

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

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*Published in:*  
Journal of Plankton Research

*DOI:*  
[10.1093/plankt/fbaa031](https://doi.org/10.1093/plankt/fbaa031)

*Publication date:*  
2020

*Document Version*  
Early version, also known as pre-print

*Citation for published version (APA):*  
van Someren Gréve, H., Jepsen, P. M., & Hansen, B. W. (2020). Does resource availability influence the vital rates of the tropical copepod *Apocyclops royi* (Lindberg, 1940) under changing salinities? *Journal of Plankton Research*, 42(4), 467-478. <https://doi.org/10.1093/plankt/fbaa031>

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1 **Journal of Plankton Research**

2 **Revised manuscript version 2**

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

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17 **RUNNING HEAD: Vital rates of a copepod under changing salinities**

18

19 **KEYWORDS: Salinity tolerance; functional response; egg production; numerical response;**  
20 **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.

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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 [at d](#)  
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<sup>-1</sup> 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<sup>-1</sup> of final PSU) acclimated during 1 h from 32 PSU to 5, 10, 20 PSU or 32

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

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

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

## 159 **Experimental procedure of short-term salinity acclimatization experiment**



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

### 169 Mortality rate

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

$$172 \quad M = \frac{n \text{ alive}_{\text{start}} - n \text{ alive}_{\text{end}}}{n \text{ alive}_{\text{start}} * .01} * t^{-1} \quad (1)$$

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

### 174 Functional feeding response

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

183 Carbon content of the prey item (*R. salina*) was determined by CHN elemental analysis. Briefly,  
 184 triplicates of ca.  $10^7$  cells were filtered onto 12 mm diameter pre-combusted GF/C filters  
 185 (Whatman), dried at  $60^\circ\text{C}$  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 the [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):

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

200 , where egg carbon produced per copepod ( $\mu g\ C\ cop^{-1}\ d^{-1}$ ) was calculated by multiplying the  
201 measured average clutch size ( $n\ eggs\ cop^{-1}$ ) by the ovigerous rate  $G$  (%) and egg carbon content ( $\mu g$   
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 ( $\mu g\ C_{eggs}\ \mu g\ C_{copepod}^{-1}\ d^{-1}$ ) 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 ( $p < 0.05$ ) 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 ( $12.6 \pm 2.1$  % mortality  $\text{h}^{-1}$ ) compared to 10, 20 and 32 PSU ( $4.6 \pm 1.4$ ,  
220  $3.6 \pm 0.7$ ,  $3.7 \pm 0.5$  % mortality  $\text{h}^{-1}$  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 ( $6.8 \pm 0.9$  % mortality  $\text{d}^{-1}$ ) and lowest mortality at 32 PSU  
223 ( $1.8 \pm 0.4$  % mortality  $\text{d}^{-1}$ , 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 ( $19.4 \pm 2.3$  %,  $p < 0.05$ ) and lowest at 32 PSU, with no significant  
231 differences in mortality between 10, 20 and 32 PSU ( $8.4 \pm 1.7$ ,  $5.7 \pm 0.8$ ,  $5.5 \pm 0.6$  % 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 ( $44.459 \pm 9.157$ ,  $36.377 \pm 21.627$  and  $48.783 \pm 10.939$  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 ( $\beta$ , 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  $\text{d}^{-1}$ ) 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 absence 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

294 described in Pan *et al.* (2016). As a result, Pan *et al.* (2016) found a sigmoidal survival curve as a  
295 function of salinities ranging from 0 to 35 PSU, with an optimum of 20 PSU. Muthupriya and Altaff  
296 (2009) however, showed a mortality of *A. royi* lowest at 12 PSU and highest at 32 PSU. In the  
297 present study, we observed an elevated mortality after acclimatization in 5 PSU but did not see any  
298 difference between 10 and 32 PSU (Fig 1 and 2). We consider this is an effect of that our *A. royi*  
299 strain has been reared at 32 PSU for several generations and therefore may have adapted to this  
300 condition, but still has kept the ability to perform well at 20 PSU. This suggests that within species,  
301 salinity tolerance is population specific or even may be related to pre-experimental rearing  
302 conditions.

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

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

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

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

#### 341 **The effect of salinity and food availability on feeding rate**

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

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



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

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

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

### 367 **The effect of salinity and food availability on egg production**

368 ~~Exposure to hypo- or hypersaline environments may reduce egg production rates in copepods due to~~  
369 ~~increased energy allocation to osmoregulation (Gaudy et al., 2000). It is therefore not surprising~~  
370 ~~that reduced egg production rates have been observed in various species when subjected to~~  
371 ~~hyposaline environments (Dutz and Christensen, 2018; Calliari et al., 2006). In the present study,~~  
372 ~~we investigated the ovigerous rate of A. royi at different salinities over a gradient in food~~  
373 ~~availability. Whereas the food availability dependent egg production of A. royi has not been~~  
374 ~~previously described, s~~ Salinity dependent egg production has been ~~scarcely~~ studied ~~in a few cases~~  
375 for ~~this Apocyclops royi species~~. Muthupriya and Altaff (2009) and Pan *et al.* (2016) tested the long-  
376 term egg production rate of acclimated *A. royi* in the presence of food and showed, similar to our  
377 observations (Fig 5), that *A. royi* is capable of maintaining egg production at a wide range of  
378 salinities (0-35 PSU) with optimal conditions varying between 12-20 PSU. Salinity above 35 PSU  
379 appeared to be unfavorable in terms of egg production (Muthupriya and Altaff, 2009). Hatching  
380 success and postembryonic development as maxima for nauplii production were observed between  
381 10-20 PSU (Lee *et al.*, 2005; Pan *et al.*, 2016) and maximum culture densities were reached at 20  
382 PSU (Pan *et al.*, 2016). In the present study the maximum ovigerous rate of *A. royi* was not  
383 significantly affected by salinity (Fig 5d), contrary to our hypothesis.

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

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

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

402 ~~The~~ *Apocyclops royi* strain used in the present study was isolated from artificial aquaculture ponds  
 403 in Taiwan. These ponds we consider representing an extreme habitat. Each of the ponds cover an  
 404 area ~~~0.7 ha and is 1 m deep~~ha, is 1 m deep, and filled with coastal water from the nearby South  
 405 China Sea. Hence, it is reasonable to assume that the copepods are not native to these ponds, but  
 406 originate from the South China Sea (Blanda *et al.*, 2015). Brackish water systems generally show  
 407 strong temporal fluctuations in environmental conditions, and the ponds *A. royi* is isolated from are  
 408 documented to fluctuate in biotic and abiotic conditions on a seasonal and even daily basis (Blanda  
 409 *et al.*, 2015, 2017). For example, oxygen levels in these ponds reach hypoxic conditions on a daily  
 410 basis and severe hypoxia on a weekly basis, with *A. royi* still thriving in these ponds (Blanda *et al.*,  
 411 2015). Salinity is variable over the season, but more interestingly, short-term drops in salinity of 6  
 412 PSU are observed due to heavy monsoon rain events. Hence, *A. royi* is exposed to abrupt salinity  
 413 changes in its natural environment and is able to can successfully maintain its population. This

414 correlates well with our results where decreasing salinities resulted in only minor increase in  
415 mortality rates and no effect on ovigerous rate (Fig 1, 2 and 5). Moreover, *A. royi* is able to upgrade  
416 their fatty acid pool to become richer in long chained fatty acids (Rayner *et al.*, 2017; Nielsen *et al.*,  
417 2019). These traits, combined with a low osmotic dependency of the vital rates of *A. royi* as shown  
418 in the present study, most likely contribute to the fact that *A. royi* can survive and is one of the  
419 predominant copepod species in Taiwanese aquaculture ponds (Blanda *et al.*, 2015; Rayner *et al.*,  
420 2015).

### 421 **Salinity tolerance of *A. royi***

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

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

432 ~~Lastly, we hypothesized that the threshold concentration for egg production (lowest food~~  
433 ~~concentration where egg production is initiated) shifts to higher food concentrations when~~  
434 ~~challenged by salinity. This hypothesis was rejected as at all tested salinities egg production was~~  
435 ~~absent when no food was present, but was initiated at the lowest food concentrations offered (Fig~~  
436 ~~5a-c).~~

### 437 **CONCLUSIONS**

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

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

#### 446 **ACKNOWLEDGEMENTS**

447 We would like to thank M.Sc. Bolette Lykke Holm Nielsen and technicians Katja Lynnerup Hansen  
448 and Anne Busk Faarborg for their help during the experimental procedure. We would also like to  
449 thank the reviewers for constructive critique to an earlier version of our manuscript.

#### 450 **FUNDING**

451 This work was supported by a Villum Foundation project no. 8960 *Acartia tonsa* Molecular  
452 PHysiology – Implementation of novel and fast tools to assess COPEpod physiological states  
453 (AMPHICOP) to B.W.H.

#### 454 **DATA ARCHIVING**

455 No supplementary data related to this article is archived.

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616 **TABLE AND FIGURE LEGENDS**

617 Table 1. Overview of the experimental work. Salinity of the final food suspension is given for each  
 618 experiment. Prey size is the average cell size ( $n \sim 30.000$ ) at start and end of the experiment (size  
 619 generally decreased during incubation). Prey concentrations are the minimum and maximum  
 620 average concentration during each incubation. All tested prey concentrations for each salinity  
 621 treatment can be derived from figures 2, 4 and 5. Temperature was monitored with 1-minute  
 622 intervals using an ONSET HOBO® Pendant temperature logger.

623

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

624

625

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

629

Equation	PSU	$a \pm \text{SE}$	$b \pm \text{SE}$	$r^2$
Mortality rate (Fig 2)	5	6.4 $\pm$ 1.1	4.9 $\pm$ 0.0	0.02
$M = a + bC$	20	2.4 $\pm$ 0.6	-2.0 $\pm$ 0.0	0.01
	32	2.1 $\pm$ 0.6	-0.0 $\pm$ 0.0	0.02
		$\beta$ (CI, 95 %)	$\alpha$ (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
		$a \pm \text{SE}$	$b \pm \text{SE}$	$r^2$
Egg prod.- ingestion rate (Fig 6)	5	3974 $\pm$ 6394	0.114 $\pm$ 0.009	0.94
$SEP = a + bl$	20	-9329 $\pm$ 7898	0.120 $\pm$ 0.012	0.86
	32	-4853 $\pm$ 4795	0.102 $\pm$ 0.006	0.95

630

631

632

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

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

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

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

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

661

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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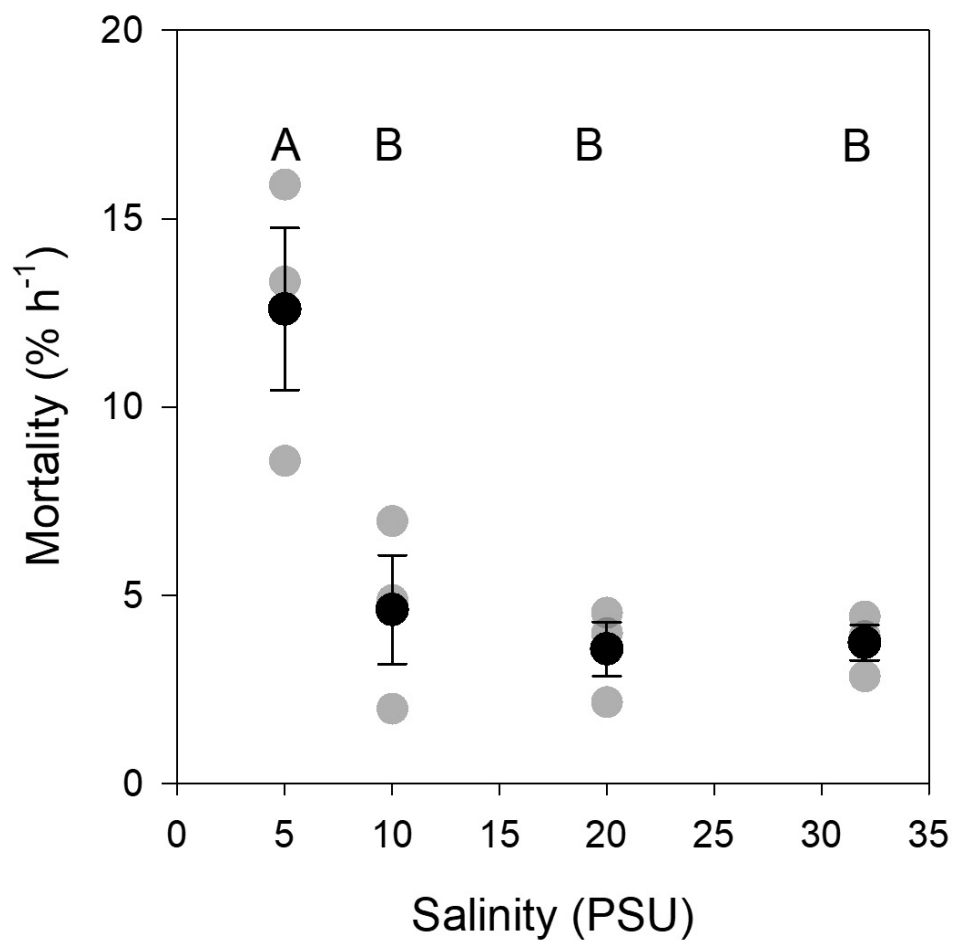


Fig 1. Mortality rate during short-term (% h<sup>-1</sup>) salinity acclimatisation of *Apocyclops royi* as function of salinity. Light dots indicate replicates and black dots are the mean value. Error bars indicate the standard error and different letters indicate statistically significant difference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

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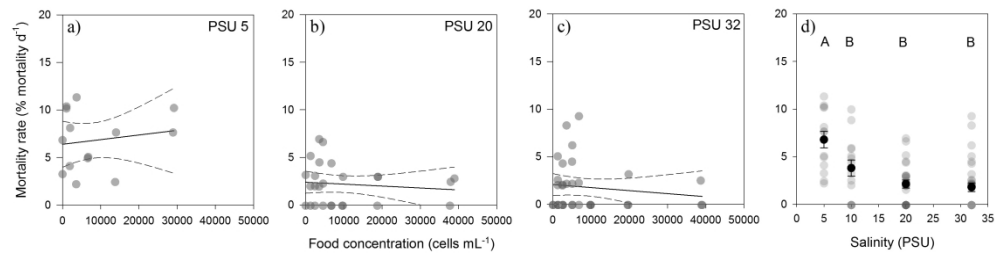


Fig 2. Food concentration dependent mortality rate of *Apocyclops royi* at different salinities (panel a-c) and average mortality (% d<sup>-1</sup>) at each tested salinity (panel d) during 24 h incubations. Light dots indicate replicates and black dots are the mean value. Discontinuous lines (panel 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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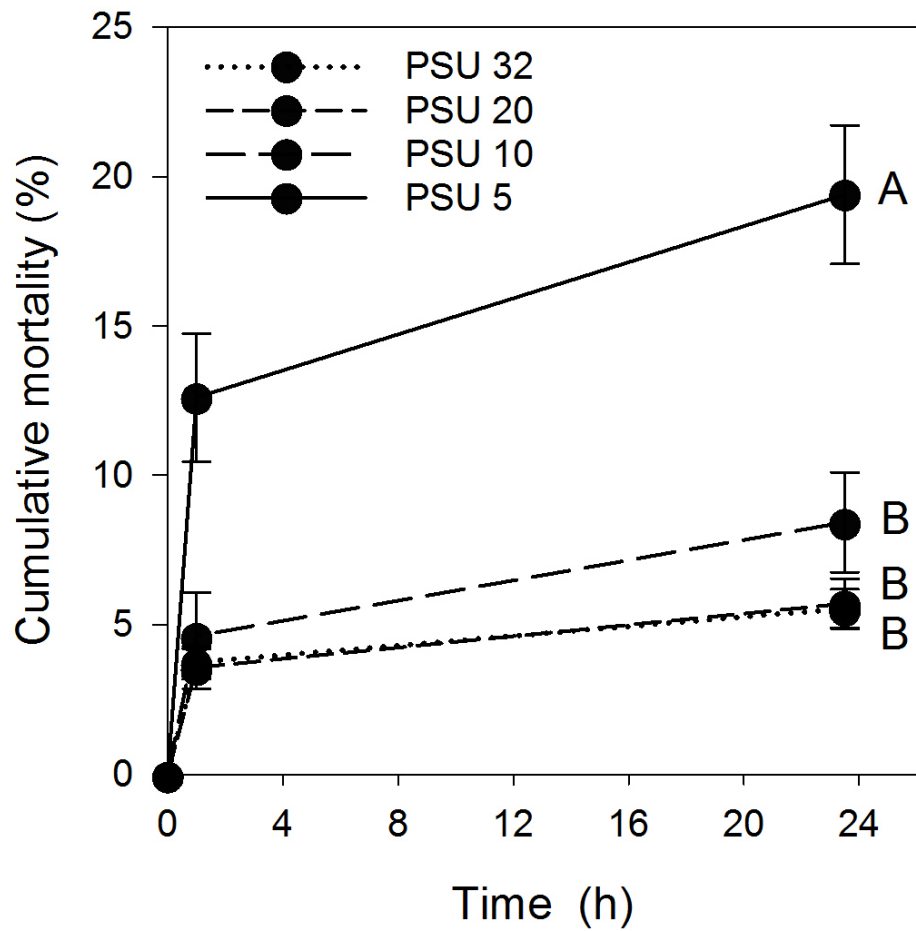


Fig 3. Cumulative mortality rates of *Apocyclops royi* at different salinities during 1h salinity acclimatization and 24h incubation experiments as a function of time. Dots are the mean value and error bars indicate the standard error. Different letters indicate statistically significant difference in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

93x92mm (300 x 300 DPI)

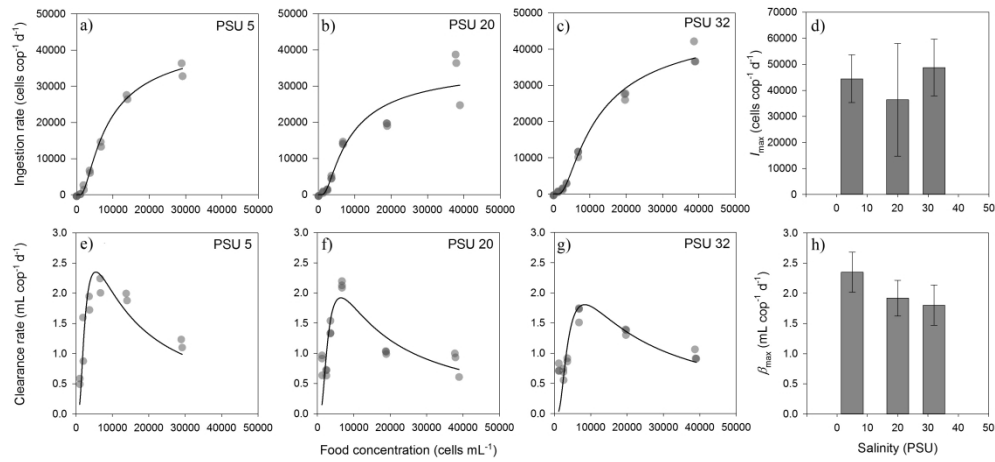


Fig 4. The functional responses of *Apocyclops royi* feeding on *Rhodomonas salina* at different salinity levels. Copepod ingestion rates (cells cop<sup>-1</sup> d<sup>-1</sup>, panel a-c) and clearance rates (mL cop<sup>-1</sup> d<sup>-1</sup>, panel e-g) are presented as function of food concentration (cells mL<sup>-1</sup>). 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 ( $\beta_{max}$ ), respectively as function of salinity; error bars indicate 95% confidence intervals. All models and parameters are presented in Table 2.

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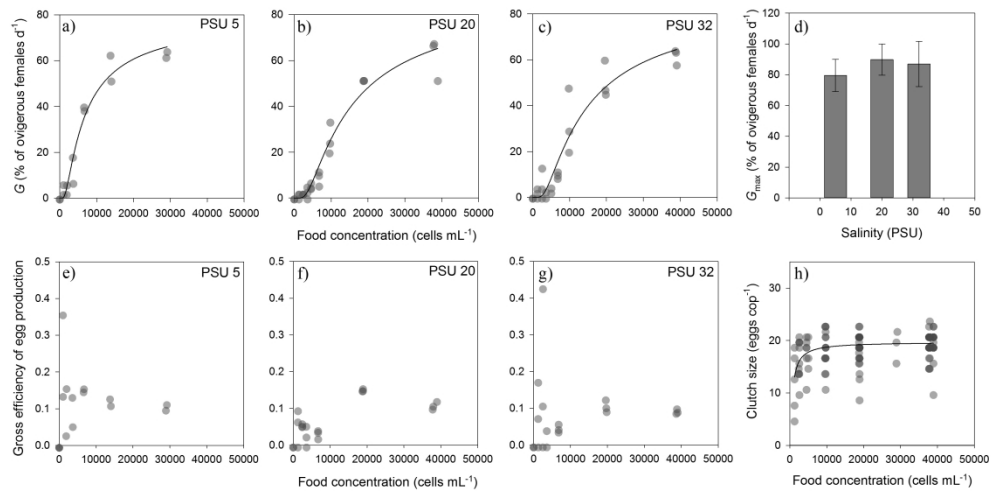


Fig 5. Food concentration dependent egg production rates of *Apocyclops royi* at different salinities. Female ovigerous rates (% of ovigerous females d<sup>-1</sup>, panel a-c) and gross efficiency of egg production rates (panel e-g) are presented as function of food concentration (cells mL<sup>-1</sup>). 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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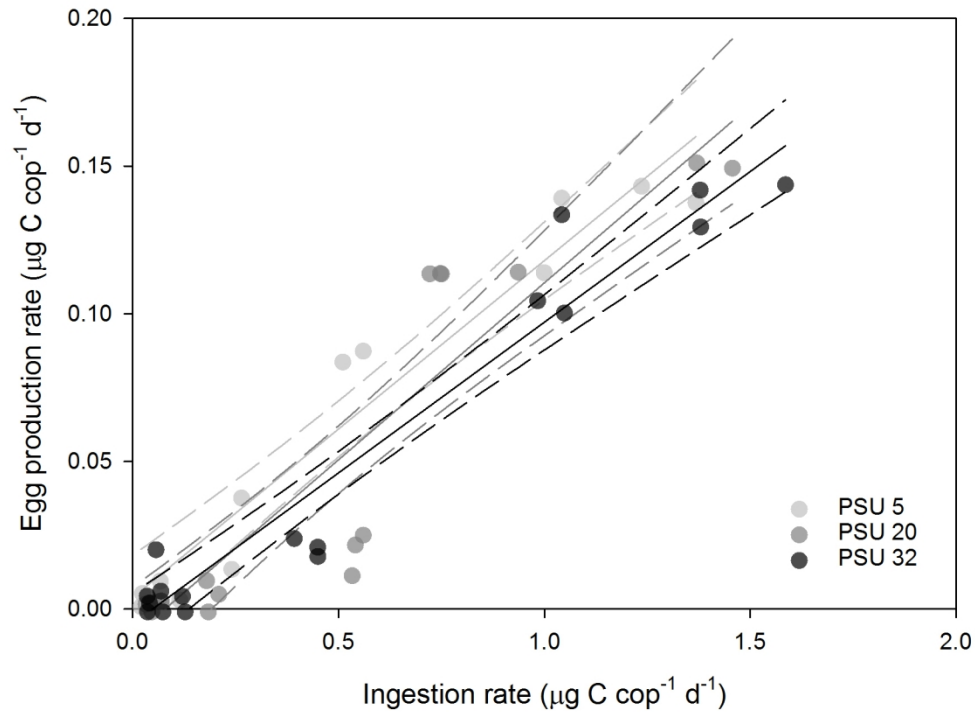


Fig 6. The specific egg production rate ( $\mu\text{g C cop}^{-1} \text{d}^{-1}$ ) is shown as function of the specific ingestion rate ( $\mu\text{g C cop}^{-1} \text{d}^{-1}$ ), where the slopes of the fitted regressions equal the estimated gross efficiency of egg production. Discontinuous lines indicate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

151x119mm (300 x 300 DPI)