) and organismal (body mass, migratory status, phylogeny) factors were accounted for, presumably because inland shorebirds that consume low salt content diets will not need to invest so much in osmoregulation when compared with coastal shorebirds, which generally feed on marine invertebrates.

In addition to possible direct energetic costs, there is evidence that the ingestion of high salt loads affect birds by reducing chick growth rates, body mass and dehydration. For instance, young captive Canada Geese *Branta canadensis* experience reduced growth rates in association with increased salinities, and nestling White Ibis *Eudocimus albus* raised on salt-loaded diets also gained less mass than those raised on a more dilute diet (Johnston & Bildstein 1990). Salinity also contributes to the loss of immune response (Gutierrez et al 2013), which may ultimately affect individual fitness (e.g. Nyström & Pehrsson 1988, Tietje & Teer 1996, Doch 1997). In this context, Gutierrez et al (2013) reported that seawater salinity had a negative effect on the immune responsiveness of Dunlins, because Phytohaemagglutinin PHA-induced swelling response was up to 56% weaker in seawater-acclimated Dunlins than in birds subjected to freshwater conditions. However, salinas are fairly productive environments with higher food availability (Lopez et al 2010), and a lower risk of infection by blood parasites than inland freshwater habitats, which are less productive and parasite-rich (Gutierrez et al 2013). Thus the availability of food and the scarcity of parasites in salinas may partly compensate the costs of living in hypersaline environments.

The degree of stress encountered by each individual in a population may imply the presence of fluctuating asymmetry (Hambly et al 2004) that is a random difference between the two sides of the body with bilateral symmetry (Van Valen 1962). This may be caused by variation and genetic stress occurring during development due to problems such as inbreeding or mutation (Balmford et al 1993), and by adverse environmental conditions that may affect the individual at any life stage (Putman & Sullivan 2000).

Fluctuating asymmetry is theoretically predicted to increase with environmental stress conditions and believed to constitute a potential biomarker in conservation (Vangestel & Lens 2011). Therefore it may occur because organisms require energy to compensate for stress (Leung et al 2000), which leads to a reduce energy for growth, precision development (Sommer 1996) and reproduction (Koehn & Bayne 1989).

Sodium chloride is the major electrolyte in extracellular body fluids of vertebrate animals, and essential functions (e.g., nerve and muscle activities) depend on constant ratios of potassium and sodium concentrations across the plasma membrane and tight regulation of the sodium concentration in the extracellular space (Hildebrandt 2001). Although NaCl is an important component for the function and survival of the organism, shorebirds in saline environments may be affected by high NaCl concentrations: if birds are not able to efficiently excrete excess salt in their blood stream they become dehydrated which affects every system in the body, and extreme levels can lead rapidly to death (Rubega & Robinson 1996). Yet at the cellular level, when birds are subjected to salt, it is important that their organism is protected. It is known that inducible stress tolerance results from numerous molecular mechanisms, of which heat shock proteins (Hsps) are collectively only one (Feder & Hofmann 1999). Heat-shock proteins are molecular chaperones that play key roles in the maintenance of cellular homeostasis under variable environmental condition and their primary role is to accompany proteins and ensure their proper function (Hill et al 2013). They assist other cellular proteins in the folding, transport, and prevention of degeneration of these other proteins in cells stressed (Osman et al 2012). Thus, the Hsps which can be produced constitutively or expressed in response to physiological, environmental or pathological (Lunz et al 2005) stress factors should be an important mechanism to cope with salt stress. Hematocrit defined as the ratio of packed volume of red blood cells to total blood volume (Dickens et al 2009), represents oxygen-carrying capacity and therefore possibly can be used to evaluate the “health” of the oxygen transport system (Dawson & Bortolotti 1997). It is used as an index of physiological performance in studies of wild bird populations (Brown 1996) reflecting the condition of birds (Saino et al 1997, Ots et al 1998). Hematocrit values can be affected by a multitude of factors, including season, hydration state, energy expenditure, nutritional condition, hormonal profile, and infection status (reviewed in Fair et al 2007). For instance, Sepp et al (2010) captured

Greenfinches *Carduelis chloris* in the wild, in Estonia, and kept in captivity for different time periods. They found that under harsh environmental conditions experienced by wild birds (i.e., predation threat, scarce resources), hematocrit are fine tuned by physiological trade-offs. Therefore, stress factors such as salt stress should influence hematocrit values.

When subjected to hypersaline environments shorebird chicks are more susceptible to suffer consequences of ingesting high salt diets than the adults. Hannam et al (2003) raised chicks of American Avocets in hypersaline environments and showed that they suffer dehydration, resulting in behavioural changes, and a reduction in body mass and growth rate. The study carried out by Hannam et al (2003) is the only study about the effects of salt stress in chicks of a wader species. However, Hannam et al (2003) fed chicks with an artificial diet that should not adequately simulate the characteristics of natural food, and somehow may have influenced levels of salt ingested. This study evaluated whether salt ingestion can affect the development of Black-winged Stilt chicks inhabiting Salinas by measuring physiological (chick growth, fluctuating asymmetry of bone, BMR, concentration of Na^+ and Cl^- ions in plasma and hematocrit) and behavioural (the ability to remove salt from prey and bill before and after ingestion by head-shaking) variables. With increasing salinity we expect a decrease in: 1) growth rates, and an increase in 2) head-shaking behaviour (by shaking the prey before ingestion and the bill after ingestion); 3) fluctuating asymmetry; 4) basal metabolic rates values; 5) concentration of Na^+ and Cl^- ions in plasma and 6) hematocrit.

Chapter 2 – Methods

2.1. Study species

Black-winged Stilt is a shorebird that feeds and breeds in a variety of habitats ranging from inland freshwater wetlands, through brackish and saline coastal environments. It can be found at dams, rice fields, marshes, estuaries and salt marshes (Osés 1993). They also often occur and breed in man-made habitats as hypersaline salt pans (Tinarelli 1990), and, in Portugal, it is known to be strongly dependent on active and/or inactive salinas as a breeding site (Neves & Rufino, 1994). Due to logistical facilities and because there exists a considerable nesting population of Black-winged Stilt, this experiment took place in Samouco salinas, which is the most extensive complex of salinas of the Tagus estuary, Lisbon, Portugal (Figure 1). We decided to study chicks because young birds of some species have a limited tolerance to incorporate salty foods in their diet (Harriman 1967, Hughes 1984, Johnston & Bildstein 1990). Therefore chicks may be more susceptible to suffer the effects of salt ingestion because their organism is still developing and they are limited in space and reflect more markedly the effects of salt.

Figure 1 – Adult Black-winged Stilts foraging and breeding in Salinas of Samouco.

2.2. Experimental design and sample collection

Chicks were captured by hand from May to July 2013 in salinas of the Tagus estuary, mainly in Samouco salinas. At least two chicks were left in the clutch. Captured chicks were marked with flag codes and distributed by four cages. Chicks of the same nestling were separated among treatments in order to minimize genetic implications. Each cage had available heat lamps, controlled vegetation for shelter, and food and water in a tray with a particular salinity**.** The four treatment levels were: 0g/l, 20g/l, 60g/l and 120g/l. Salinity levels were established by dissolving coarse salt in tap water and 0g/l treatment was tap water. We chose this range of salinities because this species can be found in conditions with different salinities from fresh to hypersaline water. Chicks were fed with fly larvae and brine shrimp (*Artemia spp*.), which is a keystone species supporting the large numbers of post-breeding and migrating waders in salinas (Dias 2009). The food and water were renovated three times a day in each cage, and salinity levels of residual and new water were measured with a conductivity meter (Table I).

Table I – Mean salinity measured ± SD of new and residual water in treatments.

Treatment	Salinity of new water	Salinity of residual water
0	1.13 ± 0.84	2.10 ± 1.95
20	21.31 ± 2.54	26.27 ± 5.24
60	59.50 ± 2.53	67.46 ± 6.85
120	119.60 ± 2.28	143.54 ± 16.91

Every other day chicks were weighed and measured (bill and both tarsi plus toe) in order to determine their growth rates and the presence of fluctuating asymmetry. Measurements were made by the same experienced person at equal time intervals and at the same period of the day after feeding chicks. Chicks were filmed once a day during two minutes while they were feeding. After three weeks in treatment, and when chicks reached at least 20 days old, they were transported to the Laboratory of the University of Extremadura, in Badajoz, to measure the basal metabolic rate during the night. At the end of this process, birds were weighed again and released in a refuge with food and water. In the following morning, at Salinas of Samouco, chicks were placed again in their respective treatments. After one day, they were color banded and a blood sample of about 70 µl was collected from de ulnar vein into a heparinized microcapillary tube with a 25 gauge needle. After blood collecting, the heparinized capillary tubes were sealed with wax and stored in a cold styrofoam box with cool packs. After blood collection chicks were placed in captivity for one more day and then released in the Salinas of Samouco.

In order to have a natural comparison for our results, 22 wild chicks were captured in the Salinas of Tagus estuary (hereafter termed saline wild chicks) and two wild chicks in Caia rice fields, an inland freshwater habitat (hereafter termed freshwater wild chicks). The mean salinity measured at salinas was approximately 67.13g/l. Wild chicks were measured and blooded to analyze ions concentrations in plasma and hematocrit, and 10 of them (eight from salinas and two from Caia rice fields) were used to measure the BMR.

We found three Black-winged Stilt dead chicks and one adult of different salinities: chicks were found in salinas at approximately 20g/l and 100 g/l and in a rice field, and the adult was found in salinas at approximately 130 g/l. We perform the dissection to evaluate and compare the size of salt glands.

2.3. Physiological and behavioural variables

To evaluate the effect of salinity on chick development we used physiological and behavioural variables. Chick growth rates can reflect the effects of salt levels ingested because chicks can quickly become dehydrated due to incapacity of salt glands for excreting the excess of salt from the bloodstream, affecting their development and resulting in reduced growth rates (Hannam et al 2003), and due to the metabolic expenses for maintaining functional salt glands that reduce the amount of energy available for nestling growth and may impact or even prove fatal to young birds (Bildstein et al 1990). For growth rates and fluctuating asymmetry calculations we used tarsi plus toe-length because it can be measured accurately in a non-invasive way (Vangestel & Lens 2011), and is a morphological trait that presents a reduced probability of being influenced by other external factors. Because it continues to grow at a faster rate in early development (Reed et al 1999) it may reflect salt stress in early nestling periods. For this species tarsi plus toe were measured instead of only the tarsi because their measurement accuracy is higher.

We defined chick growth rate as the coefficient of a regression of tarsi plus toe-length mean on chick age (Reed et al 1999), using chicks from 1 to 47 days old because chicks in captivity did not reach inflection point before 47 days old, which is consistent with the work of Reed et al 1999 that reported that *Himantopus mexicanus knudseni* Hawaiian Stilt chicks in captivity did not reach inflection point until after 42 days old in terms of mass gain. We also computed a body condition index through a regression of body mass (g) on tarsi plus toe-length mean (mm) for each treatment to analyze the growth patterns. The age of each chick was calculated using the following formula: age= -15.67+1.2821*(bill length, in mm) (Masero, unpublished). Culmen bill length has been used to age chicks of several shorebirds species in the wild (Beintema & Visser 1989) and was the parameter used for aging chicks in the experiment because its growth trajectory is fairly linear and it typically has a constant growth rate throughout the chick stage (Reed et al 1999).

The degree of fluctuating asymmetry is advocated as a potential estimate of developmental instability (Helle et al 2010) and has been demonstrated to reflect the ability of individuals to cope with different kinds of environmental stress (Parsons 1990). Several studies showed an increase in fluctuating asymmetry in response to parasites (Moller 1992), to a decrease in food availability (Carbonell & Telleria 1998), and to elevated baseline plasma levels of corticosterone (Helle et al 2010). In this study we evaluated whether stress produced by the intake of highly salty contents translates in a lack of energy available to a symmetric growth. It was studied in tarsus plus toe-length asymmetry which was computed as the absolute value of 100: (left tarsus + toe-length – right tarsus + toe length)/mean tarsus + toe length (mm), where no asymmetry = 0 (Chappell et al 2011), for chicks between 20-30 days old and raised between 10-20 days in each treatment.

There are many behavioural adaptations of organisms to cope with extreme conditions (e.g. Wingfield et al 1998, Martin & Wiebe 2004); for instance salt stress may be avoided or minimized by feeding on less salty food (Sabat 2000). In this study we analyzed the head-shake movements of chicks, which shake the prey and the bill while feeding, to evaluate how chicks behave when feeding in prey within increasing salt concentrations.

Basal metabolic rate, an adjusted and reversibly physiological parameter, is the sum of metabolic activities during the inactive period of the day in a thermoneutral environment and post-absorptive state in the inactive phase of the circadian cycle (McNab 1997, Hulbert & Else 2000, Turner et al 2004). Peaker & Linzell (1975) estimated theoretically that the energetic cost of salt gland secretion in Ducks represented 7% of their metabolic rate. Because the rate of salt gland secretion depends on the salt load that animals have experienced (Sabat 2000), an increase in salinity increases the metabolic activity of the organs and tissues responsible for osmoregulation and should contribute to increase the BMR of chicks. Gutierrez et al (2011) suggested that the development of the reversible physiological changes of salt glands exhibited by birds exposed to prolonged osmotic stress such as gland hypertrophy and increased Na^+/K^+ - ATPase activity (Hildebrandt 2001) is the cause of the observed increases in BMR. Presently, the data about the effects of salinity on increasing BMR is available only for adult shorebirds (e.g., Gutierrez et al 2012a, Gutierrez et al 2013, Gutierrez et al 2011). Thus, we aimed at understanding in what extent salinity may lead to an increase in the activity of chick’s salt glands, and consequently and increase in BMR. We expected that chick’s BMR would increase with salinity, and their salt glands will respond to salt ingested (Sabat 2000), but this mechanism would not be as efficient as in adults and chick’s will became affected by salt stress. Overall, BMR can provide information about how increasing salinity and its possible energetic consequences have an effect at the minimal energy that birds need to maintain their activities for survival, because BMR contribution is important to daily energy turnover (McNab 2002).

Acclimation to saline environments induces interstitial water and Na^+ to move into cells increasing the concentration of salts in the body fluids (Pena-Villalobos et al 2013). When the bird drinks saline water, Na^+ enters the plasma from the gut and plasma osmolality pressure, that is the concentration of solutes in moles per kilogram of solvent (mOsm/kg, (Beaufrere et al 2011)) increases, which stimulates salt gland secretion (Hughes 2003). For instance, Goldstein et al (1990) found differences in the osmoregulatory parameters of Savanaah Sparrow *Passerculus sandwichensis* populations, with those inhabiting salt marshes *P. sandwichensis beldingi* having higher plasma osmolarity than sparrows inhabiting the uplands surrounding the salt marsh. The measurement of Na^+ and Cl^- ions concentration in plasma enables us to evaluate in what to extent chicks use the extrarenal osmoregulatory function. i.e. whether they are able to avoid ingesting salt and excrete the excess from the bloodstream in order to maintain their internal concentration of solutes.

Low hematocrit (the percentage volume of red blood cells in whole blood) values may indicate anemia (Potti et al 1999), and may therefore be related to difficulties in oxygen uptake and transport (Phillips et al 1985), reflected in anabolic metabolizable energy that may affect survival. Anemia results from a reduction below the normal level of erythrocyte number or hemoglobin concentration per unit volume of blood (Harrison & Harrison 1986). Fair et al (2007) find low hematocrits in wild birds from environmental stressors, both natural and anthropogenic. Salt stress can lead to a Black-winged Stilt chicks reduced hematocrit values and manifest itself in anemia. For instance, Campbell (1994) concluded that decreases in haematocrit below 35% in caged birds could be an indication of anemia.

Although hematocrit has been used to indicate general body condition (Howlett et al 2002, Sanchez-Guzman et al 2004) we also employed hematocrit as a measurement of hydration (Vleck & Vleck 2002). Measurements of hematocrit in Black-winged Stilt chicks allow understand whether chicks can maintain their normal concentrations of solutes, because an animal’s degree of dehydration can be reflected in an increase of hematocrit (Vazquez-Galindo et al 2013). For instance, Johnston & Bildstein (1990) found high hematocrit values in nestling of White Ibis raised in high-salt diet, indicating that nestlings were presumably dehydrated and salt loaded. In addition, the increase of basal metabolic rates can lead to a dehydration state and high hematocrits. For instance Moneva et al (2008) found higher hematocrit values in White Plymouth rock *Gallus gallus domesticus* injected with 0.5 ml formalin. This could occur as a result of increased metabolism, leading to an increase in erythropoiesis as a compensatory reaction to the lack of sufficient oxygen in the tissues (Olanrewaju et al 2007), possibly because of an impaired oxygen-carrying capacity in the blood hematocrit as a marker of hydration, work load, and/or general health state (Campbell & Dein 1984, Fair et al 2007).

2.4. Laboratory analyses

In the analysis of videos each chick was observed during the first two minutes of the feeding period (after renovating the water). For each fly larvae ingested the number of head movements was counted while chicks held their prey in its bill, shaking it before ingestion, and the number of head movements after chicks had ingested the prey to expel the water in the bill.

Blood samples were centrifuged for 10 minutes at 1200 rpm at 4°C to separate the plasma from the red blood cells, resulting in the hematocrit and to measure Na^+ and Cl^- concentrations in the plasma. Hematocrit was measured as the percentage of the length of the part of the microcapillary tube occupied by red blood cells in relation to the total length of the microcapillary tube occupied by blood. After that the tube was broken in two parts (plasma and cellules). The extreme of each part was closed with wax. Both parts were stored in a single tube with an appropriated label before placing it in the fridge at -20ºC until analyzed.

The measurement of Na^+ and Cl^- ions in plasma were taken in the University of Extremadura. We used the SPOTCHEM E-Plate with the SPOTCHEM-SE and SPOTCHEM El Analyser, using a small volume of plasma to determine electrolytes. The SPOTCHEM E-Plate measures Na^+, K^+, and Cl^- simultaneously. Its reactive portion is made up of two layers: the first layer is formed by an ion selective membrane and underneath this is a silver/silver chloride layer as base electrode. On the surface of this plate, there are a sample well, a reference solution well, probe-contact holes where a potential formed on each of Na^+, K^+ and Cl^- electrodes is measured, and air bleeding holes (Worth 1998).

The BMR was measured in terms of oxygen consumption (VO_2) through a computerized flow-through respirometer. Birds were fasted for at least 3.5 h to create a post-absorptive condition (Castro et al 2008), and the BMR was measured at night during the resting period of their daily cycle (Aschoff & Pohl 1970). Birds were weighed and then placed for 3 hours in a polymethyl methacrylate (Perspex) metabolic chamber (effective volume= 4.6 l) which were in darkness and at a monitored temperature with a calibrated thermistor probe (± 0.001°C), and located in a temperature-controlled room at 27°C. The chambers received atmospheric air at a rate of 1 l min-1 from calibrated mass flow controllers (MFS-5; Sable Systems, Las Vegas, NV, USA) allowing to measure the air flow upstream. Water vapour was removed from the air stream downstream from the metabolic chambers using Drierite® columns, followed by a multiplexer (TRRM4; Sable Systems, Las Vegas, NV, USA) which allowed automatic switching between four channels. A subsample of the air was taken at 150 ml min-1 using a subsampler mass flow meter unit (SS-3; Sable System, Las Vegas, NV, USA). Using a gas analyzer (FC-10 Oxygen Analyzer; Sable Systems, Las Vegas, NV, USA) calibrated using pure nitrogen as low reference and a certified mixture of 21% O_2 as high reference, was determined the oxygen concentration which was logged at a 1 Hz sampling rate on a computer using ExpeData software (v. 1.1.25; Sable System) and a UI2 converter. Each sampling sequence started with logging ambient baseline air for 10 minutes, followed by sampling each chamber for 10 minutes with two minutes flushing the system between chambers to remove latent gases. Once this sequence was repeated three times, there were 3 records per bird per night. For determining the body bass related to BMR measures was done the mean of the initial measurement and this final measurement. According to Hill (1972), oxygen consumption was calculated on the basis of the lowest 5-minute average of O_2 consumption. The respiratory quotient (RQ) used was 0.72 assuming protein and lipid catabolism, and the metabolic rate was calculated using an energy equivalent of 20 kJ l^{-1} O_2 (Gutierrez et al 2011).

2.5. Statistical analyses

The effect of salinity (0, 20, 60,120 g/l) on chick growth rate, fluctuating asymmetry, concentration of Na^+ and Cl^- ions in plasma and hematocrit was evaluated with a GLM (one-way Anova). A GLM was also used to evaluate differences in ions concentration between saline (three treatments) and freshwater captivity chicks. A factorial Anova was used to assess differences in concentration of Na^+ and Cl^- ions and hematocrit values between saline (67.13 g/l) and freshwater wild chicks, saline (60 g/l) and freshwater captivity chicks, and their interaction. The Na^+ and Cl^- concentrations in plasma and head-shake movements per minute (before and after prey ingestion) were log transformed to attain normality and homogeneity of variances. The effect of salinity

(0, 20, 60,120 g/l) on the number of head-shake movements/minute before and after ingesting prey was analyzed with mixed models, using chick as a random factor (because each chick was observed more than once). Differences in BMR among: 1) captivity treatments and 2) saline (67.13g/l) and freshwater wild chicks and captivity freshwater and saline chicks (60g/l), were evaluated with general linear models (GLM). The mean of BMR cycles, days in treatment and body mass were log transformed. Log (BMR) was chosen a dependent variable and treatment as a fixed factor. Log (days in treatment) and log (body mass) were included as covariates since there is some variation among factors in number of days in treatment and because we need to manage mass independently from BMR. The treatment 120 was not included in statistical analysis of BMR because, besides their small sample size (n = 4 individuals), their mean body mass when starting the experiment was significantly higher than those of chicks in the treatments 20 and 60. Data is presented as mean ± SD. All statistical tests were performed with STATISTICA v8.

Chapter 3 – Results

Our results show an influence of salinity in the foraging behaviour of Black-winged Stilts but there was no effect on morphological and physiological variables (Table II).

Table II – Comparison of the morphological, physiological and behavioural parameters of Black-winged Stilt *Himantopus himantopus* chicks among the 4 salinity treatments (mean ± SD). The GLM results testing for effects of salinity on growth rate, fluctuating asymmetry, BMR, concentration of Na$^+$ and Cl$^-$ ions in plasma, hematocrit and head-shaking behaviour of chicks is presented.

	Salinity (g/l)									
Parameter	***n***	**0**	***n***	**20**	***n***	**60**	***n***	**120**	***F***	***P* =**
Growth rate (mm/day)	8	2.30 ± 0.29	7	2.69 ± 0.71	6	2.63 ± 0.29	4	2.73 ± 0.10	$F_{3,21}$= 1.38	0.28
Fluctuating asymmetry (%)	7	- 0.002 ± 0.01	7	0.004 ± 0.008	6	0.003 ± 0.007	4	0.005 ± 0.009	$F_{3,21}$= 1.77	0.20
BMR (O_2/ml)	9	1.2 ± 0.08	8	1.28 ± 0.08	9	1.16 ± 0.07	-	-	$F_{2,21}$= 0.70	0.51
Na$^+$ concentration (mmol/L)	7	144.71 ± 6.18	5	150 ± 18.01	6	154 ± 32.38	4	168.25 ± 17.84	$F_{3,18}$= 1.12	0.37
Cl$^-$ concentration (mmol/L)	7	143.71 ± 11.67	5	145 ± 9.25	6	147.17 ± 31.76	4	300.5 ± 286.06	$F_{3,18}$= 2.00	1.15
Hematocrit (%)	7	38.26 ± 3.67	5	36.53 ± 4.24	6	40.277 ± 5.26	4	41.75 ± 2.63	$F_{3,18}$ = 1.43	0.27
Head-shake (head-shake/min)										
- **Before ingesting prey**	56	0.33 ± 0.73	51	1.57 ± 1.89	27	4.84 ± 3.34	8	6.20 ± 3.62	$F_{3,22}$= 30.35	P < **0.001**
- **After ingesting prey**	56	0.24 ± 0.49	51	0.59 ± 0.92	27	3.00 ± 3.79	8	4.07 ± 3.95	$F_{3,22}$= 11.42	P < **0.001**

There was a significant influence of salinity in head-shake movements before and after chicks had ingested the prey; these movements increased markedly from salinities 0 and 20 to 60 and 120 (Table II, Figure 2). Bonferroni post-hoc test detected significant differences between all treatments (P<0.001), except between 60g/l and 120g/l. Differences were more significant between 20 and 60g/l (P < 0.001) and less significant between 0 and 20g/l (P < 0.01). Taken altogether, these results suggest that the behavioural effect occurs when changing from 20 to 60 g/l, but it does not increase further from 60 to 120 g/l.

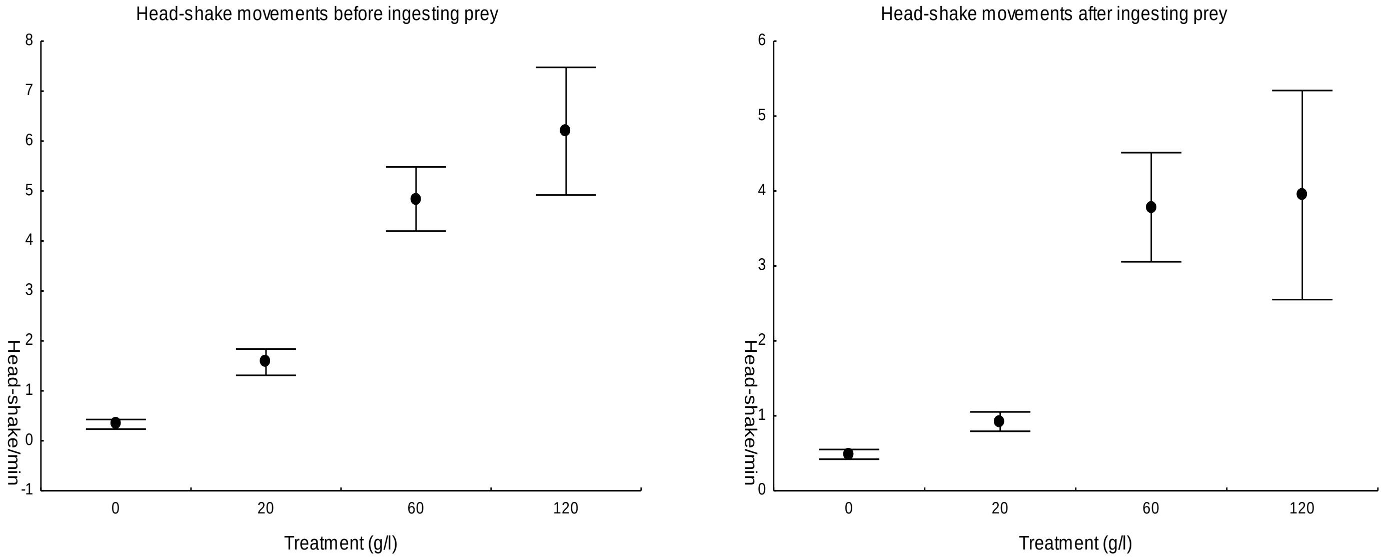

Figure 2 – The mean ± SD of Black-winged Stilt *Himantopus himantopus* chick head-shake per minute in the four different treatments, before and after chicks ingested the prey.

No effect of salinity was found on chick growth rates among treatments (Table II). Body condition index suggests that chicks had a similar growth pattern among treatments (Figure 3).

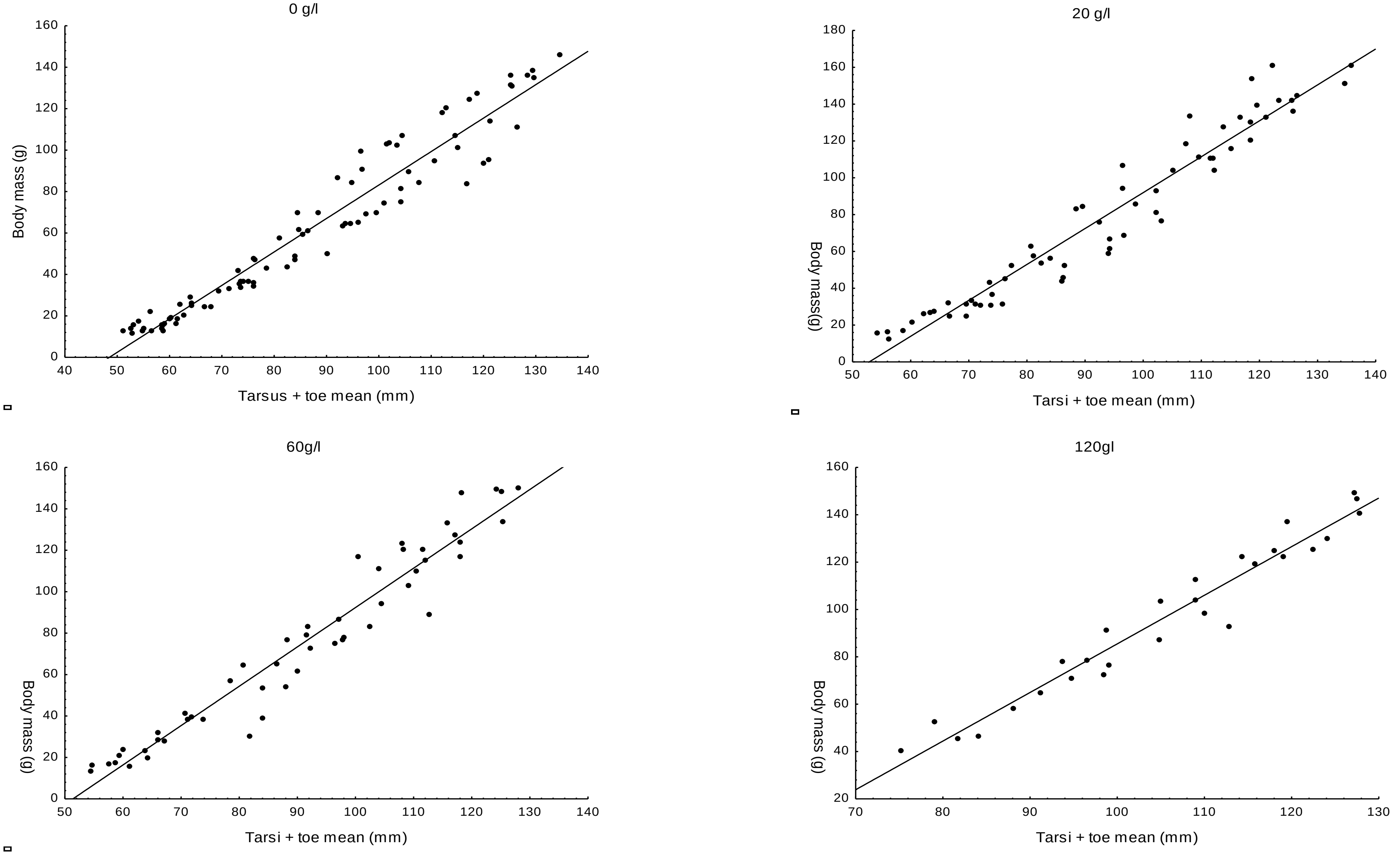

Figure 3 – The relationship between Black-winged Stilt *Himantopus himantopus* chick body mass and tarsi plus toe-length mean for the four different treatments.

Contrary to our expectations there was no effect of salinity on fluctuating asymmetry, BMR, concentration of Na^+ and Cl^- ions in plasma and hematocrit values of chicks (Table II). There was also no difference in the concentration of Na^+ and Cl^- in plasma between captivity freshwater chicks and those raised in saline treatments: $[Na^+]_{\text{freshwater captivity chicks}}$ = 144.71 ± 6.18 mmol/L, n=7, $[Na^+]_{\text{saline captivity chicks}}$ = 156.80 ± 24.31 mmol/L, n=15, $F_{1,20}$=1.39, P= 0.25; $[Cl^-]_{\text{freshwater captivity chicks}}$ = 143.71 ± 11.67 mmol/L, n=7, $[Cl^-]_{\text{saline captivity chicks}}$ = 187.33 ± 151.37 mmol/L, n=15, $F_{1,20}$= 0.6, P= 0.45. Factorial Anova did not detect an effect of salinity in the concentration of Na^+ and Cl^- in plasma between freshwater and 60 g/l raised captivity chicks, and freshwater and saline (67.12g/l) wild chicks, nor an interaction between salinity and chick state (Table III). There was also no difference in BMR when comparing captivity chicks raised in freshwater, and freshwater wild chicks: $BMR_{\text{captivity chicks}}$= 1.18 ± 0.02 O_2/ml, n=9, $BMR_{\text{wild chicks}}$= 1.16 ± 0.05 O_2/ml, n=2, $F_{1,7}$ = 0.15, P= 0.71. Salinity had no effect on BMR when comparing saline wild chicks (67.13g/l) with captivity chicks raised in 60g/l: $BMR_{\text{wild chicks}}$= 1.20 ± 0.13 O_2/ml, n=8, $BMR_{\text{captivity chicks}}$= 1.11 ± 0.12 O_2/ml, n=9, $F_{1,13}$ = 0.16, P= 0.70.

Factorial Anova did not detect differences in hematocrit values when comparing 60 g/l raised chicks and freshwater captivity chicks with saline and freshwater wild chicks, nor an interaction between salinity and chick state (Table III).

Table III – Comparison of hematocrit and concentration of Na^+ and Cl^- ions in plasma between wild and captive Black-winged Stilt *Himantopus himantopus* chicks from freshwater and saline water (mean ± SD). The factorial Anova results are presented.

	n	**Wild**	**n**	**Captive**	**n**	**Saline water**	**n**	**Freshwater**	**Anova**		
									Wild/captive	**Fresh/saline water**	**Interaction**
Hematocrit (%)	16	39.69 ± 4.31	13	39.19 ± 4.40	20	37. 73 ± 5.0	9	38.0 ±3.27	$F_{1,25}$ = 1.34 P= 0.25	$F_{1,25}$= 0.16 P= 0.70	$F_{1,25}$= 0.32 P= 0.58
Na^+ (mmol/L)	23	157.74 ± 39.14	13	149.38 ± 21.99	7	157.59 ± 38.68	9	146.11 ± 6.03	$F_{1,32}$ = 0.14 P= 0.71	$F_{1,32}$ = 0.22 P= 0.64	$F_{1,32}$ = 0.02 P= 0.88
Cl^- (mmol/L)	23	174 ± 131.7	13	145.31 ± 22.17	27	163.78 ± 118.73	9	163.22 ± 59.00	$F_{1,32}$ = 2.2 P= 0.15	$F_{1,32}$ = 1.33 P= 0.26	$F_{1,32}$ = 1.44 P= 0.24

The comparison of the size of salt glands between three Black-winged Stilt chicks found dead in freshwater, 20 g/l and 100 g/l, and one adult found in salinas with approximately 130 g/l shows all these individuals presented salt-gland of a similar size (Figure 4).

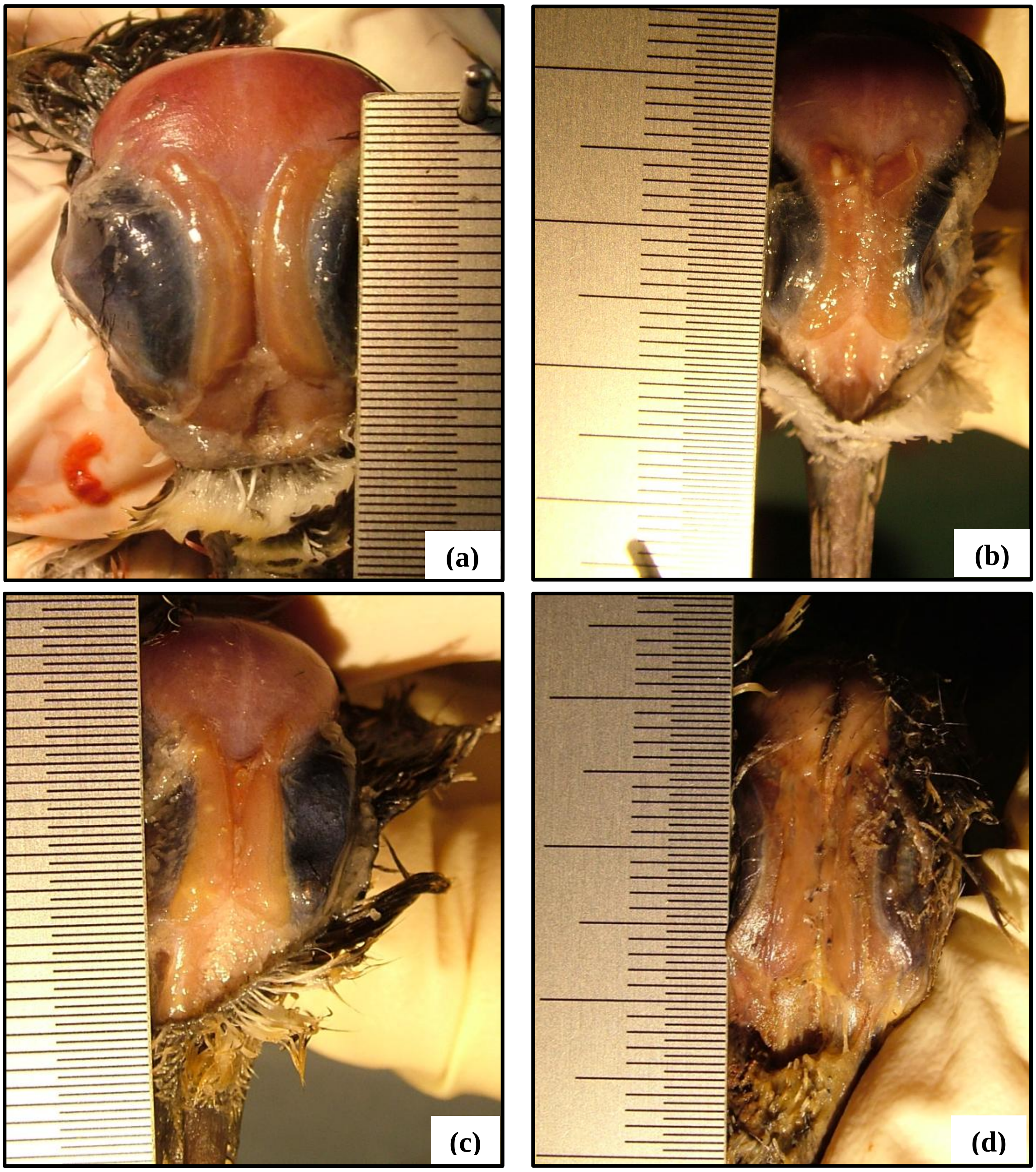

Figure 4 – The saltglands of Black-winged Stilt *Himantopus himantopus* individuals that were found dead, and made visible after removal of the head skin: (a) chick from rice fields; (b) chick from salinas with 20g/l; (c) chick from salinas with 100 g/l and (d) adult from salinas with130g/l. Note that the saltglands of the three birds have approximately the same size, and are restricted to a small area above the orbits. We cannot detect differences at color observed in the saltglands of the birds, which would indicate high vascularization to support the high rate of blood flow that occurs during salt secretion.

Chapter 4 – Discussion

This study examined the effect of salinity on physiological and behavioural parameters of Black-winged Stilt chicks. We detected only an effect on the behaviour of chicks: our expectation of an increase in the number of head-shake movements before and after the ingestion of prey with increasing salinity was verified. The increase of movements by shaking their prey held in the bill before its ingestion with increasing salinity should be related with an attempt to expel the excess of salt surrounding the prey and minimize salt ingested (Sabat 2000). The head movements after ingestion should be a strategy to expel the water that remains inside the bill to avoid swallowing it. Black-winged Stilt chicks may be able to deal with low salinities without a large increase in the head-shake movements, but when salinity increased from 20 to 60 they were forced to further increase the intensity of their behaviour to expel more frequently the salt water from their prey and bill. Therefore, it appears that chicks can respond behaviourally to a significant increase of salinity levels by maintaining a low salt ingestion. Similarly, Hannam et al (2003) found a significant increase in the head-shake behaviour of American Avocet chicks raised in three treatments of increasing salinity (freshwater, <500 µS, brackish water, 5000 µS, and hypersaline water, 50000 µS), supposedly to expel the secretions of the salt gland accumulated in the nostrils, with chicks raised in the hypersaline environment having the highest mean value. But no secretions from the salt glands were observed around the nostrils in any of the treatments. Therefore these head-shake movements were probably related to the avoidance of ingestion of high salt contents, as was verified in our study. The Black-winged Stilt behavioural strategies to avoid salt-related dehydration were expected because it has been demonstrated that some species of birds, even those that have functional saltglands, can minimize osmotic stress by choosing prey with a lower content of saline water, or even osmoregulatory hipo-osmotic prey (Nyström & Pehrsson 1988, Mahoney & Jehl (1985b) in order to decrease the intake of salt. For instance, Hughes & Winkler (1990) reported that California Gull *Larus californicus* inhabiting salt marshes, predates almost exclusively on brine shrimps and brine flies (*Ephydra hians*) which has a salt concentration of one-tenth that of saline water where they live. Similarly, Nyström and Pehrsson (1988) found that two marine species, the Long tailed Duck *Clangula hyemalis* and the Commom Scoter *Melanitta nigra*, have a preference for smaller prey, and this may be related to salt stress avoidance. They feed on mussels smaller than the maximum size possible to swallow because the proportion of sea water inside the mussels increases with increasing mussel size. Mahoney & Jehl (1985a) examined the stomach content of American Avocets from three lakes with different salinity (Mono Lake was 2314 mOsm/kg in 1983 and dropped to 2160 mOsm/kg in 1984, Great Salt Lake experienced similar conditions in 1984 and had salinities from about 4500 mOsm/kg mid-lake to about 1200 mOsm/kg around the periphery, and Lake Abert's salinity was 757 mOsm/kg in 1983) and found that birds had a lower osmolarity than that of the water where they were feeding. They suggested that Avocets avoided swallowing salt water by straining their prey. Through a combination of behavioural and anatomical adaptations in the oral cavity (such as the presence of palate papillae which lies in a small depression that prevents the tongue from making a tight contact, and any fluid that might be present would presumably be extruded laterally preventing from being swallowed), American Avocets appear to be able to expel most adherent salt water from their prey, thereby largely avoiding the problems of salt-loading and the ingestion of harmful ions.

Changes in the behaviour of birds and its interaction with some physiological features are regarded as the first responses to avoid salt stress (Sabat 2000), however we predicted that such behavioural mechanism should not be enough to maintain the internal concentration of Na^+ and Cl^- ions and osmotic balance, because chicks ingested salt remnants from the surface of the prey and by drinking salt water, and their supposed immature extra-renal osmoregulatory system should not be able to excrete the excess of ions from the bloodstream. Chicks could increase the salt gland's activity with increasing salinity to osmoregulate but their activity would not be efficient to prevent salt consequences. In addition to the energetic expenditure itself due to salt gland function, the dehydration state would decrease the energy available to growth. In addition, osmotic stress, particularly dehydration that can be considered as a stress factor, and energetic costs would affect organism function in terms of fluctuating asymmetry. However, for Black-winged Stilts none of these consequences were detected.

The energetic expenditure associated with the potential increase in the functioning of salt glands could obligate chicks to compensate and increase their basal rates, because BMR is the sum of the metabolic rates of all the organs and tissues

(Gutierrez +et al 2011), in order to have the minimum energy to survival. For instance, Gutierrez et al (2011) reported that adults Dunlins living in saltwater habitats have higher BMRs than Dunlins living in freshwater habitats. Contrary which was found by Gutierrez et al (2011), our results show that salt ingested did not imply an energetic cost that led chicks to increase their basal metabolism, apparently because the compensation behavioural mechanism between the increase of salt concentration and the increase of head shake movements to expel the excess of salt should be sufficient to avoid the ingestion of salt loads susceptible to increase the activity of salt glands. Indeed, Mahoney & Jehl (1985a) raised the possibility that the need for salt gland competence might be obviated in some species by behavioural and mechanical means of salt avoidance. The BMR is not only associated with environmental factors but also to other factors such diet and behaviour (Gutierrez et al 2012). Through the BMR comparisons between captivity and wild chicks it was possible to verify that metabolic rates were similar, which suggests that chicks of the two groups osmoregulate in the same way, and that free-living Black-winged Stilt chicks were not affected by salinity regarding their energetic demands. Contrary to Hannam et al (2003) that found hypersaline Avocet chicks with high plasma osmolality levels, indicating that chicks raised in hypersaline environments without access to freshwater quickly became dehydrated, our results show that chicks kept in saline treatments maintained a similar concentration of ions as chicks without salt in their diets**.** Because the concentration of Na^+ and Cl^- was similar for wild chicks raised in saline and freshwater environments, and similar between these chicks and captivity chicks from freshwater and saline environments, our study suggests that all captivity chicks presented a similar osmoregulatory and behaviour to that of wild chicks. Our results are consistent with those reported by Gutierrez et al (2011) who did not find differences in the concentration of ions in the plasma of adult *Calidris alpina* subjected to a freshwater (0.3±0.0‰ NaCl) brackish water (10.4±0.5‰ NaCl) and salt water (33.1±2.1‰) regimes. Contrary to what was reported by Hannam et al (2003) that found a significantly higher hematocrit in hypersaline-raised American Avocet chicks, our results show no relationship between hematocrit values and increasing salt concentration, indicating that captivity chicks had a good health condition and did not become dehydrated, which is supported by the concentration values of Na^+ and Cl^- in the plasma. In addition, the similarity between the hematocrit values of freshwater and saline water chicks suggests that there was no energetic expenditure due to salt glands function, otherwise hematocrit of saline water chicks would have been higher. Moreover, wild and captivity Black-winged Stilt chicks had similar hematocrit values, which suggests similar behaviour and physiologic features for chicks raised in the wild and in captivity. Similarly, Mahoney & Jehl (1985a) found no evidence of salt-loading in blood hematocrit of American Avocets and Wilson's phalarope *Phalaropus tricolor*.

Gray & Erasmus (1989) reported that birds exposed to excessive salinity can experience osmotic dehydration and may lose mass. For instance, severe dehydration leads to decreases in body condition in Ducklings (*Oxyura jamaicensis* and *Anas rubripes),* Goslings (*Branta canadensis)* and nestling White Ibis (Cooch 1964, Johnston & Bildstein 1990, Barnes and Nudds 1991, Stolley et al 1999ab). But our results show that growth rates were not affected by hypersaline conditions and are different to previously reported effects of highly saline conditions on growth: a) Dosch et al 1997 reported that Laughing Gulls *Larus atricilla* chicks receiving the largest salt supplements gained body weight at a significant lower rate and were smaller at fledging; b) Barnes and Nudds (1991) found in Mallard *Anas platyrhynchos* and Black Duck *A. rubipes* that duckling growth rates decreased with increasing salinity conditions, and newly hatched Ducklings did not even survive in brackish water; c) Hannam et al (2003) concluded that stress induced by salinization can decrease the proportion of time spent feeding resulting in a mismatch between energetic demand and intake, and consequently in low chick growth rates. Indeed, it is known that inland species of shorebirds that have evolved in less productive environments may have a slower pace of life compared with coastal representatives in the presence of abundant food (Gutierrez et al 2012a).

Moller (1992) reported that parasites are an important environmental stress factor, and the fowl mite *Ornithonyssus bursa* parasite directly affects the degree of fluctuating asymmetry in the elongated tail of the Swallow *Hirundo rusticaafter*. In our study, salinity levels did not reflect themselves in fluctuating asymmetry of Black-winged Stilt chick’s morphology. In our experiment the biometrics were made by the same person, but tarsi were not measured three times as suggested by Campo & Prieto

(1999). Once tarsi + toe length was measured instead of tarsi it may have implied a bias in the measure, because it deals with more than one bone.

During periods of increased stress, the role of the molecular chaperone may be amplified to minimize cell protein damage (Locke & Noble 1995, Råberg et al 1998). Therefore when animals are subjected to stressful situations, the concentrations of heat shock proteins (Hsps) in blood can increase to 20% of total cellular proteins (Bhardwaj et al 2012). In birds, the heat shock protein Hsp60 is the most widely assayed in studies of stress (e.g. Merino et al 1998, 2002), and several studies reported an increase in response to stressful environments, including high temperatures, transportation, and pathogen exposure (Hill et al 2013). Evaluating the concentration of heat shock protein in chick's blood would enable us to understand whether chicks can cope with possible negative effects of high salinities by increasing the concentration of shock proteins, but this factor was not measured in this study. For instance, Hill et al (2013) studied changes in the circulating concentrations of three heat shock proteins (Hsp60, Hsp70 and Hsp90) in wild-caught House Finches *Haemorhous mexicanus*, and found that birds sampled during a period of low-environmental stress with moderate ambient temperatures had low levels of HSP60 and modest levels of HSP70 and HSP90, when compared to birds sampled during a more stressful period (with high temperatures). However, taking into account our results, we expected that heat shock proteins would not increase in red blood cells among treatments because differences in concentrations of Na^+ and Cl^- among treatments were not significantly different, meaning that chicks could maintain their cellular homeostasis and therefore their organism would not need to increase their cells protection and repair mechanisms by increasing heat-shock protein levels.

Our results demonstrate that Black-winged Stilts chicks were able to deal with high salty diets without metabolic disadvantages by preventing salt stress through the avoidance of its ingestion. This corresponded well with the fact that salt glands did not increase their activity as salinity increased, as BMR did not increase with the increase of salinity. Increasing salt avoidance behaviour allowed chicks raised in saline environments to maintain an osmotic balance among treatments, and salt glands, although supposedly under-developed, may be able to excrete part of the excess salt ingested without increase their activity. It is known that the avian salt gland functions in osmoregulation respond to increases in plasma osmolality and volume (Rubega & Robinson 1996). Indeed, taking into account our BMRs and ions concentration results, and comparing with those from freshwater chicks, we assume that the salt glands of Black-winged Stilt chicks should function at the same rate in all treatments and did not increase their activity, due to the behavioural compensation mechanism which leads to a decrease in salt intake at higher salinities, and allowed chicks to maintain a similar internal concentrations among treatments**.** Our results demonstrate that chick's behaviour while feeding resulted in the avoidance of dehydration and energetic costs, and energy-saving contributed to maintain energy requirements for a precision and normal growth.

Hannam et al (2003) concluded that salt gland development and function in American Avocet chicks was insufficient to impact the dehydration state. They also reported that failure to gain mass and dehydration through measures of hematocrit and plasma osmolality are similar to previously reported effects of highly saline conditions on adult Kentish Plover (Purdue & Haines 1977) and White Ibis (Johnston & Bildstein 1990) suggesting inefficient salt glands. But contrary to information reported by Hannam et al (2003), Rubega & Oring (2004) found that American Avocet chicks hatch with relatively large salt glands. It is believed that the salt glands of some birds in their initial life-cycle stages are not completely developed (Sabat 2000). For instance, Hughes (1984) reported that the percent of sodium load secreted by nestling Gulls *Larus glucescens* was correlated with age and with the size of salt glands. However this pattern does not seem to be shared by all species: 1) Janes (1997) found that the Adelie Penguin *Pigoscelis adeliae* exhibited no significant ontogenetic changes in their physiological osmoregulatory capacities and 2) Hughes (1968) reported that older individuals of Common Terns *Sterna hirundo* were less efficient at excreting salt loads than younger individuals. Apparently chicks of some marine and salt marsh species have a well-developed extrarenal system at birth (Sabat 2000).

If Black-winged Stilt chicks ingest large quantities of salt water and its attendant osmotic and ionic load, they could be expected to show changes in salt gland size because the degree of its hypertrophy is usually a function of the degree of salinity (Peaker & Linzell 1975, Mahoney & Jhel 1985b). But the size of salt glands from dead chicks was similar between individuals raised in salt water and freshwater.

The salt gland of adult Black-winged Stilts appeared to be similar to that of chicks. Therefore, we predict that the adults will have the same behavioural strategies to cope with salt than chicks and their salt glands function in a similar way. Indeed, we observed that adult Black-winged Stilts foraging in hypersaline areas of Samouco showed head-shake movements while feeding, presumably to expel salt water from the bill. But their small salt gland size could also be due to the fact that adults can feed in habitats with lower salinity and drink fresh water regularly (Sabat 2000). However, this is unlikely to be the case in Samouco, as no fresh water sources are available. Moreover, salt glands of dead adult chicks not seem to be high vascularized which may means that they were not excreting high salt contents from de bloodstream in all salinities. Indeed Gutierrez (2012c) found that salt glands of a Dunlin captured from Cadiz Bay Natural Park (coastal saline habitat) were highly vascularised and cover a large part of the frontal skull region, whereas the saltglands of the freshwater Dunlin are restricted to a small area, suggesting low salt glands activity.

Mahoney & Jehl (1985a) found that newly hatched American Avocets have large or larger salt glands as a proportion of body mass, as those of adults and of obligatory marine bird species, and conclude that they hatch with salt glands that are large enough to cope with the osmoregulatory demands of saline environments. They also compared salt glands of adult American Avocets from three lakes with different salinity, and showed: 1) that salt glands were not enlarged and averaged a relatively low percentage of body weight (0.02%), and 2) no evidence of salt glands extreme hypertrophy that could be associated with the different salinities of the three lakes. They also reported that this species uses freshwater sporadically, and that it may not be required for osmoregulatory balance. It is possible to conclude that American Avocets chicks have a similar salt gland size as that of the adults, and show behavioural adaptations to maintain osmotic equilibrium and avoid the energetic costs of salt glands function. Therefore, due to the markedly behavioural strategy of Black-winged Stilts that enables chicks to excrete excess salt while feeding, it is possible that their salt glands have little competence to excrete high salt levels, but physiological studies are needed to fully address this point.

It is believed that non-tidal inland habitats are advantageous for shorebirds, given the ingestion of less saline food and protection from wind, which may subsequently reduce heat loss by convection during periods of low environmental temperatures. However shorebirds can take advantages by inhabiting coastal marine habitats, particularly salinas, because they are highly productive (Rubega & Robinson 1996), and more predictable than inland freshwater habitats (Gutierrez et al 2012a). On the other hand the salinity role in shaping parasite and pathogen distributions via indirect interaction with immune function (Gutierrez et al 2013) can have a role in the avoidance of energetic expenditure for diseases. It was reported that for waterfowl wintering in coastal lagoons, high salinities could impose energetically expensive osmoregulatory costs on such species as Redheads *Aythya americana*, Red-crested Pochards *Netta rufina*, and Northern Pintails *Anas acuta* (Woodin et al 2008). However, our study suggests that salinas are a suitable habitat for Black-winged Stilt chicks because they react to the increase of salinity levels with behavioural strategies that allow chicks to maintain their energy requirements and normal development. This is very important for young chicks because they cannot fly and search for foraging areas with lower salt conditions. Therefore, our study is important to show that salinas are indeed important breeding habitats for Black-winged Stilt populations, and contributes to explain why 70% of the population (Rufino & Neves 1991) of this species in Portugal breeds in salinas.

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