



Variability in the degree of euryhalinity of neotropical estuarine annelids

Serena Mucciolo^{a, bc, *}, Andrea Desiderato^{c, d, e}, Selene Miranda Leal^{a, f}, Maria Mastrodonato^g, Paulo Lana^a, Carolina Arruda Freire^h

^a Laboratório de Bentos, Centro de Estudos do Mar, Universidade Federal do Paraná, Av. Beira Mar s/n, 83255-976, Pontal do Paraná, Paraná, Brazil

^b Programa de Pós-Graduação em Zoologia, Departamento de Zoologia, Setor de Ciências Biológicas, Campus Politécnico, Universidade Federal do Paraná, Av. Cel. Francisco H. dos Santos 100, 81530-000, Curitiba, Paraná, Brazil

^c Katedra Zoologii Bezkręgowców i Hydrobiologii, Uniwersytet Łódzki, ul. Banacha 12/16, 90-237 Łódź, Poland

^d Laboratório de Estudos de Cnidários e Comunidades Incrustantes, Departamento de Zoologia, Setor de Ciências Biológicas, Campus Politécnico, Universidade Federal do Paraná, Av. Cel. Francisco H. dos Santos 100, 81530-000, Curitiba, Paraná, Brazil

^e Department of Functional Ecology, Alfred Wegener Institute & Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

^f Departamento de Biologia Geral, Universidade Estadual de Ponta Grossa, Av. General Carlos Cavalcanti 4748, 84030-000 Paraná, Brazil

^g Dipartimento di Zoologia, Università degli Studi di Bari Aldo Moro, Via Orabona, 4, 70125 Bari, Italy

^h Laboratório de Fisiologia Comparativa da Osmorregulação, Departamento de Fisiologia, Setor de Ciências Biológicas, Campus Politécnico, Universidade Federal do Paraná, Av. Cel. Francisco H. dos Santos 100, 81530-000, Curitiba, Paraná, Brazil

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ABSTRACT

Estuaries are highly dynamic, spatially complex systems showing large daily fluctuations in salinity. Estuarine organisms, especially small animals with limited locomotory capacities, are thus expected to have variable ecological and morphophysiological strategies. This paper evaluates the responses to salinity challenges in four species of neotropical annelids along a subtropical estuarine gradient by assessing mortality (i.e., tolerance) and changes in body weight (proxy for water fluxes). We selected the nereidids *Alitta* sp. and *Laeonereis acuta*, with broad salinity niches, and the nephtyid *Nephtys fluviatilis* and the melinnid *Isolda pulchella*, with either narrow salinity niches or a more restricted spatial range. Worms were weighed, exposed to salinities ranging from fresh water to full-strength seawater (0, 5, 15, 25, and 35) for 1, 6, 12, and 24 h, and weighed again after these exposure times. Their mortality (as absence of body movements or vascular circulation) rates were recorded to assess their ranges of tolerance to these treatments or their degree of euryhalinity. Their integument was characterized histologically to show possible morphological links to their tolerances. Both nereidids displayed stable body weights and thick integuments, compatible with the wide range of salinity tolerance and capacity to maintain body hydration. Body weight of *Alitta* sp. returned to initial values after 1 h at salinities ≥ 5 . Body weight did not change in *L. acuta* at salinities ≥ 15 ; mortalities occurred only at salinity 0 after 12–24 h. *Nephtys fluviatilis* showed a thin integument and less variation in weight throughout the whole range of tested salinities, but mortality occurred after 24 h at salinity 0, at its reference salinity (3) and at 35. *Isolda pulchella* showed a lower capacity to maintain body weight/hydration and a thin integument, compatible with its relative stenohalinity and presence restricted to polyhaline marine sectors. Its weight variation was significant at all salinities except 35; mortality occurred at extreme salinities: 0 and 35. Responses of the four species were compatible with their distribution along the estuarine gradient, as an interesting outcome of their autecological traits, morphophysiological adaptations and evolutionary histories.

1. Introduction

Estuaries are critical transition zones linking land, fresh water, and the sea, showing marked hydrographic fluctuations due to regular or stochastic events linked to seasonality, tides, and storms (Levin et al.,

2001). Regular and abrupt salinity changes (e.g., tidal fluctuations, large precipitation) drive and limit taxa abundance and distribution, affecting their dispersion and connectivity in different ways (Chang and Marshall, 2016; Oglesby, 1965b). Estuarine annelids cope with abrupt salinity variation by means of behavioural and osmoregulatory strate-

* Corresponding author at: Katedra Zoologii Bezkręgowców i Hydrobiologii, Uniwersytet Łódzki, ul. Banacha 12/16, 90-237 Łódź, Poland

E-mail addresses: serena.mucciolo@biol.uni.lodz.pl (S. Mucciolo), andrea.desiderato@biol.uni.lodz.pl (A. Desiderato), maria.mastrodonato@uniba.it (M. Mastrodonato), lana@ufpr.br (P. Lana), cafreire@ufpr.br (C.A. Freire).

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gies, such as physical avoidance and extracellular fluid and cell volume regulation (Oglesby, 1968; Preston, 2009; Tait et al., 1981). Vagile, soft-bottom annelids show diel vertical migrations (i.e., burrowing deeper during low tides) to explore fewer demanding microhabitats and microclimates (Oglesby, 1969; Preston, 2009). Alternatively, sessile annelids such as *Galeolaria caespitosa* Lamarck, 1818 (Serpulidae) may use the operculum to remain isolated from the surrounding water (Tait et al., 1981). Moreover, in annelids, these autecological strategies most likely combine with epithelial osmoregulation (Oglesby, 1965a, 1965b).

In response to an osmotic challenge from altered salinity, animals may physiologically act as osmoregulators or as osmoconformers. When they osmoregulate, they spend metabolic energy to maintain the osmolality of the extracellular fluid relatively stable, keeping an osmotic gradient with respect to the external water. This strategy spares their tissues and cells from the challenges of a highly variable extracellular fluid. When animals osmoconform, on the other hand, they are highly permeable, normally isoosmotic to the external water, and face osmotic and ionic fluxes when there is a change in the external salinity. Annelids, similar to most marine invertebrates, are generally considered osmoconformers. However, some taxa have been reported to display osmotic gradients between their coelomic fluids and the external water at low salinities in a hyperregulatory pattern, that is, able to maintain a higher osmotic concentration compared to the environment (Castellano et al., 2020; Preston, 2009; Thonig et al., 2019). In general, the tighter the capacity to transport salt through interface epithelia and the better the capacity to reduce integumental permeability, the higher the ability for “Anisosmotic Extracellular Regulation, AER. A certain control of extracellular osmolality may also be the result of the capacity of the excretory system to produce urine diluted with respect to the coelomic fluid. For instance, the nereidid *Hediste limnicola* (Johnson, 1903), usually confined to oligohaline environments, shows a relatively impermeable body together with the capacity to produce hypoosmotic urine to eliminate excess water (Oglesby, 1965b).

A higher capacity of AER means a greater osmotic stability of the extracellular fluid and less need for complementary cellular volume regulation, or the so-called “Isosmotic Intracellular Regulation”, IIR (Florkin, 1962). Tolerating salinity fluctuations (i.e., being euryhaline) requires conformers to be competent in IIR, in addition to showing avoidance behaviours (Evans, 2009; Foster et al., 2010; Freire et al., 2008; Kirschner, 1991; Willmer et al., 2005). Therefore, stenohaline osmoconformer animals, possibly less competent in regulating tissue and cell hydration and volume, are thus restricted to habitats with more stable water salinities (Lignot and Charmantier, 2015; Rivera-Ingraham and Lignot, 2017; Thonig et al., 2019).

In addition, annelid larvae exposed to hypoosmotic stress are unable to beat the cilia to swim, while adults are unable to move, both because of their swollen conditions (Lyster, 1965; Oglesby, 1968, 1969). Reduced growth rates, regenerative abilities and impaired reproduction may also be potentially related to swollen conditions and an insufficient capacity for IIR to achieve volume control (David and Williams, 2016; Neuhoﬀ, 1979; Thonig et al., 2019). Indeed, the nereidid *Alitta succinea* (Leuckart, 1847) showed lower fertilization success, developmental delays and a general asynchrony between male and female sexual maturity when exposed to low salinities (Kuhl and Oglesby, 1979).

Responses to salinity variation may differ among annelid families adapted to distinct environments (Oglesby, 1969; Whitfield et al., 2012). Most of them show a better performance at higher salinities, compatible with the hypothesis of a marine origin of the families and subsequent colonization of estuarine environments by single species (namely, saline species; Lee and Bell, 1999). Notwithstanding “polychaete” diversity, which includes more than 10,000 species (Read and Fauchald, 2020), the existing literature is still scarce, with many families completely disregarded, thus requiring further research.

In this paper, we experimentally investigated the tolerance to salinity variation and osmotic behaviour of four annelids from a subtropical Brazilian estuary, contributing to the current knowledge of the nereidids *Alitta* sp. and *Laeonereis acuta* (Treadwell, 1923), and the nephtyid *Nephtys fluviatilis* Monro, 1937, and providing a first assessment for the melinnid *Isolda pulchella* Müller, 1858. The nereidids display a broad spatial distribution in the estuary, and we expected them to better tolerate salinity variations, while the nephtyid (with a limited distribution range in oligohaline waters) and the melinnid (with a narrow salinity niche in euhaline waters) would hypothetically display a lower degree of euryhalinity. We assessed the responses to osmotic stress by assessing mortality rates and body weight variations at a range of salinities, from fresh water to full-strength seawater. In addition, we investigated whether cuticle complexity and thickness could be related to salinity tolerance.

2. Material and methods

2.1. Sampling and laboratory acclimatization

Sampling was carried out in the Paranaguá Estuarine Complex (PEC), Brazil (Table 1), a mixed estuary of ~612 km² displaying seasonal variations in salinity and temperature and gradually increasing salinities from inner to outer sectors (Marone et al., 2005).

According to the occurrence along the estuary and the expected salinity tolerance, we selected *Alitta* sp. - usually from hard, artificial substrates - and *Laeonereis acuta* - from soft bottoms, both showing broad salinity niches, being abundant in meso/polyhaline sectors of the complex (5–30). *Nephtys fluviatilis* was sampled from oligohaline sectors (0–5), and *Isolda pulchella* was sampled from euhaline sectors (30–40); these two latter species display narrower salinity niches than nereidids. Animals were sampled with a shovel or by scraping on fishermen's buoys (Table 1) under the authorization of the “Sistema de Autorização e Informação em Biodiversidade” (SISBIO, permit # 36255-1/73,627-1).

In the laboratory, the animals were acclimatized for 48 h in 1 l plastic containers with native water and sediment (3–4 cm), kept at ~20 °C, constant aeration and natural photoperiod, and fed commercial aquarium fish flakes. To prevent cannibalism, each container held up to 50 specimens, and larger animals were kept separated from the smaller ones.

2.2. Experimental design

We designed an orthogonal experiment with salinity (fixed, with five levels treatment – 0, 5, 15, 25, and 35 – plus a control with the salinity of each sampling area) and time (fixed, with four levels – 1, 6, 12, and 24 h – after the onset of osmotic shock) as factors. These salinity levels simulate the daily and seasonal salinity fluctuations in the estuary, extending to fresh water and full-strength seawater. To avoid potential pH biases, the experimental salinities were obtained by diluting the prefrozen and filtered native seawater (salinity 35) with local commercial bottled mineral water.

Table 1

Geographical coordinates of sampling points with local salinity at the time of sampling and type of substrate.

Species	Coordinates (DMS)	Salinity	Substrate
<i>Alitta</i> sp.	S25°26'7.5" W48°42'18.18"	20	Plastic debris, hard bottom, anchoring buoy
<i>Laeonereis acuta</i>	S25°30'35.52" W48°28'4.32"	28	Sand
<i>Nephtys fluviatilis</i>	S25°14'46.5" W48°24'57.72"	1	Mud
<i>Isolda pulchella</i>	S25°33'25.2" W48°18'37.4"	35	Sand

We used 120 non-sexed specimens per species (five adults for each of the 24 treatments). Before the experiment, only healthy animals were chosen under a binocular stereoscope, and regenerating specimens were used only if caudal development was sufficiently advanced, and damage occurred prior to sampling. Mature specimens were discarded. Wet body weights were obtained after extraction from water and quick dry blotting on filter paper, both before exposure to osmotic stress and at the end of each exposure. Afterward, worms were discarded. Death was detected under a binocular stereoscope when worms displayed no body movement or vascular circulation.

2.3. Data processing

To reduce intraspecific variation in size/weight for each species, relative weight variation calculated as $(\frac{w_{tx}-w_{t0}}{w_{t0}} \times 100)$ was used for the statistical analyses, with w_{t0} being the initial weight at the start of the experiments and w_{tx} the weight at a fixed time x .

Normality and homogeneity of variances were tested with the Shapiro-Wilk (Shapiro and Wilk, 1965) and Levene tests (Levene, 1960), respectively, and outliers were removed when required. Best fitting models for weight (Gaussian distribution) and mortality (binomial distribution) variations were assessed by generalized linear models (GLMs) (Nelder and Wedderburn, 1972). Models were selected with the corrected Akaike Information Criterion, AICc (Hurvich and Tsai, 1989; Sugiura, 1978) and were visually validated (Zuur and Ieno, 2016). One-way analysis of variance (one-way ANOVA; Fisher, 1925) allowed us to test, for each best model level (salinity, time, or salinity/time interactions), whether the variation in raw weights from t_0 was significant. Post hoc analysis was performed by Tukey honestly significant difference (HSD) (Tukey, 1953), and the results were considered significant when $p \leq 0.05$. All analyses and graphics were carried out using R (R Core Team, 2013), RStudio (RStudio Team, 2015) and the packages

GAD v. 1.1.1 (Sandrini-Neto and Camargo, 2011), ggplot2 (Wickham, 2016), car v. 2.1–6 (Fox and Weisberg, 2017), and MuMIn v. 1.42.1 (Barton, 2018).

2.4. Histology

We evaluated the structure and thickness of the cuticle and the epidermis using seven individuals of each species. The specimens were acclimatized for 48 h as described above and then fixed in 5% paraformaldehyde diluted in filtered water from each sampling site for 24 h. The worms were then washed, stored in 1% phosphate-buffered saline (PBS) at 4 °C, embedded in Technovit 8100 monomer (EMS, Hatfield, PA), sectioned in semithin slices 2 μm thick, and stained with metachromatic toluidine blue to assess the general tissue morphology (Mentino et al., 2014). Colours range from blue/violet (basophilic cellular structures such as nuclei) to red/purple (acidophilic cellular structures such as cytoplasm and mucins), depending on the charge of the stained tissues (Sridharan and Shankar, 2012). Cuticle thickness was standardized with the average width of the species (at segment ~15–20, parapodia not included in the measurement), measuring specimens previously anaesthetized with menthol.

3. Results

3.1. Tolerance to osmotic stress

3.1.1. Species with broad salinity niches

The best model selected for *Alitta* sp. and *Laeonereis acuta* included the interaction between salinity and time (Table S1). *Alitta* sp. gained weight at extremely low salinities (0 and 5) and lost it at salinities >21 1 h after the shock. Significant differences from the initial weight were recovered at salinities of 0, 5, 25 and 35 (Fig. 1 – S1.1) after 1 h of expo-

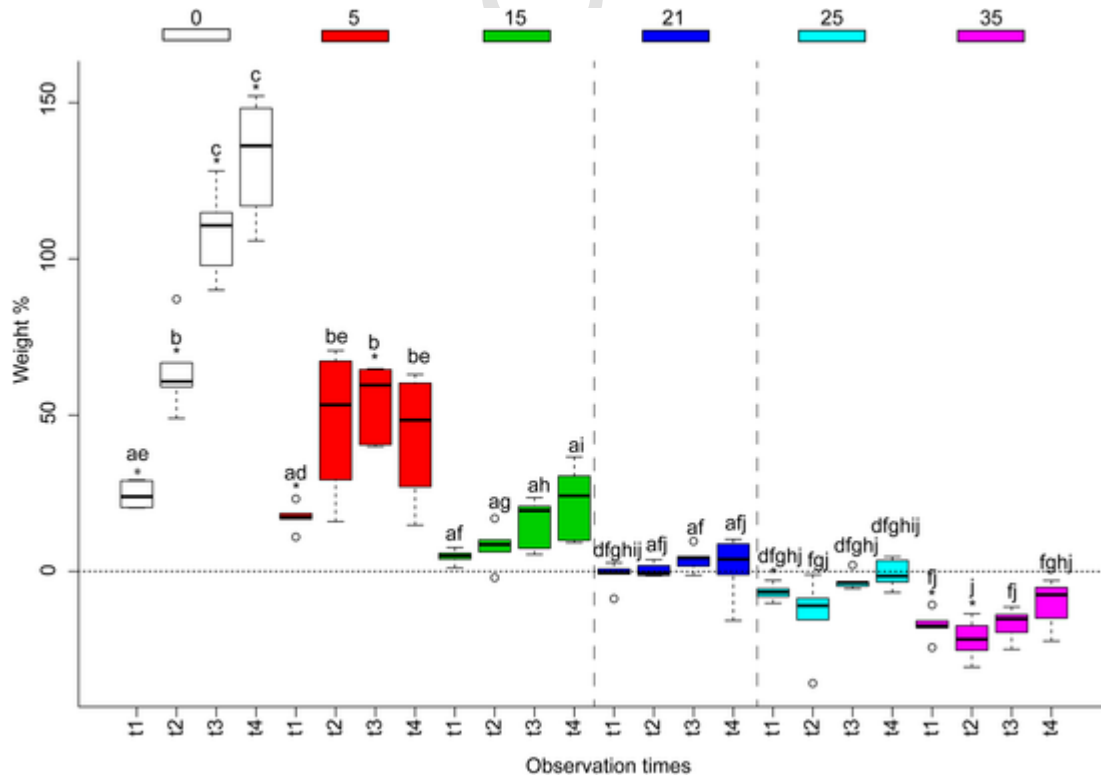


Fig. 1. Percentage of variation in body weights in the interaction between salinity and time for the annelid *Alitta* sp. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t0} . Colours referring to different salinities: white – 0, red – 5, green – 15, blue – 21, light blue – 25, purple – 35. T1 = 1 h, t2 = 6 h, t3 = 12 h, t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sure but then returned to initial levels (i.e., no significant differences from t_0 at all salinities) except for 0, which continued to increase over time (Fig. S1.1). The best models for mortality rates selected both time and salinity (but not salinity/time interaction). Mortality occurred only after 12 h at salinity 0 (Fig. S2).

The weight of *L. acuta* did not vary significantly over 24 h either at the reference salinity (31) or at 25 and 35 (Fig. 2). Weight did not vary significantly among salinity treatments after 1 h, except for the two lowest (0 and 5) vs. the two highest (31 and 35). At 0 and 5, the weight increased throughout the experiment, while at salinity 15, there was an initial increase, followed by a decrease after 12 h. Significant differences from the initial weights were obtained after each salinity exposure, except for some treatments at 25, the reference (31) and 35 (Fig. 1-S1.2). According to the AICc, mortality was independently influenced

by time and salinity. Deaths occurred after 12 h at 0 but were significant only after 24 h (Fig. S2).

3.1.2. Species with narrow salinity niches

The best model retrieved for *Nephtys fluviatilis* included salinity and time independently. Nevertheless, a second-best model only with factor salinity showed a delta difference < 2 and, thus, was chosen as the most parsimonious (Table S1).

Weight did not vary significantly at the reference salinity (3) or at salinities < 15 over 24 h (Fig. 3-S1.3), while it decreased significantly only at 25 and 35 (Fig. 3-S1.3). The best model for mortality was the one with only the time (after 24 h) with deceased specimens at 0 (the reference) and at 35 (Fig. S2).

The best model for *Isolda pulchella* included salinity and time independently (Table S1). Weight changed at each salinity (Fig. 4A) and dif-

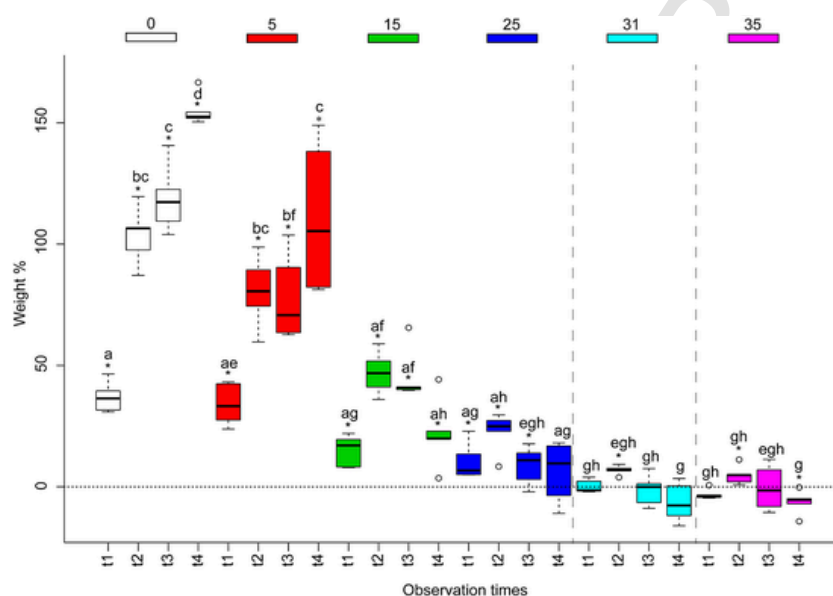


Fig. 2. Percentage of variation in body weights in the interaction between salinity and time for the annelid *Laeonereis acuta*. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t_0} . Colours referring to different salinities: white – 0, red – 5, green – 15, blue – 25, light blue – 31, purple – 35. T1 = 1 h, t2 = 6 h, t3 = 12 h, t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

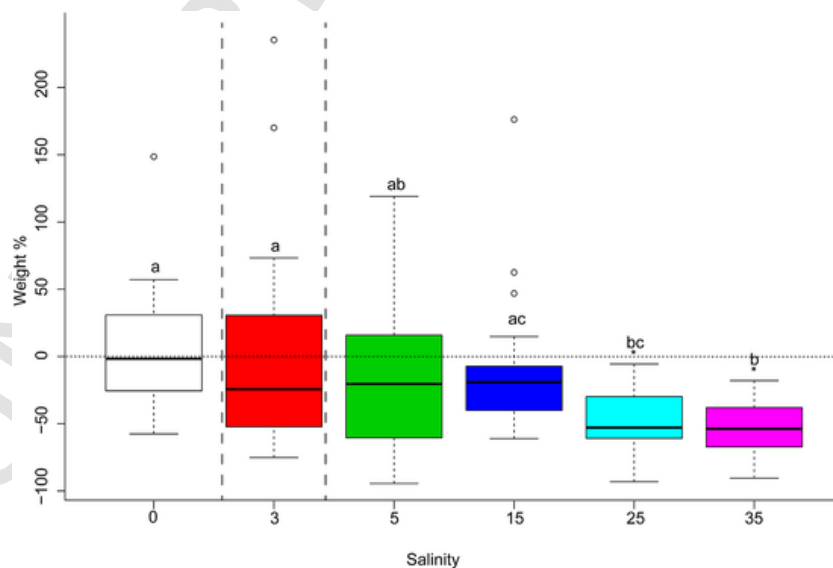


Fig. 3. Percentage of variation in body weights in *Nephtys fluviatilis* for the factor Salinity. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t_0} . T1 = 1 h; t2 = 6 h; t3 = 12 h; t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

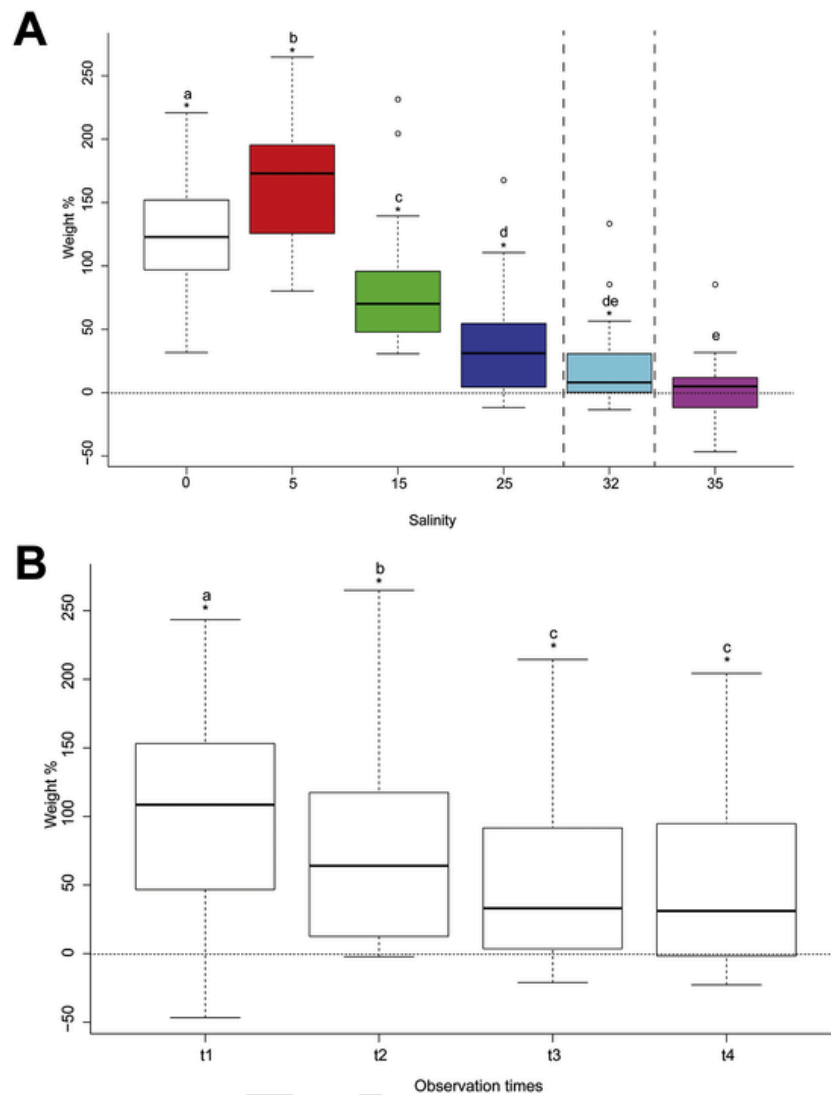


Fig. 4. Percentage of variation in body weights in *Isolda pulchella* for the factors Salinity (A) and Time (B). Reference salinity delimited by dashes. Horizontal dashed lines indicate w_0 . T1 = 1 h; t2 = 6 h; t3 = 12 h; t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

ferred significantly throughout the experiment (Fig. S1.4). Weight variations at reference (32) were similar only to those at higher salinities (25 and 35), even if the specimens at 25 showed significantly higher weights than those at 35. The largest weight increment was at salinity 5. Although there was a general gain, the highest increase was after 1 h, followed by a relatively continuous decrease and a stabilization after 12 h, never recovering the initial weight (Fig. 4B). Mortality occurred at every salinity and time, but the best model included salinity and time as independent factors. Most deaths occurred at 0 and 5, whose levels were significantly higher than those at the other salinities (Fig. S2).

3.2. Histological analysis

All species showed an integument consisting of a cuticle covering the epidermis (Fig. 5 A-D). In *Alitta* sp. the epidermis was monostratified, with homogeneous stained blue cells covered by a thick cuticle (cuticle/width = 3.3; Fig. 5 A). In *Laeonereis acuta*, the epidermis was pseudostratified, alternating secretory (i.e., glands) and non-secretory cells (i.e., epithelium). Gland cells show intense purple metachromasia, covered with a thick cuticle, thinner than *Alitta* sp. (cuticle/width = 2.6; Fig. 5 B). In *Nephtys fluviatilis*, the epidermis was monostratified with a very thin layer and homogeneously stained blue cells.

The cuticle was also thin (cuticle/width = 1.5; Fig. 5 C). In *Isolda pulchella*, the epidermis was monostratified, with homogeneously stained blue cells covered with a thin cuticle (cuticle/width = 1.8; Fig. 5 D).

4. Discussion

Our results partially supported our working hypothesis, as each target species showed distinct tolerance responses depending on the amplitude of deviation from their respective reference salinities. The euryhaline nereidids displayed morphophysiological adaptations, including weight (hydration) regulation and more complex integuments. Conversely, the less euryhaline nephtyid and melinnid showed thinner integuments, suggesting that they may rely on autecological strategies (Preston, 2009; Tait et al., 1981), putatively including avoidance and tube building, respectively.

Both nereidids controlled body water after the initial shocks over a wide range of salinities. Nereidids include species that occur from hypersaline to freshwater habitats, thus being a notable euryhaline annelid clade (Glasby and Tarmo, 2008). Coherently, this euryhalinity is achieved through a strong capacity for cell volume regulation, involving both a variation in the concentration of free amino acids (FAAs) and active/passive extrusion of ions such as Na^+ , Cl^- and Mg^{2+} (e.g.,

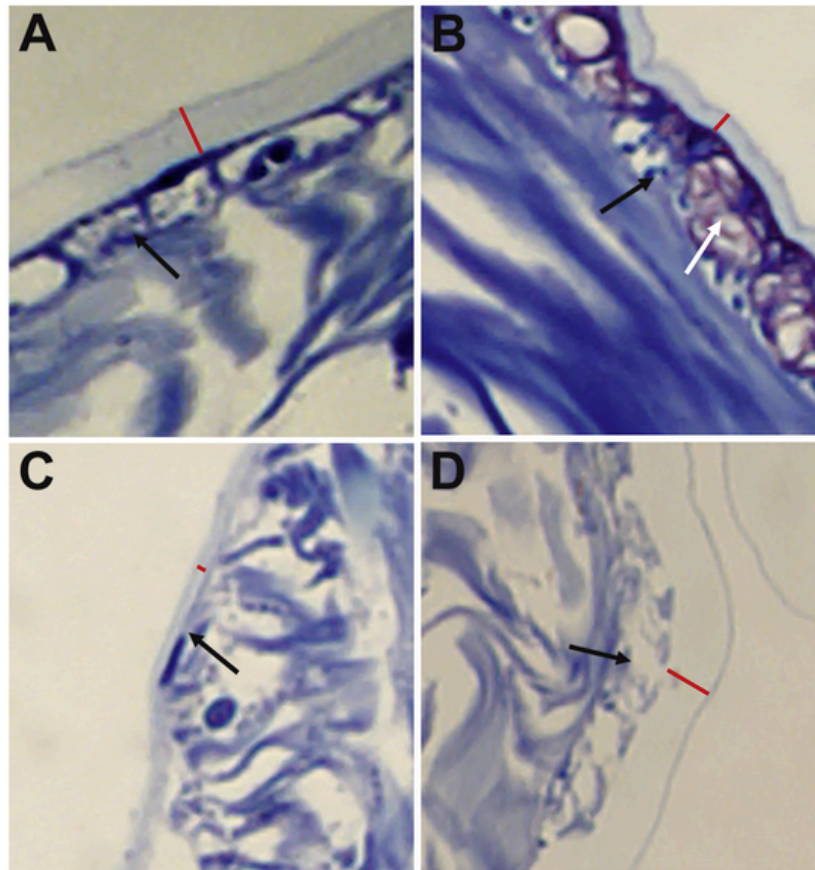


Fig. 5. Sagittal sections of the integument of the annelids *Alitta* sp. (A), *Laeonereis acuta* (B), *Nephtys fluviatilis* (C), and *Isolda pulchella* (D) stained with toluidine blue. Red bars indicate the thickness of the cuticle: (A) 11.5 μm , (B) 8.1 μm , (C) 2.1 μm , (D) 4.2 μm . Black arrows indicate epithelial cells; white arrow refers to gland cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dykens and Mangum, 1984; Freel et al., 1973; Oglesby, 1965a, 1968). Compatibly, the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ (NKCC) cotransporter is abundant and widespread along the bodies of both nereidids (Mucciolo, unpublished data).

After a first osmotic shock leading to weight change (swelling or shrinking depending on the salinity), *Alitta* sp. started to regulate, consequently stabilizing the weight, and reverting the initial disturbance in volume/weight. Hypoosmotic stress may prompt an initial swelling for 1–2 h, reaching a *plateau* characterized by cell volume regulation, followed by readjusting cell volume to resume the original volume/weight levels after 24 h (Oglesby, 1965a, 1965b, 1978; Oglesby et al., 1982), although in some cases, the final volume/weight may be higher than the initial one (Dykens and Mangum, 1984). Indeed, when exposed to a hypoosmotic shock at salinity 5, *Alitta* sp. appeared to regulate the volume while maintaining a weight higher than the initial one. Conversely, these worms were able to resume their initial weight at higher salinities after transient weight/volume loss. The death of individuals at salinity 0 resulted from excessive water influx combined with the inability to decrease the internal volume, which led to cell/body burst (Oglesby, 1969). Thus, *Alitta* sp. seem to display all the typical features related to other saline species (Lee and Bell, 1999), dealing much better with higher than lower salinities.

The response of *Laeonereis acuta* slightly differed from *Alitta* sp., although both were very much harmed by freshwater. *Laeonereis acuta* was more affected by salinity 5 than *Alitta* sp., progressively increasing in weight (i.e., swelling) to $\sim 100\%$ at the last experimental time. Conversely, *Alitta* sp. stabilized at an $\sim 50\%$ weight increase and showed a less evident time effect. *L. acuta* appears to be clearly euryhaline (Koenig et al., 1981; Oglesby, 1965a, 1965b), showing lower swelling

or shrinkage than expected from the magnitude of the challenges under hypo- and hyperosmotic conditions (50% changes relative to the isosmotic condition) (Castellano et al., 2020). These authors observed a distinct regulatory volume decrease response using isolated cells of *L. acuta* upon 50% hypoosmotic shock, which nicely fits the behaviour observed in our experiment at salinity 15. Both *Alitta* sp. and *L. acuta* died in fresh water due to continuous and uncontrolled swelling, highlighting how critical persistent freshwater (salinities < 0.5) exposure may be for the survival of marine annelids.

Alitta sp. seems to be able to swell and resist, maintaining a more constant weight, which is congruent with the hard bottom habitat in which it occurs. Contrary to what happens in soft sediment habitats, there are fewer microhabitats, such as galleries or burrows, allowing for the buffering of salinity decreases. In contrast, *L. acuta* is a soft-bottom tube dweller that is well known to “escape” salinity changes by actively burrowing deeper (Oglesby, 1969) to find a more suitable microhabitat to reduce and buffer osmotic stress, which allows for sufficient volume regulation after 24 h (Preston, 2009). Additional evidence given by the differentiated distribution of the NKCC cotransporter, a membrane protein involved in cell volume regulation (Mucciolo, unpublished data), along their bodies reflects their distinct lifestyles. NKCC is significantly more abundant along the integument of *L. acuta* than along the integument of *Alitta* sp.

The greater or lesser ability to deal with reduced salinities is clearly reflected in the distribution patterns of *Alitta* sp. and *L. acuta* along the estuarine gradient. Despite their distributions overlap, *Alitta* sp. is more abundant in mesohaline sectors, while *L. acuta* is more restricted to poly- and euhaline sectors, as previously reported in North American Atlantic estuaries (Oglesby, 1965a). Indeed, a clear distribution pattern

in congeneric species in response to different salinities has already been found in another family of annelids from the Baltic Sea (Quintana et al., 2018). The euryhalinity of both species is also supported by their thick cuticle and epidermis. Tube building annelids tend to show reduced or absent cuticles (Storch, 1988), whereas infaunal annelids from sandy bottoms show thick cuticles with epicuticular projections along the whole body (Gustavsson, 2001). A thick and less permeable integument is often associated with freshwater and estuarine species (Preston, 2009). Our results allow us to suggest that the thick, less permeable cuticle of *Alitta* sp. (relative thickness = 3.3) may contribute to increasing its tolerance to low salinities when compared to *L. acuta* (relative thickness = 2.6). Notwithstanding, *L. acuta* occupies a greater variety of habitats (e.g., sandy, and muddy substrates, oligohaline to polyhaline areas of estuaries), which may be explained by the numerous gland cells that may produce acidic mucopolysaccharides (i.e., mucus), typical of both sessile and errant annelids (Mastrodonato et al., 2005, 2006). Depending on its composition, mucus may have different roles, such as membrane structural stability, electrolyte homeostasis, mechanical and chemical defence, or reproduction (Hausen, 2005; Mastrodonato et al., 2005; Stabili et al., 2009; Storch, 1988). We thus suggest that mucus production in *L. acuta* may help buffer low-term (less than 24 h) salinity changes, even better than the strategies developed by *Alitta* sp. (also at salinity 0).

In agreement with their distributions along the estuarine gradient, *Nephtys fluviatilis* and *Isolda pulchella* were distinctly more comfortable at low and high salinities, respectively. Although showing mortality at salinities 0–3, *N. fluviatilis* had a putatively narrower salinity niche than the nereidids, being the examined species showing the lowest changes in weight across the salinities tested. Similar to typical oligohaline organisms, it was able to control its weight from fresh water up to salinities ≤ 15 . Such a weight response fits well with that of isolated cells, which did not swell upon 50% hypoosmotic shock (Castellano et al., 2020). Moreover, *N. fluviatilis* showed a significant body fluid hyperosmotic state (by ~ 120 mOsm/kg H₂O) at a salinity of 15 (Castellano et al., 2020), a significant AER signal for such a small animal, a member of a typically osmoconforming invertebrate group (Deaton, 2009). Similarly, *Hediste limnicola*, also known from low salinity waters, showed a comparable response (Oglesby, 1965a). *Nephtys fluviatilis* usually occurs in muddy bottoms in oligohaline sectors, with salinity 15 being considered its ecological salinity limit (Castellano et al., 2020; Giménez et al., 2005; Lana, 1986; Passadore et al., 2007). However, higher salinities did not significantly affect its survival, with its weight being almost stable across the tested salinities, even if switching from osmoregulation to osmoconformation at higher salinities (≥ 25). Its weight did not change significantly until salinity 5 (i.e., differing from that at 3 and 0) and was maintained (with some “readjustment”) over time. Moreover, our results show that *N. fluviatilis* may tolerate salinities of $\sim < 25$ for at least 24 h. Therefore, its restricted ecological distribution may be the result of other driving factors, such as interspecific competition and feeding or reproductive habits (e.g., Gale and Proctor, 2011; Lemieux et al., 1997).

Having a thick integument allows facing salinity changes (as shown for the two nereidids with narrower salinity niches). Thus, other strategies allowing osmotic/volume homeostasis must be in place in species with very thin cuticles and epidermises. Among them, cuticle composition interferes with osmotic and ionic permeability (e.g., Hausen, 2005; Richards, 1984), expressing ion transporters capable of generating a significant osmotic gradient (Castellano et al., 2020) and showing active avoidance. A thin and flexible integument is expected in burrowers (Gustavsson, 2001), such as nephtyids (Rouse and Pleijel, 2001). Accordingly, *N. fluviatilis* almost reaches the surface of the sediment to avoid freshwater during low tides, while it burrows deep when tides rise (Mucciolo, pers. obs.). Thus, having a thin integument is more likely a typical nephtyid trait rather than an osmoregulation mechanism, suggesting phylogenetic constraints for vagile, burrowing

lifestyles in soft bottoms, as hypothesized for clitellates (Gustavsson, 2001).

Among the four annelids, the melinnid *I. pulchella* was the least euryhaline species and the species with the highest sensitivity to seawater dilution. It displayed a more than twofold gain in water: extremely low salinities (i.e., 0 and 5) dramatically decreased its survival. Its water permeability was apparently much higher than that of the other three species, a typical feature of marine osmoconformers (Deaton, 2009). As a sessile species not able to show avoidance behaviour to cope with salinity variations, it is usually restricted to poly- and euhaline estuarine sectors. The ability to successfully respond to environmental stresses, such as hypoxia and thermal or salinity variations, is hampered in annelids with sessile habitus and soft bodies (Rivera-Ingraham and Lignot, 2017). *Terebellides parvus* Solis-Weiss, Fauchald & Blankensteyn, 1991 and *Loimia medusa* (Savigny, 1822), usually restricted to poly/euhaline sectors such as *I. pulchella*, showed a similar osmotic stress tolerance with increasing mortality rates when exposed to lower salinities (Ferraris et al., 1994; Krishnamoorthi, 1962), together with a low capacity to adjust cell or body water/volume when salinity-challenged and late volume regulation (up to 12 h in *I. pulchella*). Their bodies changed in weight according to salinity (i.e., increasing in hypoosmotic media), emphasizing their supposed marine origin and more limited distribution in estuaries (Foster et al., 2010). Evidence of their high sensitivity to salinity variations is also provided by the restricted distribution of the NKCC, expressed in the body portions in direct contact with the external environment (Mucciolo, unpublished data).

Isolda pulchella is like *N. fluviatilis* in having a thin integument, which is compatible with our results and our initial hypothesis of having a low degree of euryhalinity and a distribution restricted to high salinity environments. Thin integuments may be compensated by tube building, which provides sheltering, more efficient feeding and respiration, and salinity buffering (Fox, 1938; Kakui and Hiruta, 2017), as in *Branchiomma luctuosum* (Grube, 1870), a brackish and marine sabelid (Mastrodonato et al., 2005), and *Aulodrilus plurisetus* (Piguet, 1906), a freshwater clitellate (Gustavsson, 2001). Nevertheless, the serpulid *Pomatocerus* spp. have a cuticle thicker in the exposed than in the less exposed body regions (i.e., those usually protected by the tube) (Bubel, 1983). Therefore, it may be hypothesized that thicker integuments may provide protection against wide or abrupt salinity changes in exposed body sections. Our results suggest that tube building may be among the few traits allowing *I. pulchella* to tolerate salinity changes (even if they are lower than those shown by the other species). Although it occasionally occurs at salinities of approximately 25 along the estuarine gradient (Mucciolo, pers. obs.), our results showed a twofold increase in volume when it was removed from the tube, thus revealing late and limited cell volume regulation.

5. Conclusion

This is the first study coupling field evidence and laboratory experiments with histological analyses to investigate salinity tolerance in annelids. Our target species displayed varying responses to osmotic stress and diverse integument morphologies, in agreement with their life strategies and preferred habitats. The two nereidids showed a high degree of euryhalinity and strong cell volume regulation but were unable to tolerate fresh water. The nephtyid was distinctly less euryhaline but tolerated low salinities and fresh water. Finally, melinnid showed marine stenohaline behaviour and did not tolerate seawater dilution. The studied species was revealed to be remarkably euryhaline and showed good capacities for body water regulation. The ability to fast, efficient cell volume regulation to face salinity changes has been revealed to be crucial to achieve euryhalinity in unsteady estuarine habitats, especially for osmoconformers. Our novel approach provides a better understanding of the osmoregulatory physiology in soft-body estuarine inver-

tebrates, underlining how their distributions are modeled by adaptations including both autecological and biological traits.

Uncited references

Ebbs and Staiger, 1965
Fretter, 1955

CRediT authorship contribution statement

Serena Mucciolo: Conceptualization, Investigation, Data curation, Writing – original draft. **Andrea Desiderato:** Formal analysis, Writing – original draft. **Selene Miranda Leal:** Investigation, Writing – review & editing. **Maria Mastrodonato:** Investigation, Writing – review & editing. **Paulo Lana:** Supervision, Resources, Writing – review & editing. **Carolina Arruda Freire:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jembe.2021.151617>.

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