

Ontogeny of osmoregulation of the Asian shore crab Hemigrapsus sanguineus at an invaded site of Europe

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1	Ontogeny of osmoregulation of the Asian shore crab Hemigrapsus sanguineus
2	at an invaded site of Europe
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23	LAY SUMMARY
24	The Asian shore crab Hemigrapsus sanguineus, an invasive species in the North Atlantic coast,
25	showed a strong capacity to osmoregulate. These osmoregulation abilities over the whole life cycle
26	also constitute an important advantage over the native European competitor Carcinus maenas.

ABSTRACT

28 We studied the ontogeny of osmoregulation of the Asian shore crab Hemigrapsus sanguineus 29 at an invaded area in the North Sea. *H. sanguineus* is native to Japan and China but has successfully 30 invaded the Atlantic coast of North America and Europe. In the invaded areas, H. sanguineus is 31 becoming a keystone species as driver of community structure and the adults compete with the 32 shore crab *Carcinus maenas*. Strong osmoregulatory abilities may confer the potential to use and 33 invade coastal areas already earlier in the life-cycle. We reared larvae and first juveniles at 24°C in seawater from hatching to intermoult of each developmental stage (zoea I-V, megalopa, crab I). 34 35 We exposed each stage to a range of salinities (0-39 ppt) for 24h, and then we quantified 36 haemolymph osmolality, using nano-osmometry. In addition, we quantified osmolality in field-37 collected adults after acclimation to the test salinities for 6 days. Larvae of H. sanguineus were 38 able to hyper-osmoregulate at low salinities (15 and 20 ppt) over the complete larval development, 39 although the capacity was reduced at the zoeal stage V; at higher salinities (25-39 ppt), all larval 40 stages were osmoconformers. The capacity to slightly hypo-regulate at high salinity appeared in 41 the first juvenile. Adults were able to hyper-osmoregulate at low salinities and hypo-regulate at 42 concentrated seawater (39 ppt). H. sanguineus showed a strong capacity to osmoregulate as 43 compared to its native competitor C. maenas, which only hyper-regulates at the first and last larval 44 stages and does not hypo-regulate at the juvenile-adult stages. The capacity of H. sanguineus to 45 osmoregulate over most of the life cycle should underpin the potential to invade empty niches in 46 the coastal zone (characterized by low salinity and high temperatures). Osmoregulation abilities 47 over the whole life cycle also constitute a strong competitive advantage over C. maenas.

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⁵³ Keywords: invasive species, larva, ontogeny, osmoregulation, shore crab

INTRODUCTION

55 Understanding and predicting the process of establishment of exotic species require better 56 knowledge of the traits promoting invasion and colonization of new habitats. Physiological traits, 57 as the foundation of environmental tolerance, are central to the process of invasion and species 58 distribution (Kelley 2014; Ames *et al.*, 2020). The capacity to osmoregulate is a key adaptation for 59 the use of coastal and estuarine habitats (Péqueux 1995; Charmantier 1998; Anger 2003). 60 Osmoregulation (here referring to extracellular osmoregulation) is the capacity of an organism to 61 actively regulate the osmotic pressure of the body fluids (Péqueux 1995; Evans and Clairborne 62 2009; Charmantier et al., 2009; Lignot and Charmantier 2015; Rahi et al., 2018). Osmoregulation 63 enables organisms to achieve optimal functioning over a range of salinities, maintain the 64 concentration of essential substances, keep acid-base balance for the proper cell function (Whiteley 65 2011; Henry et al., 2012; Whiteley and Taylor 2015), and sustain growth and development (Torres et al., 2011). Osmoregulation is also likely to confer some capacity to tolerate ocean acidification 66 67 conditions (Whiteley 2011; Whiteley et al., 2018), and to facilitate evolutionary transitions from 68 the marine to semi-terrestrial habitats (Anger and Charmantier 2000).

69 Crustaceans are one of the most important groups of invaders in coastal areas (Galil et al., 70 2011). Tolerance to low salinity is a key trait within the most notable crustacean invaders (Roche 71 et al., 2009; Rudnick et al., 2005; Fowler et al., 2013); low, high, or variable salinities are 72 characteristic of intertidal zones and estuaries. In invasive crustaceans, the ontogeny of 73 osmoregulation is likely to influence the process of invasion through changes in the so-called 74 "propagule pressure" (sensu Simberloff 2009). Little propagule pressure towards coastal-estuarine 75 habitats would be expected from marine crustaceans, as they generally are stenohaline and weak 76 osmoregulators or osmoconformers over their full life cycle (i.e. the osmolality of their body fluids 77 is roughly equal to that of the surrounding fluid: Péqueux 1995; Charmantier et al., 2009). By 78 contrast, invasive estuarine species are osmoregulators at least during the adult stage (Rudnick et 79 al., 2005; Fowler et al., 2013), but the full process of invasion may also depend on the life-history 80 strategy during the larval phase. Estuarine species or those living in land-locked habitats show a 81 pattern of osmoregulation that varies according to the strategy of ontogenetic migration. For 82 instance, species retaining their larvae in the parental habitat (e.g. within estuaries or other land-83 locked water masses), are able to osmoregulate over the full life cycle (Charmantier *et al.*, 1998;

Anger and Charmantier 2000, 2011). Therefore, for an invasive species following a larval retention strategy, invasion could theoretically take place at any stage of development. By contrast, the process of invasion will be restricted to specific life stages in species following the larval export strategy, i.e. where larvae are exported to coastal waters, characterised by higher and more stable salinities. In such species, the osmoregulatory capacity, and hence the ability to use estuarine waters as habitat, is reduced or absent during the osmoconforming larval stages (Charmantier *et al.*, 2002; Cieluch *et al.*, 2004; Anger *et al.*, 2008).

91 The ontogeny of osmoregulation should also drive the capacity of an exotic species to establish 92 a self-sustaining population in a new habitat. Self-sustaining populations are key for the long-term 93 establishment, as well as for range expansion, a major characteristic of invasions (Gurevitch et al., 94 2011). Theoretically, self-sustaining populations can play a role in sustaining sink populations at 95 the range limit (Dauphinais et al., 2018; Giménez et al., 2020). In species showing larval export 96 strategy, successful establishment of local populations must rely in the capacity of larvae to 97 perform ontogenetic migrations from and to the parental habitats. However, such capacity can vary regionally depending on hydrodynamic processes driving larval transport (Schab et al., 2013; 98 99 Shanks et al., 2017), as well as the timing of reacquisition of the capacity to osmoregulate (Torres 100 et al., 2006). By contrast, in species that are able to osmoregulate over the full life cycle, the 101 establishment of local self-sustaining populations should not be restricted to specific life phases.

102 Here we report on the ontogeny of osmoregulation of the Asian shore crab Hemigrapsus 103 sanguineus. H. sanguineus is native to the Pacific coast (Japan, China and Russia; Stephenson et 104 al., 2009), but over the past 50 years, it has invaded the Atlantic coasts of North America and 105 North Europe. In North America, H. sanguineus was first recorded in the 1980's, in the Delaware 106 Bay and it subsequently expanded northwards over 10 degrees of latitude (Epifanio *et al.*, 1998; 107 Stephenson et al., 2009; Epifanio 2013; Lord and Williams 2017). In Europe, H. sanguineus was 108 first found in the Dutch delta system in 1999 (Dauvin et al., 2009) and then it expanded over the 109 North Sea (Jungblut et al., 2017, 2018; Geburzi et al., 2018) reaching also the coast of Scandinavia 110 (Karlsson et al., 2019). In the German Bight (North Sea), H. sanguineus has invaded the intertidal 111 zones of the Wadden Sea where densities average reached values of the order of 500 crabs m^{-2} 112 (Geburzi *et al.*, 2018; Fig 3, C6: size range ≤ 10 mm).

113 The impact of *H. sanguineus* on other species, including mussels and crabs, has been recorded in both the Atlantic coast of USA and in N. Europe (Stephenson et al., 2009). As a mode of 114 115 comparison, in the Atlantic coast of USA, the increase in abundance of *H. sanguineus* correlates 116 with the disappearance of another invasive species, the shore crab C. maenas (Lohrer and 117 Whitlatch 2002), which has invaded coast worldwide (Carlton and Cohen 2003, Roman and 118 Palumbi 2004) and is included in the list of the top 100 alien invasive species in the world (Global 119 Invasive Species Database 2021). In the US coast, adults of *H. sanguineus* outcompete *C. maenas* 120 through predation on juveniles and shifts in diet that ultimately drive fecundity (Jensen *et al.*, 2002; 121 Griffen *et al.*, 2011). We know less about how those species compare at their dispersive larval 122 phase, a key trait driving invasions (Hassall et al., 2008; Simberloff 2009).

123 H. sanguineus is a euryhaline crab with larvae capable to withstand salinities as low as 15 ppt 124 (Epifanio et al., 1998), although the megalopa appears to exhibit reduced salinity tolerance. In the 125 present study, we quantified the ontogeny of the capacity to osmoregulate in the five zoeal stages 126 and the megalopa, in the first crab (juvenile) stage, and in adults, thereby covering the full larval 127 and benthic life phases. We studied osmoregulation at the optimal temperature in order to quantify 128 the full osmoregulatory potential of the species. The range of salinities used are those to be 129 experienced by *H. sanguineus* populations in North Europe and associated estuarine areas 130 (Karlsson et al. 2019), including the mouth of the Elbe and Weser rivers in the German Bight 131 (surface salinities 15-32 ppt: Bils et al. 2012, Sprong et al. 2020), Dutch delta system (salinities 132 range 12-32 ppt in the Osterschelde estuary: Gerringa et al. 1998), Kattegat (salinity ~18-34 ppt). 133 Moderately low salinities may be experienced by populations located in North America (e.g. 134 mouth of Delaware Bay) and in the Western Pacific coast (range distribution in Fig. 9 of 135 Stephenson *et al.* 2009). We also tested osmoregulation at high salinities (>35 ppt) because salinity 136 can increase above that of seawater in intertidal pools in the summer. We show that *H. sanguineus* 137 has the capacity to osmoregulate over the full life cycle, an adaptation that is likely to underpin the 138 invasion capacity, enhancing the dispersal ability and the capacity to deal with competitors such 139 as C. maenas.

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MATERIALS AND METHODS

142 Collection of adults and larval rearing of *Hemigrapsus sanguineus* (De Haan, 1835)

Experiments were carried out with adults and larvae from the local population of Helgoland and Sylt (North Sea, German Bight) during the reproductive period. Egg-carrying females were collected in intertidal habitats and kept in the Helgoland laboratory in 2 L aquaria with oxygenated and filtered ($0.2 \mu m$) natural seawater (32.5 ppt). Aquaria were placed in a temperature-controlled room at 24°C with a 12:12 h light:dark cycle.

148 Experiments with adults were based on field-collected individuals. Experiments with larvae and 149 first stage juveniles were run using standard methods of larval rearing (Charmantier *et al.*, 2002; 150 Torres et al., 2020, 2021b). Larvae were reared in filtered aerated natural seawater in groups of 50 individuals in 500 ml glass bowls (density: 0.1 larva ml⁻¹) in a temperature-controlled room at 151 152 24°C, 12:12 h light:dark cycle. Daily, rearing bowls were rinsed and cleaned, water was changed, 153 dead individuals were removed, and larvae were fed with freshly hatched Artemia sp. nauplii. Prior 154 to experiments, larvae moulting to each stage were separated daily from the cultures and kept in 155 additional bowls in order to obtain larvae with the same moulting age. Survival was tested at each 156 stage at all salinities. Animals were checked at regular intervals and determined as dead when not 157 moving after being repeatedly touched with a probe.

158 Experiments

Osmolality was adjusted with a salinometer (Cond 3110 SET1, WTW GmbH, Weilheim, Germany) by diluting natural seawater (32.5 ppt = $966 \pm 1 \text{ mOsm kg}^{-1}$) with appropriate amounts of tap water. Water (samples of 20 µl) was then checked to achieve the appropriate osmolality using a micro-osmometer (Model 3MO, Advanced Instruments, Needham Heights, MA, USA); conversion factors between osmolality and salinity are as follows: 3.36 ppt $\approx 100 \text{ mOsm kg}^{-1}$; 29.7 mOsm kg⁻¹ ≈ 1 ppt.

Haemolymph osmolality was quantified using nano-osmometry (Charmantier *et al.*, 1998); osmoregulatory capacity (OC) was calculated as the difference between the haemolymph and medium osmolalities. Osmoregulatory capacity was quantified at intermoult; freshly hatched or recently moulted larvae or juveniles were separated from cultures and kept in vessels for 50% of the expected stage duration when they were checked visually (based on preliminary experiments). At the appropriate time, larvae of each stage were placed in petri dishes at the appropriate test 171 salinities and kept for 24 h (see below, Fig. 1). Before proceeding with the measurements, larvae 172 were quickly rinsed in deionised water and gently dried on a filter paper. They were then 173 submersed in mineral oil in order to avoid evaporation and desiccation (any remaining water was 174 aspired using a first micropipette). Samples of haemolymph (sample volume ~ 30 nl) were taken 175 from the heart by inserting a second micropipette into the body. Adults were placed in individual 176 aquaria and maintained in the test salinities. Prior to haemolymph sampling, their moulting stage 177 was checked through microscopic examination of pleopod setae (Drach 1939; Drach and 178 Tchernigovtzeff 1967) and only crabs in intermoult stage C were retained. After 6 days of exposure 179 (see below, Fig. 1), the crabs were rinsed with deionized water and dried with filter paper. 180 Haemolymph was sampled with a micropipette inserted at the basis of a posterior pereiopod, and 181 it was quickly transferred into mineral oil to avoid evaporation. For all experimental stages, 182 haemolymph osmolality was determined with reference to the medium osmolality on a Kalber-183 Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA).

184 Data analysis

185 For the range of osmolalities between 15 and 39 ppt, data of osmoregulatory capacity by stage 186 was analysed through two-way factorial ANOVA (Underwood 1997) after confirmation that 187 variance did not show evidence of heterogeneity (Cochran test, p = 0.38) and residuals showed 188 normal distribution (qq-plot of within cell residuals). Both the first juvenile (C I) and adults 189 survived at lower salinities than the larval stages, but we did not record measurements for the C I 190 in seawater. Therefore, the comparison of osmoregulatory capacities was carried out through 191 separate ANOVAs considering (1) megalopa and first juvenile, and (2) first juvenile vs. adult. In 192 addition, for adult crabs, we also considered responses under pure freshwater (i.e. 0.03 ppt, shown 193 as <1 ppt in Fig. 3) and 10 ppt, which were analysed along with the responses at the remaining 194 osmolalities using one-way ANOVA. Differences among treatment combinations were determined 195 using the Student-Newmann-Keuls test.

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RESULTS

198 The **percentage survival** after exposure to the test salinities for 24 h or 6 days for larvae and 199 juvenile or adults, respectively, is given in Table 1. Recall that different time-periods between

200 adults and larvae were needed to achieve stability for measurements of osmoregulation. 201 Differences between those time-periods do not explain the contrasting differences in survival rates 202 between larvae and adults. Larvae did not survive at 10 ppt or lower salinities while survival was 203 60 - 100% at 20 ppt or higher salinities. Advanced zoeal stages and the megalopa showed moderate 204 to low survival rates at 15 ppt. However, adults survived in all tested salinities until 6 days, even 205 if there was only one survivor in freshwater (< 1 ppt). Hence, comparisons based on instantaneous 206 mortality rates, would show even a stronger contrast between larval and adult mortality at different 207 salinities.

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209 Table 1. Hemigrapsus sanguineus. Percent survival of the tested stages according to the 210 different salinities, after exposing each larval stage and the first juvenile for 24 h, and the adults 211 for 6 - 7 days. The number of individuals at the start of the exposure was 10 for all larval stages 212 and 5 for juveniles (in the tested salinities); except at 15 ppt for zoea III and IV (n = 15), zoea V 213 and megalopa (n = 20). The number of adults at the start of the exposure was 10 for all salinities, 214 except at freshwater (n = 20), 5 ppt (n = 15), and 15 and 38,7 ppt (n = 12). NT: not tested (due to 215 low number of individuals reaching C I, we prioritised the other salinities; indicated with an 216 asterisk in Fig. 3).

Osmolality (mOsm kg ⁻¹)	1	145	290	434	581	730	942	1146
Salinity (ppt)	<1	5	10	15	20	25	32.5	38.7
ZI	0	0	0	100	100	100	100	100
ZII	0	0	0	100	100	100	100	90
ZIII	0	0	0	33	100	100	90	100
ZIV	0	0	0	33	100	100	100	100
ZV	0	0	0	25	100	100	100	100
\mathbf{M}	0	0	0	30	80	70	60	60
CI	NT	NT	100	100	80	100	NT	100
Adults	5	20	60	100	100	90	90	75

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Note: Survival of adults was 100 % at all salinities except at <1 and 5 ppt : 5 and 20 %, respectively (i.e. animals that survived the first 24 hours, also survived until the osmoregulation measurements were performed).

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The **acclimation time** after an abrupt change in salinity was determined in zoea V as a representative for the larval stages, and in adults, using for each of these stages a salinity for which

- survival was high. We determined the acclimation time for zoea V and adults at the selected
- salinity (a salinity for which survival was high: preliminary experiments).For zoea V, such tests
- showed that haemolymph osmolality and osmoregulatory capacity stabilised after less than 10
- hours of exposure; for adults, stabilization occurred after ca. 4 5 days depending on salinity (Fig.
- 1). The period of exposure of larvae (24 h) and adults (6 days) to the different salinities was
- established after these tests and was used for the subsequent experiments.



Figure 1. *Hemigrapsus sanguineus*. Osmoregulatory capacity (OC) change through time. Haemolymph osmolality was measured in zoea V (upper panel) and adults (bottom panel) after transfer from seawater (32.5 ppt) to 20 ppt (blue) and to 15 ppt (red) and 39 ppt (green), respectively. Values are shown as means \pm standard error (n = 6 - 8 for Z V; n = 3 - 5 for adults for each measurement).

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The **ability to osmoregulate** is illustrated by variations of haemolymph osmolality (Fig. 2) and of osmoregulatory capacity (OC; Fig. 3) according to salinity for each tested stage. These results revealed significant variations with salinity showing a pattern consistent with hyper- and hyporegulation (Fig. 2), but such capacity varied considerably among stages (significant interaction stage by salinity: Fig. 3, Table 2). The zoea I-IV and the megalopa were able to hyper-regulate within the range of $434 - 581 \text{ mOsm } \text{kg}^{-1}$ (15 to 25 ppt) and hyper-osmoconformed in the range of 730 - 1146 mOsm kg⁻¹ (25 to 38.7 ppt). The zoea V, by contrast showed a weak capacity to osmoregulate, evident only at 581 (20 ppt).



272 Figure 2. Hemigrapsus sanguineus. Variations in the haemolymph osmolality in different life-273 cycle stages in relation to the osmolality (bottom X-axis) and salinity (upper X-axis) of the medium 274 at 24 °C. Acclimation time was 24 h. for larval and crab I stages, and 6 days for adults. Values are shown as average values \pm standard error. For zoeal stages I-III: n = 9 - 10, IV-V: n = 5; for 275 276 megalopa: n = 6 - 8, for crab I: n = 4 - 5; for adults: n = 9 - 12, except for crabs exposed to <1 ppt: 277 n = 1, 5 ppt: n = 3 and 10 ppt: n = 6. Zoeal stages are shown in red (Z I: stars, Z II: diamonds, Z 278 III: triangles, Z IV squares, and Z V: circles); megalopa (M) is shown in green squares; first 279 juvenile crab (C I) is shown in light blue circles and adult is shown in dark blue circles. Note that 280 standard errors may be smaller than symbols.

282 First juvenile crabs (C I) differed in their osmoregulatory capacity with respect to the megalopa 283 and adults but the difference depended on salinity (Stage:Salinity: comparison with megalopa: $F_{3,38} = 53.5$, p <10⁻¹²; comparison with adult: $F_{4,60} = 19.8$, p <10⁻⁹). At 15 and 20 ppt, juveniles 284 285 were stronger hyper-regulators than the megalopa while they did not differ significantly in 286 osmoregulation at 25 ppt; in addition, at 39 ppt, juveniles showed on average a weak capacity to 287 hypo-regulate. When compared to adults, juveniles were weaker hyper-regulators. In addition, at 288 39 ppt the osmoregulatory capacity was not significantly different from zero: two out of 5 crab I 289 juveniles had positive values of OC (20 and 40) while other two had negative values.

Adults had comparatively the strongest osmoregulatory capacity of all tested stages (Fig. 3), and, at least 5% managed to survive in freshwater (<1 ppt), while 20 - 60% survived at 5 and 10 ppt, respectively (Table 1). They hyper-osmoregulated in the range 434 - 730 mOsm kg⁻¹ where the osmoregulatory capacity increased three times with respect to the larval stages. Adults also hypo-osmoregulated at 1146 mOsm kg⁻¹ where survival was 75% (Table 1).

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Table 2. *Hemigrapsus sanguineus*. Analysis of variance evaluating the effect of salinity and stage in the haemolymph osmolality during the life cycle. This analysis considered all zoeal stages, the megalopa and the adult for salinities ranging between 15 ppt (= 434 mOsm kg⁻¹) and 39 ppt (= 1146 mOsm kg⁻¹). Abbreviations: df: degrees of freedom; MS: mean squares, F: Fisher statistics, p: p-value.

	df	MS	F	Р
Stage (S)	6	38955	163	<10-4
Osmolality (O)	4	125477	526	<10-4
S:0	24	24765	104	<10-4
Residual	280	239		

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Figure 3. *Hemigrapsus sanguineus*. Variations in osmoregulatory capacity (OC) at different life-cycle stages in relation to the osmolality (bottom X-axis) and salinity (upper X-axis) of the medium at 24 °C. Values are shown as average values \pm standard error (replicate numbers as in Figure 2). Zoeal stages (ZI–ZV): red bars; megalopa (M): green bars; Juvenile I crabs (CI): light blue bars; adults crabs (A): dark blue bars; missing bars at < 15 ppt (434 mOsm kg⁻¹) denote 100% mortality in all larval stages; asterisks instead of bars show salinities not tested for CI.

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DISCUSSION

We found that *Hemigrapsus sanguineus* was able to osmoregulate over the entire life cycle, a trait that is likely to contribute to the invasion potential. To our knowledge, there is no native crab species in the North Sea with the capacity to osmoregulate across the full life cycle. Such capacity is not present in larvae of *C. maenas* (Cieluch *et al.* 2004, Torres *et al.* 2021a), the most important 329 native crab occupying intertidal and estuarine zones of North Europe and in addition to being a 330 global invader (Carlton and Cohen 2003, Roman and Palumbi 2004). The osmoregulatory pattern 331 of H. sanguineus is only comparable to the one exhibited by larvae of Eriocheir sinensis (Cieluch 332 et al. 2007), another invasive crab found in the North European Seas (Ojaveer et al. 2007). The 333 crab E. sinensis is also included in the top 100 invasive alien species of the world (Global Invasive 334 Species Database 2021). Hence, given the realised invasion of *E. sinensis*, which includes the 335 Baltic Sea, the osmoregulatory pattern reported in this paper is indicative of the strong potential 336 of *H. sanguineus* to establish coastal populations in the newly invaded areas. Whether such full 337 potential is realised over the full life cycle will depend on other factors, such as temperature, which 338 is known to modulate osmoregulatory capacity in crustaceans (Torres et al. 2021a) and drive 339 salinity tolerance in the larvae of *H. sanguineus* (Epifanio *et al.*, 1998). Hence, whether the full 340 potential is currently realised will depend on latitude, with those local populations present at lower 341 latitude being nearer such potential. However, the realised potential is likely to increase in the 342 future, especially in the Channel and Southern North Seas where isotherms are shifting at a speed 343 of 100 km per decade (Burrows *et al.* 2011), temperatures have increased at a rate of $\sim 0.4 - 0.8$ °C per decade (Huthnance et al. 2016) and are expected to increase another 2 - 3 °C by 2100 344 345 (Shrum et al. 2016). Our data on acute exposure (Table 1) and those of Epifanio et al. (1998) on 346 chronic exposure suggest that larvae of *H. sanguineus* may close the life cycle in habitats of 347 moderately low salinity (> 15 ppt) in situations where larvae experience temperatures in the range 348 of 18 - 25 °C. Adults show a much wider thermal tolerance as shown for instance by populations 349 in the Wadden Sea where winter temperature drops to < 10 °C.

350 Adults of *H. sanguineus* showed a strong capacity to hyper-regulate in diluted seawater over a 351 range of salinities (0 - 20 ppt) although survival rates at the lowest tested media was low (Table 352 1). Our results confirm the hyper-regulatory pattern in our exotic local populations (Sylt and 353 Helgoland, North Sea), as well as the time adults needed to achieve full osmoregulation (> 4 days), 354 found by Watanabe (1982) for a native population (Hokkaido, Japan) and by Hudson et al., (2018) 355 for a population of North America (Connecticut, USA). The capacity to hyper-regulate in H. sanguineus (maximum of ~ 400 - 470 mOsm kg⁻¹ at 5 ppt and in fresh water was lower than that 356 of strong osmoregulators such as Armases miersii and Neohelice granulata (up to 600 mOsm kg⁻¹ 357 358 at 1 ppt: Charmantier *et al.*, 1998, 2002), which occur respectively in rocky intertidal and estuaries 359 pools.

360 We also found in the adult a significant capacity to hypo-regulate at high salinities (38.5 ppt). 361 We do not have strong evidence showing that the capacity to hypo-osmoregulate is already present 362 at the first juvenile stage; slight hypo-regulation was detected in two individuals but also slight 363 hyper-regulation was found in other 2 individuals. In any case, the capacity to hypo-osmoregulate 364 must be clearly established at more advanced juvenile stages. Hypo-osmoregulation in adults was 365 not found by Watanabe (1982) and not studied by Hudson et al., (2018); the latter study did not 366 quantify osmoregulation at concentrated seawater. Differences between our finding and that of 367 Watanabe (1982) may be due to either different temperatures used to acclimate crabs in the 368 laboratory (this study = $24 \degree C$, Watanabe = $15\degree C$), different range of salinities or genetic variation 369 among populations. The pattern of hyper-hypo regulation is however consistent with that found in 370 other species of the same superfamily (e.g. Sesarma meinerti: Gross et al., 1966; S. curacaoense: 371 Anger and Charmantier 2000; A. miersii: Charmantier et al., 1998; N. granulata: Charmantier et 372 al., 2002).

373 Most larval stages of *H. sanguineus* (except the zoea V) had the capacity to osmoregulate over 374 the range 15 - 25 ppt; this pattern and the high survival in response to acute exposure to low salinity 375 (Table 1) are partially consistent with experiments reporting larval salinity tolerance (Epifanio et 376 al., 1998). Such experiments also report a reduction in tolerance of the megalopa, but perhaps the 377 reduction reflects carry-over effects from the zoea V, which showed a reduced capacity to 378 osmoregulate. This ontogenetic pattern of osmoregulation of *H. sanguineus* appears to be 379 intermediate between species showing retention strategy, where the osmoregulatory capacity is 380 maintained or increased along the larval phase (e.g. Charmantier et al., 1998; Anger and 381 Charmantier, 2000), and those showing export strategy, with osmoconforming zoea II-IV stages 382 (Charmantier et al., 2002; Cieluch et al., 2004; Anger et al 2008). Laboratory experiments have 383 shown that *H. sanguineus* larvae exhibit behaviours consistent with an export strategy (Cohen et 384 al., 2015). However, the capacity to osmoregulate in the zoeal stages should provide H. sanguineus 385 larvae opportunities to exploit estuarine habitats as no other known crab with such strategy. In 386 those species, the timing of successfully crossing of salinity gradients should be constrained by 387 the re-acquisition of the capacity to osmoregulate (e.g. see Torres et al., 2006) but our study shows 388 that such constraint is weaker in *H. sanguineus*.

The pattern of osmoregulation in *H. sanguineus* compares well with the competitor, the European shore crab *Carcinus maenas*, also with an export strategy (Queiroga and Blanton 2004). 391 Larvae of both species share a trait that is critical for invasion in a context of global warming: that 392 tolerance to low salinity increases with temperature (C. maenas: Spitzner et al., 2019; Torres et 393 al., 2020, 2021a; H. sanguineus: Epifanio et al., 1998). However, H. sanguineus appear to have 394 an advantage in three main points: First, the pattern of hyper-hypo regulation found in the adults 395 for *H. sanguineus* is not present in *C. maenas*; hence, hypo-regulation may provide a competitive 396 advantage to *H. sanguineus*, in intertidal habitats where salinity may increase over summer months 397 at low tide, since it increases survival at high salinity through ion excretion. Second, most larval 398 stages of *H. sanguineus* osmoregulate while most stages of *C. maenas* do not (zoea II-IV stages 399 do not osmoregulate: Cieluch et al., 2004). Third, at high temperatures (e.g. 24 °C), the capacity to osmoregulate of the zoea I is lower in C. maenas (e.g. zoea I: 55 - 60 mOsm kg⁻¹: Torres et al. 400 401 2021a) as compared to *H. sanguineus* (~ 100 mOsm kg⁻¹, this study). Thus, in temperate estuaries, 402 at the time of the initiation of the migration from estuarine to open waters, first stage larvae of H. 403 sanguineus are better equipped than those of C. maenas. The advantage of C. maenas over H. 404 sanguineus might occur at low temperatures (≤ 18 °C).

In synthesis, we have found two critical traits that make *H. sanguineus* stand-up as compared with competitors (including the global invader *C. maenas*) and with other species with similar lifehistory strategy and habitat. First, adults are hyper-osmoregulators at low salinity and hyporegulate at high salinity; this combination makes them a species well adapted to intertidal zones and especially for the use of tidal pools at latitudes where salinity can increase during the summer season. Second, the capacity to osmoregulate is present along most of the larval phase, which provides opportunities for use and invasion of estuarine waters.

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- 421 **Conflicts of interest:** The authors declare that they have no conflicts of interests.
- 422 Compliance with Ethical Standards: The research presented in this paper complies with the
- 423 guidelines from the directives 2010/63/EU of the European parliament and of the Council of 22^{nd}
- 424 September 2010 on the protection of animals used for scientific purposes.
- 425 **Data accessibility:** All data for this paper will be available from PANGAEA ®Data Publisher
- 426 <u>https://www.pangaea.de</u>

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1 FIGURE LEGENDS

Figure 1. *Hemigrapsus sanguineus*. Osmoregulatory capacity (OC) change through time. Haemolymph osmolality was measured in zoea V (upper panel) and adults (bottom panel) after transfer from seawater (32.5 ppt) to 20 ppt (blue) and to 15 ppt (red) and 39 ppt (green), respectively. Values are shown as means \pm standard error (n = 6 - 8 for Z V; n = 3 - 5 for adults for each measurement).

7 Figure 2. Hemigrapsus sanguineus. Variations in the haemolymph osmolality in different life-8 cycle stages in relation to the osmolality (bottom X-axis) and salinity (upper X-axis) of the medium 9 at 24 °C. Acclimation time was 24 h. for larval and crab I stages, and 6 days for adults. Values are 10 shown as average values \pm standard error. For zoeal stages I-III: n = 9 - 10. IV-V: n = 5; for 11 megalopa: n = 6 - 8, for crab I: n = 4 - 5; for adults: n = 9 - 12, except for crabs exposed to <1 ppt: 12 n = 1, 5 ppt: n = 3 and 10 ppt: n = 6. Zoeal stages are shown in red (Z I: stars, Z II: diamonds, Z 13 III: triangles, Z IV squares, and Z V: circles); megalopa (M) is shown in green squares; first 14 juvenile crab (C I) is shown in light blue circles and adult is shown in dark blue circles. Note that 15 standard errors may be smaller than symbols.

Figure 3. *Hemigrapsus sanguineus*. Variations in osmoregulatory capacity (OC) at different life-cycle stages in relation to the osmolality (bottom X-axis) and salinity (upper X-axis) of the medium at 24 °C. Values are shown as average values \pm standard error (replicate numbers as in Figure 2). Zoeal stages (ZI–ZV): red bars; megalopa (M): green bars; Juvenile I crabs (CI): light blue bars; adults crabs (A): dark blue bars; missing bars at < 15 ppt (434 mOsm kg⁻¹) denote 100% mortality in all larval stages; asterisks instead of bars show salinities not tested for CI.