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**Does resource availability influence the vital rates of the tropical copepod
Apocyclops royi (Lindberg, 1940) under changing salinities?**

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RUNNING HEAD: Vital rates of a copepod under changing salinities

KEYWORDS: Salinity tolerance; functional response; egg production; numerical response; zooplankton

21 ABSTRACT

22 Functioning of invertebrates inhabiting coastal ecosystems is challenged by strong temporal
23 fluctuations in salinity. We investigate how food availability influences vital rates in the tropical
24 cyclopoid copepod *Apocyclops royi* under different salinities (5-32 PSU). We hypothesized that i)
25 mortality decreases and egg production rate increases with food availability, ii) that under
26 suboptimal salinity mortality increases and the egg production rate is reduced and iii) the threshold
27 concentration for egg production (lowest food concentration where egg production is initiated)
28 shifts to higher food concentrations when challenged by salinity. Surprisingly, *A. royi* survived,
29 [fed](#)ingested food, and produced eggs at all tested salinities. Mortality rate was however dependent
30 on salinity level, but not on food availability. Mortality increased ($\sim 12\% \text{ h}^{-1}$) during short-term (1 h)
31 salinity acclimatization to 5 PSU and during the following 24 h incubations ($\sim 5\% \text{ d}^{-1}$) compared to
32 higher salinities. Feeding- and egg production rates increased with food availability up to an
33 optimum at all salinity levels, with no effect of salinity on [the](#) lowest food concentration initiating
34 egg production. This reveals a high salinity tolerance by *A. royi* and may partly explain why this
35 particular copepod is so successful compared to its congeners in occupying extreme habitats.

36

37 INTRODUCTION

38 Living as a zooplankton in near shore environments such as estuaries, lagoons or intertidal areas can
39 be considered a challenge because of regular or rapid salinity oscillations (McCallen *et al.*, 1998;
40 Rivera-ingraham and Lignot, 2017). How spatiotemporal variation in salinity affect zooplankton
41 depends on their physiology and functioning under fluctuating salinity. Therefore salinity is a
42 shaping environmental factor for distribution of species inhabiting these environments and the
43 overall near shore ecosystem structure and functioning (Peterson and Ross, 1991; Henry *et al.*,
44 2002; Hauton, 2016).

45 Dominant zooplankton in these physically rapidly changing environments are species of copepods,
46 often below mm-sized euryhaline crustaceans. There is substantial evidence that most copepods
47 inhabiting these environments do not strictly conform to the external salinity but have
48 osmoregulatory capacities to various degrees. In adult species of all the dominant orders of free
49 living copepods, Calanoida, Cyclopoida and Harpacticoida hypo- and hyperregulation of the
50 internal osmotic pressure and ion balance (Battaglia and Bryan, 1964; Bayly, 1969; Farmer, 1980;
51 Roddie *et al.*, 1984; McCallen *et al.*, 1998) or body density (Svetlichny and Hubareva, 2014), non-
52 isometric to the external conditions, has been observed, ~~non-isometric to the external conditions~~.
53 This indicates the process of active ion regulation.

54 Different from osmoconformation, osmoregulation is an energetically expensive process as ion-
55 transport is particularly ATP consuming (Hand and Hardewig, 1996; Bradly, 2009). Furthermore,
56 elevated aerobic metabolism (mitochondrial activity) associated with exposure to salinity changes
57 may cause oxidative stress by compromising cell functionality and it requires energy to restore the
58 cellular redox balance (reviewed in Rivera-Ingraham and Lignot, 2017). Thus adjusting to osmotic
59 stress infers increased energy expenditure and hence, less energy availability for other basic
60 physiological functions of the copepod e.g. reproduction (Chen *et al.*, 2006).

61 It is therefore not surprising that studies investigating the effect of salinity changes on the vital rates
62 of copepods reveal increased respiration rates (Miliou and Moraitou-Apostolopoulou, 1991; Dutz
63 and Christensen, 2018), decreased reproductive output and even increased mortality under sub-
64 optimal salinities (Lance, 1963; Cervetto *et al.*, 1999; Lee *et al.*, 2005; Chen *et al.*, 2006; Svetlichny
65 and Hubareva, 2014). However, the osmotic dependence of these rates, or 'salinity tolerance',
66 varies greatly among species (Gaudy *et al.*, 1982; Bergmans and Janssens, 1988; Lee *et al.*, 2005).

67 The constant energy requirement for osmoregulation in some copepods infers that their
68 osmoregulatory capacity may be restricted under food-limited conditions and [can be](#) compensated
69 when resources are available. This is supported by Rippingale and Hodgkin (1977), who showed
70 that longevity of the calanoid copepod *Sulcanus conflictus* increases at hyperosmotic salinities when
71 food was available. Similarly Hammock *et al.* (2016) showed that survival of the calanoid copepod
72 *Eurytemora affinis* increases at hypo- or hyperosmotic salinities when resource levels are high.
73 Further, they measured a relatively high food consumption rate at sub-optimal salinities when
74 resources were abundant, possibly to compensate for the elevated metabolic cost due to
75 osmoregulation. Thus, the few studies available on the effect of food presence on the copepods vital
76 rates under hypo- or hyperosmotic salinities suggest that [saturated-increased](#) resource availability
77 increases [their](#) tolerance to sub-optimal salinities. In the present study, we experimentally
78 investigate if and how food availability influences the vital rates of the cyclopoid copepod
79 *Apocyclops royi* at a range of salinities, expected to cover from sub-optimal to optimal salinities.

80 This small, euryhaline copepod inhabits tropical and sub-tropical estuaries (Muthupriya and Altaff,
81 2009; Su *et al.*, 2005) and coastal saline ponds (Blanda *et al.*, 2015; Dhanker and Whang, 2013).
82 Salinity is an important environmental variable [here, as in](#) the coastal waters of (sub)tropical
83 regions, [which](#) are characterized by dilution due to heavy rainfall and by evaporation dominated
84 incidents (Muthupriya and Altaff, 2009).

85 *A. royi* is regularly exposed to abrupt and extreme salinity changes and presumably also to
86 fluctuating food abundance due to heavy rainfall (Blanda *et al.*, 2015). Although previous studies
87 on *A. royi* have shown [it](#) tolerates and reproduces over a wide range in salinity (0-35 PSU)
88 (Muthupriya and Altaff, 2009; Pan *et al.*, 2016), no data is available on the effect of food
89 concentration on other vital rates of this species as a function of [differences in](#) salinities. Here we
90 test the hypothesis that due to presumed energetic expenses for osmoregulation and oxidative stress
91 at sub-optimal salinities vital rates are affected. Specifically we hypothesize that under sub-optimal
92 salinities i) the mortality rate increases and maximum egg production rate [are](#) reduced; ii)
93 mortality decreases and egg production rate [increases](#) with food availability; and iii) that the
94 threshold concentration for egg production (lowest food concentration where egg production is
95 initiated) shifts to higher food concentrations when challenged by salinity. This is the first study
96 describing the mortality, feeding and egg production responses for *A. royi* as a function of a wide
97 span of food concentrations at different salinities. Our results are highly relevant for understanding

98 how ~~euryhaline copepod the~~ vital rates of a euryhaline copepod respond to salinity changes under
99 food-limited ~~and~~ or eutrophicated conditions.

100

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101 METHOD

102 Experimental organisms

103 *Apocyclops royi* was originally obtained from Tungkang Biotechnology Research Center, Taiwan,
104 but was received from culturing facilities at the LOG-Marine Station of Wimereux, France (Pan *et*
105 *al.*, 2016). The copepods were kept in continuous cultures at Roskilde University (Roskilde,
106 Denmark) at 25 °C in 0.2 µm filtered seawater (FSW) and acclimated from 20 PSU to 32 PSU in at
107 least 111 days (~10 generations). *A. royi* was kept in 70 L buckets, gently aerated and fed daily [atd](#)
108 libitum with the marine cryptophyte algae *Rhodomonas salina* (strain code K-1487). *R. salina* was
109 kept in culture at 18 °C in 20 L plastic bags in 0.2 µm FSW at 32 PSU in exponential growth by
110 daily dilution with FSW amended with a modified 0.1 % F/2 medium deprived for cobalt chloride
111 (Guillard, 1975; Thoisen *et al.*, 2018). *R. salina* proved to have a suitable cell size (~7 µm, Table 1)
112 for feeding all developmental stages of *A. royi* and to be sufficiently nutritious to use as sole food
113 source for maintaining the copepod cultures. Further, *R. salina* has a high salinity tolerance, with
114 growth rates close to 1 d⁻¹ at salinities between 5 – 50 PSU (Jepsen *et al.*, 2018).

115 Experimental procedure of incubation experiments

116 We determined the mortality rates, feeding rates and ovigerous rates by initiating experiments with
117 non-egg carrying females at four salinity levels (5, 10, 20 and 32 PSU), each at 7 different food
118 concentrations (Table 1, exp. 1-4) using bottle incubations (Frost, 1972; Kiørboe *et al.*, 1985). Food
119 concentrations were chosen to ensure reaching a tendency to saturation of [the](#) ingestion rate of the
120 copepods according to other studies (e.g. Almeda *et al.*, 2017) and trial experiments. Due to
121 unexplainable cell degradation of *R. salina* during the incubation experiment at 10 PSU, we were
122 not able to determine cell concentrations and feeding rates at this salinity level, thus only data on
123 mortality rate at that particular salinity was further analyzed.

124 Prior to each bottle incubation experiment a mix of males and females were gently separated from
125 the stock culture with a 200 µm mesh and starved in 0.2 µm FSW (32 PSU) for 19 h to ensure
126 complete gut evacuation and to evoke fertilization of the females. One hour prior to the start of the
127 incubation period of each tested food concentration, copepods were gradually acclimated to the
128 experimental salinity to avoid an acute salinity shock (Pan *et al.*, 2016). Briefly, copepods were
129 gradually (~ -1.7% min⁻¹ of final PSU) acclimated during 1 h from 32 PSU to 5, 10, 20 PSU or 32

PSU, respectively. To reach the desired salinity, temperature acclimated deionized water (0 PSU) was increasingly added and culture water was removed from the animals using a programmable peristaltic pump (Jebao® DP-4). Previously, dilution of seawater with deionized water [havehas](#) shown not to cause reduced physiological performances by the calanoid copepod *Acartia tonsa* (Jepsen *et al.*, 2018). *R. salina* was acclimated to the desired experimental salinity with steps of ± 5 PSU per day ~~also~~ by addition of deionized water, [similar to like in](#) Jepsen *et al.* (2018), and to the experimental temperature in 2 h prior to start of the experiments. Hence, there are reasons to believe that neither of our experimental organisms suffered from e.g. low calcium ion concentration.

Food suspensions were prepared by successive dilution of the highest food concentration with 0.2 μm FSW and amended with 3.5 mL L⁻¹ modified F/2 algal growth medium to avoid differential growth of *R. salina* between treatments due to nutrient excretion by the copepods. For each food concentration 12 Pyrex glass bottles (300 mL) were filled with the suspension. Three bottles were used to measure the initial concentration, three bottles to measure algal growth during the incubation (controls) and three bottles, with copepods added, served as experimental treatments (experimental bottles). From the acclimated copepods only live, non-egg bearing females were selected under a dissection microscope and distributed over the experimental bottles (35-50 females per bottle, Table 1). The number of copepods added per bottle varied depending on food concentration, to assure an approximately 30 % reduction of *R. salina* at the end of the incubation. The control and experimental bottles were sealed with a screw cap and placed on a slowly rotating plankton wheel (0.6 rpm) in dark for 24 h at 25 °C. At the end of the experiment, the content of each bottle was filtered through a 100 μm mesh to retrieve all copepods, which were consequently checked for mortality and egg-sac production.

Food concentrations (cells mL⁻¹) of the initial, control and experimental bottles were determined using a Beckman Coulter Multisizer 4e. The salinity of the food suspension was measured at the start of each experiment with an Atago S/Mill-E hand-held refractometer with a resolution of 0.5 units. Prosome length (Table 1) of live copepods ($n = 25$, immobilized by cooling) were measured at termination of each experiment from digital images taken with a Nikon SMZ 18 stereomicroscope mounted with a Nikon DS-Fi2 camera, using [the imaging processing software](#) NIS-Elements [Imaging Software](#).

Experimental procedure of short-term salinity acclimatization experiment

To investigate copepod resilience to the short-term salinity acclimatization prior to the start of the previous [described](#) incubation experiments, we conducted an additional experiment (Table 1, exp. 5) where we determined copepod mortality after a 1 h acclimatization period. Briefly, a mix of males and females were separated from the stock culture with a 200 µm mesh and starved in 0.2 µm FSW (32 PSU) for 19 h. Prior to the salinity acclimatization three replicates of 50 non-egg carrying females were prepared per treatment. The procedure for salinity acclimatization was similar as described previously for experiment 1-4. Copepod mortality (copepods were considered dead when a response to mechanical stimuli was absent) was determined 30 minutes after the acclimatization period with a dissection microscope.

169 Mortality rate

Mortality rate (M , % t^{-1}) of *A. royi* after short-term salinity acclimatization and after the 24 h incubation experiments was calculated as

$$172 \quad M = \frac{n_{alive_{start}} - n_{alive_{end}}}{n_{alive_{start}} * 0.01} * t^{-1} \quad (1)$$

173 , where t is the incubation period in hours or days.

174 Functional feeding response

The ingestion rate, clearance rate and average food concentration during the incubation experiments were calculated according to Frost (1972). The sigmoidal shape of the observed feeding response suggested the presence of a feeding threshold, below which the copepod reduces its feeding rate. Therefore, a Holling type III functional response model was fitted to the measured ingestion and clearance rates (Table 2). This is similar to Schultz and Kiørboe (2009) and van Someren Gréve *et al.* (2017), where [model parameter](#) β is the maximum clearance rate (mL d^{-1}), C is the prey concentration (cells mL^{-1}) and α is the prey concentration at the maximum clearance rate. The maximum ingestion rate, I_{max} , was calculated as $\alpha\beta e^1$ ($\text{cells cop}^{-1} \text{d}^{-1}$).

Carbon content of the prey item (*R. salina*) was determined by CHN elemental analysis. Briefly, triplicates of ca. 10^7 cells were filtered onto 12 mm diameter pre-combusted GF/C filters (Whatman), dried at 60 °C for 24 h and analyzed by a Thermo Fisher Scientific FLASH 2000

186 Organic Elemental Analyzer. A methionine standard curve was used to obtain concentrations of C
187 and N.

188 **Ovigerous rate**

189 Directly after termination of each feeding experiment, the presence of egg sacs was determined
190 under a dissection microscope. Similar to Rayner *et al.* (2017) we calculated the female ovigerous
191 rate (G , % of ovigerous females d^{-1}) for each replicate by

$$192 \quad G = \frac{\text{ovigerous copepods}_{end}}{\text{copepods alive}_{end} * 0.01} * t^{-1} \quad (2)$$

193 The dependence of the ovigerous rate on the food concentration was described by [thea](#) model
194 similar to Kiørboe *et al.* (1982) (see Table 2), where G_{max} equals the maximum ovigerous rate (% of
195 ovigerous females d^{-1}), C the food concentration (cells mL^{-1}) and b a constant.

196 **Gross efficiency of egg production**

197 The gross efficiency of egg production was calculated from the functional response and ovigerous
198 rate observations according to Peterson (1988):

$$199 \quad E = \frac{C_{eggs} \text{ cop}^{-1} d^{-1}}{C_{ingested} \text{ cop}^{-1} d^{-1}} \quad (3)$$

200 , where egg carbon produced per copepod ($\mu g \text{ C cop}^{-1} d^{-1}$) was calculated by multiplying the
201 measured average clutch size ($n \text{ eggs cop}^{-1}$) by the ovigerous rate G (%) and egg carbon content (μg
202 C egg^{-1}). The egg carbon content was calculated from measured egg diameter ($n=20$ per
203 experiment) and the egg diameter to carbon equation for copepods derived by Uye and Sano (1995).

204 Further, the maximum weight-specific fecundity ($\mu g \text{ C}_{eggs} \mu g \text{ C}_{copepod}^{-1} d^{-1}$) was calculated from the
205 maximum total egg carbon produced per copepod and measured copepod sizes ($n=25$ per
206 experiment, Table 1), using the length-dry weight relationship for *A. royi* by Chang and Lei (1993)
207 and dry weight-carbon relationship for copepods according to Kiørboe and Sabatini (1995).

208 **Statistics**

209 We conducted an analysis of variance (ANOVA and Tukey post-hoc test) to determine the
210 significance level ($p \leq 0.05$) of differences in mortality between salinity levels using the software
211 SPSS Statistics 20. All models (Table 2) were fitted to the observational data using the software
212 Sigmaplot 14.0. To compare differences in the vital rates (ingestion rate, clearance rate, maximum
213 ovigerous rate, gross efficiency of egg production) between salinities for non-linear models fitted to
214 the experimental data, we calculated the Wald confidence intervals (95%) for each model
215 parameter.

216

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217 RESULTS

218 The mortality rate of *A. royi* during short-term (1 h) salinity acclimatization after a 19 h starvation
 219 period was highest at 5 PSU (12.6 ± 2.1 % mortality h^{-1}) compared to 10, 20 and 32 PSU (4.6 ± 1.4 ,
 220 3.6 ± 0.7 , 3.7 ± 0.5 % mortality h^{-1} respectively, Fig 1). During the following 24 h incubation, with or
 221 without food, the average mortality showed a similar salinity dependent trend, with highest
 222 measured mortality rates at 5 PSU (6.8 ± 0.9 % mortality d^{-1}) and lowest mortality at 32 PSU
 223 (1.8 ± 0.4 % mortality d^{-1} , Fig 2d). Both during the short-term (1 h) acclimatization and following
 224 incubation a significantly higher mortality was observed at PSU 5 compared to 10, 20 and 32 PSU
 225 ($p < 0.05$). No significant differences in mortality were observed between 10, 20 and 32 PSU
 226 though. Food availability did not appear to influence the mortality of *A. royi* during the incubations
 227 (Fig 2a-c).

228 The cumulative mortality of *A. royi* during the experiment (mortality during 1 h salinity
 229 acclimatization + 24 h incubation with/without food) was consequently highest and statistically
 230 significantly different at 5 PSU (19.4 ± 2.3 %, $p < 0.05$) and lowest at 32 PSU, with no significant
 231 differences in mortality between 10, 20 and 32 PSU (8.4 ± 1.7 , 5.7 ± 0.8 , 5.5 ± 0.6 % respectively, Fig
 232 3).

233 The feeding rate of *A. royi* varied depending on food concentration at all tested salinities (Fig 4a-c,
 234 e-f). The ingestion rate increased with food concentration towards saturation. The estimated
 235 maximum ingestion rates (I_{\max}) did not differ (overlapping 95 % CL) between the tested salinities
 236 (44.459 ± 9.157 , 36.377 ± 21.627 and 48.783 ± 10.939 cells $\text{cop}^{-1} \text{d}^{-1}$ at 5, 20 and 32 PSU respectively,
 237 Fig 4d). The observed clearance rate was very low at the lowest tested food concentrations and
 238 increased with food concentration up to an optimum (β , Table 2) followed by a decrease with
 239 increasing food availability, indicating *A. royi* exhibited a typical Holling type III functional feeding
 240 response (Fig 4e-g). The estimated maximum clearance rate did not differ (95 % confidence
 241 intervals overlap) between salinities, (Fig 4h). Model parameters of the fitted functional response
 242 model to the observations are shown in Table 2.

243 The ovigerous rate of *A. royi* (% of ovigerous females d^{-1}) varied with food concentration in a
 244 similar fashion as the observed feeding rate (Fig 5a-c). Generally, the percentage of egg carrying
 245 females increased with increasing food availability towards a maximum. No differences in
 246 maximum ovigerous rates were observed between salinities (61.5-61.9 %, Fig 5a-c), neither in the

247 estimated maximum ovigerous rates (Fig 5d, Table 2). The observed maximum weight-specific
248 fecundity was equal to $0.176 \mu\text{g } C_{\text{eggs}} \mu\text{g } C_{\text{copepod}}^{-1} \text{ d}^{-1}$, regardless the treatment.

249 Egg production was completely absent when no food was present, but was initiated at all tested
250 salinities at the lowest food concentrations offered (Fig 5a-c). Contrary to the ovigerous rate, the
251 clutch size was largely independent of food availability (Fig 5h) and only decreased at the lower
252 end of the tested concentrations. There did not appear to be a correlation between food
253 concentration and the gross efficiency of egg production (Fig 5e-f). However, egg production was
254 positively correlated to ingestion rate (Fig 6), showing a gross efficiency of egg production of *A.*
255 *royi* ranging between 10-12 %, with no significant differences between salinities (parameter *a*,
256 Table 2). Model parameters of the fitted models to the observations for ovigerous rate, clutch size
257 and gross efficiency of egg production are shown in Table 2.

258 Overall, our observations suggest that during and after a short-term acclimatization (1 h) to low
259 saline water, mortality of *A. royi* increased compared to ~~when~~ exposed to 32 PSU. However,
260 short-term acclimatization did not compromise the ability to feed and produce eggs within the
261 following 24 h incubations. Further, food availability did not influence the mortality rate, but
262 strongly influences sd feeding rate and egg production in a sigmoidal fashion.

263

264 DISCUSSION

265 The physiological cost of regulating body osmolarity and ion balance in coastal copepods has been
 266 investigated and quantified extensively during the past decades. This has been conducted by
 267 monitoring end-points at different levels e.g. in terms of mortality, growth rate, reproduction rate
 268 and metabolic rate. These studies indicate that increased energy allocation [due](#) to osmoregulation
 269 significantly compromise energy investment in growth and reproduction and the overall viability of
 270 embryonic, naupliar, and adult life stages (e.g. Devreker *et al.*, 2007; Dutz and Christensen, 2018).
 271 [However](#), ~~the~~ presence of an adequate food source may, in part, cover the increased energetic
 272 needs imposed by osmoregulation and thereby dampen the negative physiological response by
 273 copepods (Rippingale and Hodgkin, 1977), [but](#). ~~However~~, multiple stressor experiments on salinity
 274 tolerance, taking into account the effect of resource availability are rare, particularly for copepods.
 275 This is surprising as estuarine species often reside in environments with strong temporal variations
 276 in both abiotic and biotic factors (Devassy and Goes, 1988; Madhu *et al.*, 2007; Martinez *et al.*,
 277 2011). In the present study, we investigated the physiological response to different salinities of the
 278 small, tropical copepod *A. royi* exposed to different food concentrations. ~~*A. royi* exhibit a relatively~~
 279 ~~short generation time of approximately 10 d under the present cultivation conditions with food in~~
 280 ~~excess. We showed that salinity affects the mortality rate of *A. royi*, during short term (1 h) salinity~~
 281 ~~acclimatization and a following 24 h incubation period, but not the ovigerous rate. Moreover, food~~
 282 ~~availability did not influence the osmotic dependence of mortality, feeding rate or ovigerous rate of~~
 283 ~~this species.~~

284 The effect of salinity and food availability on mortality

285 Acclimatization or plasticity and adaption to different abiotic factors in marine copepods are poorly
 286 understood (Lee and Petersen, 2003). It varies greatly between species (Calliari *et al.*, 2008) and
 287 within species and may depend on acclimatization period and biotic factors, such as food
 288 availability (Dutz and Christensen, 2018; Lindley *et al.*, 2011). We observed the highest mortality
 289 of *A. royi* directly after each short-term [salinity](#) acclimatisation (Fig. 1) and somewhat lower
 290 mortality during the following 24 h incubation, regardless the absense or presence of food (Fig. 2a-
 291 d). Overall, mortality of *A. royi* in our study was highest when exposed to the lowest tested salinity
 292 (5 PSU, Fig 1, 2d and 3) [as hypothesized](#). In our experiments, we acclimatized *A. royi* by changing
 293 from 32 PSU during 1 h to either 20, 10 or 5 PSU. We used [a](#) similar acclimation method as

described in Pan *et al.* (2016). As a result, Pan *et al.* (2016) found a sigmoidal survival curve as a function of salinities ranging from 0 to 35 PSU, with an optimum of 20 PSU. Muthupriya and Altaff (2009) however, showed a mortality of *A. royi* lowest at 12 PSU and highest at 32 PSU. In the present study, we observed an elevated mortality after acclimatization in 5 PSU but did not see any difference between 10 and 32 PSU (Fig 1 and 2). We consider this is an effect of that our *A. royi* strain has been reared at 32 PSU for several generations and therefore may have adapted to this condition, but still has kept the ability to perform well at 20 PSU. This suggests that within species, salinity tolerance is population specific or even may be related to pre-experimental rearing conditions.

~~Excess food~~ Food availability has previously shown to reduce mortality of copepods exposed to hypersaline environments (Rippingale and Hodgkin, 1977; Hammock *et al.* 2016). This elevated salinity tolerance is supported by a study by Lindley *et al.* (2011) on the species *Apocyclops panamensis*, a close relative to *A. royi*. They investigated the effect of short-term (3 h) acclimatization to a hypersaline environment, from 6.6 PSU to 30 PSU (Lindley *et al.*, 2011). They and showed a significant increase of the intracellular free amino acid (FAA) pool in the animals and an even higher increase when the copepods were offered additional FAAs (Lindley *et al.*, 2011). FAAs, such as proline, alanine, glycine and taurine have been shown to be major osmolytes in marine invertebrates (Helland *et al.*, 2000). Their observed built up of FAAs in *A. panamensis* could be dedicated to protein catabolism, but increase of intracellular FAAs also depends on food intake (amino acids) presumably accounting for their observed larger FAA pool when the copepods were enriched with FAAs (Farmer and Reeve, 1978). ~~The effect of food availability on mortality of copepods in hypersaline environments has been demonstrated by Hammock et al. (2016), who observed increased mortality of the euryhaline copepod Eurytemora affinis with increasing salinity at low food availability, compared to high food availability.~~

~~Contrary to our hypothesis, food availability did not influence the osmotic dependence of mortality of A. royi (Fig 2a-c). This may be partly explained by the fact that in our study on the other hand,~~ the copepods were exposed to hyposaline environments. Adjusting the internal osmolarity to a lowered external salinity requires down-regulation of the intracellular FAA pool (Farmer and Reeve, 1978). Protein synthesis and enhanced excretion account for the decrease of the FAA pool and this mechanism is even active in the absence of food (Farmer and Reeve, 1978). ~~This may partly explain, contrary to our hypothesis, the absence of an effect of food availability on mortality~~

of *A. royi* in response to lower salinity (Fig 2a-c). In order to understand the underlying mechanism of osmoregulation in *A. royi*, it would therefore be interesting to investigate in future studies changes in FAA concentrations in *A. royi* as an effect of decreasing external salinity and functional feeding response.

Another possible explanation of the absence of an effect of food availability on mortality could be a decrease in energetic expenses related to foraging activity at low food concentrations, allowing energy allocation to energy demanding osmoregulatory processes, such as ion transport (Hand and Hardewig, 1996; Bradly, 2009) and restoring the cellular redox balance (reviewed in Rivera-Ingraham and Lignot, 2017). The observed functional response of *A. royi* follows a Holling type III response (Fig 4) suggesting that feeding activity ceases when food availability is limited. Optimal foraging theory predict such a response for actively foraging zooplankton (Kjørboe *et al.*, 2018) in order to reduce the energetic cost for searching for food at low food conditions. In fact, for various marine copepod species such a behavioral response has been observed, which can be dedicated to reduced swimming activity at low food concentrations (Kjørboe, 2016). However, no direct observational studies exist on *A. royi* foraging tactics and the effect of food availability on foraging behavior verifying such a behavioral response.

341 The effect of salinity and food availability on feeding rate

The effect of salinity on the feeding rate of copepods has been scarcely studied. Feeding rate may be increased under iso-osmotic conditions in order to compensate for the increased metabolic demand imposed by osmoregulation (Gaudy *et al.* 2000). On the other hand, exposure to extreme iso-osmotic conditions may reduce the predatory capabilities of an animal, thereby reducing the animals' feeding rate (Hammock *et al.*, 2016; Rivera-Ingraham and Lignot, 2017).

Calliari *et al.* (2008) investigated the effect of instantaneous salinity reduction on the feeding rate of *A. tonsa* and *A. clausi* and showed a substantial decrease in both ingestion rate and clearance rate of both species when salinity was lowered from 32 PSU to 14 and 4 PSU, respectively. Their results indicate that sudden lowering of salinity significantly decreases the feeding rate by these species and thereby potentially influence the plankton dynamics in the coastal system these species reside. The euryhaline species *Eurytemora affinis* on the other hand, showed an opposing response and considerably increased its consumption rate when exposed to increased salinity. However, when

exposed to increased salinity under low food levels, *E. affinis* did not increase its feeding rate and their growth was reduced (Hammock *et al.*, 2016).

We did not observe a significant decrease or increase in either ingestion or clearance rate with decreasing salinity (Fig 4), which may be together with the relatively low observed mortality rates an indicator for the overall high salinity tolerance of *A. royi* exposed to lower salinities. In comparison, the mortality rates observed for *A. tonsa* and *A. clausi* which lowered their feeding rate in hyposaline environments (Calliari *et al.*, 2008) were much higher (31.3 and 20 %, respectively) than observed for *A. royi* (from 1.8 to 6.8 %) (Fig 1 and 2).

A possible reason for the absence of increased feeding rates in *A. royi* exposed to hyposaline conditions could be differences in osmotic regulation mechanisms, where down-regulation of the FAAs may be independent of feeding, contrary to up-regulation (synthesis) of FAAs as in *E. affinis* exposed to hypersaline environments, which requires food uptake (Farmer and Reeve, 1978; Hammock *et al.*, 2016).

The effect of salinity and food availability on egg production

Exposure to hypo or hypersaline environments may reduce egg production rates in copepods due to increased energy allocation to osmoregulation (Gaudy *et al.*, 2000). It is therefore not surprising that reduced egg production rates have been observed in various species when subjected to hyposaline environments (Dutz and Christensen, 2018; Calliari *et al.*, 2006). In the present study, we investigated the ovigerous rate of *A. royi* at different salinities over a gradient in food availability. Whereas the food availability dependent egg production of *A. royi* has not been previously described, Salinity dependent egg production has been scarcely studied in a few cases for this *Apocyclops royi* species. Muthupriya and Altaff (2009) and Pan *et al.* (2016) tested the long-term egg production rate of acclimated *A. royi* in the presence of food and showed, similar to our observations (Fig 5), that *A. royi* is capable of maintaining egg production at a wide range of salinities (0-35 PSU) with optimal conditions varying between 12-20 PSU. Salinity above 35 PSU appeared to be unfavorable in terms of egg production (Muthupriya and Altaff, 2009). Hatching success and postembryonic development as maxima for nauplii production were observed between 10-20 PSU (Lee *et al.*, 2005; Pan *et al.*, 2016) and maximum culture densities were reached at 20 PSU (Pan *et al.*, 2016). In the present study the maximum ovigerous rate of *A. royi* was not significantly affected by salinity (Fig 5d), contrary to our hypothesis.

Further, we did not observe an affect of salinity on the threshold concentration for egg production, thus rejecting our hypothesis that the threshold concentration for egg production would shifts to higher food concentrations when exposed to sub-optimal salinities. Egg production was, regardless salinity treatment, initiated at the lowest food concentrations offered (Fig 5a-c) and showed, as hypothesized a food density dependency in reproductive output principally similar to its congeners (e.g. Berggreen *et al.*, 1988; Sabatini and Kiørboe, 1994). The maximum weight-specific fecundity of *A. royi* measured here also did not vary between salinities ($0.18 \pm 0.0 \mu\text{g C}_{\text{eggs}} \mu\text{g}^{-1} \text{C}_{\text{copepod}} \text{d}^{-1}$) and is relatively high compared to similar sized egg sac carrying copepods, but low compared to broadcast spawners (Kiørboe and Sabatini, 1995).

Gaudy *et al.* (1982) observed a similar absence of response in egg production over a wide range in salinity. They did not observe variation in ovigerous rate of the exceptionally euryhaline harpacticoid copepod species *Tisbe holothuriae*. However, the majority of copepod species tolerate a much narrower salinity range before egg production is reduced (e.g. Hall and Burns, 2002; Holste and Peck, 2006; Dutz and Christensen, 2018). We did not measure egg hatching success or larval development to assess the salinity effect on the full life-cycle and population dynamics of *A. royi*. From the few studies that exist these are more salinity dependent than egg production and may vary greatly depending on the copepod strain used (Lee *et al.*, 2005; Pan *et al.*, 2016).

Ecological implications **Success of *A. royi* in an extreme habitat**

The *Apocyclops royi* strain used in the present study was isolated from artificial aquaculture ponds in Taiwan. ~~These ponds we consider representing an extreme habitat.~~ Each of the ponds cover an area $\sim 0.7 \text{ ha}$ ~~and is 1 m deep~~, is 1 m deep, and filled with coastal water from the nearby South China Sea. Hence, it is reasonable to assume that the copepods are not native to these ponds, but originate from the South China Sea (Blanda *et al.*, 2015). Brackish water systems generally show strong temporal fluctuations in environmental conditions, and the ponds *A. royi* is isolated from are documented to fluctuate in biotic and abiotic conditions on a seasonal and even daily basis (Blanda *et al.*, 2015, 2017). For example, oxygen levels in these ponds reach hypoxic conditions on a daily basis and severe hypoxia on a weekly basis, with *A. royi* still thriving in these ponds (Blanda *et al.*, 2015). Salinity is variable over the season, but more interestingly, short-term drops in salinity of 6 PSU are observed due to heavy monsoon rain events. Hence, *A. royi* is exposed to abrupt salinity changes in its natural environment and ~~is able to~~ can successfully maintain its population. This

correlates well with our results where decreasing salinities resulted in only minor increase in mortality rates and no effect on ovigerous rate (Fig 1, 2 and 5). Moreover, *A. royi* is able to upgrade their fatty acid pool to become richer in long chained fatty acids (Rayner *et al.*, 2017; Nielsen *et al.*, 2019). These traits, combined with a low osmotic dependency of the vital rates of *A. royi* as shown in the present study, most likely contribute to the fact that *A. royi* can survive and is one of the predominant copepod species in Taiwanese aquaculture ponds (Blanda *et al.*, 2015; Rayner *et al.*, 2015).

Salinity tolerance of *A. royi*

Here we tested the hypothesis that due to presumed energetic expenses for osmoregulation and oxidative stress at sub-optimal salinities vital rates of *A. royi* are affected. Firstly, we hypothesized that under sub-optimal salinities the mortality rate increases, and maximum egg production rate are reduced. This hypothesis was partly rejected as mortality rate increased when exposed to a lowered salinity during salinity acclimatization and incubation period (Fig 1, 2 and 3), but egg production was not reduced (Fig 5).

Second, we hypothesized that mortality decreases, and egg production rate increases with food availability. Egg production increased with increasing food availability (Fig 4) as expected from previous research on food dependency of egg production. However, we did not observe a decrease in mortality rate with increasing food availability (Fig 2).

Lastly, we hypothesized that the threshold concentration for egg production (lowest food concentration where egg production is initiated) shifts to higher food concentrations when challenged by salinity. This hypothesis was rejected as at all tested salinities egg production was absent when no food was present, but was initiated at the lowest food concentrations offered (Fig 5a-c).

CONCLUSIONS

In the present study we experimentally investigated the physiological response of the tropical copepod *Apocyclops royi* to different salinities under varying food availability. We showed that the tropical copepod *A. poeyelops royi* is a euryhaline species. Its mortality rate increased during and after short-term (1 h) acclimatization to low salinity (5 PSU), whereas the individual feeding- and

ovigerous rate was not affected at all during our 24 h exposure experiments. Food availability directly influenced the ovigerous rate and feeding rate of *A. royi* in a sigmoidal manner, but did not influence the threshold concentration for egg production or the mortality rate of this species when exposed to sub-optimal salinities.

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DATA ARCHIVING

No supplementary data related to this article is archived.

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TABLE AND FIGURE LEGENDS

Table 1. Overview of the experimental work. Salinity of the final food suspension is given for each experiment. Prey size is the average cell size ($n \sim 30,000$) at start and end of the experiment (size generally decreased during incubation). Prey concentrations are the minimum and maximum average concentration during each incubation. All tested prey concentrations for each salinity treatment can be derived from figures 2, 4 and 5. Temperature was monitored with 1-minute intervals using an ONSET HOBO® Pendant temperature logger.

Experiment no.	Salinity	Copepods per 300 mL bottle	Prosoma length	Prey size	Prey concentration	Temperature
	‰	n	$\mu\text{m} \pm \text{SD}$	$\mu\text{m ESD} \pm \text{SD}$	cells mL^{-1}	$^{\circ}\text{C} \pm \text{SD}$
1	32	35-50	504 \pm 43	6.9 \pm 0.8	0-42.388	25.7 \pm 1.2
2	20	35-50	501 \pm 38	7.4 \pm 0.8	0-38.943	26.0 \pm 0.9
3	10	35-50	489 \pm 38	n.a.	n.a.	26.6 \pm 0.5
4	5	40-50	507 \pm 28	7.8 \pm 1.3	0-36.576	26.9 \pm 0.4
5	5, 10, 20, 32	50	495 \pm 30	-	0	26.9 \pm 0.6

Table 2. Model parameters ±SE or 95 % confidence intervals (CI) for all equations fitted to the observational data for mortality rate, feeding rate (ingestion and clearance rate), ovigerous rate, clutch size and egg production as function of ingestion rate for *Apocyclops royi* at different salinities.

Equation	PSU	$a \pm SE$	$b \pm SE$	r^2
Mortality rate (Fig 2)	5	6.4±1.1	4.9±0.0	0.02
$M = a + bC$	20	2.4±0.6	-2.0±0.0	0.01
	32	2.1±0.6	-0.0±0.0	0.02
		β (CI, 95 %)	α (CI, 95 %)	
Ingestion rate (Fig 4a-d)	5	2.32 (2.14-2.49)	7056 (6261-7850)	0.99
$I = \alpha \beta e^{1 - \frac{a}{c}}$	20	1.87 (1.45-2.29)	7157 (4954-9360)	0.92
	32	1.77 (1.62-1.91)	10156 (8865-11446)	0.99
Clearance rate (Fig 4e-h)	5	2.35 (2.02-2.68)	5424 (4294-6553)	0.67
$F = \frac{\alpha \beta}{C} e^{1 - \frac{a}{c}}$	20	1.92 (1.62-2.22)	6448 (5133-7762)	0.48
	32	1.80 (1.50-2.10)	8379 (6641-10117)	0.15
		G_{max} (CI, 95 %)	b (CI, 95 %)	r^2
Ovigerous rate (Fig 5a-d)	5	79.5 (69.0-90.0)	5413 (4020-6806)	0.96
$G = G_{max} e^{-b/C}$	20	89.8 (79.7-100.0)	12457 (10422-14492)	0.98
	32	87.0 (72-3-101.7)	11696 (8715-14677)	0.96
Clutch size (Fig 5h)	5,20,32	19.7 (19.1-20.4)	527 (319-733)	0.2
		$a \pm SE$	$b \pm SE$	r^2
Egg prod.- ingestion rate (Fig 6)	5	3974±6394	0.114±0.009	0.94
$SEP = a + bI$	20	-9329±7898	0.120±0.012	0.86
	32	-4853±4795	0.102±0.006	0.95

Fig 1. Mortality rate during short-term ($\% \text{ h}^{-1}$) salinity acclimatisation of *Apocyclops royi* as function of salinity. Light dots indicate replicates and black dots are the mean value. Error bars indicate the standard error and different letters indicate statistically significant difference in average mortality between salinities.

Fig 2. Food concentration dependent mortality rate of *Apocyclops royi* at different salinities (panel a-c) and average mortality ($\% \text{ d}^{-1}$) at each tested salinity (panel d) during 24 h incubations. Light dots indicate replicates and black dots are the mean value. Discontinuous lines (panel a-c) indicate the 95% confidence intervals for each fitted regression. Regression parameters are shown in Table 2. Error bars (panel d) indicate the standard error and different letters indicate statistically significant difference in average mortality between salinities.

Fig 3. Cumulative mortality rates of *Apocyclops royi* at different salinities during 1h salinity acclimatization and 24 h incubation experiments as a function of time. Dots are the mean value and error bars indicate the standard error. Different letters indicate statistically significant difference in average mortality between salinities.

Fig 4. The functional responses of *Apocyclops royi* feeding on *Rhodomonas salina* at different salinity levels. Copepod ingestion rates ($\text{cells cop}^{-1} \text{ d}^{-1}$, panel a-c) and clearance rates ($\text{mL cop}^{-1} \text{ d}^{-1}$, panel e-g) are presented as function of food concentration (cells mL^{-1}). Black solid lines are Holling type III model fits to the experimental observations. Panel d and h show model estimates of the maximum ingestion rate (I_{\max}) and maximum clearance rate (β_{\max}), respectively as function of salinity; error bars indicate 95% confidence intervals. All models and parameters are presented in Table 2.

Fig 5. Food concentration dependent egg production rates of *Apocyclops royi* at different salinities. Female ovigerous rates ($\% \text{ of ovigerous females d}^{-1}$, panel a-c) and gross efficiency of egg production rates (panel e-g) are presented as function of food concentration (cells mL^{-1}). Black solid lines are model fits to the experimental observations. Panel d shows model estimates of the maximum ovigerous rate (G_{\max}) and panel h food concentration dependent clutch size, respectively as function of salinity. Error bars indicate 95% confidence intervals. Models fitted to the observational data and model parameters are presented in Table 2.

662 **Fig 6.** The specific egg production rate ($\mu\text{g C cop}^{-1} \text{ d}^{-1}$) is shown as function of the specific
663 ingestion rate ($\mu\text{g C cop}^{-1} \text{ d}^{-1}$), where the slopes of the fitted regressions equal the estimated gross
664 efficiency of egg production. Discontinuous lines indicate the 95 % confidence intervals for each
665 fitted regression. Regression parameters are shown in Table 2.

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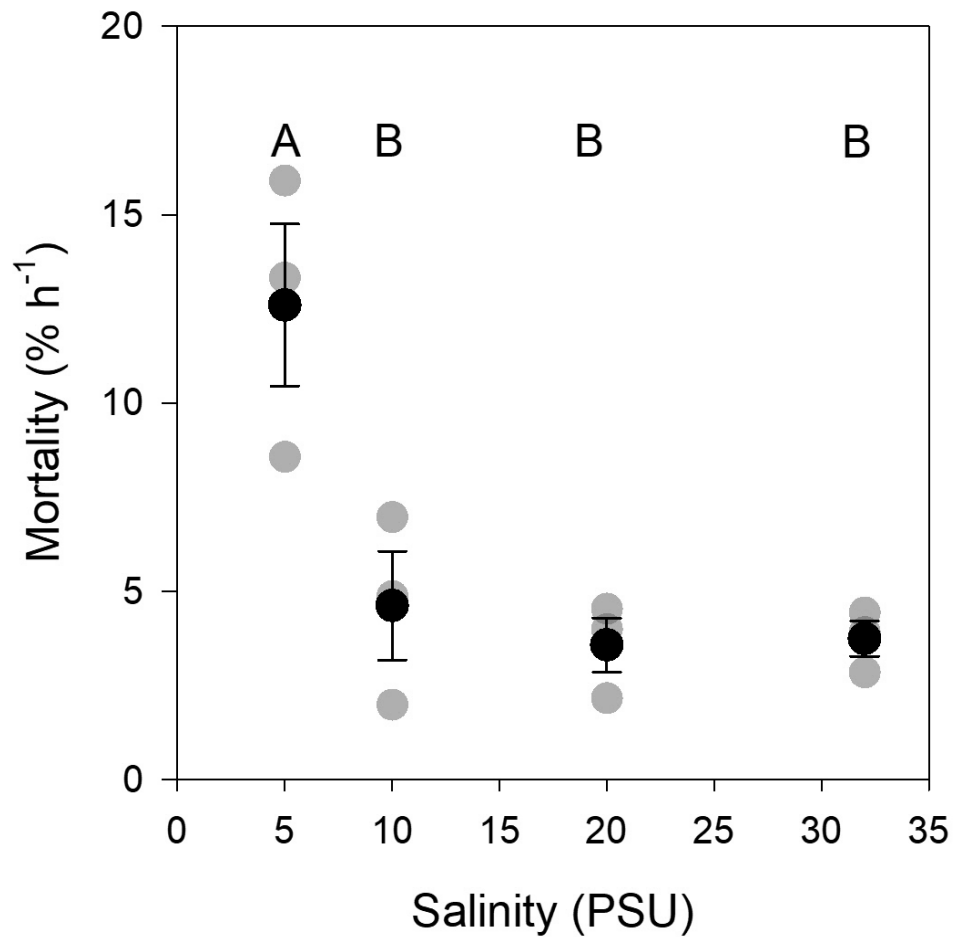


Fig 1. Mortality rate during short-term (% h⁻¹) salinity acclimatisation of *Apocyclops royi* as function of salinity. Light dots indicate replicates and black dots are the mean value. Error bars indicate the standard error and different letters indicate statistically significant difference in average mortality between salinities.

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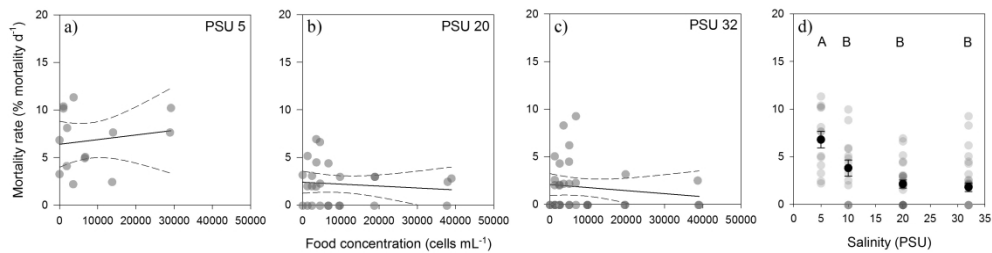


Fig 2. Food concentration dependent mortality rate of *Apocyclops royi* at different salinities (panel a-c) and average mortality (% d-1) at each tested salinity (panel d) during 24 h incubations. Light dots indicate replicates and black dots are the mean value. Discontinuous lines (panel a-c) indicate the 95% confidence intervals for each fitted regression. Regression parameters are shown in Table 2. Errorbars (panel d) indicate the standard error and different letters indicate statistically significant difference in average mortality between salinities.

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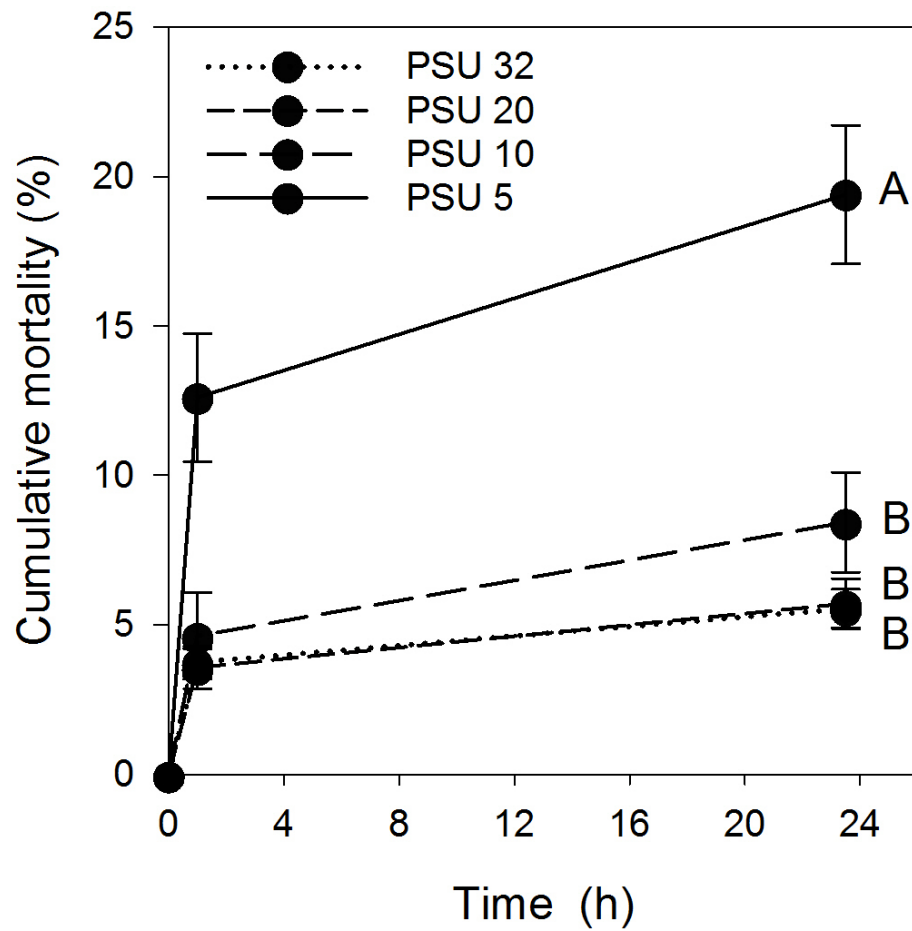


Fig 3. Cumulative mortality rates of *Apocyclops royi* at different salinities during 1h salinity acclimatization and 24h incubation experiments as a function of time. Dots are the mean value and error bars indicate the standard error. Different letters indicate statistically significant difference in average mortality between salinities.

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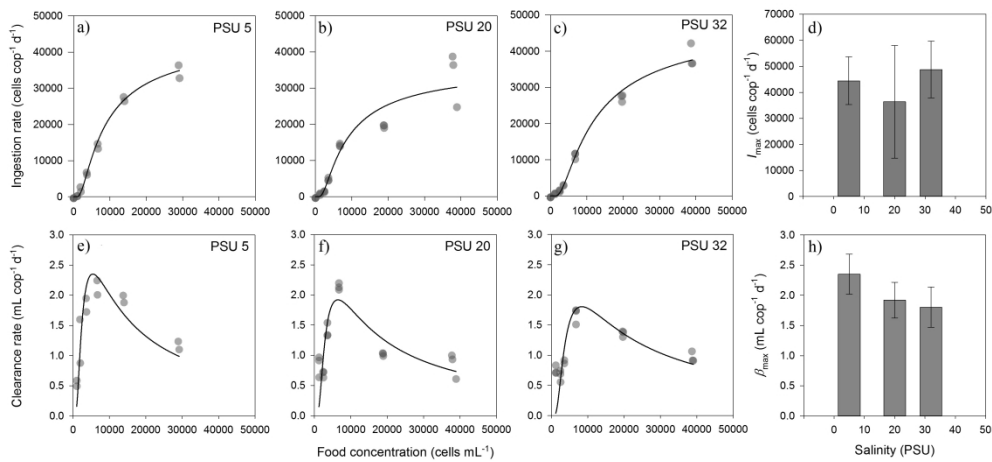


Fig 4. The functional responses of *Apocyclops royi* feeding on *Rhodomonas salina* at different salinity levels. Copepod ingestion rates (cells cop⁻¹ d⁻¹, panel a-c) and clearance rates (mL cop⁻¹ d⁻¹, panel e-g) are presented as function of food concentration (cells mL⁻¹). Black solid lines are Holling type III model fits to the experimental observations. Panel d and h show model estimates of the maximum ingestion rate (I_{max}) and maximum clearance rate (β_{max}), respectively as function of salinity; error bars indicate 95% confidence intervals. All models and parameters are presented in Table 2.

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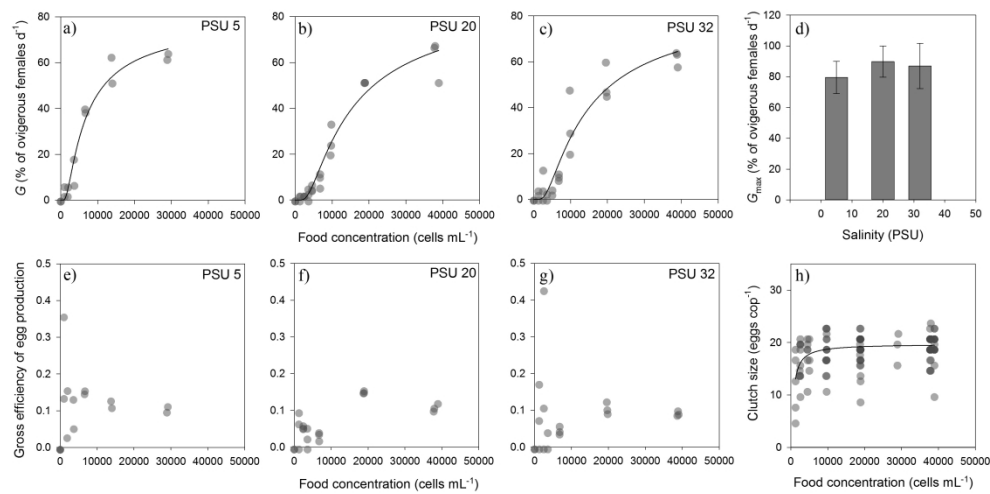


Fig 5. Food concentration dependent egg production rates of *Apocyclops royi* at different salinities. Female ovigerous rates (% of ovigerous females d⁻¹, panel a-c) and gross efficiency of egg production rates (panel e-g) are presented as function of food concentration (cells mL⁻¹). Black solid lines are model fits (to the experimental observations). Panel d shows model estimates of the maximum ovigerous rate (G_{max}) and panel h food concentration dependent clutch size, respectively as function of salinity. Error bars indicate 95% confidence intervals. Models fitted to the observational data and model parameters are presented in Table 2.

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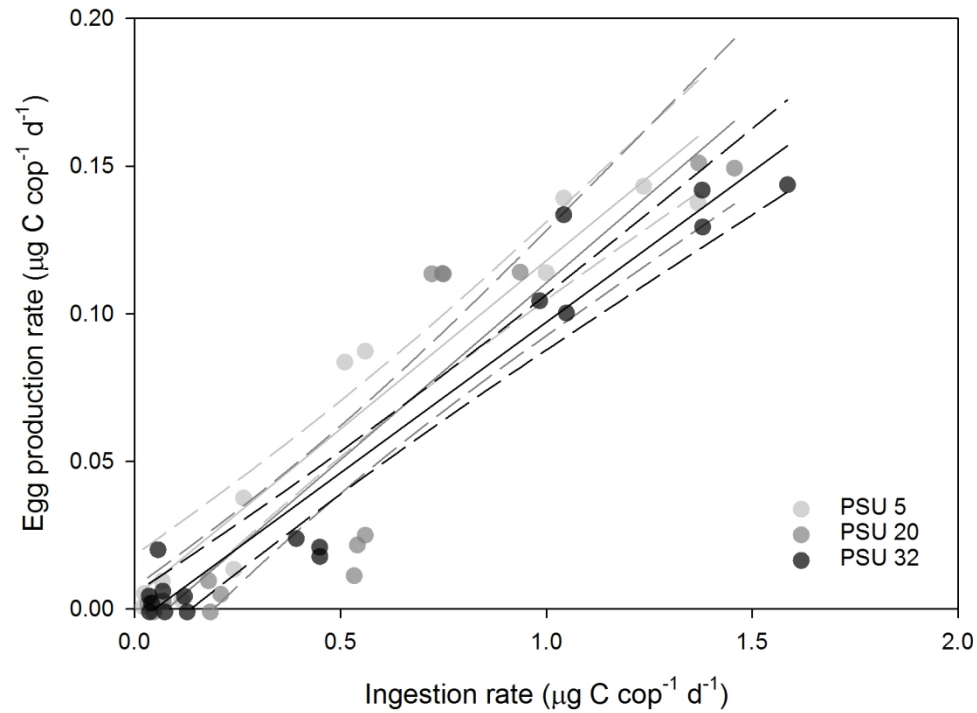


Fig 6. The specific egg production rate ($\mu\text{g C cop}^{-1} \text{ d}^{-1}$) is shown as function of the specific ingestion rate ($\mu\text{g C cop}^{-1} \text{ d}^{-1}$), where the slopes of the fitted regressions equal the estimated gross efficiency of egg production. Discontinuous lines indicate the 95% confidence intervals for each fitted regression. Regression parameters are shown in Table 2.

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