Tilapia Prolactin Cells are Thermosensitive Osmoreceptors

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12   **Abstract**

13   Prolactin (PRL) cells within the *rostral pars distalis* (RPD) of the euryhaline teleost

14   tilapia, *Oreochromis mossambicus*, rapidly respond to a hyposmotic stimulus by releasing

15   two distinct PRL isoforms, PRL_{188} and PRL_{177}. Here, we describe how environmentally

16   relevant temperatures affect the release and mRNA levels of PRL_{188} and PRL_{177} from RPDs

17   and dispersed PRL cells. When applied under isosmotic conditions (330 mOsm/kg), a 6 °C

18   rise in temperature stimulated the release of PRL_{188} and PRL_{177} from both RPDs and

19   dispersed PRL cells under perifusion. When exposed to this same change in temperature,

20   ~50% of dispersed PRL cells gradually increased in volume by ~8%, a response partially

21   inhibited by the water channel blocker, $HgCl_2$. Following their response to increased

22   temperature, PRL cells remained responsive to a hyposmotic stimulus (280 mOsm/kg). The

23   mRNA expression of *transient potential vanilloid 4*, a Ca^{2+}-channel involved in

24   hyposomotically-induced PRL release, was elevated in response to a rise in temperature in

25   dispersed PRL cells and RPDs at 6 and 24 h, respectively; prl_{188} and prl_{177} mRNAs were

26   unaffected. Our findings indicate that thermosensitive PRL release is mediated, at least

27   partially, through a cell-volume dependent pathway similar to how osmoreceptive PRL

28   release is achieved.

29

30   **Introduction**

31   Prolactin (PRL) is a pleiotropic hormone secreted by the pituitary gland that exerts

32   over 300 physiological functions in vertebrates[1]. In fishes, PRL coordinates the activities of

33   multiple osmoregulatory organs such as the gill, gastrointestinal tract, urinary bladder, and

34   kidney[1,2,3,4]. PRL is especially vital to promoting physiological phenotypes that enable fishes

35   to inhabit dilute freshwater (FW) environments because it stimulates active ion absorption

36   and inhibits passive water gain[2,3]. Consistent with facilitating FW acclimation via their

37   secreted hormones, PRL cells of euryhaline fishes, such as the Mozambique tilapia

38   (*Oreochromis mossambicus*), are highly sensitive to physiologically relevant changes in

39   extracellular osmolality. A fall in extracellular osmolality initiates the rapid release of two

40   isoforms of tilapia PRL (PRL_{188} and PRL_{177}) that are encoded by separate genes[5,6].

41   Hyposmotically-induced PRL release is triggered by an increase in cell volume[7,8] followed by

42   the entry of extracellular Ca^{2+} into PRL cells through the stretch-gated transient potential

43   vanilloid 4 (TRPV4) channel[9,10]. The operation of this osmosensory system to control

44   hormone release continues to provide the basis for employing tilapia PRL cell as a model to

45   investigate osmoreceptors in vertebrates[11,12,13,14]. In mammals, the organum vasculosum of

46   the lamina terminalis transduces changes in osmotic conditions into an endocrine response

47   through vasopressinergic neurons[15]; these neurons, however, reside among other cell types of

48   the supraoptic and paraventricular nuclei with their axons projecting a considerable distance

49 to the posterior pituitary where vasopressin is released into the bloodstream[16,17]. By contrast,

50 tilapia PRL cells form a nearly homogeneous mass that comprises the *rostral pars distalis*

51 (RPD), thereby making them highly amenable to *in vitro* experimental paradigms[18].

52   Euryhaline fishes can typically tolerate large fluctuations in environmental salinity,

53 while eurythermal fish can survive a wide range of ambient temperatures. Mozambique

54 tilapia are both euryhaline and eurythermal given their tolerance of salinities ranging from

55 FW to greater than double-strength seawater and temperatures between 14 and 38 °C[19,20]. An

56 indispensable aspect of osmotically-regulated hormone release from PRL cells is a change in

57 cell volume, which increases and decreases as extracellular osmolality falls and rises,

58 respectively[7,8,9,21]. The hyposmotically-induced increase in PRL cell volume requires the

59 operation of aquaporin 3 (AQP3)[22], an aquaglyceroporin involved in fluid transport and the

60 regulation of water-permeability of cell membranes in various vertebrate tissues[23]. Tilapia

61 AQP3 expressed in *Xenopus* oocytes induces an increase in osmotic water permeability,

62 which can be inhibited by mercury chloride ($HgCl_2$)[24]. $HgCl_2$ is a potent AQP blocker that

63 also inhibits hyposmotically-induced cell volume changes and PRL release from dispersed

64 tilapia PRL cells[22].

65   Members of the transient receptor potential (TRP) family of channels, including

66 TRPV4, are sensitive to changes in osmolality, mechanical stress, small molecules, and

67 temperature[25]. For instance, the thermal sensitivity of TRPV1 underlies mammalian

68 thermoregulatory cooling through sweat production by promoting preemptive vasopressin-

69 mediated renal water reabsorption[26]. Moreover, TRPV4, which is also localized in

70 thermosensitive regions of the brain, is activated by warming in *Xenopus* oocytes and human

71 embryonic kidney 293 expression systems[27]. *trpv4* gene transcripts are elevated in the brain

72 and pituitary of chum salmon (*Oncorhynchus keta*) following a rise in temperature[28].

3

73 Knowing that TRPV4 is highly expressed in tilapia RPD[10], we hypothesized that PRL cells

74 are also directly thermosensitive. Under this scenario, PRL cells would respond to

75 fluctuations in environmental (and internal) temperature to modulate hormone release. This

76 would constitute an entirely new facet of how PRL cells are controlled in a poikilothermic

77 model. Moreover, inasmuch as cell volume changes provide a means to precisely regulate

78 osmotically-sensitive PRL release, we hypothesized that thermal stimulation of tilapia PRL

79 cells may also trigger cell volume changes. A link between a rise in temperature and an

80 increase in cell volume has been previously observed in various cell models. For example,

81 hyperthermic conditions within mouse mastocytoma P815 cells and rapid shifts to higher

82 temperatures in human leukemia cells promote increases in cell volume[29,30]. A connection

83 between thermal stimuli and changes in cell volume in tilapia PRL cells would provide a

84 mechanism that allows for the integration of thermal and osmotic stimuli to support adaptive

85 patterns of PRL secretion.

86   In the present study, we employed RPDs and dispersed PRL cells in both perifusion

87 and static incubation systems to determine how temperature impacts PRL release and whether

88 cell volume changes are coincident with thermal PRL responses. We also evaluated the

89 effects of temperature on the expression of prl_{188}, prl_{177}, and $trpv4$ in RPDs and PRL cells.

90 Further, we assessed whether thermally-induced changes in cell volume are sensitive to

91 mercury to probe the involvement of AQP3. Together, these experiments describe the

92 relationship between thermal sensitivity, cell volume changes, and hormone secretion in a

93 vertebrate osmoreceptor.

94

95 **Results**

96 <u>Effects of temperature on PRL_{188} and PRL_{177} release from static incubations</u>

4

97   A two-way ANOVA detected significant individual and interaction effects of

98   incubation temperature and time on the release of both PRL_{188} and PRL_{177} from RPDs (Figs.

99   1A and 1B). As early as 6 h, the release of both PRLs was elevated at 30 °C compared with

100   both 25 °C and 20 °C; there were no differences in the release of PRLs between the two

101   lowest incubation temperatures at 6 h. By 24 h of incubation, however, a temperature-

102   dependent pattern of PRL release was observed; the release of PRL_{188} and PRL_{177} was

103   highest at 30 °C followed by 25 °C, which in turn, was higher than release at 20 °C (Figs. 1A

104   and 1B, respectively). PRL_{188} released at 30 °C was 8-fold higher than that released at 20 °C

105   at 24 h (Fig. 1A). Similarly, PRL_{177} released at 30 °C was 6-fold higher than that released at

106   20 °C over the same time course (Fig. 1B).

107   The effects of temperature on PRL release from PRL cells are shown in Figures 1C

108   and 1D. In this experiment, we compared PRL release between cells incubated at 26 °C and

109   32 °C for 1 and 6 h. Temperature had no effect on the release of both PRL_{188} and PRL_{177} at 1

110   h (Figs. 1C and 1D). While there was no effect of temperature on PRL_{177} release at 6 h (Fig.

111   1D), PRL_{188} release rose by over 2-fold during the same period (Fig. 1C).

112

113   <u>Effects of temperature on PRL_{188} and PRL_{177} release from perifused PRL cells</u>

114   Two experimental treatments, distinguished by their exposure to either baseline

115   temperature (26 °C) or increased temperature (32 °C), were compared. There were significant

116   effects of treatment, time, and an interaction on PRL_{188} (Fig. 2A). A significant difference in

117   PRL_{188} release between control and temperature-exposed cells was observed between 50 and

118   65 min (Fig. 2A). PRL_{188} release from the control group increased to ~364% relative to the

119   average PRL release at the first 5 time points (baseline) when cells were exposed to

120   hyposmotic media. PRL_{188} release from the temperature group increased by ~400% relative

5

121 to baseline between 50 and 60 min, before subsiding to ~350% above baseline by 70 min.

122 Finally, PRL_{188} release increased by ~500% relative to baseline 10 min after cells were

123 exposed to hyposmotic media.

124 PRL_{188} release at time 0 was compared to all other time points within each treatment

125 from the experiments shown in Figure 2A (Table S1). PRL_{188} release from the control cells

126 increased during hyposmotic-stimuli (80-95 min), whereas PRL_{188} release of temperature-

127 exposed cells increased earlier, between 50-75 min of perifusion and during exposure to a

128 hyposmotic stimulus (75-105 min).

129 There were significant effects of treatment and time on PRL_{177} (Fig. 2B). Significant

130 differences in PRL_{177} between control and temperature-exposed cells were observed at 50 and

131 55 min (Fig. 2B). PRL_{177} release from the control group increased by ~445% relative to

132 baseline when cells were exposed to 280 mOsm/kg. PRL_{177} release within the temperature

133 group increased by ~240% relative to baseline at 50 min before subsiding to ~165% above

134 baseline by 65 min. Finally, PRL_{177} release increased by ~400% relative to baseline when

135 cells were exposed to hyposmotic media for 10 min.

136 PRL_{177} release at time 0 was compared to all other time points within each treatment

137 for the experiments shown in Figure 2B (Table S2). PRL_{177} release from the control cells

138 increased during exposure to a hyposmotic stimulus (70-100 min), while PRL_{177} release from

139 temperature-exposed cells increased earlier, at 50-55 min of perifusion and during exposure

140 to a hyposmotic stimulus (80-100 min) (Table S2).

141

142 Effects of temperature on PRL cell volume

143 Three experimental groups, distinguished by their exposure to either baseline

144 temperature (26 °C; control group), high temperature (32 °C; high temperature group), or

6

145 high temperature in the presence of the water channel blocker, $HgCl_2$ ($HgCl_2$ group), were

146 compared. While 100% of the viable cells responded to a hyposmotic stimulus, ~50% of the

147 cells exposed to a thermal stimulus responded in an identical fashion as the control cells

148 whereas ~50% responded to the thermal stimulus. The ~50% of cells that were responsive to

149 a thermal-stimulus were used for comparing controls versus high temperature and $HgCl_2$

150 groups. Cell volumes of the control group increased by ~20% relative to the average cell

151 volume from the first 6 time points (cell volume baseline) when cells were exposed to

152 hyposmotic media at 26 °C (Fig. 3A, white circles). Cell volumes in the high temperature

153 group gradually increased and peaked at ~8% relative to baseline between 30 and 70 min

154 (Fig. 3A, black squares). Cell volume remained at ~8% above baseline up to 15 min

155 following re-exposure to baseline conditions. Then, at 105 min, cell volume decreased from

156 ~8% to ~5% above baseline, where it remained stable until 135 min before decreasing further

157 to ~3% above baseline between 135 and 140 min. Finally, cell volume increased by ~20%

158 relative to baseline when cells were exposed to hyposmotic media. When cells were exposed

159 to 32 °C for 60 min in the presence of $HgCl_2$, cell volume remained steady for the first 5 min

160 and then gradually increased, peaking at ~5% above baseline between 35 and 75 min and

161 maintaining at ~5% above baseline for the remainder of the temperature exposure with $HgCl_2$

162 (Fig. 3A, grey triangles). Cell volume was maintained at ~5% above baseline between 90 and

163 110 min, before gradually decreasing to ~2% above baseline by 140 min. Lastly, cells

164 exposed to hyposmotic media increased in cell volume by ~20% relative to baseline (Fig. 3A,

165 grey triangles).

166   The cell volume responses reported in Figure 3A were combined according to the

167 treatments within each experimental group for statistical analysis and expressed as the

168 NCCV. There were significant effects of treatment, time, and an interaction on NCCV (Fig.

7

169   3B). Temperature-induced NCCV was significantly different between all treatments (Fig.

170   3B). The high temperature group had a NCCV of ~7% above baseline, a value that was

171   significantly higher than that of control and $HgCl_2$ groups. The $HgCl_2$ group had a NCCV of

172   ~3% above baseline during exposure to high temperature, a value intermediate to that of

173   control and high temperature groups. NCCV was significantly different between all

174   treatments during the recovery baseline (Fig. 3B); the NCCV of the high temperature group

175   was higher than that of both control and $HgCl_2$ groups. There was no significant difference in

176   NCCV between treatments during the final 30 min of the perifusion with hypoosmotic media;

177   the NCCV of all three treatments increased by ~15% relative to baseline during that period

178   (Fig. 3B). The NCCV induced by exposure to 32 °C was higher than the baseline but lower

179   than the NCCV induced by hyposmotic media (Fig. 3B).

180

181   <u>Effects of temperature on prl_{188}, prl_{177}, and $trpv4$ mRNA levels</u>

182   Unlike release, the expression of prl mRNAs from RPDs was not altered by

183   elevations in incubation temperature at 24 h (Fig. 4A and 4B). By contrast, the mRNA

184   expression of $trpv4$ was increased by over 2-fold in RPDs incubated at 30 °C compared with

185   those held at 20 °C and 25 °C (Fig. 4C). Consistent with the 24 h RPD incubations, prl_{188} and

186   prl_{177} expression in dispersed PRL cells was not affected by incubation temperature during a

187   6 h static incubation (Figs. 4D and 4E). $trpv4$ expression in PRL cells increased 2-fold at 32

188   °C compared with cells incubated at 26 °C (Fig. 4F).

189

190   **Discussion**

191   The regulation of PRL and other pituitary hormones by environmental stimuli has

192   remained understudied. The paucity of information stems from the lack of suitable model

193 systems that are normally subject to wide and frequent excursions in the stimulus being

194 studied and that allow for the homogeneous isolation of specialized cells with known

195 secretory output. In mammals, tight homeothermic and osmoregulatory control ensures that

196 homeostasis is rigorously maintained; deviations in internal temperature and fluid osmolality

197 are minimized. Many fishes, on the other hand, are capable of withstanding acute internal

198 variations in osmolality, and possibly temperature. In Mozambique tilapia, for example, non-

199 lethal deviations in plasma osmolality have ranged from 300 to 450 mOsm/kg within 6 h of

200 transfer from FW to brackish water[31]. In light of PRL's diverse actions in fishes, and the

201 poikilothermic nature of Mozambique tilapia, we hypothesized that PRL cells are

202 thermosensitive and may integrate thermal fluctuations in the environment with one or more

203 of the physiological actions that PRL imparts. Here, we report for the first time that release of

204 both tilapia PRLs (PRL_{188} and PRL_{177}) from the pituitary is induced by an environmentally

205 relevant increase in temperature. Moreover, our findings indicate that the PRL cell sensory

206 mechanism(s) responding to thermal stimuli share similarities to those that transduce osmotic

207 stimuli into PRL release through changes in cell volume. In light of the established role of

208 PRL cells as osmoreceptors, these findings suggest that the integration of osmotic and

209 thermal stimuli is carried out in a single cell type that secretes products with pleiotropic

210 actions.

211   Previous studies established the direct osmotic control of PRL cell function

212 [7,31,32,33,34,35]. We have also provided evidence that the regulation of PRL_{188} and PRL_{177} release

213 by extracellular osmolality is modulated by a series of endocrine factors[36]. In the current

214 study, we discovered that the regulation of PRL cells is directly affected by temperatures that

215 are within the range of environmental conditions (20 to 32 °C) normally experienced by

216 Mozambique tilapia[20]. While specific physiological drivers underlying PRL's release in a

9

217 temperature-dependent manner remain subject to further investigation, there are a number of

218 temperature-dependent processes that are regulated, at least in part, by PRL. For example, in

219 addition to exerting hyperosmoregulatory actions required for survival in FW, PRL also

220 directs growth and reproduction[37,38]. In tilapia, growth is enhanced by elevations in water

221 temperature[39] and PRL_{177} exhibits somatotropic activity[40]. In several species of fish, PRL

222 affects migration, binds to gonads, varies with sex steroids and breeding cycle, stimulates

223 steroidogenesis and gonadogenesis, and induces reproductive behaviors such as mouth

224 brooding, nesting, nest fanning, and fry defense[38]. In *O. mossambicus*, the PRL receptor is

225 expressed in the gonads[41] and serum levels of PRL change across the reproductive cycle[42]. In

226 the congeneric Nile tilapia (*O. niloticus*), pituitary and plasma PRL levels are highest during

227 vitellogenesis[43]. Given that spawning in tilapia is induced by increased temperatures[44], we

228 propose that a direct transduction mechanism links environmental temperature with the

229 release of a hormone, in this instance PRL, that supports reproduction.

230   The PRL cell is equipped with a sensory mechanism to transduce osmotic stimuli

231 into hormone release via the cell-volume dependent control of Ca^{2+} entry through TRPV4

232 channels. Hence, we hypothesized that PRL cells employ a similar mechanism to couple

233 thermal stimuli with a change in cell volume. In the current study, both thermal- and

234 hyposmotic-stimuli lead to an increase in PRL release and cell volume; however, the timing

235 of the responses to these stimuli varied. The difference in osmotic and thermal responses may

236 be attributed to the distinct nature of the stimuli and their subsequent effects on the cell

237 membrane. In static incubations, both RPDs and dispersed PRL cells responded to a rise in

238 temperature by 6 h (Fig. 1). By contrast, PRL cells in static incubation responded to a

239 hyposmotic stimulus as early as 1 h[21]. Similarly, in perifusion incubations, PRL release

240 increased 35 min after initial exposure to a rise in temperature, while that response occurred

10

241 within 5 min following exposure to hyposmotic conditions (Fig. 2). Interestingly, ~50% of

242 PRL cells gradually increased in cell volume in response to the rise in temperature, peaking at

243 ~8% after 30 min of exposure to an elevated temperature, whereas all PRL cells exposed to a

244 hyposmotic stimulus increased in volume by ~15% within 15 min (Fig. 3A). Weber and

245 colleagues[8] showed that the magnitude of PRL release is related to the rate of increases in cell

246 volume. Media containing urea, a solute that permeates the cell membrane at a slower rate

247 than water, led to slower rates of cell volume increase and PRL release compared with

248 standard hyposmotic media[8]. Therefore, PRL release from dispersed PRL cells is tied to a cell

249 volume increase via water moving across the cell membrane rather than a direct response to

250 extracellular osmolality *per se*. Similarly, the delayed PRL response to a rise in temperature

251 was consistent with a gradual and protracted thermally-induced change in cell volume.

252 Thermal effects on cell volume increase have been previously reported in human leukaemia

253 K562 cells[29]. While a rise in temperature also resulted in an increase in cell volume, the

254 magnitude of thermal stimulation in this study differs from the current experiment given that

255 the thermal shock applied, from 37 to 46 °C, far exceeded the physiological range and

256 resulted in much faster responses (within seconds) coupled with gradual lowering of cell

257 viability[29]. Irrespective of differences in experimental approach, temperature-induced cell

258 volume changes may be attributable to the sensitivity of the plasma membrane and/or

259 cytoskeleton to heat. Supra-physiological increases in temperature lead to modifications in

260 the cytoskeleton, changes in membrane fluidity, and to increased membrane surface due to

261 loss of membrane invaginations in mammalian cells[45,46]. We propose these thermally-induced

262 cellular modifications may also underlie changes in the volume of tilapia PRL cells exposed

263 to a rise in temperature within a physiological range. While future investigations are

264 warranted to resolve whether these modifications do in fact occur in tilapia PRL cells, our

11

265 current findings suggest that subtle and gradual changes in cell volume play a role in

266 thermally-stimulated PRL secretion. This role was further supported by the attenuation of the

267 thermally-induced increase in cell volume with $HgCl_2$ (Fig. 3B). Partial suppression of cell

268 volume increases by $HgCl_2$, an AQP3 blocker also shown to inhibit hyposmotically-induced

269 cell volume increase from PRL cells[22], suggests a role for the AQP3 water channel in

270 thermally-induced cell volume increases. Together with the established role that PRL cell

271 volume plays as a trigger for hyposmotically-induced PRL release, the current findings

272 indicate that thermally-induced PRL release is also activated, at least in part, by a cell-volume

273 dependent mechanism.

274   Heterologously expressed TRPV4 mediates Ca^{2+} influx in response to both

275 hyposmotic and hyperthermal stimuli[27,47]. The activation of Ca^{2+} influx through TRPV4

276 channels is identical to the mechanism described in hyposmotically-induced PRL release[9,10].

277 The expression of $trpv4$ mRNA, however, is downregulated by hyposmotic stimuli and by

278 pharmacological induction of Ca^{2+} influx, responses that reflect negative feedback loops

279 involved in both the maintenance of intracellular Ca^{2+} homeostasis and the attenuation of

280 osmosensitivity[48]. Here, $trpv4$ expression increased following an elevation in temperature

281 (Fig. 4) indicating further regulation of PRL cell sensitivity to thermal stimuli. Interestingly,

282 patterns of prl gene expression did not generally follow hormone release patterns; prl_{188} and

283 prl_{177} expression was unresponsive to a rise in temperature. This observation suggests that a

284 thermally-induced change in cell volume and the associated release of PRLs, while delayed

285 relative to hyposmotically-induced PRL release, may be short-lived. Further studies

286 combining hyposmotic and thermal stimuli in PRL cells will further clarify the disparate

287 effects of these stimuli on the expression of prl_{188}, prl_{177}, and $trpv4$.

288   PRL_{188} and PRL_{177} differ in the magnitude of their responses to environmental

289 osmolality[31,49,50,51]. This could be partially attributed to isoform-specific regulation of their

290 associated gene transcripts[31]. Similar to osmotic responses, the responses of PRL_{188} to rises in

291 temperature were more robust than those of PRL_{177} over all incubation systems tested. While

292 the regulatory effect of environmental stimuli on the functions of PRL is intricate and not

293 fully understood, the current study is the first to link an established cell model of

294 osmoreception with thermosensitivity, expanding our understanding of the sensory capacity

295 of tilapia PRL cells. The underlying cellular mechanisms responding to these stimuli, in turn,

296 manifest physiological consequences at the organismal scale through the myriad actions of

297 PRL (Fig. 5). One such consequence may include the enhanced control of osmoregulatory

298 organs by PRL when its release is thermally-stimulated. Indeed, elevated environmental

299 temperatures are known to impact osmotic homeostasis[52,53]. Other metabolically demanding

300 functions that are both stimulated by rises in temperature and by PRL, such as growth and

301 reproduction, may be impacted by the thermal responsiveness of PRL cells. The current study

302 paves the way to resolve the molecular mechanisms that enable integration of osmotic and

303 thermal stimuli by the pituitary of poikilotherms.

304

305 **Materials and methods**

306 <u>Animals</u>

307   Mature Mozambique tilapia (*O. mossambicus*) of mixed sexes and sizes (0.4-1.0 kg)

308 were obtained from stocks maintained at the Hawaiʻi Institute of Marine Biology, University

309 of Hawaiʻi (Kaneohe, HI) and at Mari's Garden (Mililani, HI). Fish were reared in outdoor

310 tanks with a continuous flow of FW under natural photoperiod and fed ~5% of their body

311 weight per day with trout chow pellets (Skretting, Tooele, UT). Fish were anesthesized with

312 2-phenoxyethanol (0.3 ml/l, Sigma Aldrich, St. Louis, MO) and euthanized by rapid

313   decapitation. All experiments were conducted in accordance with the principles and

314   procedures approved by the Institutional Animal Care and Use Committee, University of

315   Hawaiʻi. The study was designed in accordance with ARRIVE guidelines.

316

317   <u>Static incubations of RPDs</u>

318   Following euthanasia, RPDs of *O. mossambicus* were dissected from the pituitary

319   gland and placed individually into a single well of 48-well plates containing 300 µl of

320   isosmotic medium (330 mOsm/kg)[54]. After preincubation for 2 h at 25 ºC, the RPDs were

321   rinsed with incubation medium and incubated for 24 h at 20, 25, or 30 °C under saturated

322   humidity (eight RPDs per experimental group). At the end of 1, 6, and 24 h of incubation,

323   media were collected, diluted 20 times with radioimmunoassay (RIA) buffer (0.01 M PBS

324   containing 1% bovine serum albumin and 0.1% Triton X-100), and stored at -20 ºC until

325   analysis. Following the final collection of media at 24 h, the RPDs were collected in 500 µl

326   of TRI Reagent (MRC, Cincinnati, OH) and stored at -80 ºC prior to RNA extraction.

327

328   <u>PRL cell dispersion</u>

329   Dispersed PRL cells were prepared from RPDs dissected from *O. mossambicus* as

330   previously described[7,21,54] with minor modifications. Briefly, RPDs were treated with 0.125%

331   (wt/vol) trypsin (Sigma-Aldrich) dissolved in PBS and placed on a gyratory platform set at

332   120 rpm for 30 min to allow for complete cell dissociation. The cells were centrifuged for 5

333   min at 1200 rpm and the supernatant decanted and discarded; cells were resuspended and

334   triturated in trypsin inhibitor (0.125% wt/vol; Sigma-Aldrich) to terminate the trypsin

335   treatment. Cells were washed with PBS twice and then re-suspended in isosmotic medium.

336   Cell viability and yield were determined following the methods recently described[54].

337

<u>Static incubations of PRL cells</u>

339   Dispersed PRL cells were preincubated in 100 µl of isosmotic media (~124,000 cells

340   per well; eight replicates per treatment) at 26 ℃. The cells were then rinsed with incubation

341   media and incubated for 1 and 6 h at 26 and 32 ℃ under saturated humidity. At the end of 1

342   and 6 h of incubation, 10 µl of media were collected, diluted 20 times with RIA buffer, and

343   stored at -80 ℃. Following the final collection of media at 6 h, 100 µl of TRI Reagent (MRC,

344   Cincinnati, OH) was added to each well. The cells and TRI Reagent were then transferred to

345   1.5 ml tubes containing 500 µl of TRI Reagent and stored at -80 ℃ before RNA extraction

346   and gene expression analyses.

347

<u>Perifusion and PRL release</u>

349   Dispersed PRL cells were added to poly-L-lysine (Sigma; 0.1 mg/ml)-coated

350   chambers as previously described[7,54] with minor modifications. The chamber consisted of two

351   rectangular coverslips (22 x 50 mm) joined by 100% silicone around the edges with cut

352   hypodermic needles (23 gauge) inserted at each end to form an inlet and outlet. The chamber

353   volume ranged from 200 to 400 µl and accommodated an average of $300,000 \pm 50,000$ PRL

354   cells (12 different chamber preparations). To stabilize perifusion conditions, the chambers

355   were first submerged in a water bath at 26 °C and pre-perifused with isosmotic media for 60

356   min. After pre-perifusion, the chambers for the control group were perifused for an additional

357   70 min under the same conditions to establish a baseline prior to exposure to hyposmotic

358   media (280 mOsm/kg) for 35 min.

359   Following pre-perifusion, the temperature-exposed group chambers were perifused

360   for 15 min under isosmotic media at 26 °C to establish a baseline of PRL release, placed in a

361   32 °C water bath with isosmotic media for 30 min, returned to 26 °C with isosmotic media

362   for 25 min, and perifused with hyposmotic media at 26 °C for the final 35 min. The desired

363   internal temperature of the chambers (32 or 26 °C) was reached within a minute. Both

364   experimental runs were replicated 6 times. Media were perifused at a rate of 50 ± 4 μl/min.

365   The perifusate was collected manually every 5 min in 1.5 ml microcentrifuge tubes and

366   stored at -80 °C until analyzed.

367

368   <u>Perifusion and PRL cell volume</u>

369   Dispersed PRL cells were resuspended in isosmotic media and added to customized

370   perifusion chambers similar to ones used in the PRL release experiments above, with minor

371   modifications. The chamber consisted of two rectangular coverslips (22 x 30 mm or 22 x 50

372   mm) joined with silicone around the edges, with cut hypodermic needles (23 gauge) serving

373   as the inlet and outlet, and an insulated thermocouple (Omega 5SRTC-TT-K-40-36-ROH,

374   USA) inserted and connected to a computer for the monitoring of temperature (Fig. S1). The

375   chamber volume ranged from 200 to 400 μl and accommodated an average of $600,000 \pm$

376   100,000 PRL cells. The chamber was mounted on a heating stage with a controller for

377   modulating temperature (BoliOptics SG13701211, Rancho Cucamonga, CA) on top of an

378   inverted microscope (Nikon Diaphot TMD Inverted Microscope, Japan) stage equipped with

379   a 100x oil-immersion objective lens (Nikon, Japan). Internal temperature of the chamber was

380   maintained via manual operation of the heating stage. Heating and cooling the chamber to the

381   desired internal temperature (32 or 26 °C) required ~8 min.

382   All treatments were pre-perifused with isosmotic media at 26 °C for 60 min. The

383   control group was perifused with isosmotic media at 26 °C for 145 min followed by exposure

384   to hyposmotic media at 26 °C for the final 30 min. The temperature-exposed group was

16

385   perifused in isosmotic media at 26 °C for 25 min, exposed to 32 °C in isosmotic media for 60

386   min, returned to isosmotic media at 26 °C for 60 min, and exposed to hyposmotic media at 26

387   °C for the final 30 min. Finally, the HgCl₂-treated group was perifused in isosmotic media at

388   26 °C for 25 min, perifused with 10 μM HgCl₂ isosmotic media at 26 °C for 10 min (not

389   shown) prior to exposure to 32 °C in 10 μM HgCl₂ isosmotic media for 60 min, returned to

390   isosmotic media at 26 °C for 60 min, and exposed to hyposmotic media at 26 °C for the final

391   30 min. Each perifusion experiment for cell volume determination was replicated 2-3 times.

392

393   <u>Cell volume determination</u>

394   During the perifusion incubation period, cell images were captured every 5 min and

395   cell volume was estimated as previously described[7] with minor modifications. The area of

396   each cell was generated using the ImageJ software (Bethesda, MD) by tracing the perimeter

397   of the cell from digitally captured images. Areas (A) were obtained in pixels and transformed

398   into square micrometers. Cell volume (V) was estimated from the area shown in the equation

399   below, assuming the cell to be a perfect sphere:

400    $$r = \sqrt{A/\pi}, \; and \; V = 4/3\pi(r^3)$$

401   Cell volume was expressed as a percent change from the baseline set at 100%. The baseline

402   value was taken as the mean of the volume calculated from the first 6 time points at the initial

403   25 min exposure in isosmotic media at 26 °C.

404

405   <u>Radioimmunoassay (RIA)</u>

406   PRL₁₈₈ and PRL₁₇₇ levels in the collected media samples were measured by

407   homologous RIA[51,55] using primary antibodies (antisera) raised in rabbit against PRL₁₈₈ and

408   PRL₁₇₇ (anti-PRL₁₈₈ and anti-PRL₁₇₇, respectively) and secondary antibody raised in goat

409   against rabbit IgG (anti-rabbit IgG; Sigma-Aldrich) as recently described and validated[54].

410   Dilutions employed for anti-PRL$_{188}$, anti-PRL$_{177}$, and anti-rabbit IgG were, 1:35000, 1:8000,

411   and 1:100, respectively.

412

413   <u>Quantitative real-time PCR (qRT-PCR)</u>

414   Total RNA was extracted from RPDs and PRL cells frozen in TRI Reagent

415   following the manufacturer's protocol. Total RNA (150 ng from PRL cells and 400 ng from

416   RPDs) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit

417   (Thermo Fisher Scientific, Waltham, MA). The levels of reference and target genes were

418   determined by the relative quantification method using a StepOnePlus real-time qPCR system

419   (Thermo Fisher Scientific). The qPCR reaction mix (15 µl) contained Power SYBR Green

420   PCR Master Mix (Thermo Fisher Scientific), 200 nmol/l forward and reverse primers, and 1-

421   3.5 µl of cDNA. PCR cycling parameters were as follows: 2 min at 50 °C, 10 min at 95 °C

422   followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Primer sequences are listed in

423   Table S3. After verifying that levels did not vary across treatments, 18S ribosomal RNA was

424   used as the reference to normalize target genes. Data are expressed as mean fold change ±

425   SEM ($n = 8$) from a given control group.

426

427   <u>Statistics</u>

428   Data for PRL release from RPDs were analyzed by two-way ANOVA with

429   incubation temperature and time as main effects. Values were normalized by body weight as

430   a surrogate marker for pituitary and RPD weight[54] and expressed as a fold-change relative to

431   the 20 °C group. There were no differences in the body weight of donor animals between

432   treatment groups (data not shown). Data for PRL release from static incubations of dispersed

18

433   PRL cells were analyzed by Student's t-test. Data for PRL release from perifused PRL cells

434   were analyzed by two-way ANOVA with treatment and time course as main effects. Changes

435   in cell volume were analyzed by two-way ANOVA with treatment and stimulus as main

436   effects. Cell volume data were converted to net change in cell volume (NCCV) by subtracting

437   the mean cell volume over the 25 min baseline from the mean cell volume change during

438   subsequent incubation periods. mRNA expression data from 24 h RPD static incubations

439   were analyzed by one-way ANOVA; data from 6 h PRL cell static incubations were analyzed

440   by Student's t-test. Significant effects were followed up by protected Fisher's LSD test.

441   When necessary, data were log-transformed to satisfy normality and homogeneity of variance

442   requirements prior to statistical analysis. All statistics were performed using Prism 6

443   (GraphPad, La Jolla, CA) and data are reported as means ± SEM.

444

445   **Data Availability**

446   The datasets generated during and/or analyzed during the current study are available from the

447   corresponding author on reasonable request.

448

449

450

451   **References**

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625 **Author Contributions:** Y.Y., J.P.B., A.P.S. conceived the research idea and designed the

626 experiments; D.W.W., G.H.T.M., F.T.C.B., Y.Y., J.P.B., A.P.S. performed the research;

627 D.W.W., G.H.T.M., F.T.C.B., Y.Y., A.P.S. conducted data analyses and interpretation;

628 A.P.S. contributed reagents/analytic tools to the project; D.W.W., J.P.B., A.P.S. wrote the

629 paper. All authors have read, edited and approved the final manuscript.

630

631 **Competing Interest Statement:** The authors declare no competing interests.

632

633 **Figure legends**

634 **Figure 1.** Effects of temperature on the release of PRL_{188} (A) and PRL_{177} (B) from RPDs at

635 1, 6, and 24 h and on the release of PRL_{188} (C) and PRL_{177} (D) from PRL cells at 1 and 6 h.

636 In panels A and B, data are expressed in ng/ml per gram body weight ± SEM ($n = 7$-8).

637 Effects of incubation time and temperature were analyzed by two-way ANOVA

638 ([***]Significant at $P < 0.001$). Incubation temperature effects were followed up by protected

639 Fisher's LSD test. Symbols not sharing the same letter are statistically significant across

640 temperatures at $P < 0.05$. In panels C and D, data are expressed in $\mu g/10^5$ cells ± SEM ($n = 7$-

641 8). Effects of incubation time and temperature were analyzed by Student's t-test at each time

642 point ([*]Significantly different at $P < 0.05$).

643

644 **Figure 2.** Effects of temperature increase (from 26 to 32 °C) and reduction in osmolality

645 (from 330 to 280 mOsm/kg) on the release of PRL_{188} (A) and PRL_{177} (B) from dispersed PRL

646 cells within 105 min. Vertical lines indicate a change in temperature or osmotic conditions.

647 Symbols represent mean percent change PRL release ± SEM ($n = 5$-6). Treatment and time

648 effects were analyzed by two-way ANOVA ([***]Significant at $P < 0.001$; [**]Significant at $P <$

649   0.01) followed by protected Fisher's LSD test. Means with daggers (†) indicate significant

650   differences from parallel controls at each time point ($^†P < 0.05$, $^{††}P < 0.01$).

651

652   **Figure 3**. Effects of temperature and osmolality on the change in volume (A) and NCCV (B)

653   of dispersed PRL cells within 175 min. Vertical lines indicate a change in stimuli. Symbols

654   represent means expressed as percent change from an average cell volume of the first 6 time

655   points ± SEM ($n = 10\text{-}12$) (Fig 3A). The bars represent NCCV during temperature (32 °C,

656   330 mOsm/kg), recovery (26 °C, 330 mOsm/kg), and hyposmotic stimuli (26 °C, 280

657   mOsm/kg) as a % change from the baseline. Data were analyzed by two-way ANOVA

658   followed by protected Fisher's LSD test. Means not sharing the same letter are statistically

659   significant at $P < 0.05$. Means between all stimuli within each treatment not sharing the same

660   lowercase letter and all treatments within each stimulus not sharing the same uppercase letter

661   are significantly different.

662

663   **Figure 4.** Effects of temperature on the mRNA expression of prl_{188} (A), prl_{177} (B), and $trpv4$

664   (C) in RPDs at 24 h and prl_{188} (D), prl_{177} (E), and $trpv4$ (F) in PRL cells at 6h. RPDs and

665   PRL cells were incubated in 330 mOsm/kg media in temperatures ranging from 20 to 32 °C.

666   In panels A-C, data are expressed as fold-change from 20 °C ± SEM ($n = 6\text{-}8$) and analyzed

667   by one-way ANOVA followed by protected Fisher's LSD test. Bars not sharing the same

668   letters are significantly different at $P < 0.05$. In panels D-F, data are expressed as fold-change

669   from the 26 °C group ± SEM ($n = 7\text{-}8$) and analyzed by Student's t-test (*Significant at $P <$

670   0.05).

671

672   **Figure 5.**   Diagram summarizing the cell-volume mediated effects of a rise in temperature

673   or a fall in extracellular osmolality on tilapia PRL_{188} and PRL_{177} release.
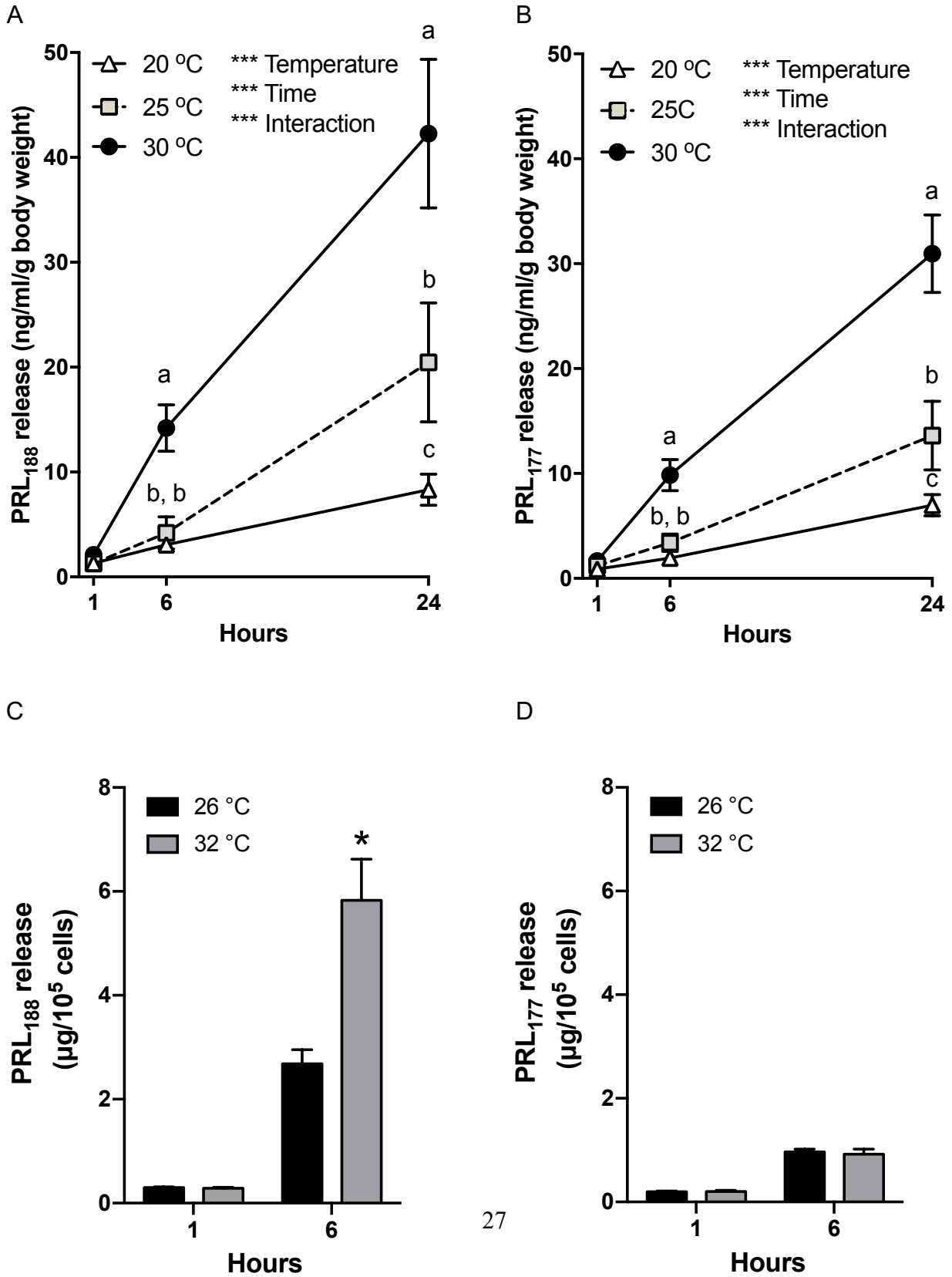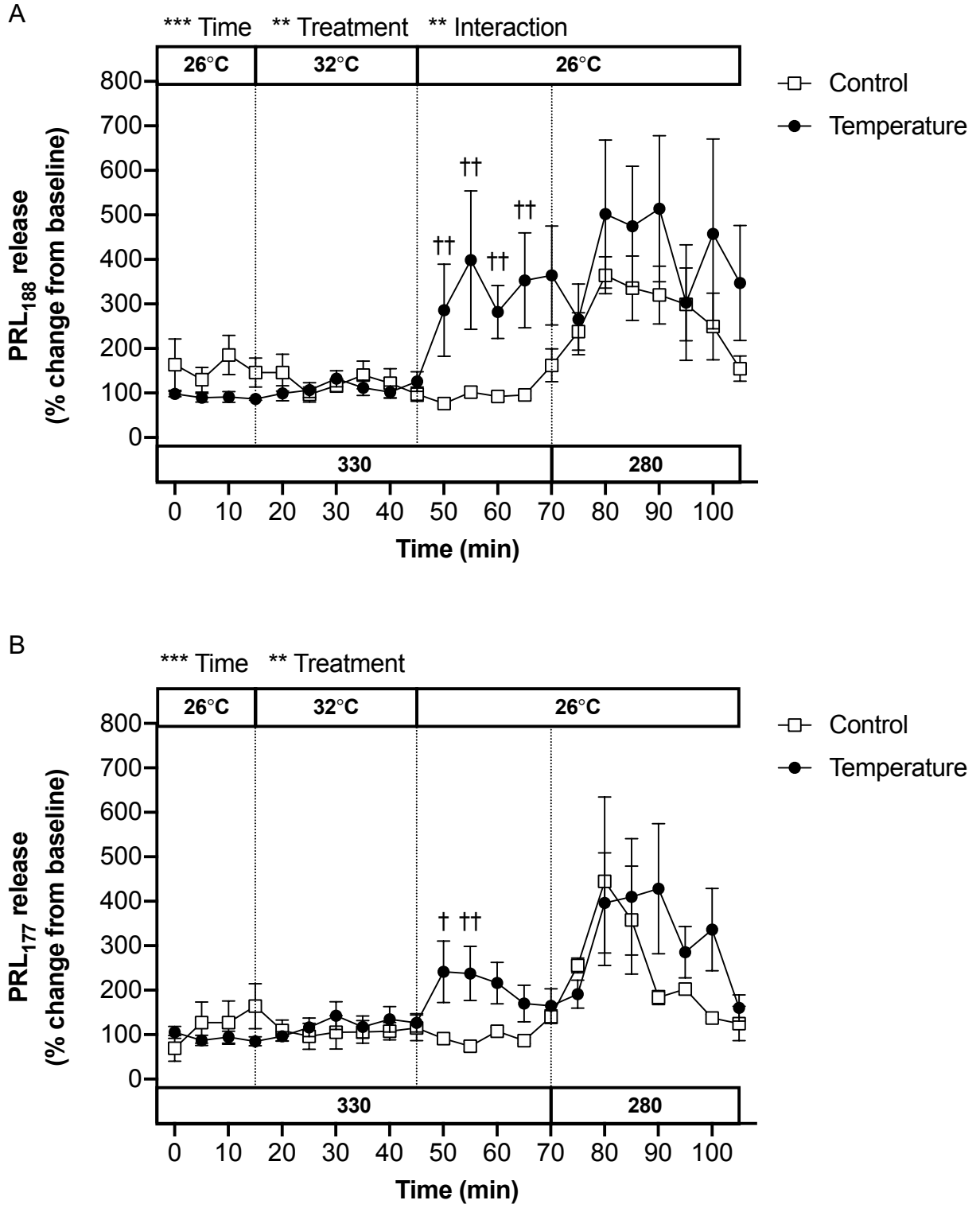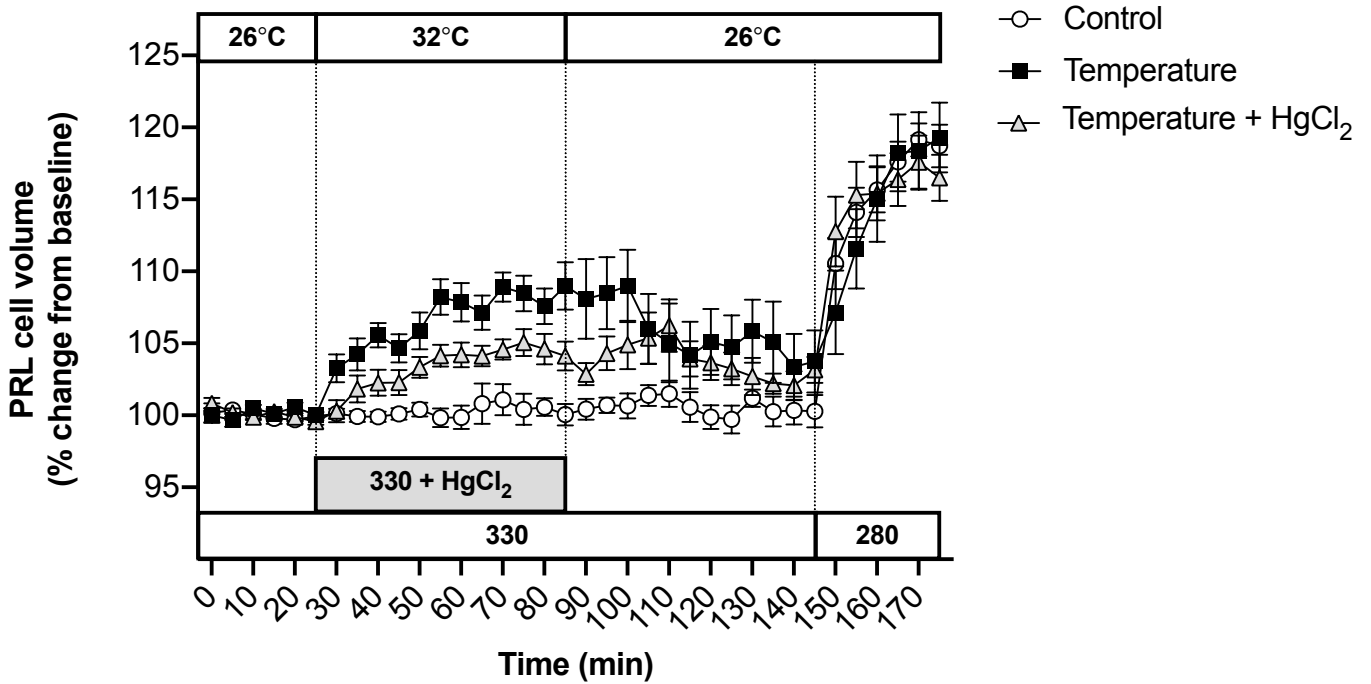
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Figure 1

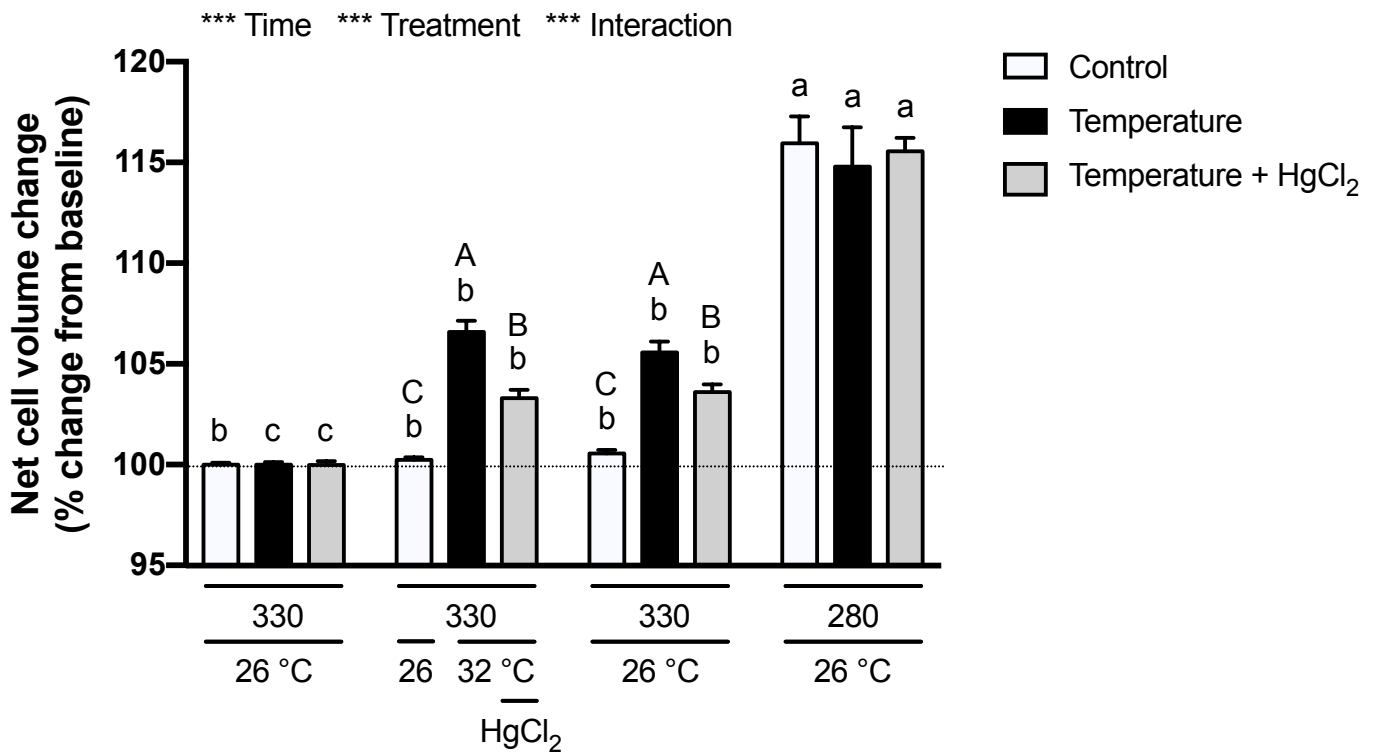
A

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C

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Figure 2

A

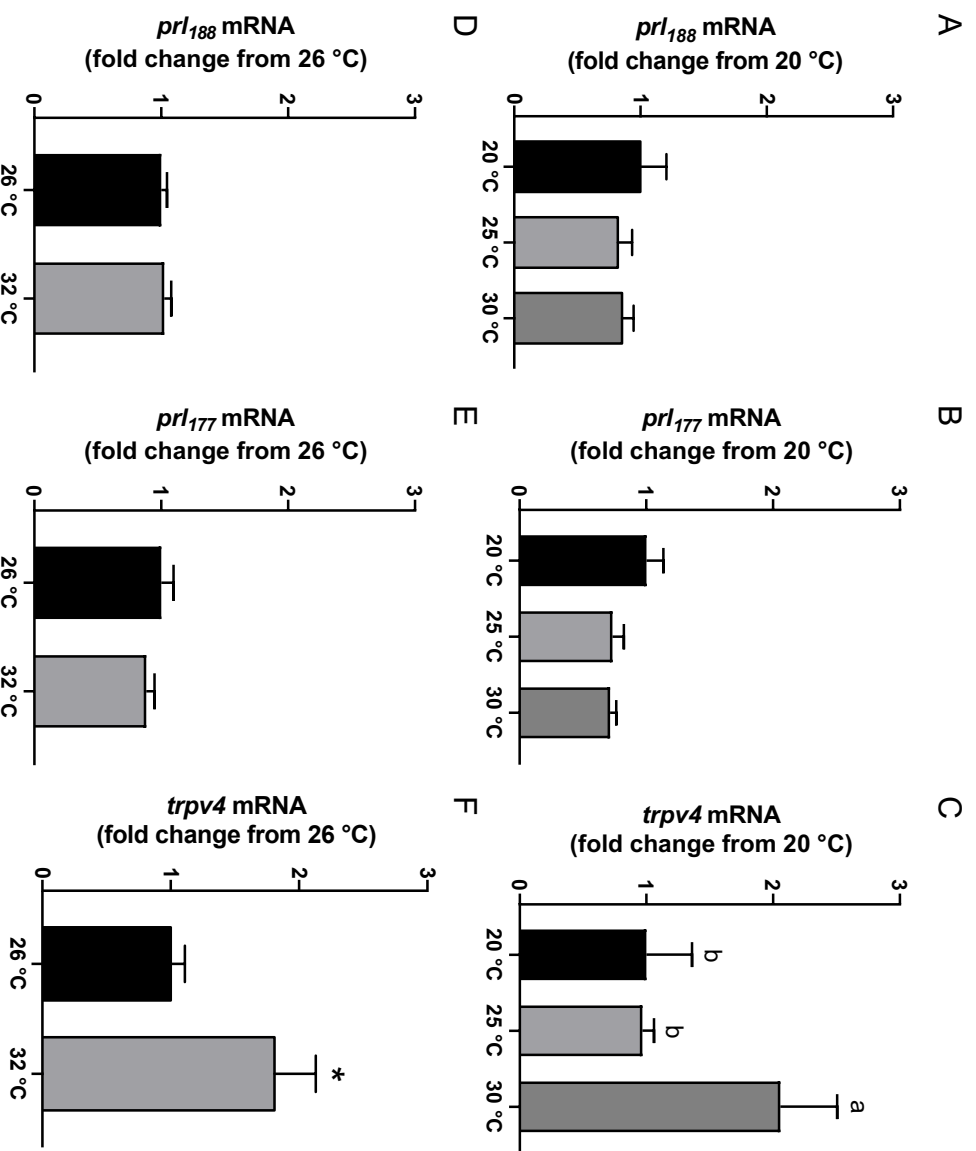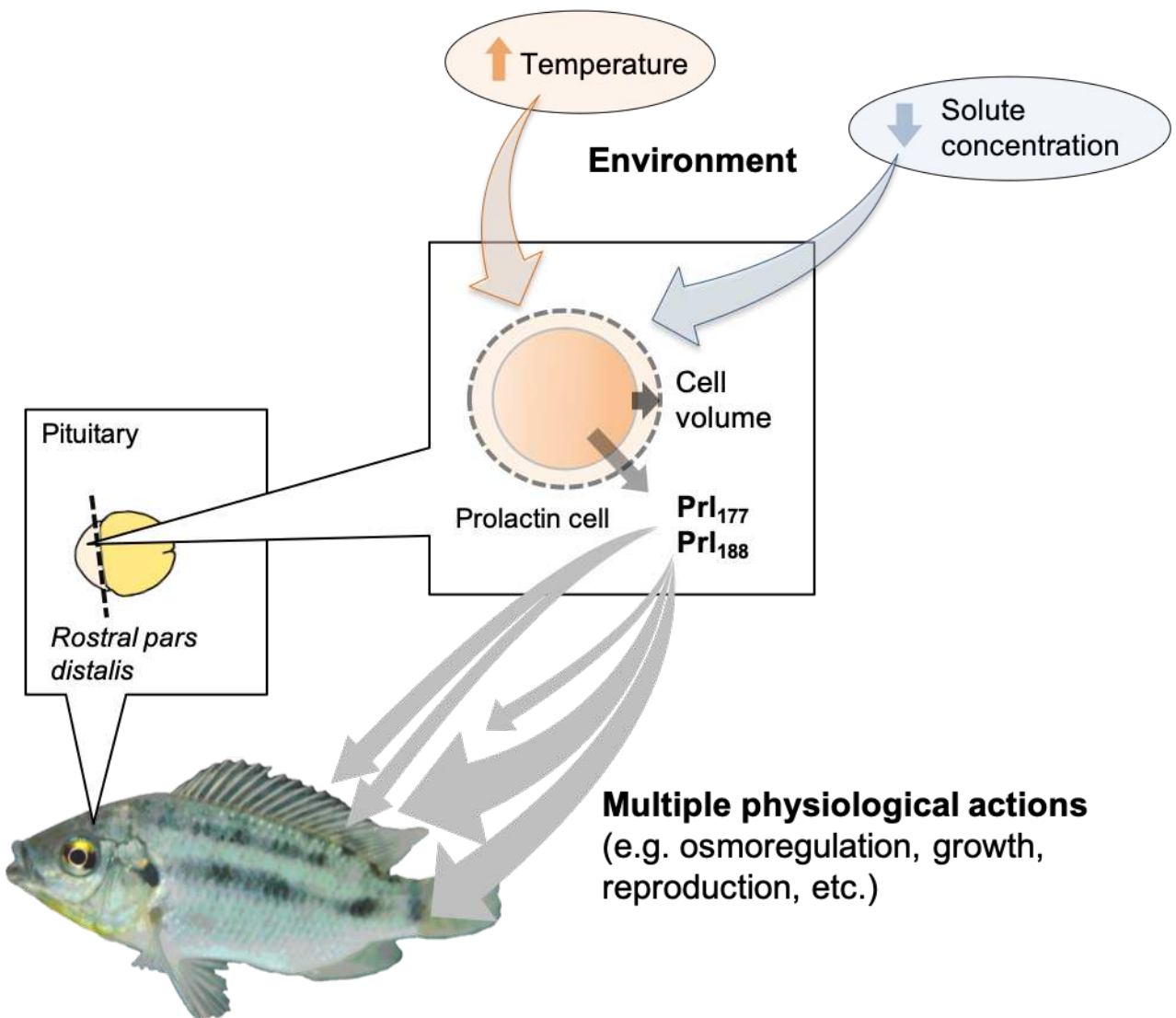B

Figure 3

A

B

Figure 4

30

Figure 5

Supplementary Files

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