

1 **Rapid body colouration change in *Oryzias celebensis***
2 **as a social signal for intraspecific competition**

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16

17 **Abstract**

18

19 In some species, such as chameleon and cichlid fish, rapid body colouration changes
20 (within seconds or minutes) serve as visual social signals in male-male competition.
21 This study investigated the relationship between aggressive behaviour and body
22 colouration changes in *Oryzias celebensis*, an Indonesian medaka fish. We analysed
23 aggressive behaviours and corresponding body colouration changes during attack
24 events in a controlled laboratory setting using groups of 3 adult fish in a small tank. In a
25 triadic relationship consisting of 2 males and a female, males with blackened markings
26 attacked more frequently than males without blackened markings and females.
27 Additionally, we observed that the males with blackened markings were seldom
28 attacked by males without blackened markings and females. These tendencies
29 persisted even in groups consisting of 3 males. Our results suggest that the blackened
30 markings in male *O. celebensis* not only indicate the level of aggression but also serve
31 as a social signal to suppress attacks by other individuals.

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34 Introduction

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36 Across a wide range of taxa from vertebrates to invertebrates, animals such as fish,
37 amphibians, reptiles, and cephalopods possess the ability to change their body
38 colouration in response to external factors [1-4]. In most species, body colouration
39 changes are primarily under hormonal control, but in some species of cephalopods,
40 reptiles, and fish, neural systems directly control the chromatophore changes to
41 respond very rapidly (within seconds). In chameleon males, rapid body colouration
42 changes serve as social signals to indicate social status [2]. Chameleon males that are
43 defeated in male-male competition rapidly change their colouration from bright yellow to
44 brown [2]. Very few studies have investigated the neural mechanisms underlying rapid
45 body colouration changes using molecular genetics.

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47 In the present study, we used a medaka fish, *Oryzias celebensis*, endemic to southwest
48 Sulawesi, Indonesia, as an experimental model. Medaka fish (family Adrianichthyidae)
49 are widely distributed in East and Southeast Asia, and 20 of the 39 Adrianichthyidae
50 species are endemic to Sulawesi [5, 6]. These fish exhibit significant diversification in
51 sexually dimorphic traits such as morphology and body colouration, making them an
52 excellent model for exploring the evolutionary genetic mechanisms underlying sexual
53 dimorphism [7]. For one of these endemic species, *O. celebensis*, the reference
54 genome assembly was generated in a previous study [8]. Interestingly, some male *O.*
55 *celebensis* exhibit distinctive blackened markings on their fins and sides (figure 1a), and
56 the colouration of these markings changes rapidly over a short period within a few
57 seconds. Here we established a behavioural experimental paradigm that allows
58 consistent observation of aggressive behaviours with stable monitoring of their body
59 colouration changes. Using this behavioural paradigm, we investigated the relationship
60 between aggressive behaviours and body colouration changes.

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66 Results

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68 To investigate the experimental conditions that can affect attacks and body colouration
69 changes in *O. celebensis*, we examined the numbers of attacks and the patterns of
70 body colouration changes in small tanks under the following 3 conditions: an
71 algae-covered tank containing 2 males and 1 female (triadic); an algae-covered tank
72 containing 3 males (3-males); and a transparent tank with no algae containing 2 males
73 and 1 female (transparent). The number of attacks in each trial did not significantly differ
74 between the triadic ($n = 13$) and 3-males ($n = 12$) conditions (Mann-Whitney U test: $Z =$
75 -0.22 , $p = 0.84$) (figure 1e), indicating that the fish attack other fish irrespective of the
76 presence of females. In contrast, neither attack behaviour nor black colouration
77 changes were observed in the transparent condition ($n = 10$) (figure 1b), indicating that
78 the algae-covered walls of the tank were required for the emergence of both the attacks
79 and the black colouration changes.

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81 To investigate whether body colouration correlates with attack frequency, we recorded
82 the number of attacks and the associated body colouration during these attack events
83 for each individual under the triadic condition. The number of attacks by males with
84 blackened markings was higher than that by males without blackened markings or
85 females [generalized linear mixed model (GLMM) followed by Tukey's post hoc test:
86 black(+)-black(-), estimate = 2.49, SE = 0.952, $p = 0.0244$; black(+)-female, estimate =
87 4.89, SE = 1.122, $p < 0.0001$; black(-)-female, estimate = 2.40, SE = 1.056, $p = 0.0598$]
88 (figure 2a). We found similar tendencies in the 3-males condition [GLMM followed by
89 Tukey's post hoc test: black(+)-black(-), estimate = 4.88, SE = 1.01, $p < 0.0001$] (figure
90 2b). These findings indicate that the *O. celebensis* males with blackened markings
91 exhibited higher aggression toward different conspecific individuals. To determine
92 whether the susceptibility to attacks varies with body colouration, we recorded the
93 number of attacks each individual received, as well as their body colouration at the time
94 of the attack in the triadic condition. The number of attacks received did not differ
95 significantly in relation to body colouration [GLMM followed by Tukey's post hoc test:
96 black(+)-black(-), estimate = 0.378, SE = 0.574, $p = 0.788$; black(+)-female, estimate =
97 0.0811, SE = 0.473, $p = 0.984$; black(-)-female, estimate = -0.296, SE = 0.474, $p =$
98 0.806] (figure 2c). We also found no significant difference between the males with and
99 without blackened markings in the number of attacks received under the 3-males
100 condition [GLMM followed by Tukey's post hoc test: black(+)-black(-), estimate = 0.132,
101 SE = 0.361, $p = 0.715$] (figure 2d).

102

103 Next, to examine whether there are biases in the body colouration of the individuals
104 attacked, we analysed the directions of the attack events. In the case of the triadic
105 condition, the observed attack values on males with blackened markings (figure 2f),
106 males without blackened markings (figure 2g), and females (figure 2h) differed
107 significantly from the expected values (figure 2e) [chi-square test: attacks on males with
108 blackened markings: $\chi^2_{2, 84} = 29.491$, $p < 0.0001$; attacks on males without blackened
109 markings: $\chi^2_{2, 131} = 145.61$, $p < 0.0001$; attacks on females: $\chi^2_{1, 265} = 8.0128$, $p =$
110 0.004645]. In the 3-males condition, the observed attack values on males with
111 blackened markings (figure 2j) and males without blackened markings (figure 2k) also
112 differed significantly different from the expected value (figure 2i) [chi-square test: attacks
113 on males with blackened markings: $\chi^2_{1, 278} = 12.887$, $p = 0.0003308$; attacks on males
114 without blackened markings: $\chi^2_{1, 196} = 18.279$, $p < 0.0001$]. These findings revealed that
115 males with blackened markings were predominantly targeted by other males with
116 blackened markings, while attacks from males without blackened markings or females
117 were rare. On the other hand, males without blackened markings were attacked not only
118 by males with blackened markings but also by other males without blackened markings
119 and females. Additionally, females under the triadic condition experienced a similar
120 attack frequency as males.

121

122 Discussion

123 The findings of the present study demonstrated that male *O. celebensis* with blackened
124 body markings exhibited increased aggression toward other members of the same
125 species. In most animals, limited resources such as food, territory, and mates can drive
126 intraspecific competition for their access [9]. The fact that females were often targeted in
127 the triadic condition suggests that the aggressive behavior noted in the present study
128 stems more from competition over resources such as food, rather than from
129 male-to-male competition for mates [10]. In these intraspecific competitions,
130 non-contact aggressive displays may eliminate escalation to physical contact [11, 12]. In
131 some animals, visual threat signals that are specific patterns of behaviors indicate
132 aggressive motivation to assist in resolving conflicts [13-15]. Our findings suggest that
133 the blackened markings on *O. celebensis* males may function as visual cues to signal
134 dominance and fighting ability, thereby aiding in the resolution of disputes over
135 resources.

136 The link between changes in body colouration and behaviour has been
137 explored in various species. For example, distinct colouration patterns and behavioural

138 displays in the cichlid fish (*Astatotilapia burtoni*) act as visual signals reflecting their
139 state of aggression, which in turn can inhibit the actions of other conspecifics [16, 17,
140 18]. These changes in colouration and behavior, however, typically take from a few
141 minutes to a day to manifest, possibly due to hormonal influences [19-20]. In contrast,
142 rapid colouration changes occurring within seconds, as seen in chameleons, octopuses,
143 and wrasses, act as immediate, short-term signals of aggression [21-23]. Considering
144 that the body colouration of *O. celebensis* can change within a minute, this species may
145 possess a neural mechanism for exhibiting these visual social signals. In teleost fish,
146 neurotransmitters such as noradrenaline and adenosine control the colouration
147 changes of melanophores [24, 25], suggesting a potential peripheral system for
148 displaying the visual signals. *O. celebensis* could be a promising candidate for applying
149 genome editing techniques, as demonstrated in Japanese medaka (*O. latipes*) [26, 27].
150 With the availability of a reference genome assembly of *O. celebensis* [8], this species
151 offers a new avenue for probing the molecular and neural mechanisms behind
152 intraspecific communication through rapid colouration changes using advanced
153 molecular and genetic methods such as optogenetics.

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155 **Material and Methods**

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157 **Fish and housing conditions**

158

159 *Oryzias celebensis* (the Ujung pandang strain) was provided by the National
160 Bioresource Project (NBRP) medaka (RS278; <https://shigen.nig.ac.jp/medaka/>). Fish
161 were maintained in groups in a glass tank (60 cm x 30 cm x 36 cm [height]) containing
162 approximately 30 to 40 individuals with a roughly 1:1 male-to-female ratio, and fed
163 nauplii of brine shrimp or powdered food once a day between 12:00 pm and 1:00 pm. All
164 fish were hatched and bred in our laboratory. Sexually matured male and female
165 medaka 3–15 months of age were subjected to behavioural trials. The water
166 temperature was ~29 °C and light was provided by LED lights for 14 h per day
167 (08:00–22:00).

168

169 **Behavioural trials**

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171 We observed aggressive behaviours among 3 adult fish in an acrylic tank (24 cm x 14
172 cm x 15 cm) for 3 consecutive days (figure 1c) under the following 3 conditions: the

173 triadic condition consisting of 2 males and 1 female in each tank covered with algae on
174 the wall; the 3-males condition consisting of 3 males in each tank covered in algae on
175 the wall; the transparent condition consisting of 2 males and 1 female in each
176 transparent tank without algae on the wall. We used adult fish housed for 2 or more
177 days under the conditions described above. A video of each behavioural trial was
178 recorded twice a day at 10:00 (morning) and 17:00 (evening) using a digital camera (Go
179 Pro Hero9 Black) (figure 1c). On the first morning during the assay (between 9:30 and
180 10:00), test fish were randomly transferred from the aquarium to each experimental tank.
181 Fifteen minutes after starting the video recording, the test fish were transferred to a
182 small bag with a zip-lock closure, and then photos of the whole body of the test fish
183 were taken using a digital camera (TG-6, Olympus) for individual identification by their
184 fin shapes and pigmentation patterns of the body surface. After obtaining the photo,
185 each fish was returned to their experimental tank. From the first 15 minutes of the video
186 recordings, we quantified aggressive behaviours that were defined as a rapid swim
187 toward a target and the target fleeing as previously described in Japanese medaka (*O.*
188 *latipes*) [28, 29]. We also described the direction and timing of each attack event, and
189 then determined the body colouration of each test fish while it attacked or was attacked
190 by the other fish. In this study, a male whose markings blackened at least once in each
191 video recording was considered as a male with blackened markings.

192

193 **Statistical analysis**

194

195 We performed a Mann-Whitney U test using the "wilcox_test" function in the *coin*
196 package version 1.4.3 implemented in R version 4.2.3 for comparisons of the number of
197 attacks per trial across conditions [30]. Also, to examine whether attack frequencies and
198 attack susceptibility vary based on body colouration, we compared the body colouration
199 with the number of attacks performed and the number of attacks received using R
200 version 4.2.3 with generalized linear mixed models by negative binominal distributions
201 with a log link function using the "glmmTMB" function in the package *glmmTMB* version
202 1.1.7 [31]. Body colouration of each individual while attacking on while being attacked
203 by other individuals was included as a fixed factor, and experiment tank numbers were
204 included as random factors. For a post hoc test, P values adjusted with Tukey's method
205 were calculated using the package *emmeans* version 1.8.5.

206 To investigate the possible biases in the body colouration of the individuals that
207 were attacked, we aggregated the number of attacks for each body colouration and
208 analysed the directions of attack events. We set the total ratio of the total number of

209 attacks as an expected value, and the ratio of the total number of attacks categorized by
210 the directions of attack events as observed values. Significant differences between the
211 observed values and expected ones were analysed using the chi-square test
212 implemented in R version 4.2.3.

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214 **References**

215

- 216 1. Bachmann JC, Cortesi F, Hall MD, Marshall NJ, Salzburger W, Gante HF 2017
217 Real-time social selection maintains honesty of a dynamic visual signal in
218 cooperative fish, *Evolution Letters*. **1**, 269–278.
219
- 220 2. Whiting MJ, Holland BS, Keogh JS, Noble DWA, Rankin KJ, Stuart-Fox D. 2022
221 Invasive chameleons released from predation display more conspicuous colors. *Sci*
222 *Adv.* **8**, eabn2415.
223
- 224 3. Camargo CR, Visconti MA, Castrucci AML. 1999 Physiological color change in the
225 bullfrog, *Rana catesbeiana*. *J Exp Zool.* **283**, 160-169.
226
- 227 4. Messenger JB. 2001 Cephalopod chromatophores: neurobiology and natural
228 history. *Biol Rev.* **76**, 473-528.
229
- 230 5. Mokodongan DF & Yamahira K. 2015 Origin and intra-island diversification of
231 Sulawesi endemic Adrianichthyidae, *Mol. Phylogenet. Evol.* **93**, 150–160.
232
- 233 6. Yamahira et al. 2021 Mesozoic origin and ‘out-of-India’ radiation of ricefishes
234 (Adrianichthyidae), *Biol. Lett.* **17**, 20210212.
235
- 236 7. Sumarto et al. 2020 Latitudinal variation in sexual dimorphism in a freshwater
237 fish group, *Biological Journal of the Linnean Society*, **131**, 898–908.
238
- 239 8. Ansai S, Mochida K, Fujimoto S, et al. 2021 Genome editing reveals fitness effects
240 of a gene for sexual dichromatism in Sulawesian fishes. *Nat Commun.* **12**, 1350.
241

- 242 9. Clutton-Brock TH & Huchard E. 2013 Social competition and selection in males and
243 females. *Phil. Trans. R. Soc. B.* **368**, 20130074.
244
- 245 10. Andersson M. 1994 Sexual Selection. Princeton University Press.
246
- 247 11. Mesterton-Gibbons M, Heap Sm. 2014 Variation between self- and mutual
248 assessment in animal contests. *American Naturalist.* **183**, 199-213.
249
- 250 12. Peake TM, Mcgregor PK. 2004 Information and aggression in fishes. *Animal*
251 *Learning & Behavior.* **32**, 114–121.
252
- 253 13. Dey Cody J, Dale James & Quinn James S. 2014 Manipulating the appearance of a
254 badge of status causes changes in true badge expression. *Proc. R. Soc. B.* **281**,
255 20132680.
256
- 257 14. Rohwer S. 1982 The evolution of reliable and unreliable badges of fighting ability.
258 *American Zoologist.* **22**, 531-546.
259
- 260 15. Tibbetts EA, Dale J. 2004 A socially enforced signal of quality in a paper wasp.
261 *Nature.* **432**, 218-222.
262
- 263 16. Leong CY. 1969 The quantitative effect of releasers on the attack readiness of the
264 fish *Haplochromis burtoni* (Cichlidae, Pisces). *Z. Vergl. Physiol.* **65**, 29-50.
265
- 266 17. Chen CC, Fernald, RD. 2011 Visual Information Alone Changes Behavior and
267 Physiology during Social Interactions in a Cichlid Fish (*Astatotilapia burtoni*). *Plos*
268 *one.* **6**, e20313.
269
- 270 18. Theis A, Salzburger W, Egger, B. 2012 The Function of Anal Fin Egg-Spots in the
271 Cichlid Fish *Astatotilapia burtoni*. *Plos one.* **7**, e29878.
272
- 273 19. White SA, Nguyen T, Fernald RD. 2002 Social regulation of gonadotropin-releasing
274 hormone. *J Exp Biol.* **205**, 2567–2581.
275
- 276 20. Burmeister SS, Jarvis ED, Fernald RD. 2005 Rapid Behavioral and Genomic
277 Responses to Social Opportunity. *PLOS Biology.* **3**, e363.

278

279 21. Stuart-Fox D, Moussalli A 2008 Selection for Social Signalling Drives the Evolution
280 of Chameleon Colour Change. *PLoS biology*. **6**, e25.

281

282 22. Scheel D, Godfrey-Smith P, & Lawrence M. 2016 Signal use by octopuses in
283 agonistic interactions. *Current Biology*. **26**, 377-382.

284

285 23. Dawkins MS & Guilford T. 1993 Color and pattern in relation to sexual and
286 aggressive behavior in the bluehead wrasse *Thalassoma bifasciatum*. *Behavioural*
287 *Processes*. **30**, 245-251.

288

289 24. Kumazawa T, Fujii, R. 1984 Concurrent releases of norepinephrine and purines by
290 potassium from adrenergic melanosome-aggregating nerve in tilapia *Comp.*
291 *Biochem. Physiol.* **78C**, 263-266.

292

293 25. Miyashita Y, Kumazawa T, Fujii R. 1984 Receptor mechanisms in fish
294 chromatophores. VI. Adenosine receptors mediate pigment dispersion in guppy
295 and catfish melanophores *Comp. Biochem. Physiol.* **77C**, 205-210.

296

297 26. Ansai S, Kinoshita M 2014 Targeted mutagenesis using CRISPR/Cas system in
298 medaka. *Biol Open*. **3**, 362–371.

299

300 27. Murakami Y, Ansai S, Yonemura A, et al. 2019 Correction to: An efficient system for
301 homology-dependent targeted gene integration in medaka (*Oryzias latipes*).
302 *Zoological Lett.* **5**, 22.

303

304 28. Kawabata, M. 1954 Socio-ecological studies on the killi-fish, *Aplocheilus latipes* I.
305 General remarks on the social behavior. *Japanese Journal of Ecology* **4**, 109–113.

306

307 29. Kagawa N. 2013 Social rank-dependent expression of arginine vasotocin in distinct
308 preoptic regions in male *Oryzias latipes*. *J Fish Biol.* **82**, 354–363.

309

310 30. Hothorn T, Hornik K, van de Wiel MA, Zeileis A. 2006 “A Lego system for
311 conditional inference.” *The American Statistician*, **60**(3), 257-263.
312 doi:10.1198/000313006X118430 <<https://doi.org/10.1198/000313006X118430>>.

313

314 31. Brooks ME, Kristensen K, Van Benthem KJ, Magnusson A, Berg CW, Nielsen A,
315 Skaug HJ, Maechler M and Bolker BM. 2017 glmmTMB Balances Speed and
316 Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling.
317 The R Journal, 9(2), 378-400
318

319 **Ethics**

320
321 The work in this paper was conducted using protocols specifically approved by the
322 Animal Care and Use Committee of Tohoku University (Permit Number: 2022LSA-003).
323 All efforts were made to minimize animal suffering, following the NIH Guide for the Care
324 and Use of Laboratory Animals.
325

326 **Authors' contributions**

327
328 Conceived and designed the experiments: RU, SA, HT. Performed the experiments: RU.
329 Analysed the data: RU, SA, HT. Contributed reagents/materials/analysis tools: SA, HT.
330 Wrote the paper: RU, SA, HT. All authors gave final approval for publication.
331

332 **Competing interests**

333
334 We declare we have no competing interests.
335

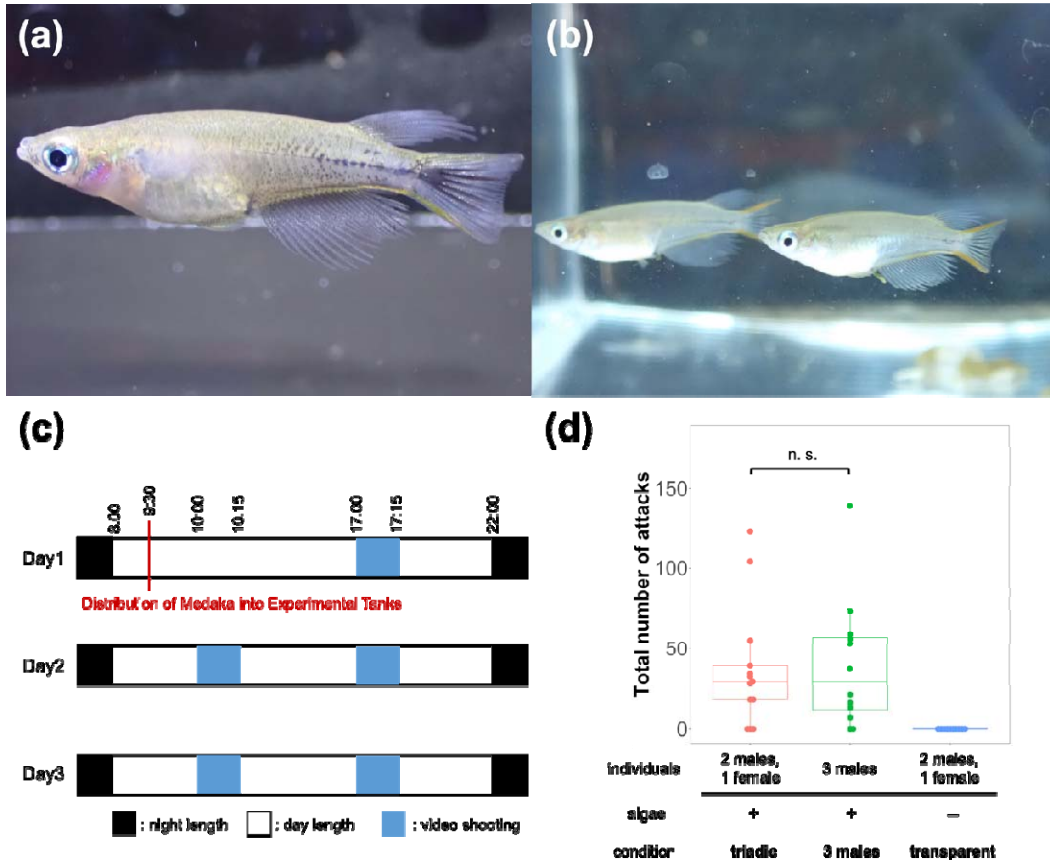
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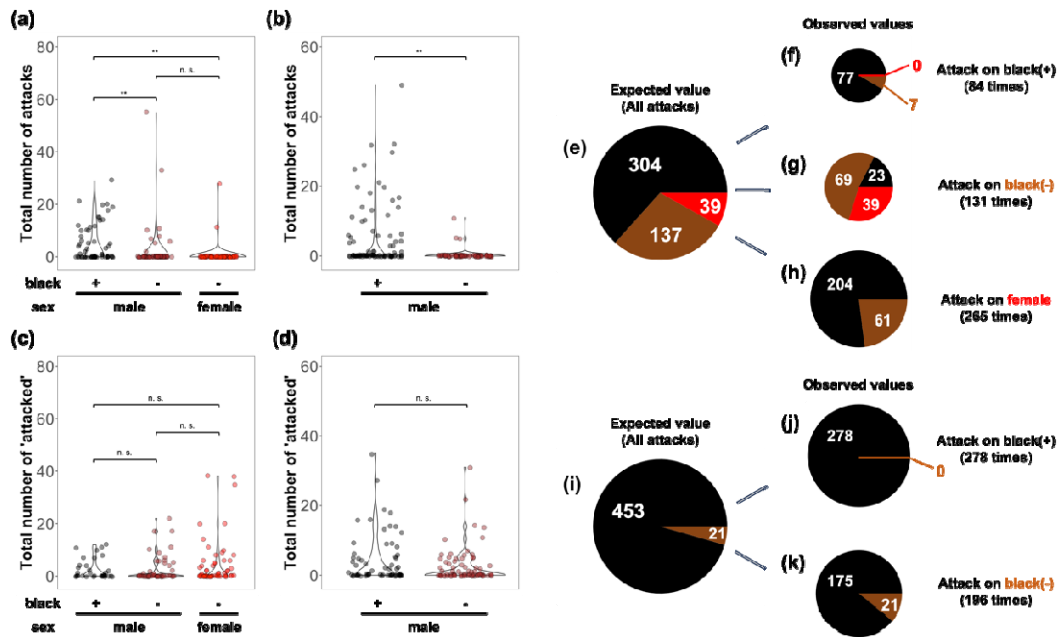


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350 **Figure 1.** Attacks and body colouration changes in *Oryzias celebensis*. Representative
351 images of male *O. celebensis* with blackened markings (a) and without blackened
352 markings (b). (c) Timetable for the triadic behavioural assays. (d) Number of attacks
353 under the different experimental conditions; algae-covered tank with 2 males and 1
354 female (triadic), algae-covered tank with 3 males (3-males), and transparent tank with 2
355 males and 1 female (transparent). Attacks occurred irrespective of the presence of
356 females in the algae-covered tanks, and did not occur at all in the transparent tank
357 under these experimental conditions. $**p < 0.01$ and not significant (n.s.) according to
358 the Mann-Whitney *U* test.

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361

362 **Figure 2.** Relationship between attacks and male body colouration. (a, b) Number of
 363 attacks under the triadic (2 males and a female) (a) and 3-males (b) conditions. Number
 364 of attacks by males with blackened markings was much higher than that by males
 365 without blackened markings and females. (c, b) Number of attacks received under the
 366 triadic (c) and 3-males (d) conditions. The number of attacks received did not differ
 367 significantly between conditions. * $p < 0.05$, ** $p < 0.01$, and not significant (n.s.)
 368 according to generalized linear mixed models followed by Tukey's post hoc test. (e-k)
 369 Pie charts showing directions of attack events under the triadic and 3-males conditions.
 370 The left pie chart represents the total number of attacks. The 3 pie charts on the right
 371 represent the division of the left pie chart based on the direction of the attacks. The
 372 black, brown, and red slices represent males with blackened markings, males without
 373 blackened markings, and females, respectively. The size of the slice in the pie charts
 374 reflects the number of attacks received.

375