Androgen receptors rapidly modulate non-breeding aggression in male and female weakly electric fish (*Gymnotus omarorum*)

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ABSTRACT

The South American weakly electric fish, *Gymnotus omarorum,* displays territorial aggression year-round in both sexes. To examine the role of rapid androgen modulation in non-breeding aggression, we administered acetate cyproterone (CPA), a potent inhibitor of androgen receptors, to both male and females, just before staged agonistic interactions. Wild caught fish were injected with CPA and, 30 min later, paired in intrasexual dyads. We then recorded the agonistic behavior which encompasses both locomotor displays and emission of social electric signals. We found that the CPA had no discernible impact on the levels aggression or the motivation to engage in aggressive behavior for either sex. However, CPA specifically decreased the expression of social electric signals in both males and female dyads. The effect was status-dependent as it only affected subordinate electric behaviour, the emission of brief interruptions in their electric signalling ("offs"). We also identified, in both sexes, the presence of three subtypes of androgen receptors located in pacemaker nucleus, the hindbrain region known for its role in controlling the rate of electric signalling in Gymnotiformes. This study is the first demonstration of a direct and rapid androgen effect mediated *via* androgen receptors on non-breeding aggression. Elucidating the mechanisms involved in non-breeding aggression in this teleost

26   model allows us to better understand potentially conserved or convergent neuroendocrine

27   mechanisms underlying aggression in vertebrates.

INTRODUCTION

Agonistic encounters are a ubiquitous and important feature of animal social behavior (Nelson, 2005; Kennedy, 2022). Such interactions can escalate into aggressive behavior that may include threatening displays as well as physical attacks (Hardy and Briffa, 2013). The neuroendocrine mechanisms regulating animal aggression is a critical area of research because it can provide insight into the evolution and maintenance of social systems. Furthermore, elucidating these mechanisms can help in the development of more effective treatments for behavioral disorders marked by heightened aggression (Flanigan and Russo, 2019).

To achieve this broad understanding of aggression, it is essential to expand the contexts in which animal aggressive behavior is studied. Most research has examined reproductively active males who engage in sexual as well as aggressive behavior. Female agonistic behavior, with the exception of maternal aggression, has been less studied, despite evidence that females also express aggression during agonistic encounters (Duque-Wilckens and Trainor, 2017; Rosvall et al., 2020). Here, we examine territorial aggression during the non-breeding season, when gonads are regressed and thus circulating sexual steroids are low, in both males and females.

Across vertebrates, steroid hormones, such as androgens and estrogens, play a crucial role in regulating aggressive behavior in both sexes (Adkins-Regan, 2013). Steroid hormone receptors can act as transcription factors, altering gene expression and modulating neural circuits underlying aggressive behavior, mostly during the breeding season (Simon & Lu, 2006; but see: Vullioud et al., 2013). This genomic action of steroids occurs over a long timeframe, taking hours to days (Beato et al., 1995; Mangelsdorf et al., 1995). However, steroids can also exert rapid effects (seconds to minutes) on cellular activity, including changes in ion channel activity

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51   and intracellular signaling cascades, that ultimately affect social behavior (Lösel & Wehling,

52   2003; Foradori et al., 2008; Heimovics et al., 2015; Schwartz et al., 2016; Balthazart, 2021).

53   Although aggression is generally more common during the breeding season, ecological

54   pressures can also lead some species to display aggression uncoupled from reproduction

55   (Caldwell et al., 1984; Logan & Wingfield, 1990; Soma et al., 1999; Hau et al., 2004; Batista et

56   al., 2012; Vullioud et al., 2013). However, compared to what is known about breeding

57   aggression relatively little is known about the hormonal regulation of such aggression outside the

58   breeding season. In vertebrates ranging from fish to birds and mammals, non-breeding

59   aggression is expressed when the gonads are regressed and persists in castrated animals

60   (Caldwell et al., 1984; Wingfield, 1994; Jalabert et al., 2015). Nevertheless, steroid hormones

61   appear to play a crucial role in regulating non-breeding aggression (Trainor et al., 2007; Soma et

62   al., 2015; Munley et al., 2018; Silva et al., 2020). The source of active steroid hormones during

63   the non-breeding season has been shown to be the brain (Wingfield et al., 2018, 2019; Quintana

64   et al., 2021). Steroid neurosynthesis can be *de novo* from cholesterol or by local conversion of

65   weak circulating androgenic precursors (e.g., androstenedione or dehydroepiandrosterone) into

66   active androgens and estrogens (e.g., dihydrotestosterone or estradiol) (Diotel et al., 2018). This

67   localized steroid brain production has been postulated to be beneficial, particularly in a non-

68   breeding context, for avoiding the costs associated with high plasma sex steroid levels, such as

69   actions of steroids in peripheral tissues and suppression of the immune system (Casto et al.,

70   2001; Wingfield et al., 2018).

71   It is well documented that estrogens rapidly modulate the expression of non-breeding

72   aggression, as evidenced by studies showing a reduction in aggression after acute inhibition of

73   estrogen synthesis (Soma et al., 2000a, 2000b; Trainor et al., 2007; Jalabert et al., 2015;

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74   Zubizarreta et al., 2020a). However, the role of androgens in this context is less clear. Previous

75   studies investigating the effects of androgens have used chronic treatments, and these have failed

76   to demonstrate significant effects on non-breeding aggression (Sperry et al., 2010; but see:

77   Vullioud et al., 2013). This apparent lack of direct effect by chronic androgen treatments has led

78   some researchers to hypothesize that androgens act only as precursors for estrogen

79   neurosynthesis during the non-breeding season (Soma et al., 2015; Munley et al., 2018).

80   However, androgens may also exert direct, rapid effects, and this possible mechanism should be

81   thoroughly tested.

82   We explored the rapid effects of androgen in non-breeding aggression in a species of

83   weakly electric fish from South American, *Gymnotus omarorum*, a well-studied model that

84   offers several advantages. *G. omarorum* is a seasonal breeder with year-round territorial

85   aggression in both males and females (Black-Cleworth, 1970; Batista et al., 2012; Jalabert et al.,

86   2015; Perrone et al., 2019; Silva et al., 2020; Zubizarreta et al., 2020a). During the non-breeding

87   season, castrated males fight vigorously, with no significant difference from sham-operated

88   controls (Jalabert et al., 2015). Just as in non-breeding models of mammals and birds, acute

89   blockade of estrogen synthesis in *G. omarorum* decreases aggression in both sexes (Jalabert et

90   al., 2015; Zubizarreta et al., 2020a). Moreover, dominant fish express aromatase and androgen

91   receptor transcripts at higher levels than subordinate fish 36 hours after a stable dominance

92   hierarchy is established (Eastman et al., 2020).

93   One advantage of electric fish for studying neuroendocrine mechanisms of behavior is

94   that they exhibit stereotyped and easily quantified electrocommunication signals (Dunlap et al.,

95   2017; Freiler and Smith, 2023). These electric social signals are generated by the electric organ

96   and regulated by a well-described hindbrain nucleus known as the pacemaker nucleus (Bennett,

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97   1971; Tokunaga et al., 1980; Elekes & Szabo, 1981, 1985; Dye & Meyer, 1986; Dunlap et al.,

98   2017). The pacemaker nucleus (PN) fires spontaneously and controls the basal electric organ

99   discharge (EOD) rate in all gymnotiform weakly electric fish. The PN is composed of two types

100   of neurons: electrotonically coupled intrinsic pacemaker cells and projecting relay cells (Bennett,

101   1971; Elekes & Szabo, 1981, 1985; Dye & Meyer, 1986). The basal activity of the PN is

102   modified by pre-pacemaker inputs to produce transient EOD frequency modulations with distinct

103   temporal dynamics that are used in social interactions (Black-Cleworth, 1970; Hopkins, 1972,

104   1974a, 1974b, 1988; Hagedorn & Heiligenberg, 1985; Hagedorn, 1988; Kawasaki &

105   Heiligenberg, 1989; Hupé & Lewis, 2008; Perrone et al., 2009).

106   In *G. omarorum*, social electric signals during non-breeding aggression include a rapid (<

107   50 ms) and transient elevation of the electric organ discharge (EOD) called a "chirp" and a

108   longer temporary (200-1000 ms) interruption of the EOD called an "off" (Fig. 1). In agonistic

109   contexts in *G. omarorum*, these two electric signals are considered submissive signals as they are

110   only emitted by subordinate individuals (Batista et al., 2012). Fish also use the EOD to reflect

111   dominant/subordinate post resolution hierarchy; dominant fish have higher EOD rates than

112   subordinates (Fig. 1; Silva et al., 2013). Hence, aggression can be quantified through the

113   measurement of the EOD and its modulations, as well as the assessment of agonistic locomotor

114   behavior. The rigorous characterization of the agonistic behavior of *G. omarorum* makes it a

115   highly valuable model to trace the underlying neuroendocrine modulation of aggression,

116   allowing a comparative vision, potentially identifying conserved or convergent mechanisms in

117   vertebrates.

118   In this study, we examine the role of rapid androgen actions on non-breeding aggression

119   separately in both males and females. We treated fish with an antagonist of various androgen

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120   receptors and, after 30 min, recorded aggressive behaviors in same-sex dyadic encounters. We

121   found that such treatment exerted specific actions on submissive signaling (offs) without

122   affecting other social signals or locomotor behaviors in both sexes. In addition, we localized

123   androgen receptors in a brain region, the pacemaker nucleus, which is crucial for the expression

124   of social electric signals.

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126   **MATERIALS AND METHODS**

127   **Animals**

128   Experiments were conducted using adult (> 13.5 cm in body length and > 10.1g in body

129   weight) male and female *Gymnotus omarorum* captured from Laguna del Sauce, Maldonado,

130   Uruguay (34°51′S, 55°07′W) using the method described by Silva et al., (2003). Fish were

131   housed in 500 L outdoor tanks, with a maximum of nine fish per tank. These housing conditions

132   were established in previous studies conducted in our lab (Batista et al., 2012; Jalabert et al.,

133   2015; Quintana et al., 2016; Perrone et al., 2019; Zubizarreta et al., 2020a). All fish were kept in

134   individual compartments within the tanks that allowed them to perceive their neighbors but

135   prevented any physical interaction. The tanks were maintained with natural winter photoperiod

136   and temperature, with a conductivity range of 100 to 200 µS/cm. Aquatic plants were added to

137   the tanks to provide a natural habitat, and each fish had its own shelter. The fish were fed twice a

138   week with *Tubifex tubifex*. Fish were kept under these partitioned housing conditions for 2-4

139   weeks to prevent agonistic experiences (Hsu et al., 2006). Neighboring fish were not used in

140   same behavioral or molecular experiment. All experiments were carried out during the non-

141   breeding season (June–September).

142   **Agonistic behavior experiments**

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143   Because *G. omarorum* is a nocturnal species in its natural environment (Migliaro et al.,

144   2018), we staged the agonistic encounters at night under infrared illumination. We used a gate

145   protocol with two fish separated by a glass gate. This same protocol has been used in (Batista et

146   al., 2012; Jalabert et al., 2015; Perrone and Silva, 2018; Zubizarreta et al., 2020a). Fish were

147   placed in test tanks ($55 \times 40 \times 25$ cm) 4 h prior to the encounter to allow them to acclimate (Fig.

148   1). Both individuals of the dyads received the same treatment: either an acute peritoneal injection

149   of cyproterone acetate (CPA, 20 µl per gram body weight; Sigma-Aldrich) or vehicle (corn oil;

150   Sigma-Aldrich) 30 min before the gate was lifted. This treatment blocks the rapid effects of

151   androgens on androgen receptors. CPA, unlike other androgen receptor antagonists, fully blocks

152   androgen receptors (Crawford et al., 2018) and has been reported to be more effective than other

153   androgen receptor antagonists in teleost brains (Wells and Van Der Kraak, 2000). In addition,

154   CPA has been demonstrated to effectively block the androgen receptors which acts through rapid

155   mechanisms in other fish species (Remage-Healey and Bass, 2007). Finally, CPA blocks the

156   actions of chronic testosterone treatment on the electric signaling of *G. omarorum* (Zubizarreta et

157   al., 2020a), indicating its efficacy in our study species.

158   After lifting the gate, evaluation and conflict phases occurred, and the fish were allowed

159   to interact until 10 min after the conflict resolution. We considered a conflict resolved when the

160   subordinate fish retreats three times without attacking back, as has been previously reported

161   (Batista et al., 2012; Jalabert et al. 2015; Perrone and Silva, 2018; Perrone et al., 2019;

162   Zubizarreta et al., 2020a). Dyads that took more than 25 min to resolve conflict were excluded

163   from the study. The locomotor behaviors were recorded on video and social electric signals were

164   recorded by two pairs of electrodes placed orthogonally in the tank. We quantified, latency to the

165   first attack, duration of the conflict, attacks from both dominant and subordinate fish during the

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166 conflict and post-resolution phases, as well as the emission of offs, chirps and EOD basal rate.

167 These variables of agonistic behavior has been previously described (Black-Cleworth, 1970;

168 Batista et al., 2012).

169 The individuals used for agonistic encounters measured 10.1-67.2 g in body mass and

170 13.5- 28.2 cm in body length. We formed male-male and female-female dyads with a body

171 weight difference ranging from 5 to 20%. There were no significant differences in body weight

172 between treated dyads and controls in either sex (control males: 25.09 ± 13.11 g; control

173 females: 23.25 ± 12.16 g; CPA males: 20.55 ± 13.34 g; CPA females: 19.18 ± 13.35g; mean $\pm$

174 SD), in body length (control males: 19.42 ± 3.76 g; control females: 17.78 ± 2.89 g; CPA males:

175 17.41 ± 3.40 g; CPA females: 17.36 ± 3.43 g; mean $\pm$ SD) nor in the weight difference between

176 the big and the small fish in the dyad (control males: $13,38 \pm 3,95$ %; control females: $15,39 \pm$

177 $8,12$ %; CPA males: $15,00 \pm 5,55$ %; CPA females: $17,40 \pm 5,12$%; mean $\pm$ SD). Furthermore,

178 weight between dominants and subordinates from different dyads overlap (CPA male dominant:

179 $20,13 \pm 12,07$ g; CPA male subordinate: $19,22 \pm 16,02$ g; CPA female dominant: $25,21 \pm 15,16$

180 g; CPA female subordinate: $24,11 \pm 14,87$ g; mean $\pm$ SD). Taking into consideration all these

181 factors, we rule out size as a variable that might explain the effect of the treatment.

182 **Androgen receptor expression in the brain**

183 The pacemaker nucleus (PN) and its bordering regions were assessed by PCR to detect

184 the expression of 2 classical androgen receptors: androgen receptor alpha (ARα) and androgen

185 receptor beta (ARβ), and a membrane androgen receptor that acts through rapid mechanism (Zip-

186 9). We employed three male and three female specimens, which did not have any social

187 interaction prior to sacrifice. The PN was identified following Pouso et al., (2017) and dissected

188 using the Palkovits punch (300 um) technique (Palkovits, 1973). The tissue was weighed (0.33

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189 mg), and the total RNA was extracted using the RNAqueous™-Micro Total RNA Isolation kit

190 (Ambion: AM1931) following the manufacturer's instructions. The RNA was reverse-transcribed

191 into cDNA using the SuperScript™ III First-Strand Synthesis System (Thermo Fisher). Primers

192 for ARα, ARβ and Zip-9 were designed from the *G. omarorum* transcriptome (Eastman et al.,

193 2020) using Primer3 (http://primer3.ut.ee/). We used the following primers: ARα (sense, 5-

194 AGGACCTTGGACATACTGGC-3 and antisense, 5-GGCGCAAACATGATACTCGA-3;

195 expected product size: 148); ARβ (sense, 5-AAAACAGCAAAACGGAGCAG-3 and antisense,

196 5-ACCACTGTGTGCCACTTCTG-3; expected product size: 100); Zip-9 (sense, 5-

197 AGCTGCACACTGGTTTGAGA-3 and antisense, 5-GCTCGAAAATCACCACCACG-3;

198 expected product size: 100). We conducted PCR using a final volume of 25 µL, consisting of 5

199 µL of cDNA sample, 0.75 µL of each the reverse and forward primers (0.5 µM), 0.15 µL of Taq

200 Polymerase (Invitrogen), 0.5 µL of dNTP mix, 2.5 µL PCR Buffer, 0.75 µL of MgCl2 and

201 completed final volume with H_2O. The cycle program was 94°C for 3 min, followed by 35

202 cycles of 94°C for 45 s, 60°C for 30 s and 72°C for 45 s; followed by a final extension of 72°C

203 for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose gel with SYBR™

204 Safe DNA Gel Stain and Buffer TAE 1X. We used the GeneRuler 50 bp DNA Ladder (Thermo

205 Scientific) to estimate product size. A negative control containing 5 µL milli-Q water instead of

206 sample was conducted for every gene. Gene presence was detected using iBright CL1500

207 Imaging System (Invitrogen).

208 **Statistics**

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210 In our study, the effect of cyproterone acetate (CPA) on motor and electric measures was

211 examined using the Mann-Whitney U test. This non-parametric statistical test was chosen due to

212 the observed non-conformity of the data with a Gaussian distribution, as confirmed by the

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213 Anderson-Darling test. Additionally, the correlation between the frequency of post-resolution

214 attacks and post-resolution offs was assessed using the non-parametric Spearman test.

215 Furthermore, the effect of CPA on the suppression of post-resolution offs compared to controls

216 was evaluated using Fisher's exact test. The data were presented as mean ± standard error of the

217 mean (SEM) and analyzed using GraphPad Prism software (version 8.4.3). Statistical

218 significance was considered at a p-value < 0.05.

219 **Ethics approval**

220 All animal procedures conducted in this study adhered to the guidelines and

221 recommendations set forth by the Uruguayan National Commission of Animal Experimentation

222 (CNEA). The housing and manipulation of animals for these experiments at the Instituto de

223 Investigaciones Biológicas Clemente Estable were approved by La Comisión de Ética en el Uso

224 de Animales (CEUA IIBCE 001/02/2012 - 003/10/2020).

225

226 **RESULTS**

227 **Rapid androgenic actions on agonistic behavior: motor patterns**

228 Acute treatment with CPA did not significantly affect any motor parameters analyzed in

229 either male or female subjects (Figs. 2, 3). Neither the latency to the first attack (females: p =

230 0.80; males: p = 0.48) nor the conflict duration (females: p = 0.45; males: p = 0.89) in treated

231 dyads was significantly different from that of the control group. The frequency of attacks by

232 dominant fish during the conflict was similar to that of controls in both sexes (females: p = 0.92;

233 males: p = 0.83), as was the frequency of attacks by the subordinate during conflict (females: p =

234 0.31; males: p = 0.85). There was no significant difference in the frequency of attacks emitted by

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235   dominants during the post-resolution phase (females: p=0.69; males: p=0.80). Subordinates did

236   not emit attacks during this phase in any group.

237   **Rapid androgenic actions on agonistic behavior: emission of electric submissive signals**

238   Both control and CPA-treated dyads emitted the three electric submissive signals (off,

239   chirps and EOD rank) in both sexes (Figs. 4, 5). During the conflict phase, there were no

240   significant differences between CPA-treated fish and controls in the emission of off (females: p =

241   0.84; males: p = 0.58) or chirps (females: p = 0.67; males: p = 0.14). However, during the post-

242   resolution phase, CPA-treated fish of both sexes displayed an off rate that was significantly

243   lower than control fish (Figs. 4C, 5C; females: p = 0.03; males: p = 0.03). Moreover, the number

244   of post-resolution offs almost disappeared in CPA-treated individuals compared to controls, as

245   depicted by rates valued at 0 (Fig. 6C, 6D; females: p = 0.03; males p = 0.0098). During this

246   phase, there were no significant effects of treatment on chirps (females: p = 0.73; males: p >

247   0.99) or in the post-resolution EOD frequency rank between dominants and subordinates

248   (females control: p = 0.002; females CPA: p = 0.04; males control: p = 0.03; males CPA: p =

249   0.047).

250   **Effect of CPA on post-resolution attack-off correlation**

251   In control fish of both sexes, the expression of post-resolution attacks by dominant fish

252   correlated positively with the emission of offs by subordinate fish during the post-resolution

253   phase (Fig. 6A, 6B; males: $r^2 = 0.49$, p = 0.04; females: $r^2 = 0.55$, p = 0.006). This correlation

254   between post-resolution attacks and offs disappeared in CPA-treated dyads (Fig. 6A, 6B; males:

255   $r^2 = 0.008$, p = 0.99; females: $r^2 = 0.006$, p = 0.85).

256   **Androgen receptor expression in the PN**

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257   Three androgen receptor types (ARα, ARβ and Zip-9) were present in the PN and its

258   close surroundings in both sexes (Fig 7). There were a single band with the expected size

259   (expected sizes: ARα: 148 bp, ARβ: 100 bp and Zip-9 100 bp).

260

261   **DISCUSSION**

262   Our study on electric fish is the first in vertebrates to demonstrate that androgens can

263   exert rapid effects during non-breeding aggression. We found three novel results: (1) androgens

264   have fast acting effects on the expression of social signals, (2) this effect is status-dependent and

265   (3) androgen receptors are located in the brain region that control the expression of these social

266   signals. Specifically, we show that, in wild-caught electric fish, androgen receptors regulate the

267   expression of social signals in subordinate fish following the resolution of conflict and the

268   establishment of hierarchy.

269   Subordinate behavior and its underlying mechanisms have been relatively understudied

270   (Reddon et al., 2021). However, examining this behavior and its hormonal modulation is

271   important for broader comprehension of agonistic encounters and, ultimately, for understanding

272   the evolution of social communication (Reddon et al., 2021; Freiler & Smith, 2023). Our

273   experimental model has several advantages for investigating the hormonal modulation of non-

274   breeding aggressive behavior. All fish were collected from the same natural habitat and had

275   minimal period in captivity. In our protocol, fish competed for space as the limited resource,

276   which is relevant to the natural behavior of this species. Our well-established gate protocol

277   permitted free movement of both contenders throughout the experiment and allowed us to

278   observe the locomotor and electrical signaling behavior of both dominants and subordinates.

279   **Androgen receptors mediate effects on agonistic behavior**

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280   Non-breeding territorial agonistic behavior has been characterized extensively in *G.*

281   *omarorum*. In intrasexual encounters, males and females show equivalent levels of aggression

282   (Batista et al., 2012; Quintana et al., 2016). The primary predictor of dominance is body size,

283   with heavier animals more likely becoming dominants (Batista et al., 2012). Winners control

284   territories for at least 36 hours, with subordinate fish relegated to the periphery of the tank

285   (Perrone et al., 2019). During these territorial conflicts, the number of attacks initiated by the

286   dominant and subordinate fish is positively correlated, indicating an aggressive escalation

287   (Zubizarreta et al., 2015). Fish that lose the conflict broadcast their subordination through motor

288   and electrocommunication behaviors, and we found that androgens contribute to the post-

289   resolution phase of this dynamic. The subordinate fish ends the fight by retreating without

290   counterattacking. During conflict phase subordinate fish may also emit off and occasionally

291   chirps. If the dominant fish persists in attacking, the subordinate fish continues to produce offs

292   and increases its chirps. Additionally, during the post-resolution phase, subordinates signal their

293   rank with a lower EOD rate compared to the dominant (Batista et al., 2012).

294   We found that the initial stages of the conflict were unaffected by CPA treatment to both

295   individuals in both male and female dyads. The motivation to fight (latency to first attack), the

296   aggressive levels of dominants and subordinates during the contest, and the dominant fish's

297   continued aggression towards the subordinate after conflict resolution were similar in CPA-

298   treated dyads and control-injected dyads (Figs. 2, 3). Similarly, the timing of subordination

299   (contest duration; Figs. 2B, 3B), the emission rate of chirps, and the establishment of an EOD

300   rank were all unaffected (Figs. 4, 5). A previous study from our lab also found the lack of effect

301   on CPA on the dynamics of non-breeding aggression in female dyads (Zubizarreta et al., 2020a).

302   Unlike in the present study, researchers found no effect of CPA on the production of offs,

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303 however they quantified signals globally across the whole encounter, without discriminating pre

304 and post resolutions phases, and their dose of CPA was half of the one used in the present study.

305 Both these factors may have contributed to the apparent lack of CPA effect in the previous study.

306 In the present study, the emission of offs during the post-resolution phase in both sexes was

307 dramatically reduced in CPA-treated individuals, decreasing practically to zero (Figs. 4C, 5C,

308 6C, 6D).

309 Status dependency of androgen-receptor mediated effects

310 　　　　Only subordinates and not dominants changed their electrical signaling in response to

311 CPA injections, indicating that dominant and subordinate fish use androgen receptors in different

312 ways to regulate electrogeneration activity. This finding aligns with previous research in white

313 footed mice, where androgens rapidly modulate the expression of social signals in subordinates

314 but not in dominants (Fuxjager et al., 2015). In *G. omarorum*, a rapid status-dependent hormonal

315 response was also demonstrated previously in studies of AVT effect on aggression (Perrone and

316 Silva, 2018). AVT treatment caused only subordinates to increase their production of offs. This

317 similar status-dependent response to experimental AVT and AR manipulation suggests that AR

318 and AVT pathways might converge on the same intracellular processes that regulate pacemaker

319 excitability. However, unlike AR, AVT also affects chirps and EOD rank, indicating that these

320 pathways can also be separable in neurons underlying electric signaling. Rapid, status-dependent

321 effects of both AR and AVT have also been demonstrated in the social signaling of vocal fish

322 (Goodson and Bass, 2000; Remage-Healey and Bass, 2006). In addition, AVT promotes a rapid

323 brain production of androgens in frogs (Do-Rego et al., 2006). In *G. omarorum* AVT positive

324 fibers are found near the PN (Pouso et al., 2017). This suggests a potential interaction between

325 AVT and androgen receptors in the generation of offs in subordinate *G. omarorum*. Thus, future

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326 studies examining interactions between AVT and fast acting AR in fish may be fruitful for

327 understanding status-dependent hormonal regulation of social behavior.

Androgen receptor expression

329 We found the expression of three subtypes of androgen receptors in the PN (Fig. 7).

330 Among these receptors, two are considered classical androgen receptors (ARα and ARβ). Many

331 teleost fish have two paralogs of the androgen receptor gene (ARα and ARβ) that are products of

332 an early whole genome duplication in teleost evolution (Glasauer and Neuhauss, 2014) (the

333 mammalian AR is likely homologous to ARβ in fish; Harbott et al., 2007). In other teleosts, ARα

334 and ARβ can have different effects in physiology and behavior (Alward et al., 2020; Hoadley et

335 al., 2022). The well documented rapid actions of androgen receptors in fish can be mediated by

336 either of these classical receptors translocated to the plasma membrane as has been shown in

337 other vertebrates (Foradori et al., 2008; Wilkenfeld et al., 2018). In the PN of *G. omarorum,* we

338 also found the expression of a third, non-conventional androgen receptor, Zip-9. Zip-9 is a

339 membrane G-protein-coupled receptor that binds to androgens and promotes zinc influx and

340 rapid signaling (Berg et al., 2014; Thomas et al., 2018). Zip-9's role in social behavior has not

341 been explored yet, but our localization of this receptor in the PN raises the possibility that it

342 might contribute to social electric signaling in *G. omarorum*. In *G. omarorum*, offs are produced

343 by a GABAergic input into the PN (Comas et al., 2019), and in other systems, GABA release

344 and GABA receptors are modulated by zinc (Xie & Smart, 1993; Wang et al., 2001). Given that

345 Zip-9 is a membrane androgen receptor that promotes zinc influx, it is a promising candidate to

346 modulate the production of offs.

Similar effects of androgenic effects on behavior in males and females

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348   Males and females responded similarly to androgen receptor blockade, and all three AR

349   subtypes are expressed in the PN of both males and females. This similarity between sexes

350   agrees with reports showing that *G. omarorum* has no sexual difference in non-breeding

351   aggressive locomotor or electric displays (Batista et al., 2012; Quintana et al., 2016) or in the

352   estrogenic modulation of aggression (Silva et al., 2020). Inhibition of estrogen synthesis

353   decreases the motivation to fight equally in males and females (Jalabert et al., 2015; Zubizarreta

354   et al., 2020a). Field observations show that, in the non-breeding season, both males and females

355   guard territories of the same size (Zubizarreta et al., 2020b), and this similarity in territorial

356   behavior may explain the sexual monomorphism in non-breeding aggression and its hormonal

357   regulation. Moreover, both non-breeding males and females have androgens both in circulation

358   and in the brain, making them available to exert rapid actions (Zubizarreta and Jalabert et al.,

359   unpublished results). Interestingly, androgens affecting non-breeding aggression have been

360   shown to be non-gonadal, as castrated males display aggression indistinguishable from controls

361   (Jalabert et al., 2015).

362   **Separate rapid effects of androgen and estrogen on aggression**

363   In the present work, we found that androgen receptors rapidly modulate submissive

364   signals without affecting the aggressiveness of the conflict or the establishment of the dominance

365   hierarchy in both sexes. This lack of androgenic effect on conflict contrasts with the effects of

366   estrogens on non-breeding aggressive behavior in male (Jalabert et al., 2015) and female *G.*

367   *omarorum* (Zubizarreta et al., 2020a). This action of estrogens as rapid modulators of non-

368   breeding aggression in *G. omarorum* aligns with similar observations in mammals and birds

369   (Heimovics et al., 2015). Based on these results with estrogens, researchers have proposed that

370   androgens may act as hormonal precursors for local estrogenic brain synthesis to modulate non-

17

371 breeding aggression (Soma et al., 2015; Munley et al., 2018). As precursors to estrogen,

372 androgens can be synthesized in the periphery (Soma & Wingfield, 2001; Scotti et al., 2008;

373 Rendon et al., 2015; Heimovics et al., 2016); or even locally in certain brain regions (Pradhan et

374 al., 2010; Diotel et al., 2018). But androgens in the brain might also have a direct action (de

375 Bournonville et al., 2020), binding to their own receptors and causing fast modulations in social

376 signaling (Remage-Healey & Bass, 2004; Fernández-Vargas, 2017). Our results in *G. omarorum*

377 are consistent with the hypothesis that androgens can act in two distinct ways to modulate non-

378 breeding agonistic behavior: 1) acting as precursors for rapid brain estrogen synthesis, crucially

379 influencing the initial phases of agonistic encounters (Jalabert et al., 2015; Zubizarreta et al.,

380 2020a) and 2) binding to androgen receptors to regulate the expression of submissive signals in a

381 status-dependent manner.

382 **Possible neural mechanisms underlying rapid androgen receptor-mediated effects on**

383 **aggression**

384   Among gymnotiform electric fish, social electric signals are produced by a conserved

385 neural pathway. Neurons in prepacemaker nuclei project to the PN to modulate its basal

386 discharge rate and produce distinct transient social signals (Kawasaki and Heiligenberg, 1989;

387 Zupanc and Maler, 1997; Metzner, 1999; Dunlap et al., 2017). Our localization of three AR types

388 in the PN indicate that it is a likely site of the behavioral action of CPA, however the

389 prepacemaker nuclei might also be an additional target. The specific type of EOD modulations

390 (offs, chirps and EOD rate rank) depends on which PN cell types and which the neurotransmitter

391 receptors are activated (Kawasaki and Heiligenberg, 1989, 1990; Spiro, 1997; Quintana et al.,

392 2011, 2014; Borde et al., 2020).

18

393   Our present study demonstrating that AR blockade affects offs but not chirps and EOD

394   rate rank is consistent with the notion that these three submissive social signals are generated

395   through separable neural mechanisms (Comas et al., 2019). In *G. omarorum*, offs are produced

396   *via* GABA receptors on pacemaker cells, which cause brief interruptions in PN firing (Comas et

397   al., 2019). Given the specific effect of CPA on offs, we suggest that ARs exert their effect by

398   enhancing GABA-mediated hyperpolarization in pacemaker cells. The rapid effects of CPA,

399   occurring only 30 min after injection, indicates that the effect of AR blockade is probably not

400   genomic and, instead, mediated through the direct action of membrane or intracellular AR

401   receptors on ion currents (Foradori et al., 2008; Wilkenfeld et al., 2018). Such rapid, androgen-

402   induced hyperpolarization of cells has been reported in mammalian endothelial cells, in this case,

403   *via* an enhancement of calcium-dependent K+ currents (Tep-areenan et al., 2002; Unemoto et al.,

404   2007; Perusquía and Stallone, 2010; Ruamyod et al., 2017). Nevertheless, this possible

405   mechanism will need to be tested in future experiments in *G. omarorum*.

406

Conclusions

408   Androgen receptor blockage rapidly modulates the generation of social electric signals during

409   non-breeding agonistic encounters. This effect is consistent across both sexes, dependent on

410   social status, and likely mediated through androgen receptors located in the pacemaker nucleus

411   of the brainstem. Combined with previous studies, these findings indicate that androgens rapidly

412   regulate non-breeding aggression directly by binding to androgen receptors and indirectly by

413   serving as a precursor in aromatase-mediated estrogenic pathways.

414

Figure legends

416   Figure 1: Agonistic behavior of *G. omarorum*. The illustration depicts the three stages of non-

417   breeding territorial behavior and the social electric signals emitted throughout. Intrasexual dyadic

418   interactions are carried out in a gate protocol. The dyad displays a first phase of evaluation once

419   the gate is lifted. The first attack signals the beginning of the aggressive phase, which ends when

420   one contender subordinates retreating three times without attacking back. This subordinate

421   animal may also produce social electric signals, most likely offs, which are interruptions in the

422   emission of the electric organ discharge. Once the conflict is resolved, the dominant patrols his

423   space and may continue attacking the subordinate fish. The subordinate continues to emit offs,

424   and may also emit chirps, an additional subordination signal. During the post resolution phase,

425   dominants discharge at higher frequency than subordinates, indicating a rate rank. In the

426   experiment of behavioral pharmacology treatment and control fish were injected 30 minutes

427   before the lifting of the gate. Color coding: purple indicates the large and eventual dominant fish;

428   green indicates the small and eventually subordinate fish.

429

430   Figure 2: Effect of CPA on male aggressive behavior (n controls = 9; n CPA = 8). (A) Latency to

431   first attack. (B) Contest duration. (C) Contest attack rate from dominant fish and (D) from

432   subordinate. (E) Post-resolution attack rate by the dominant fish. Controls are depicted as black

433   circles, while CPA treated individuals with red. Data are presented as mean ± SEM.

434

435   Figure 3: Effect of CPA on female aggressive behavior (n controls = 15; n CPA = 9). (A)

436   Latency to first attack. (B) Contest duration. (C) Contest attack rate from dominant fish and (D)

437   from subordinate. (E) Post-resolution attack rate by the dominant fish. Controls are depicted as

438   black circles, while CPA treated individuals with red. Data are presented as mean ± SEM.

439

440   Figure 4: Effect of CPA on male submissive electric signals (for controls, n = 9; for CPA, n = 8).

441   Emissions of (A) offs during contest. (B) chirps during contest. (C) offs during post-resolution

442   (D) chirps during post-resolution from subordinate. (E) EOD rank between dominant and

443   subordinates during the pre-conflict and the post-resolution phases, filled circles represent

444   dominants and open circles subordinate. Controls are depicted as black circles, while CPA

445   treated individuals with red. Data are presented as mean ± SEM. Asterisks represent significant

446   differences (* = $p < 0.05$).

447

448   Figure 5: Effect of CPA on female submissive electric signals (for controls, n = 9; for CPA, n =

449   8). (A) Emission of off during contest. (B) Emission of chirps during contest. (C) Emission of

450   offs during post-resolution (D) Emission of chirps during post-resolution from subordinate. (E)

451   EOD rank between dominant and subordinates during the pre-conflict and the post-resolution

452   phases, filled circles represent dominants and open circles subordinate. Controls are depicted in

453   black, while CPA treated individuals with red. Data are presented as mean ± SEM. Mann–

454   Whitney U-test was performed. Asterisks represent significant differences (* = $p < 0.05$; ** = p

455   < 0.01).

456

457   Figure 6: Effect CPA on post-resolution offs (A) Relationship between post-resolution attacks

458   initiated by dominant individuals and the emission of post-resolution offs by subordinates. (B)

459   Effect of CPA on the absence of post-resolution offs, the percentage of dyads in which the

460   subordinate did not express offs during the post-resolution phase in shown. Controls are depicted

461 in black, while CPA-treated individuals are indicated in red. Asterisks represent statistically

462 significant differences (* = $p < 0.05$; ** = $p < 0.01$).

463

464 Figure 7: Gene expression of androgen receptors in the PN of males and females. (A) Androgen

465 receptor alpha; (B) Androgen receptor beta; (C) Androgen membrane receptor, Zip-9. In addition

466 to the ladder, each other lane corresponds to either a male (♂), a female (♀) fish or negative

467 control (-)

468 **Acknowledgements**

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Figure 1:

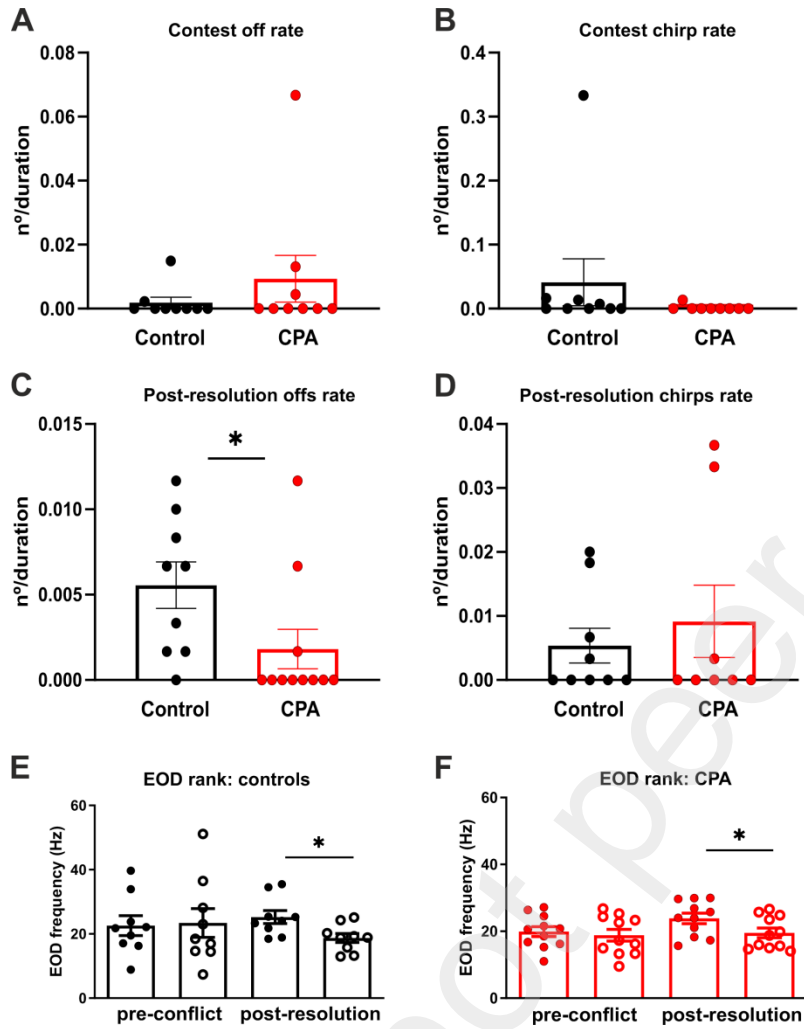Agonistic Behavior *of G. omarorum*

23

486   **Figure 2:**
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489 **Figure 3:**
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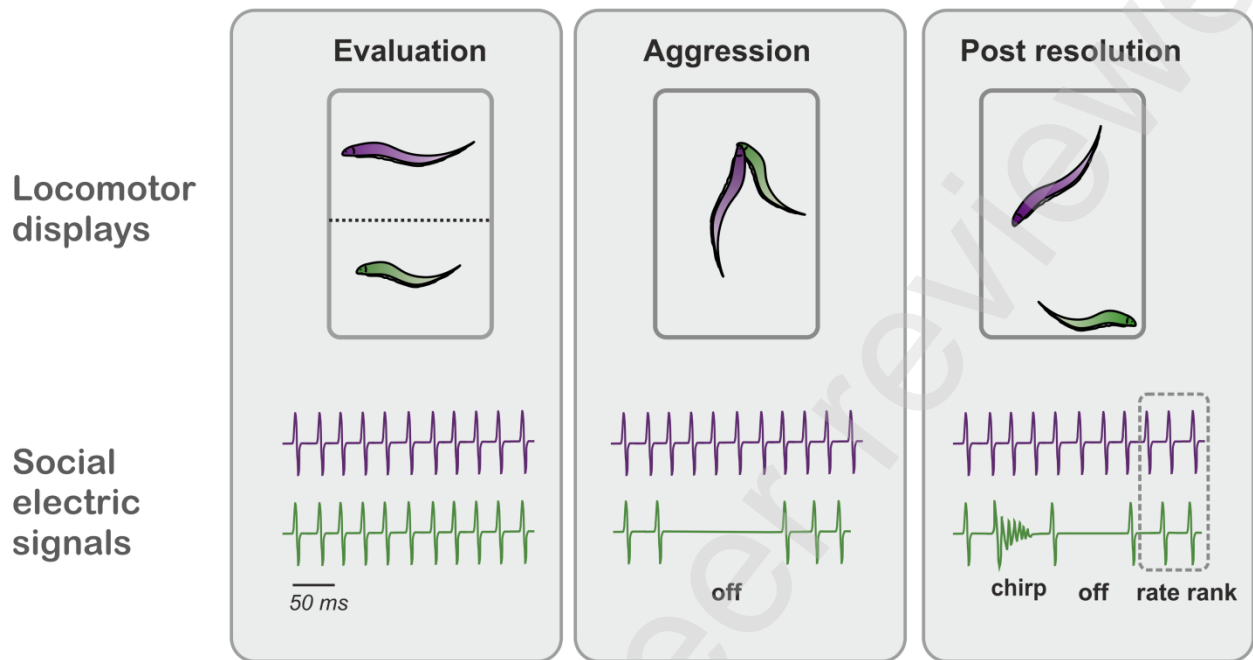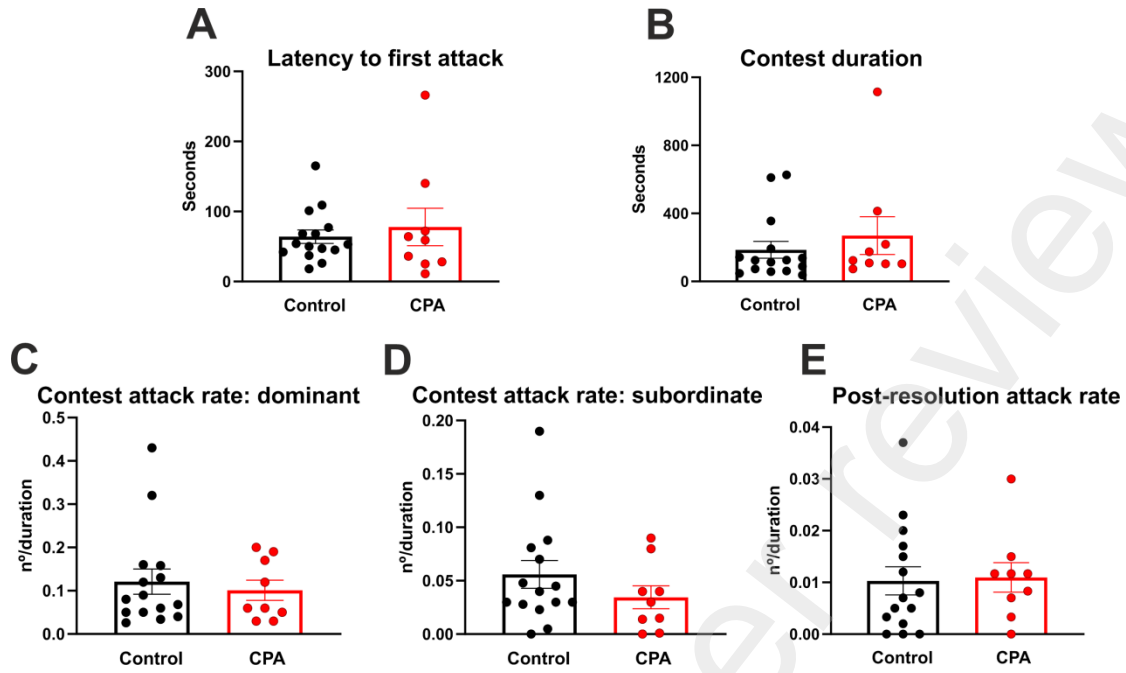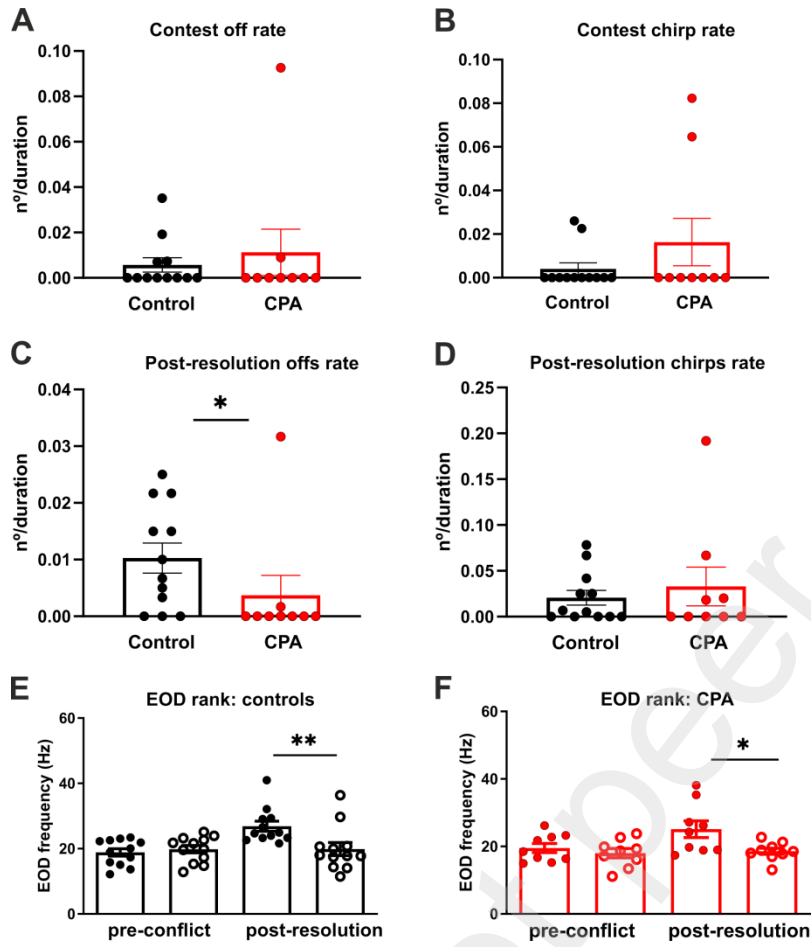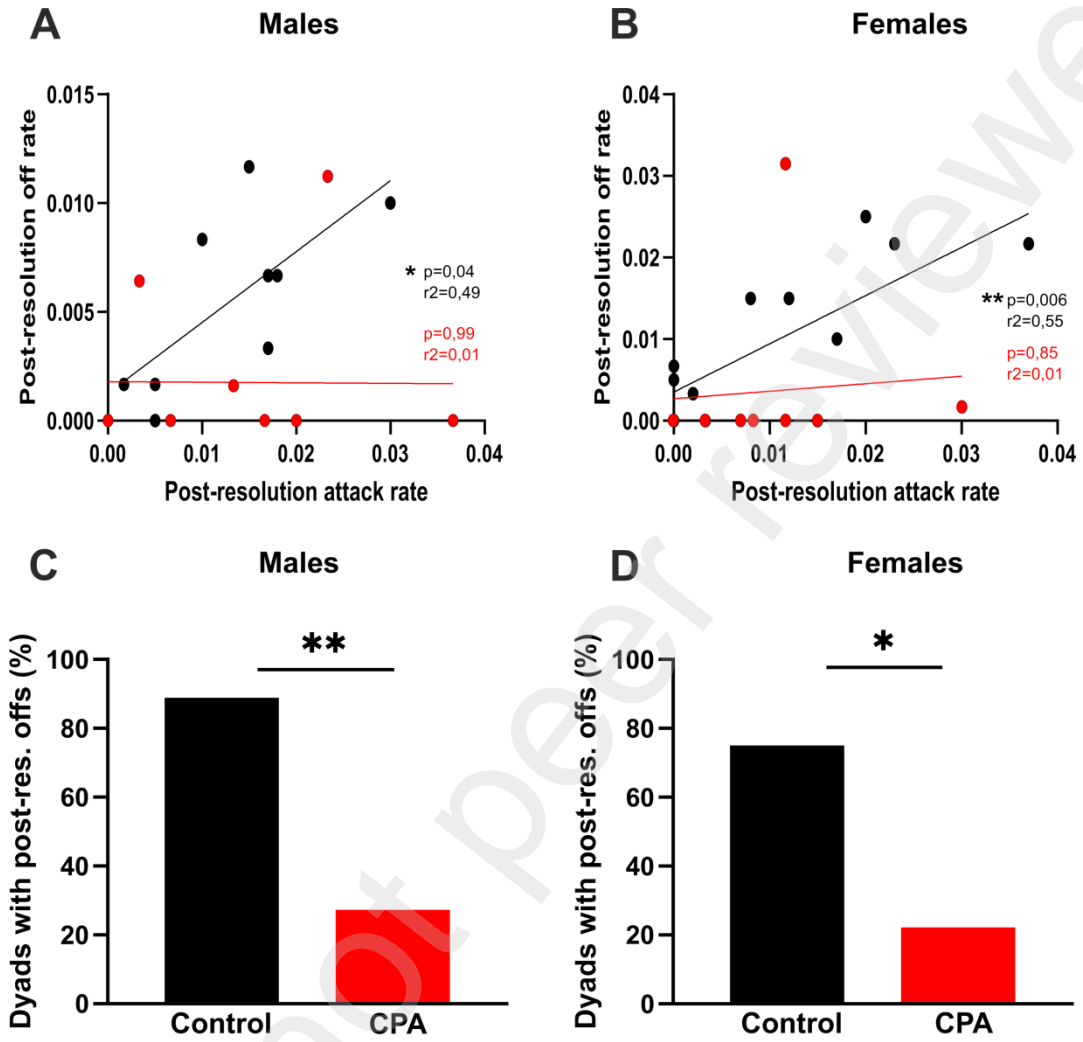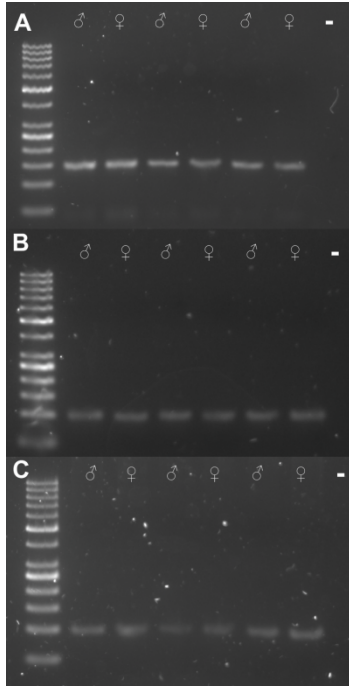
492   **Figure 4:**
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501 **Figure 7:**
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