Adaptation to water salinity changes: phenotypic plasticity meets and moulds carry-over effects in sea rock-pool mosquitoes

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Abstract

Organisms living in highly variable environments are expected to exhibit a strong phenotypic variability within populations due to plastic responses to environmental fluctuations. Yet, this phenomenon is not commonly seen in nature, since compensatory mechanisms can promote phenotypic stability. Here, we hypothesized a potential compensatory effect that could result from the interaction between behavioural and morphological trait plasticity. These traits are highly responsive to environmental factors, and compelling research has demonstrated that behavioural plasticity can impact individual life history traits. In microcosm experiments, we analysed plasticity in body size, larval and pupal behaviour and carry-over effect across developmental stages in the sea rock-pool mosquito *Aedes mariae* in response to water salinity changes. Analyzing morphological traits, we found that larvae raised under increasing salinity were smaller than those raised under constant conditions. Additionally, a smaller body size at the pupal stage was observed, which indicated the presence of carry-over effects from the larval to pupal stages. However, no differences were observed in adult sizes between the two salinity conditions. When examining behavioural traits, we observed that higher salinity promoted plastic changes in larval activity behaviour and pupal diving behaviour. In particular, pupae exposed to increased salinity spent 20.6% less time underwater and performed fewer abdominal contractions than those under constant conditions. Since pupal energy expenditure is proportional to the time spent underwater, we suggest that the plastic pupal behaviour promoted compensatory energy allocation to growth, thereby reducing the carry-over effects from the pupal to the adult stage. This study emphasizes that plasticity at multiple traits during development can impact carry-over effects, ultimately leading to the convergence of adult phenotypes.

Introduction

Phenotypic plasticity is the ability of a given genotype to express different phenotypes in response to different environmental conditions (Pigliucci 2005). In organisms with complex life-cycles, phenotypic changes during a previous life stage can impact later stages, leading to within-generation carry-over effects (Beckerman et al. 2002, Giménez 2004, 2006, O'connor et al. 2014). In the damselfly *Ischnura elegans*, exposure to heat waves during larval development affects adult size-related traits and wing shape (Arambourou et al. 2017). Similarly, it has been documented that food availability, predator exposure, competition or pollution experienced in the larval stages might impact post-metamorphic performance(West-Eberhard 1989, Masel and Siegal 2009, Potter et al. 2011, Gray 2013, Garcia et al. 2017, Evans et al. 2021, Snell-Rood and Ehlman 2021, Zamora-Camacho et al. 2022).

In organisms living in highly variable environments, a strong phenotypic variability within populations is expected due to plastic responses to natural environmental variation and carry-over effects(West-Eberhard 1989, Snell-Rood and Ehlman 2021). However, this is not commonly observed in nature. Therefore, it has been shown that organisms would evolve the ability to produce a consistent phenotype regardless of environment variation by compensatory effects (*i.e*. Phenotypic stability) (Masel and Siegal 2009, Metcalfe 2024). Individuals exposed to adverse circumstances, such as reduced food availability or quality, can accelerate their growth if circumstances improve before development is completed, reaching the same size as the individuals who didn't experience food shortage (Metcalfe 2024).

Compelling studies showed that plastic response to environmental changes can occur at multiple traits of an organism (*i.e.* multivariate plasticity) (Schlichting 1989, Foster et al. 2015). Interestingly, some investigations showed that one trait could potentially alter the development and expression of a second trait (reviewed in Nielsen and Papaj 2022). For example, the *Battus philenor* caterpillar responds plastically to high temperatures by changing body colouration from black to red and by leaving host plants to seek cooler locations as a thermal refuge (Nice and Fordyce 2006). Red colouration has been shown experimentally to reduce the frequency of refuge-seeking, and the interaction between the two plastic traits occurs through the effect of colour on body temperature (Nielsen et al. 2018). Accordingly, we can expect that phenotypic stability in organisms living in highly variable environments can also arise from the interaction between plasticity at multiple traits. However, studies are needed to document plasticity and possible compensatory effects at multiple traits and across multiple stages during development.

Here, we focused on the potential compensatory effects which might arise from the interaction between behavioural and morphological trait plasticity using as study system the sea-rock pool mosquito *Aedes mariae*. Behavioural and morphological traits are remarkably sensitive to environmental conditions (Snell-Rood 2013), and compelling studies showed that behavioural plasticity can influence individual life history traits such as body size or shape (Van Buskirk 2002, Dzialowski et al. 2003, Benard 2004, Nielsen and Papaj 2022). We used the sea-rock pool mosquito, *Aedes mariae*, as study system. This species develops in the water of rock pools go through four distinct life cycle stages (*i.e.* egg, larvae, pupae

and adults) (Clements 1992) in the supralittoral zone along the western Mediterranean coast (Coluzzi et al. 1974, Tripet et al. 2004). Rock pools are extremely variable habitats, experiencing high salinity variation over extremely reduced time lapses from freshwater to hyperaline water conditions (Morris and Taylor 1983, Rosenfeld et al. 2019). Thus, this organism-environment system is ideal for exploring multivariate plasticity, carry-over effects across metamorphic boundaries, and adult phenotype stability. By manipulating salinity conditions during larval development in microcosmos and analysing morphological and behavioural traits, we specifically aimed to evaluate (i) the carry-over effect across metamorphosis of plasticity in larval traits and (ii) the interactions between plastic traits in determining the adult phenotype.

Material and Methods

Sampling and experimental conditions

Experiments were carried out on individuals of *Ae. mariae* obtained from eggs collected in July 2020 from supralittoral rock pools of San Felice Circeo, Italy (41°13'18.77 "N, 13° 4'5.51"E). We designed two experimental treatments based on field data collected during the reproductive season of the species. In the first treatment, individuals were maintained at a constant 50 ‰ salinity (50 g/l) throughout the experiment (hereafter constant condition, C). In the second treatment, larvae were exposed to increasing salinity from 50 ‰ to 150 ‰, with an increase of 10 ‰ per day throughout the experiment (salinity condition, hereafter S). The experiments were run in a climate chamber set to 26 ± 1 °C, 14 h light and 10 h dark regime. Experimental larvae were fed daily with 1 mg of cat food (Imam et al. 2014).

Behavioural and morphological trait measurements

Second instar larvae were individually placed into plastic trays (12x12x7 cm) filled with 200 ml of tap water, previously salted with aquarium salt (Tetra Marine Seasalt). Then, we randomly assigned individuals to constant or salinity developmental treatments (N = 30 x treatment). We placed larvae individually to follow the development of each individual across its life cycle from the L2 instar stage to adulthood.

For the larval stage, we measured the fourth instar larvae's body size and spontaneous activity. Morphometric measures were obtained by taking digital pictures using a stereomicroscope Leica EZ4W at magnification 1x of all individuals within two hours from the ecdysis. Subsequently, using the open-access software IMAGEJ we measured the width and length of the head, thorax, abdomen, and total body (Bar and Andrew 2013). Larval spontaneous activity was analysed by video-recording larvae for 10 min in their housing containers using a camera Canon TG-6. Individuals were tested randomly respect to treatment and within 12 hours from the ecdysis between 8:00 and 13:00. A single operator (G.C.), blindly respect to the treatment, manually scored videos using the software BORIS (Behavioral Observation Research Interactive Software). Larval behaviours in the ethogram consisted of resting, browsing, and thrashing.

Here, the resting category includes the "underwater/still" and "float/suspension feed" behaviours described by Walker & Merritt (1991). The larva is positioned underwater or at the water surface with the respiratory siphon attached to the air-water interface and the body hanging obliquely into the water column.

The larva actively feeds in this position by beating its lateral palatal brushes. In browsing behaviour, the larva brushes the wall or the bottom of the container with its mouthparts and moves along the container surface. During browsing, the larva feeds on the bottom of the plastic tray. This category included the "brush wall" and "float/substrate brush" behaviours by Walker and Merritt (1991). During thrashing, the larva moves through the water column by energetic lateral flexions of the body (Juliano and Reminger 1992). This category included the "wriggle swim" behaviour described by Walker and Merritt (1991). This behaviour is the characteristic wriggling motion that mosquito larvae make when they swim.

For the pupal stage, we measured the pupal body size and diving behaviour from control and salinity treatments. Morphometric measures were obtained by taking digital pictures as described for the larval stage. We measured cephalo-thorax length and width (Bar and Andrew 2013, Ukubuiwe et al. 2018) and determined the sex of individuals by checking the last cephalo-thorax segment (Clements 1992). The diving behaviour was investigated by placing pupae individually in a plastic tray (10x10 cm by 9 cm in height) filled with the water of the treatment they belong to. Pupae are lighter than water and float passively at the surface when undisturbed (Romoser and Lucas 1999). Following a physical disturbance, pupae respond by diving into deeper water and then floating back to the surface to breathe (Awasthi et al. 2012). After allowing the pupa to acclimate for 10 minutes, we applied a mechanical stimulus by touching the water surface by using a homemade automated mechanical harm to automatically deliver and standardize the stimulus («Arduino Uno»; http://www.arduino.cc). Individuals were tested randomly respect to treatment and within 12 hours from the ecdysis between 8:00 and 13:00. We recorded pupae diving behaviour at 30 frames/s with an Olympus TG-6 camera placed in front of the experimental tray. Diving tests were carried out in a lightbox with a single led light (intensity of 1400 lux) situated above the experimental tray. Recording started when the mechanical stimulus was delivered and ended when the pupae returned to the surface. Videos were analyzed by a single operator (G.C.) blindly respect to the treatment using the software BORIS (Behavioral Observation Research Interactive Software). The following measures were obtained: i) the time spent underwater after the stimulus; ii) the time spent in the upper, medium and lower part of the water column (1-3 cm, 3-6 cm, and 6-9 cm from the water surface, respectively); iii) the number of abdominal movements during the immersion.

For the adult stage, we measured the percentage of emerging adults and wing length, as a proxy for body size (Van Handel and Day 1989). Adults left wing was dissected within 24 hours from emergence, and wing length from the axillary incision to the apex of the wing, excluding the marginal fringe (Nasci 1986), was obtained from digital pictures using the software IMAGEJ.

Data analysis

Generalized linear models (GLMs) were carried out to assess the contribution of salinity treatment in explaining larval and pupal morphology, larval spontaneous activity, pupal diving behaviour and adult wing length. We applied principal component analysis (PCA) on fourth-instar larvae morphometric measures and subsequently performed GLM on the first two principal components separately as dependent variables. The analysis was run using the *glm* function in R with a Gaussian distribution and with identity link function. For

spontaneous activity of fourth instar larvae, we applied GLMs with Gaussian distribution and with identity link for resting behaviour and with Gamma distribution and inverse link for browsing and thrashing behaviours.

For pupal traits, we applied GLM with a Gaussian distribution and with identity link function for cephalo-thorax width and length and for the number of movements/time spent underwater and GLM with a Gamma distribution and inverse link for time spent underwater in diving behaviour and for the percentage of time spent in the upper, medium and lower part of the water column. To assess the effect of treatments on the number of abdominal contractions in diving behaviour, we applied GLM with a negative binomial distribution. The wing length of adults was analysed using GLM with a Gaussian distribution and with identity link function.

We relied on the Akaike information criterion (AIC) to identify the distribution type and the link function that improved the model fitting. We checked for the effect of sex because of the sexual dimorphism in this mosquito species (Cordeschi et al. 2024); however, for models where there was no evidence for an effect of sex based on likelihood ratio test (LRT), we did not include this term in the final models (see Table S1)

All statistical analyses were performed in R vers.4.1.2 (http://www.R- project.org/).

Results

Larval traits

The first PCA component explained 76.9% of the total variance and discriminated against individuals on body size. The second component explained 13.9 % of the variance and detected differences in body shape (Table 1).

The GLM conducted on larval body size (PC1) showed significant differences between treatments (Table 2). Fourth instar larvae grown up in control treatment were bigger than larvae in increasing salinity treatment (Fig. 1). We found no effect of treatment on the larval shape (PC2; Table 2).

The activity behaviour of larvae varied significantly between treatments (Fig. 2). Increasing salinity caused an increase in resting by 7.22% and reduced browsing behaviour by 6.31% compared to the control treatment, while different salinity concentrations did not affect thrashing behaviour (Table 2).

Pupal and adult traits

GLMs on morphological pupal traits showed an effect of treatment for cephalo-thorax width (Table 2). Pupae developing in salinity treatment showed a narrower cephalo-thorax than pupae in the control treatment (Fig. 3). Instead, no effect of treatments was observed for cephalo-thorax length that differed only between sexes (Tab. 2 and Table S1).

We observed a significant effect of treatment on time spent underwater (Table 2). In increasing salinity conditions, pupae spent 20.6 % less time in immersion than pupae from control after the stimulus (Fig. 4A). Analyzing the time spent in the three areas of the water column, pupae in increasing salinity conditions spent less time in the lower and medium part and more time in the upper part than control treatment pupae (Table 2

and Fig. 4B). Moreover, pupae in salinity treatment performed a higher number of movements/time underwater (Fig. 4C) and fewer abdominal movements than control pupae (Table 2 and Fig. 4D).

The wing length did not vary between adults from the two treatments but only between sexes (Table 2, Table S1 and Fig. 3B).

Discussion

Phenotypic plasticity in the larval stage in response to increasing salinity

Aedes mariae larvae showed plasticity at both behavioural and morphological traits under the increased salinity condition. We found they were smaller than the larvae developed under constant conditions. Empirical studies showed that mosquito species living in brackish or saline water maintain the correct level of osmolarity by accumulating amino acids in the hemolymph (Garrett and Bradley 1987, Patrick and Bradley 2000), by increasing the anal papillae size (Surendran et al. 2018) or by producing less permeable cuticles (Bradley 1987). All these mechanisms require substantial energy allocation and could trade off with body size (Clark et al. 2004), accounting for the larval size reduction observed in *Ae. mariae* larvae, as also suggested in other species (Nayar 1969, Clark et al. 2004).

Interestingly, by analyzing the activity behaviour of *Ae. mariae* larvae, we observed that larvae in the salinity treatment spent more time resting than larvae in the control treatment. While resting on the water surface, the larvae filter and drink water, and it was observed, in saltwater mosquito species, that oral water intake countered osmotic loss through the cuticle (Merritt et al. 1992). For example, in *Culex tarsalis*, when larvae were transferred in higher salinity, the drinking rate increased by over 50% compared to the freshwater value (Patrick and Bradley 2000). Accordingly, behavioural plasticity observed in *Ae. mariae* could contribute to their smaller size under increasing salinity conditions, along with the energy allocation to osmoregulation.

Carry-over effect across metamorphosis

By analysing morphological traits in both larvae and pupae, we observed a carry-over effect. The reduced size of the larvae developed under increasing salinity resulted in the reduced width of the cephalo-thorax in pupae. Similar results were observed in other mosquito species (Nayar 1969, Clark et al. 2004, Arduino et al. 2015). For example, in *Ae. aegypti*, larval exposure to higher salinity conditions led to pupal mass reduction (Clark et al. 2004). The carry-over effect between larval and pupal stages is expected. Indeed, mosquitoes feed in the larval stages but not as pupae, so energy reserves stored during the larval development constitute a crucial component of the body condition and survivorship of both pupae and emerged adults (Lucas and Romoser 2001). However, by analysing adult wing size no differences were observed between the two treatments. Therefore, while we observed carry-over between larval and pupal stages, we unexpectedly did not find it between pupal and adult stages.

In the diving experiments, we observed that the *Ae. mariae* pupae developed under the increasing salinity condition, spent less time submerged, remained more in the surface zone of the water column and performed fewer abdomen contractions than the pupae developed under constant salinity conditions. Notably,

mosquito pupae do not feed, while diving is an active, energy-consuming behaviour. Indeed, being positively buoyant because of gas in the ventral air space, pupae need to counterbalance buoyancy with continual body movements to remain underwater, resulting in an energy expenditure proportional to the time spent underwater (Romoser and Lucas 1999, Lucas and Romoser 2001). Lucas and Romoser (2001) found that energy expenditure was significantly higher in newly emerged adults of the mosquitoes *Aedes aegypti* and *Aedes albopictus* that were repeatedly forced to dive as pupae than adults from pupae held at rest. Based on the above arguments, if *Ae. mariae* pupae developed under increased salinity conditions consume lesser energy than pupae developed under constant salinity conditions, we could expect a lack of carry-over in body size between pupal and adult stages, as actually observed.

Conclusions

Phenotypic plasticity is a major determinant of individual phenotype, and much research has been devoted to studying the resulting phenotype. However, recent studies highlight how developmental plasticity might lead to an adjusted developmental trajectory but to an unaltered final phenotype (Metcalfe 2024). As a consequence, these plastic changes in developmental trajectories could remain hidden as they are not evident in the final phenotype. Here, we extended this idea to plasticity at multiple traits across multiple stages. Taken together, our results support that salinity conditions induce phenotypic plasticity at both behavioural and morphological traits in *Ae. mariae* immature stages. Carry-over in body size occurs between larval and pupal, but not adult stages. Our findings of pupal plasticity in diving behaviour suggest that behavioural plasticity at the pupal stage smoothed the difference in body size observed in immature stages. Thus, plasticity at multiple traits in immature stages can affect carry-over and, ultimately, lead to the convergence of the adults' phenotypes. The interactions between multiple plastic traits in determining adult phenotype is an important but overlooked area. This study highlights that the role of plasticity in building adult phenotype can remain concealed without studying plasticity at multiple traits across all life stages and considering trait plasticity interactions.

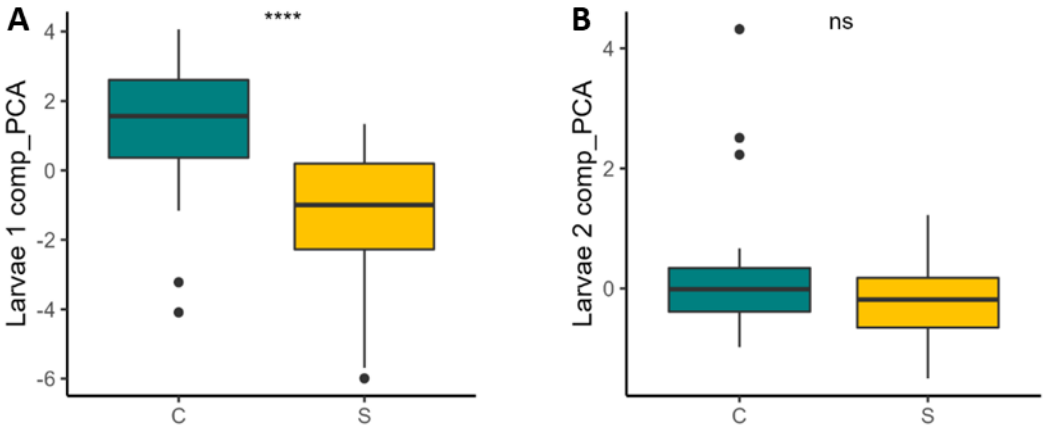

Figure 1: Morphometric analyses of larval stage. PCA first and second component are shown under constant (C; N = 27) and increasing salinity (S¸ N = 26) conditions. Significance levels: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, ns = $P > 0.05$. Boxplots show median values (middle line), interquartile range (box) and range values including some outliers (dots which extend beyond the min and max of the boxplot).

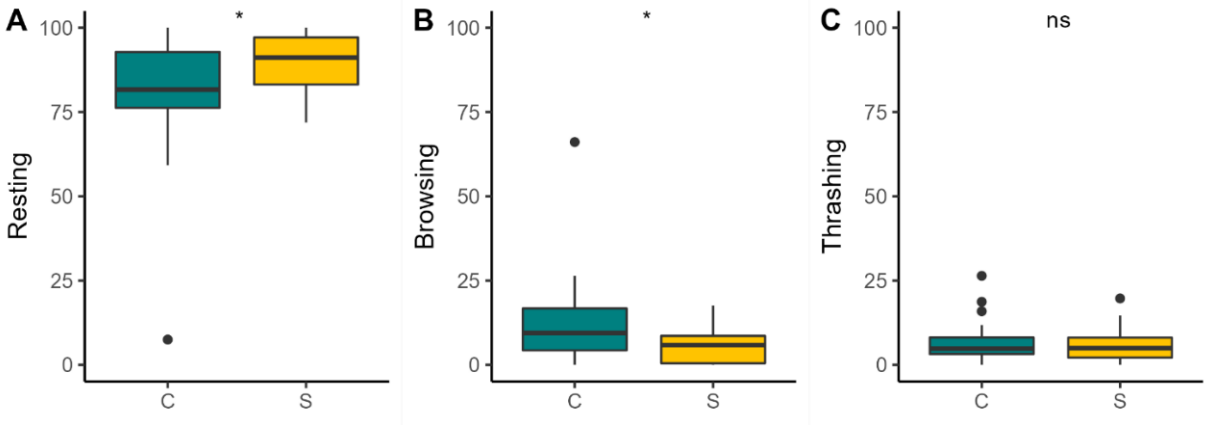

Figure 2: Larval activity behaviour. Time spent in resting (A), browsing (B), and thrashing (C) of fourth instar larvae under constant (C; N = 27) and increasing salinity (S¸ N = 25) conditions. Significance levels: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, ns = $P > 0.05$. Boxplots show median values (middle line), interquartile range (box) and range values including some outliers (dots which extend beyond the min and max of the boxplot).

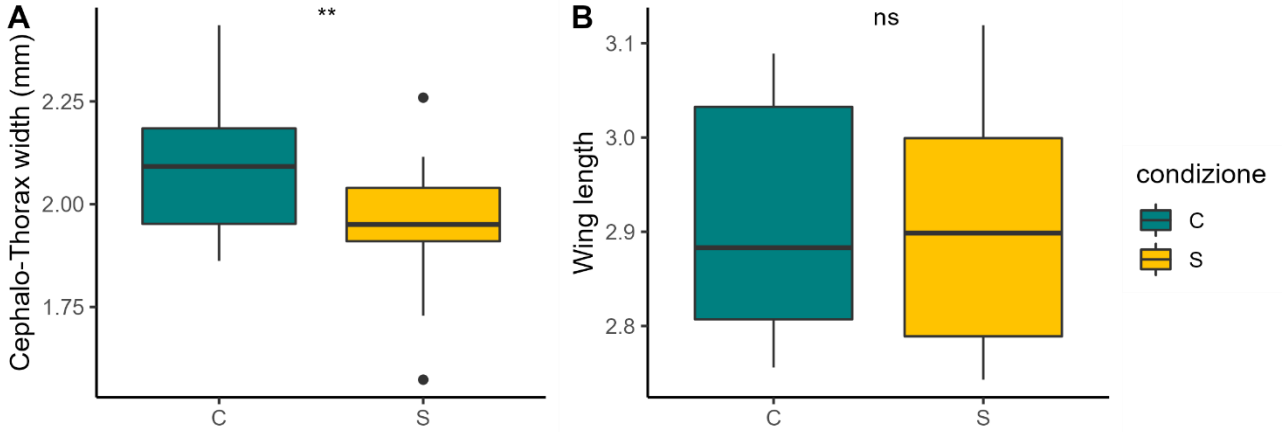

Figure 3: Cephalo-thorax width of pupae and wing length of adult mosquitoes under constant (C; N = 20) and increasing salinity (S; N = 20) conditions. Significance levels: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, ns = $P > 0.05$. Boxplots show median values (middle line), interquartile range (box) and range values including some outliers (dots which extend beyond the min and max of the boxplot).

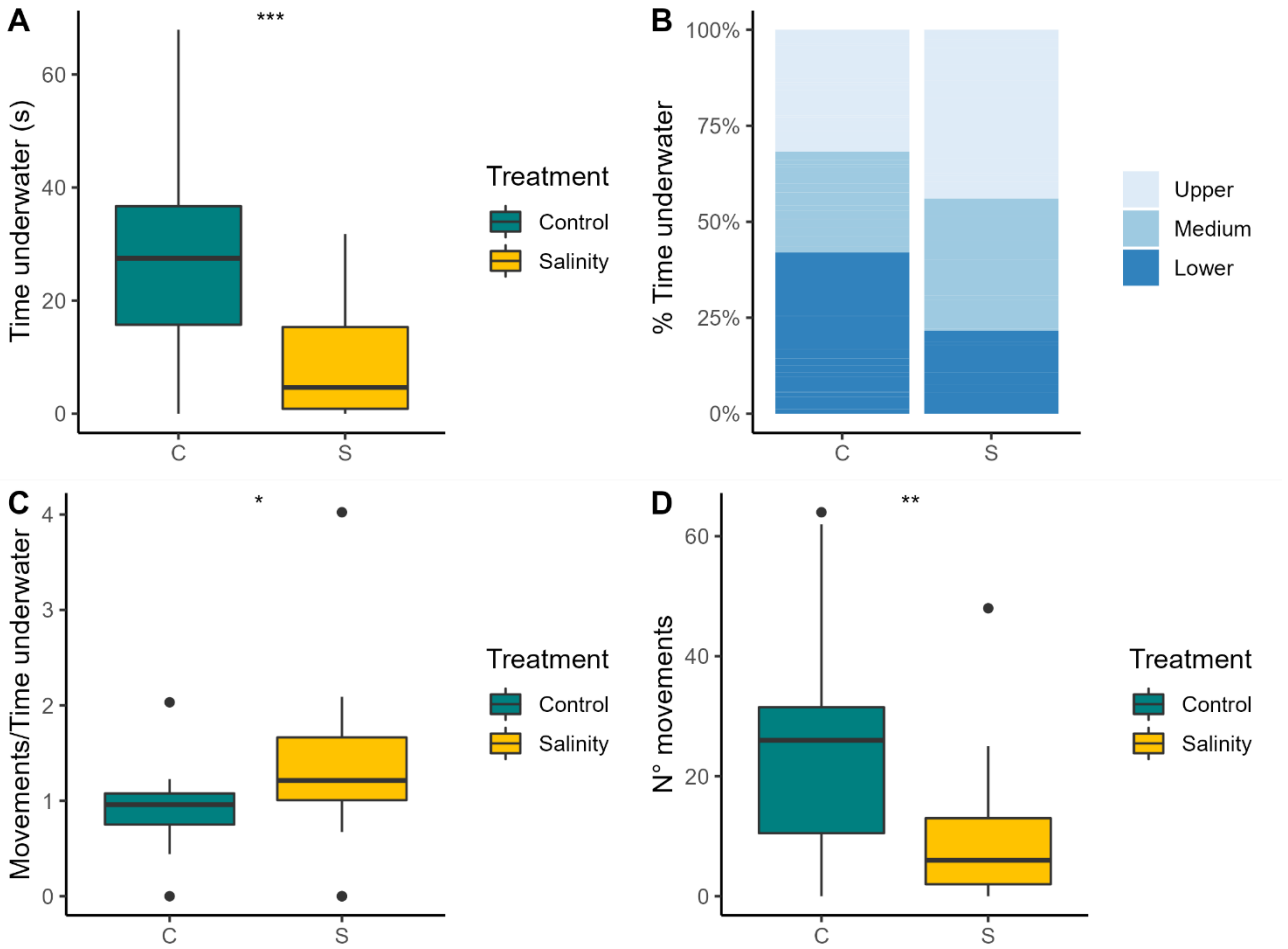

Figure 4: Diving behaviour of pupae. A: time spent underwater of pupae under constant (C; N = 20) and increasing salinity (S; N = 21) conditions. B: time spent in upper, middle and lower water column part during diving behaviour of pupae. C: abdominal movements and time spent underwater ratio. D: number of abdominal movements of pupae Significance levels: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, ns = $P > 0.05$. Boxplots show median values (middle line), interquartile range (box) and range values including some outliers (dots which extend beyond the min and max of the boxplot).

Table 1. Results of PCA analysis on morphometric measures of fourth instar larvae.

	Eigenvalues	PC1	PC2
4th instar larvae			
Proportion of total variance explained		0.769	0.139
	Head width	0.318	0.285
	Thorax width	0.423	
	Abdomen width	0.418	-0.134
	Head length	0.153	0.914
	Thorax length	0.412	-0.192
	Abdomen length	0.413	-0.149
	Total length	0.426	

Table 2. Outcome of generalized linear models using treatment as fixed factor. Significant *P*-values are shown in bold.

	Trait	Coefficient	Standard error	*t*-value	*P*-value
Larvae	Morphology- PC1	-2.583	0.532	-4.849	**< 0.001**
	Morphology - PC2	-0.460	0.266	-1.728	0.090
	Spontaneous activity- Resting behaviour	0.077	0.038	2.02	**0.048**
	Spontaneous activity- Browsing behaviour	9.876	3.914	2.523	**0.014**
	Spontaneous activity- thrashing behaviour	2.220	3.988	0.557	0.58
Pupae	Cephalo-thorax width	-0.134	0.048	-2.781	**0.008**
	Cephalo-thorax length	-0.032	0.044	-0.731	0.469
	Time underwater	0.090	0.026	3.449	**0.001**
	lower	0.517	0.244	2.114	**0.040**
	medium	0.257	0.088	2.911	**0.005**
	upper	0.184	0.049	3.706	**<0.001**
	N of movements/ Time under water	0.415	0.204	2.034	**0.048**
	Abdominal movements	-0.952	0.313	-3.037	**0.002**
Adults	Wing length	-0.001	0.038	-0.036	0.971

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