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1 # The decision of male medaka to mate or fight depends on two
2 complementary androgen signaling pathways

3

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11 ## Abstract

12

13 Adult male animals typically court and attempt to mate with females, while attacking other males. Emerging
14 evidence from mice indicates that neurons expressing the estrogen receptor ESR1 in behaviorally relevant
15 brain regions play a central role in mediating these mutually exclusive behavioral responses to conspecifics.
16 However, the findings in mice are unlikely to apply to most other vertebrates, where androgens — rather
17 than estrogens — have been implicated in male behaviors. Here we report that male medaka (*Oryzias*
18 *latipes*) lacking one of the two androgen receptor subtypes (Ara) are less aggressive toward other males and
19 instead actively court them, while those lacking the other subtype (Arb) are less motivated to mate with
20 females and conversely attack them. These findings indicate that, in male medaka, the Ara- and Arb-
21 mediated androgen signaling pathways facilitate appropriate behavioral responses, while simultaneously
22 suppressing inappropriate responses, to males and females, respectively. Notably, males lacking either
23 receptor retain the ability to discriminate the sex of conspecifics, suggesting a defect in the subsequent
24 decision-making process to mate or fight. We further show that Ara and Arb are expressed in intermingled
25 but largely distinct populations of neurons, and stimulate the expression of different behaviorally relevant
26 genes including galanin and vasotocin, respectively. Collectively, our results demonstrate that male teleosts
27 make adaptive decisions to mate or fight as a result of the activation of one of two complementary androgen
28 signaling pathways, depending on the sex of the conspecific that they encounter.

29

30 **Keywords:** aggression; androgen receptor; decision-making; mating; teleost

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31 # Introduction

32

33 Across species, adult males typically court and attempt to mate with females, while directing aggression
34 toward other males. The question of how these mutually exclusive behavioral responses to conspecifics are
35 generated has long been a topic of great interest (1, 2). The underlying neural mechanisms, while extensively
36 studied in flies (3, 4), have generally remained elusive in vertebrates. Recent studies in mice, however, have
37 yielded important findings: neurons expressing the estrogen receptor (ESR) subtype ESR1 in the medial
38 preoptic area (MPOA) and the ventrolateral part of the ventromedial hypothalamus (VMHvl), which evoke
39 mating and aggression, respectively, inhibit each other to ensure mutually exclusive displays of these
40 behaviors (5, 6).
41 Nevertheless, the specific role of estrogen/ESR1 signaling in these neurons is still unclear. It has been
42 consistently shown in rodents that estrogen/ESR1 signaling is essential for male behaviors (7–9).
43 Specifically, testosterone secreted from the fetal testis is converted to estradiol-17β (E2) in the developing
44 brain, which then acts through ESR1 to organize the neural substrates that later mediate male-typical
45 behaviors. In addition, adult testicular testosterone, after conversion to E2, activates the neural substrates
46 through ESR1 to achieve male-typical behaviors (1, 9–12). Despite these well-established findings, the
47 neural mechanisms by which estrogen/ESR1 signaling mediates male behaviors, including its target genes,
48 are largely undefined (12).
49 More importantly, the neural circuits that mediate social behaviors, including mating and aggression
50 (often referred to as the "social behavior network"), seem to be highly conserved across vertebrates (13,
51 14); in many non-rodent species including humans, most birds, and teleost fish, however,
52 androgen/androgen receptor (AR) signaling — rather than estrogen/ESR signaling — has been implicated
53 in male behaviors (i.e., testicular androgens act directly through AR without conversion to estrogens) (15–
54 18). This suggests that the neural underpinnings of male-typical behavioral responses observed in mice do
55 not apply to these species.
56 In teleosts, 11-ketotestosterone (11KT), which cannot be converted to estrogen, is the primary testicular
57 androgen, and treating females with 11KT as adults effectively induces male-typical behaviors, including
58 courtship and aggression (18–21). These facts suggest that both organization and activation of the neural
59 substrates for male behaviors rely solely on androgen/AR signaling in adulthood. As such, teleosts provide
60 simple and easy-to-manipulate model systems for studying the neural and hormonal mechanisms underlying
61 male-typical behavioral responses. Most teleost species have two AR subtypes, Ara and Arb, resulting from
62 a whole-genome duplication that occurred early in the teleost lineage (22, 23). The role of each AR in
63 testicular development and secondary sexual characteristics has recently been studied in AR-deficient
64 models of cichlid (*Astatotilapia burtoni*) and medaka (*Oryzias latipes*) (24, 25). In the present study, we

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65 have studied the role of androgen/AR signaling in male-typical behavioral responses by investigating the
66 behavior of AR-deficient male medaka toward conspecifics. We find, to our surprise, that males deficient in
67 Ara frequently court other males, while males deficient in Arb attack females. Our findings provide evidence
68 that two functionally distinct AR signaling pathways act in a complementary manner in males to generate
69 appropriate behavioral responses during social encounters.

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70 ## Results

71

72 **Androgen/Ara signaling facilitates male aggression toward other males.** We generated medaka
73 deficient in *ara* and *arb* using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-
74 associated protein 9 (Cas9) genome editing. To ensure the reproducibility of the observed behavioral
75 phenotypes, two independent medaka lines were established for *ara* (Δ326 and Δ325; Fig. S1) and for *arb*
76 (Δ10 and Δ11; Fig. S2) and used for subsequent analyses. Note that this paper follows the nomenclature of
77 teleost ARs based on their orthology/paralogy relationships (23, 25), and the designations Ara and Arb are
78 opposite to those in our earlier publications (e.g., 19, 26–29).

79 We first used these medaka to investigate the consequences of impaired Ara- and Arb-mediated androgen
80 signaling on male aggression toward other males. The aggressive behavior of teleosts, including medaka,
81 involves five types of behavioral act: chases, fin displays, circles, strikes, and bites (Fig. 1A) (19, 30). Tests
82 of aggressive behavior among grouped *ara*-deficient (*ara*$^{-/-}$) males revealed that they engaged in all of these
83 aggressive acts less frequently than their wild-type (*ara*$^{+/+}$) siblings (although not significantly for some
84 acts) in both the Δ326 and Δ325 lines (Fig. 1 B–D). In contrast, similar behavioral testing in *arb*-deficient
85 (*arb*$^{-/-}$) males showed no significant difference in the frequency of any aggressive acts between these males
86 and their wild-type (*arb*$^{+/+}$) siblings in both the Δ10 and Δ11 lines (Fig. 1 B, E, and F). Taken together, these
87 results indicate that male aggression toward other males is primarily facilitated by androgen/Ara signaling.

88

89 **Androgen/Arb signaling facilitates male mating with females.** Next, we assessed the impact of
90 impaired Ara- and Arb-mediated androgen signaling on male mating with females. The mating behavior of
91 medaka follows a stereotyped pattern, wherein a series of followings, courtship displays, and wrappings by
92 the male leads to spawning (31, 32) (Fig. 2A). Tests of the mating behavior of *ara*$^{-/-}$ males paired with a
93 stimulus female showed that they initiated followings, courtship displays, and wrappings with latencies
94 comparable to those of *ara*$^{+/+}$ males in both the Δ326 and Δ325 lines (Fig. 2 B–D). In addition, the majority
95 (>80%) of *ara*$^{-/-}$ males spawned during the test period (Fig. 2 C and D). These observations indicate that
96 loss of androgen/Ara signaling does not affect male mating with females.

97 Conversely, behavioral testing in *arb*$^{-/-}$ males of the Δ10 line paired with a stimulus female revealed
98 significantly longer latencies to initiate wrappings and spawn, with less than 10% of them spawning
99 successfully (Fig. 2 E and F). Similar results were obtained in the Δ11 line, where *arb*$^{-/-}$ males showed
100 significantly longer latencies to followings and courtship displays, in addition to wrappings and spawning,
101 and none of them spawned in the test period (Fig. 2 E and G). These observations suggest that loss of
102 androgen/Arb signaling renders males less motivated to mate with females.

103 However, given that *arb*$^{-/-}$ males lack male-typical secondary sexual characteristics in fin morphology

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104 (25), we considered that their mating defects might be the result of females being less sexually attracted to
105 *arb*$^{-/-}$ males. To address this possibility, their mating behavior was tested again using females deficient in
106 the ESR subtype Esr2b, which are completely unreceptive to male courtship (20), as stimulus females. This
107 test, which enabled us to assess the motivation of males to mate with females without the influence of female
108 receptivity, revealed that *arb*$^{-/-}$ males were indeed less motivated to mate with females. Specifically, *arb*$^{-/-}$
109 males showed significantly longer latencies to initiate followings, courtship displays, and wrappings, and
110 significantly fewer courtship displays and wrapping attempts than *ara*$^{+/+}$ males in both the Δ10 (Fig. 2 H–
111 J) and Δ11 lines (Fig. S3 A–C). Similar tests on *ara*$^{-/-}$ males showed that they did not differ significantly
112 from *ara*$^{+/+}$ males in any measure (Fig. S3 D–H). Collectively, these findings indicate that the motivation of
113 males to mate with females is primarily facilitated by androgen/Arb signaling.
114

115 **Adult androgen/AR signaling elicits male-typical behavioral responses even in females.**
116 Female teleosts, when treated with 11KT as adults, display male-typical aggressive and mating behavior
117 while retaining their ovaries (18–21, 33). We considered that, if the above conclusions apply to females as
118 well as males, the male-typical aggressive and mating behaviors observed in 11KT-treated females are likely
119 to be elicited through Ara and Arb, respectively. To address this possibility and further verify our preceding
120 findings, we investigated whether *ara*$^{-/-}$ (Δ326 line) and *arb*$^{-/-}$ (Δ10 line) females treated with 11KT as
121 adults exhibit male-typical aggressive and mating behaviors.

122 In tests of aggressive behavior towards a stimulus male, none of the 11KT-treated *ara*$^{-/-}$ females
123 exhibited any aggressive acts, whereas more than 60% of the control 11KT-treated *ara*$^{+/+}$ females did (Fig.
124 S4 A and B); in addition, there were significant differences between the two genotypes in the frequency of
125 fin displays and bites (Fig. S4C). In contrast, more than 50% of 11KT-treated *arb*$^{-/-}$ females exhibited
126 aggressive acts towards a stimulus male, and these females did not differ significantly from the control *arb*$^{+/+}$
127 females in the frequency of any acts (Fig. S4 D–F). We further examined aggressive behavior among
128 grouped females treated with 11KT, but hardly any aggressive acts were observed regardless of strain or
129 genotype (Fig. S4 G–I), probably because these females were not fully masculinized in appearance and did
130 not recognize each other as targets for attack.

131 In tests of mating behavior toward a stimulus female, 11KT-treated *ara*$^{-/-}$ females initiated followings,
132 courtship displays, and wrappings with latencies comparable to those of *ara*$^{+/+}$ females (Fig. S4 J and K). In
133 contrast, only a fraction of 11KT-treated *arb*$^{-/-}$ females performed followings or courtship displays to the
134 stimulus female, and their latencies to these acts were significantly longer than those of *arb*$^{+/+}$ females (Fig.
135 S4 L and M). Furthermore, none of the *arb*$^{-/-}$ females engaged in wrapping (Fig. S4M). Together, these
136 results suggest that Ara- and Arb-mediated androgen signaling in adulthood elicit male-typical patterns of
137 aggression and mating, respectively, even in females (i.e., regardless of genetic or phenotypic sex).

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138

139 ***ara*-deficient males frequently attempt to mate with other males.** Curiously, in examining the
140 aggressive behavior of *ara*$^{-/-}$ males, we noted that they frequently exhibited mating acts to other males. To
141 explore this observation further, we tested and quantified the mating behavior of *ara*$^{-/-}$ males paired with a
142 stimulus male (Fig. 3 A and B). The results showed that *ara*$^{-/-}$ males performed courtship displays and
143 attempted wrappings to the stimulus male more frequently and had a shorter latency to wrapping attempts
144 as compared with their *ara*$^{+/+}$ siblings (although not significantly for some results) in both the Δ326 and
145 Δ325 lines (Fig. 3 C–F). These data collectively indicate that *ara*$^{-/-}$ males not only are less aggressive toward
146 other males but also frequently attempt to mate with them.

147 What behavioral mechanisms, then, underlie the male-directed mating attempts of *ara*$^{-/-}$ males? We
148 considered that there are three possible explanations: 1) *ara*$^{-/-}$ males cannot discriminate the sex of
149 conspecifics and consequently misidentify males as females; 2) they can discriminate sex, but cannot make
150 appropriate decisions about how to respond to male conspecifics; and 3) they are excessively sexually
151 aroused and seek to mate with conspecifics regardless of their sex. As this last possibility seemed unlikely
152 because *ara*$^{-/-}$ males were not overly motivated to mate with females, we assessed the first and second
153 possibilities by investigating the sex discrimination ability of *ara*$^{-/-}$ males in a three-chamber test with a
154 stimulus female in one side chamber and a stimulus male in the other (Fig. 3G). Similar to *ara*$^{+/+}$ males,
155 *ara*$^{-/-}$ males spent significantly more time near the stimulus female than near the male (Fig. 3 H and I),
156 suggesting that *ara*$^{-/-}$ males can discriminate the sex of conspecifics.

157 Unexpectedly, *ara*$^{-/-}$ males spent even more time near the female than did *ara*$^{+/+}$ males (Fig. 3 H and I).
158 This observation could be explained by assuming that *ara*$^{-/-}$ males either have an increased preference for
159 females or avoid contact with other males. Given that *ara*$^{-/-}$ males were not more motivated to mate with
160 females and were less aggressive toward other males, their decreased propensity to attack other males
161 probably led them to spend relatively more time near the female. We tested this presumption by an additional
162 three-chamber test in which the stimulus male was removed to leave an empty side chamber. Whereas *ara*$^{+/+}$
163 males spent significantly more time near the female in the absence than in the presence of the stimulus male,
164 *ara*$^{-/-}$ males showed no difference in their behavior with or without the stimulus male (Fig. 3 J and K), again
165 suggesting a reduced propensity of *ara*$^{-/-}$ males to attack other males. In summary, *ara*$^{-/-}$ males actively
166 court and attempt to mate with other males even though they retain the ability to discriminate the sex of
167 conspecifics. Thus, androgen/Ara signaling presumably prevents the maladaptive decision to engage in
168 mating with other males.

169 We also investigated the mating behavior of *arb*$^{-/-}$ males toward a stimulus male. In contrast to *ara*$^{-/-}$
170 males, *arb*$^{-/-}$ males performed courtship displays and attempted wrappings less frequently and with longer
171 latencies as compared with *arb*$^{+/+}$ males (Fig. S5). Therefore, *arb*$^{-/-}$ males are less inclined than *arb*$^{+/+}$ males

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172 to mate with males or females, suggesting that mating behavior toward females and males is stimulated by
173 common neural substrates involving androgen/Arb signaling.
174
175 ***arb*-deficient males attack females.** In examining the mating behavior of *arb*$^{-/-}$ males, we also noted
176 that they attacked females (in general, male medaka rarely attack females). To explore this observation, we
177 tested and quantified the aggressive behavior of *arb*$^{-/-}$ males toward a stimulus female (Fig. 4 A and B).
178 While none of the *arb*$^{+/+}$ males exhibited aggressive acts toward the stimulus female, more than half of the
179 *arb*$^{-/-}$ males from both the Δ10 and Δ11 lines did (Fig. 4 C and D). Furthermore, they exhibited all five
180 types of aggressive act rather than specific acts, with significant increases in chases, fin displays, circles,
181 and bites in the Δ10 line, and in chases and bites in the Δ11 line (Fig. 4 E and F). These results demonstrate
182 that *arb*$^{-/-}$ males not only are less motivated to mate with females, but also are aggressive toward them.
183 *ara*$^{-/-}$ males, on the other hand, did not show any aggressive acts toward the stimulus female (Fig. S6 A–
184 C).
185 This finding prompted us to investigate the sex discrimination ability of *arb*$^{-/-}$ males, as we did for *ara*$^{-/-}$
186 males. In the three-chamber test with a stimulus male in one side chamber and a stimulus female in the other,
187 *arb*$^{-/-}$ males, as well as *arb*$^{+/+}$ males, spent more time near the stimulus female; in addition, the degree of
188 time spent near the female was comparable between *arb*$^{+/+}$ and *arb*$^{-/-}$ males (Fig. 4 G–I). Thus, *arb*$^{-/-}$ males
189 attack females despite being able to discriminate the sex of conspecifics, suggesting that androgen/Arb
190 signaling prevents the maladaptive decision to attack females.
191 We further noticed that the individual *arb*$^{-/-}$ males that engaged more frequently in mating acts toward
192 the stimulus females also showed more frequent aggressive acts toward them (e.g., compare the *arb*$^{-/-}$ males
193 in rows 1–3 versus row 4 in Fig. 4B). Analysis of the correlation between the frequency of aggressive acts
194 and wrapping attempts indeed showed a positive correlation between them (Fig. 4J). In addition, none of
195 the *arb*$^{-/-}$ males initiated aggressive acts prior to mating acts (Fig. 4K and Fig. S6 D and E); when they
196 spawned, however, all of them turned to aggressive acts immediately (within 3 min) (4/4 and 3/3 individuals
197 in the Δ10 and Δ11 lines, respectively; e.g., see the *arb*$^{-/-}$ male in row 3 in Fig. 4B). Coupled with the
198 finding in mice and flies that interactions with females increase intrasexual aggression in males (34, 35),
199 these observations led us to assume that *arb*$^{-/-}$ males may be aggressive during the consummatory phase of
200 mating. We tested this idea by assessing the aggression of *arb*$^{-/-}$ males paired with *esr2b*-deficient females,
201 which are unreceptive to male courtship and prevent males from proceeding to the consummatory phase
202 (20). As expected, *arb*$^{-/-}$ males showed scarcely any aggressive acts toward the *esr2b*-deficient females (Fig.
203 S6 F–J), thereby supporting our assumption.
204 In summary, these results demonstrate that *arb*$^{-/-}$ males are aggressively aroused upon mating with
205 females, suggesting partially shared neural substrates for mating and aggression. Androgen/Arb signaling

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206 may act on these shared neural substrates to promote a state of sexual arousal, while simultaneously
207 suppressing aggressive arousal, toward females.
208
209 ***ara* and *arb* are preferentially expressed in different neurons.** Overall, the above findings indicate
210 that androgen signaling via Ara and Arb mediates behavioral responses to male and female conspecifics,
211 respectively. This raises the new question of how Ara and Arb might mediate such different behavioral
212 responses, despite sharing common ligands, binding sites, and even expression sites in the medaka brain
213 (26). To answer this question, we analyzed the coexpression of *ara* and *arb* at the cellular level by double
214 in situ hybridization, which showed that these genes are indeed expressed together in many brain regions,
215 but mainly in distinct populations of intermingled neurons. Neurons coexpressing *ara* and *arb* accounted
216 for less than 30% of all neurons expressing these genes in any brain nucleus (28% and 23% in the PPa and
217 NPT, respectively), and were rarely seen in the Vs/Vp (4.5%), PMp (2.8%), PPp (4.0%), and NVT (4.4%)
218 (Fig. 5A and Fig. S7A; see Table S1 for abbreviations of brain nuclei). These data suggest that the Ara and
219 Arb signaling pathways mediate different behavioral responses by acting independently of each other in
220 different populations of neurons.
221 This notion was further supported by the results of in situ hybridization analysis of *arb* expression in
222 $ara^{-/-}$ male brains and *ara* expression in $arb^{-/-}$ male brains, which revealed that the expression of either
223 gene in any brain nucleus did not differ significantly between these males and their wild-type siblings (Fig.
224 S7 B and C). Loss of one AR subtype, therefore, does not lead to a likely functional compensation via
225 upregulation of the other paralogous subtype, again suggesting the independence of the Ara and Arb
226 signaling pathways.
227
228 **The two androgen/AR signaling pathways stimulate different behaviorally relevant genes.**
229 Given our above observations, we explored which downstream neural events are elicited by Ara and Arb to
230 achieve aggression and mating. First, to determine which brain nuclei expressing Ara and Arb are relevant
231 to the display of these behaviors, we analyzed the expression of *fos* in each brain nucleus of males that
232 mated with females and males that attacked other males, and compared the results with those of solitary
233 males that neither mated nor attacked. In situ hybridization showed that *fos* expression was significantly
234 higher in many brain regions of males that mated as compared with solitary males. These regions included
235 the Vd/Vs/Vp in the ventral telencephalon, PMp, PPa, and PPp/PMm/PMg in the preoptic area, VM in the
236 thalamus, and NAT, NVT, and NPT in the hypothalamus, most of which are components of the social
237 behavior network (13, 14) (Fig. 5B and Fig. S8). For males that attacked other males, significantly higher
238 *fos* expression was found in the Vd/Vs/Vp, PPa, NVT, and NPT (Fig. 5B and Fig. S8). These results suggest
239 that one or more of these brain nuclei are sites of action of Ara and Arb signaling for aggression and mating,

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240 respectively.

241 Next, we explored which effector genes act downstream of Ara and Arb signaling in these brain nuclei.
242 We focused on two neuropeptide genes, *vt* (encoding vasotocin) and *gal* (encoding galanin), which have
243 been implicated in aggression and mating in many vertebrates, including medaka and other teleosts (19, 36,
244 37). In medaka, *vt* and *gal* are expressed in an androgen-dependent, and hence male-biased, manner in the
245 posterior part of the NVT (pNVT) and PMp (pPMp), respectively (19, 21, 38), where increased *fos*
246 expression was observed in our in situ hybridization analysis (Fig. 5B). Because *vt* neurons in the pNVT
247 express *ara* but not *arb*, and *gal* neurons in the pPMp express *arb* but not *ara* (19, 21), we hypothesized
248 that Ara signaling might facilitate male-directed aggression by inducing *vt* expression in the pNVT, and Arb
249 signaling might facilitate mating with females by inducing *gal* expression in the pPMp.

250 We first tested these hypotheses by examining the expression of *vt* and *gal* in the brains of $ara^{-/-}$ and
251 $arb^{-/-}$ males and females by in situ hybridization. The male-specific expression of *vt* in the pNVT was almost
252 completely abolished in $ara^{-/-}$ males (Fig. 5 C and D), but no significant differences between $ara^{+/+}$ and
253 $ara^{-/-}$ males were observed in other brain nuclei (Fig. S9A). In contrast, the levels of *vt* expression in $arb^{-/-}$
254 males were similar to those in $arb^{+/+}$ males in the pNVT (Fig. 5 E and F), but slightly decreased in the
255 PMp/PPa/PMm/PMg (Fig. S9 B and C). Expression of *gal* in the pPMp remained intact in $ara^{-/-}$ males (Fig.
256 5 G and H), but was reduced in $arb^{-/-}$ males to approximately one-third of the level in $arb^{+/+}$ males (Fig. 5
257 I and J). In both $ara^{-/-}$ males and $ara^{-/-}$ females, reduced *gal* expression was instead observed in the
258 aPMp/PPa and NAT/NVT/NRL (Fig. S9 D–F), while in $arb^{-/-}$ males, no significant changes in *gal*
259 expression were found outside the pPMp (Fig. S9G). These results demonstrate that the expression of *vt* in
260 the pNVT and that of *gal* in the pPMp are critically and exclusively dependent on Ara and Arb signaling,
261 respectively, and imply that the loss of this *vt* and *gal* expression might account for the behavioral deficits
262 observed in $ara^{-/-}$ and $arb^{-/-}$ males, respectively.

263 Finally, we tested our hypothesis by administering Vt and Gal peptides to $ara^{-/-}$ and $arb^{-/-}$ males,
264 respectively, to explore whether their behavioral deficits could be rescued. As predicted, Vt-treated $ara^{-/-}$
265 males engaged in aggressive acts more frequently relative to non-treated controls, with significant
266 differences for fin displays and circles (Fig. 5 K and L). By contrast, Gal treatment had no significant effect
267 on any measure of mating or aggression in $arb^{-/-}$ males (Fig. S10). Taken altogether, our results suggest that
268 *vt* acts downstream of Ara signaling in the pNVT to mediate its stimulatory effects on male-directed
269 aggression; however, it remains to be determined whether *gal* mediates the behavioral effects of Arb
270 signaling.

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271 # Discussion

272

273 Herein, we have explored the behavioral consequences of the loss of two AR subtypes in male medaka.
274 Our results reveal, to our surprise, that males lacking Ara are less aggressive toward, and instead actively
275 court, other males, while those lacking Arb are less motivated to mate with, and instead attack, females.
276 These findings signify that, in male medaka, Ara- and Arb-mediated androgen signaling facilitate
277 appropriate behavioral responses, while simultaneously suppressing inappropriate responses, to male and
278 female conspecifics, respectively. It thus follows that the adaptive behavioral responses of males are shaped
279 by the complementary action of two distinct androgen signaling pathways. Up until this study, mouse *trpc2*,
280 which encodes a cation channel critical for signal transduction in the vomeronasal organ, has been the only
281 known vertebrate gene that, when deleted, reliably causes males to court other males or attack females (39,
282 40). Male mice lacking *trpc2* are unable to discriminate between male and female conspecifics, indicating
283 that this gene plays an essential role in sex discrimination (39, 40). In contrast, male medaka lacking *ara* or
284 *arb* retain the ability to discriminate sex; therefore, these two genes most probably act in the circuitry
285 controlling decision-making rather than in sex recognition.

286 Our results also suggest that Ara and Arb signaling inhibit each other to ensure mutually exclusive
287 displays of mating and aggression. Considering that Ara and Arb suppress male-directed mating behavior
288 and female-directed aggression, respectively, while facilitating aggression and mating, respectively, we
289 postulate that Ara inhibits Arb signaling to prevent male–male mating, while Arb inhibits Ara signaling to
290 prevent males attacking females. This reciprocal inhibition of Ara and Arb signaling probably involves
291 interactions that occur between neurons, rather than within a single neuron, because Ara and Arb are
292 expressed primarily in different neurons. This system evokes parallels with the reciprocal inhibition between
293 *Esr1*-expressing neurons in the MPOA and VMHvl of male mice that functions in the decision to choose
294 whether to mate or fight (5, 6). Such neuronal interactions involving sex steroid signaling may be conserved
295 in vertebrates and represent a general mechanism by which males decide whether to mate or fight, although
296 the specific sex steroid and receptor involved in this mechanism is like to vary among species. Given that
297 both the PMp and NAT – the putative teleost homologs of the rodent MPOA and VMH, respectively (13,
298 41) – express *ara* and/or *arb*, the brain nucleus associated with this mechanism may be also conserved
299 across species. This idea needs to be explored in future studies.

300 It may be relevant to note here that *Esr1*-expressing neurons in the mouse MPOA and VMHvl also
301 express *Ar* (42). This raises the question of whether androgen/AR signaling may play a critical role in
302 mediating male behavioral responses in mice as well as in medaka. In mice, however, the role of
303 androgen/AR signaling in male behaviors is rather limited: unlike in many other vertebrates, it only
304 increases the extent of behaviors (43); and indeed, male mice lacking *Ar* in the brain do not frequently court

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305 other males or attack females (44, 45). Instead, male mice lacking *Esr1* in inhibitory neuronal populations
306 show aggression, albeit not significant, toward females (46). Therefore, it can be still concluded that the
307 specific sex steroid signaling pathway playing a causal role in male behaviors varies among species.
308 Furthermore, a recent study in male cichlid demonstrated that Ara is essential for mating, while either Ara
309 or Arb is sufficient for aggression (24), suggesting that species differences may exist even among teleosts.
310 Another significant finding in this study is that the Ara and Arb signaling pathways are activated in
311 females in response to exogenous 11KT administration, producing male-typical aggressive and mating
312 behaviors, respectively. This finding suggests either that the functional neural circuits through which these
313 signaling pathways direct male-typical behavioral responses during social encounters exist in the normal
314 female brain, or that these circuits are rapidly organized by exogenous 11KT. Whichever is the case, these
315 circuits must be one of the neural substrates through which changes in the sex steroid milieu of adult teleosts
316 effectively reverse their sex-typical behaviors. Among vertebrates, teleosts are exceptionally sexually plastic
317 in their behavior as adults; however, there is accumulating evidence to suggest that other species, including
318 rodents, also have some degree of plasticity (e.g., 47–49). Further research in teleosts is likely to shed light
319 on the neural basis of sexual differentiation and plasticity of sex-typical behaviors in vertebrates.
320 With the exception of some species such as rodents, androgen/AR signaling is essential for the
321 expression of male-typical behaviors (15–18). Nevertheless, limited information is available on the
322 downstream effectors that mediate the behavioral effects of androgen/AR signaling, including the direct
323 targets of AR (50). Our current studies suggest that the neuropeptide Vt (vasotocin) acts downstream of Ara
324 signaling in the pNVT to mediate its stimulatory effects on male-directed aggression. Importantly, *vt* is
325 expressed exclusively in males in the pNVT and Ara can directly activate the transcription of *vt* (21); thus,
326 it is highly likely that *vt* serves as a male-specific, direct target of Ara for male-typical aggression. The
327 teleost NVT is considered homologous to the rodent anterior hypothalamus (AH), a known major site of
328 action of VT relevant to male behaviors (13, 41). Studies in hamsters, for example, have shown that
329 androgen administration increases the amount of VT peptide in the AH while concurrently increasing
330 aggression, and that administration of a VT receptor antagonist to the AH diminishes androgen-induced
331 aggression (51, 52). These observations in hamsters seem to be comparable to the present results in medaka.
332 Hence, the neural mechanism whereby androgen/AR signaling in the pNVT/AH stimulates male-typical
333 aggression via VT may be conserved across species.
334 We also showed that another neuropeptide, Gal (galanin), is a target of Arb signaling in the pPMp, but
335 found no evidence that Gal mediates the behavioral effects of Arb signaling. In our previous study, loss of
336 *gal* in medaka resulted in a marked reduction in male–male aggressive chases, suggesting that *gal* is
337 involved in male aggression (19). However, the present results show that *arb*-deficient males engage
338 normally in aggressive chases despite reduced *gal* expression in the pPMp. A recent study in midshipman

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339 fish (*Porichthys notatus*) suggests that Gal neurons in the pPMp play a role in male mating behavior (37),
340 but our treatment of *arb*-deficient males with Gal did not restore their impaired mating behavior. One
341 possible explanation for these discrepancies is that the reduction in *gal* expression in the pPMp of *arb*-
342 deficient males (down to approximately one-third) was not sufficient to affect behavior. Further studies are
343 needed to pinpoint the downstream effectors of Arb signaling.

344 In conclusion, our findings reveal that male medaka make adaptive decisions to mate or fight as a result
345 of the activation of one of two functionally distinct androgen/AR signaling pathways, depending on the sex
346 of the conspecific that they encounter. Furthermore, our findings suggest that these pathways inhibit each
347 other to ensure mutually exclusive displays of mating and aggression. Although the relevance of
348 androgen/AR signaling in vertebrate male behaviors has long been recognized, to our knowledge, this study
349 is the first to show that androgen/AR signaling stimulates male-typical mating and aggression while
350 simultaneously suppressing these opponent behaviors toward inappropriate targets. Last but not least, the
351 findings that the deficiency of a single gene in medaka can cause male–male mating (in the case of *ara*; this
352 study) or female–female mating (in the case of *esr2b*; 20) provide important insights into the neural
353 substrates underlying sexual orientation and their evolutionary aspects.

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Materials and Methods

Animals. Wild-type and *ara*-, *arb*-, and *esr2b*-deficient medaka (20) on the d-rR genetic background were raised at 28ºC with a 14-hour light/10-hour dark photoperiod. They were fed with live *Artemia nauplii* and dry food (Otohime; Marubeni Nisshin Feed, Tokyo, Japan) 3–4 times a day. Sexually mature, spawning adults (aged 2–6 months) were used in all experiments and assigned randomly to experimental groups. The fish used in each experiment were age-matched, co-housed siblings to control for genetic and environmental confounding. Tissue sampling was consistently performed 1–3 hours after light onset. All experimental procedures involving animals were performed in accordance with the University of Tokyo Institutional Animal Care and Use Committee guidelines.

Generation of gene-deficient medaka. *ara*- and *arb*-deficient medaka were generated by CRISPR/Cas9-mediated genome editing. Two and one CRISPR RNAs (crRNAs) were designed to target the predicted DNA-binding domains of *ara* and *arb* (22), respectively (Fig. S1A for *ara*; Fig. S2A for *arb*). The crRNAs and trans-activating crRNA (tracrRNA) were synthesized by Fasmac (Kanagawa, Japan) and injected along with Cas9 protein (Nippon Gene, Tokyo, Japan) into the cytoplasm of embryos at the one- or two-cell stage. Upon reaching adulthood, injected fish were outcrossed with wild-type fish, and the resulting progeny were tested for target site mutations by T7 endonuclease I assay (53), followed by direct sequencing. For *ara* targeting, two founders were identified that reproducibly produced progeny with deleterious mutations: one produced progeny carrying a 327-bp deletion/1-bp insertion (Δ326), which removed both the DNA- and ligand-binding domains; the other produced progeny carrying a 338-bp deletion/13-bp insertion (Δ325), which removed the N-terminal half of the DNA-binding domain (Fig. S1). Similarly, for *arb* targeting, two founders were identified that produced progeny carrying, respectively, 10-bp and 11-bp deletions (Δ10 and Δ11), both of which removed the DNA- and ligand-binding domains (Fig. S2). For both *ara* and *arb*, progeny from each founder were intercrossed to establish two independent mutant lines. Each line was maintained by intercrossing heterozygotes to obtain wild-type and homozygous siblings for experimental use. All fish were genotyped by PCR amplification of the target locus, followed by agarose gel electrophoresis (*ara*-deficient lines) or high-resolution melting analysis (*arb*-deficient lines) using the primers and probe listed in Table S2.

Aggressive behavior test. Intrasexual aggressive behavior among grouped fish was assessed as described by Yamashita et al. (19). In brief, four fish of the same sex and genotype (*ara*- or *arb*-deficient fish or wild-type siblings), unfamiliar with one another, were placed together in a 2-liter rectangular tank 1 hour after light onset (2 hours after treatment in tests using Vt and Gal). After acclimation to the test tank

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388 for 1 min, fish were allowed to interact for 15 min. All interactions were recorded with a digital video camera
389 (HC-V360MS, HC-VX985M, or HC-W870M; Panasonic, Tokyo, Japan), and the total number of each
390 aggressive act (chase, fin display, circle, strike, and bite) displayed by the four fish in the tank was counted
391 manually from the video recordings. In all video analyses, the researcher was blind to the fish genotype and
392 treatment.

393 Aggressive behavior was also tested individually by pairing each fish with a stimulus fish. On the day
394 before behavioral testing, a focal (*ara*- or *arb*-deficient fish or wild-type siblings) and stimulus (wild-type
395 male in the tests shown in Fig. S4 A–F; wild-type female in Fig. 4 A–F, J, and K and Fig. S6 A–E; and
396 *esr2b*-deficient female in Fig. S6 F–J) fish were placed in a 2-liter rectangular tank with a perforated
397 transparent partition separating them. The exception was the test using 11KT-treated females, where each
398 focal fish received 11KT continuously and was not introduced to the test tank until the day of testing to
399 ensure the effectiveness of 11KT. The partition was removed 1 hour after light onset, and fish were allowed
400 to interact for 15 min while their behavior was recorded as described above. The interaction time was
401 increased to 30 min in the tests using stimulus females (wild-type or *esr2b*-deficient), because females were
402 attacked relatively infrequently. The number of each aggressive act performed by the focal fish, and the
403 percentage of focal fish exhibiting any aggressive act were calculated from the video recordings. In tests
404 where the focal and stimulus fish were of the same sex, a small portion of the caudal fin of the stimulus fish
405 was clipped to distinguish them.

406 Regarding the behavior of *arb*-deficient males toward wild-type stimulus females, the correlation
407 between the number of aggressive acts during the interaction period and the number of wrapping attempts
408 in the first 10 min of interaction, and the latency from the first mating act to the initiation of aggressive acts
409 were further analyzed (note that the correlation analysis was not performed for *arb*-deficient males of the
410 Δ11 line, because only 4 of 11 males showed wrappings). In these analyses, courtship displays and wrapping
411 attempts, in addition to aggressive acts, were time-stamped in the first 15 min of interaction and used to
412 create the raster plots shown in Fig. 4B.

413

414 **Mating behavior test.** *ara*- and *arb*-deficient fish and wild-type siblings were tested for mating behavior
415 toward a stimulus fish (wild-type female in the tests shown in Fig. 2 B–G, Fig. S4 J–M, and Fig. S10A;
416 *esr2b*-deficient female in Fig. 2 H–J and Fig. S3; or wild-type male in Fig. 3 A–F and Fig. S5) using
417 essentially the same procedure as described above for aggressive behavior, with the following exceptions.
418 The partition was removed after 2 hours of treatment in the tests using Vt- and Gal-treated males. The
419 interaction time was increased to 30 and 120 min in the tests using, respectively, *esr2b*-deficient females as
420 the stimulating fish and 11KT-treated females as the focal fish because the 15-min interaction time was
421 insufficient.

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422 The latencies of the focal fish to initiate followings, courtship displays, and wrappings, and to spawn
423 were calculated from video recordings. In tests using *esr2b*-deficient females and wild-type males (which
424 were unreceptive to male courtship and did not spawn) as stimulus fish, the number of courtship displays
425 and wrapping attempts refused by the stimulus fish was calculated instead of the latency to spawn. In tests
426 where the focal and stimulus fish were both males, aggressive acts, in addition to courtship displays and
427 wrapping attempts, were time-stamped in the first 5 min of interaction and used to create the raster plots
428 shown in Fig. 3B. Note that the latency to initiate followings was not calculated in these tests because it was
429 sometimes difficult to distinguish between followings for mating and aggressive chases.
430
431 **Drug treatment.** Females of the *ara*-deficient (Δ326) and *arb*-deficient (Δ10) lines were treated with 100
432 ng/ml of 11KT (Denis Pharma, Tokyo, Japan) by immersion in water for 14–16 days and then tested for
433 aggressive and mating behaviors as described above. In separate experiments, *ara*-deficient (Δ326) and *arb*-
434 deficient (Δ10) males were treated intraperitoneally with 1 µl of an 8 µg/ml solution of synthetic medaka Vt
435 and Gal peptides (Scrum, Tokyo, Japan), respectively, or with vehicle (phosphate-buffered saline) alone 1–
436 1.5 hours after light onset. Aggressive and mating behaviors were evaluated 2 hours after treatment as
437 described above.
438
439 **Three-chamber test.** The test apparatus consisted of a rectangular tank (165 mm long by 190 mm wide,
440 filled with water to 30 mm) divided into three chambers by two perforated transparent partitions positioned
441 to give a central (test) chamber of 105 mm and two side chambers of 30 mm in length (Fig. 3 G and J and
442 Fig. 4G). Focal males (*ara*- and *arb*-deficient males or wild-type siblings) were individually placed in the
443 test chamber, while a wild-type stimulus female, unfamiliar to the focal males, was placed in one side
444 chamber. The other side chamber either contained a wild-type unfamiliar male or was left empty. After
445 acclimation for 1 min, the focal males were allowed to swim freely in the test chamber for 10 min while
446 their behavior was recorded. All tests were conducted 1–3 hours after light onset. The position of the
447 stimulus male and female was alternated every two trials to control for any side-preference bias. The location
448 of the focal males was tracked from the video recordings and the time spent in each location was calculated
449 using UMATracker (54). The resulting data were used to generate heat maps for visual analysis and to
450 calculate the difference in time spent near the stimulus male versus the stimulus female.
451
452 **Analysis of *fos* expression.** The focal fish (wild-type male) was placed alone or paired with a wild-
453 type stimulus female or male in a 2-liter rectangular tank with a perforated transparent partition separating
454 them. The partition was removed to allow fish to interact 0.5–1.5 hours after light onset on the following
455 day. The brain of each paired focal male was sampled 30 min after it spawned with the stimulus female or

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456 exhibited any aggressive act toward the stimulus male, and analyzed for *fos* expression by in situ
457 hybridization (see below). The brains of solitary males were sampled 30 min after removal of the partition
458 and processed likewise.
459
460 **Single-label in situ hybridization.** DNA fragments corresponding to nucleotides 16–1030 (1015 bp) of
461 the *ara* cDNA (GenBank accession number: NM_001122911), 53–1233 (1181 bp) of the *arb* cDNA
462 (NM_001104681), 20–1223 (1204 bp) of the *fos* cDNA (NM_001252234), 1–845 (845 bp) of the *vt* cDNA
463 (NM_001278891), and 5–533 (529 bp) of the *gal* cDNA (LC532140) were PCR-amplified and transcribed
464 in vitro to generate digoxigenin (DIG)-labeled cRNA probes using T7 RNA polymerase and DIG RNA
465 Labeling Mix (Roche Diagnostics, Basel, Switzerland).
466 Single-label in situ hybridization was performed as described previously (28). In brief, brains dissected
467 from males of the *ara*-deficient (Δ326) line (for analysis of *arb*, *vt*, and *gal*), *arb*-deficient (Δ10) line (*ara*,
468 *vt*, and *gal*), and wild-type strain (*fos*) were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin.
469 Serial sections (10-μm thick) were cut in the coronal plane and hybridized with one of the DIG-labeled
470 probes described above. Hybridized probes were detected using an alkaline phosphatase-conjugated anti-
471 DIG antibody (RRID: AB_514497; Roche Diagnostics) with nitro blue tetrazolium/5-bromo-4-chloro-3-
472 indolyl phosphate (NBT/BCIP) substrate (Roche Diagnostics). The color was allowed to develop for 1 hour
473 (*gal*), 6 hours (*fos* and *vt*), or overnight (*ara* and *arb*). Brain nuclei were identified using medaka brain
474 atlases (55, 56). Images were acquired with a virtual slide microscope (VS120; Olympus, Tokyo, Japan),
475 and the total area of expression signal in each brain nucleus was calculated using Olyvia software (Olympus).
476
477 **Double-label in situ hybridization.** The above-mentioned *ara* and *arb* cRNA probes were labeled,
478 respectively, with fluorescein using T7 RNA polymerase and Fluorescein RNA Labeling Mix (Roche
479 Diagnostics) and with DIG as described above.
480 Wild-type male brains were fixed in 4% PFA, embedded in paraffin, and coronally sectioned (10-μm
481 thick). The sections were hybridized simultaneously with the fluorescein-labeled *ara* and DIG-labeled *arb*
482 probes. Fluorescein was detected with a horseradish peroxidase-conjugated anti-fluorescein antibody
483 (RRID: AB_2737388; PerkinElmer, Waltham, MA) and visualized by using the TSA Plus Fluorescein
484 System (PerkinElmer); DIG was detected with a mouse anti-DIG antibody (RRID: AB_304362; Abcam,
485 Cambridge, UK) and visualized using the Alexa Fluor 555 Tyramide SuperBoost Kit, goat anti-mouse IgG
486 (Thermo Fisher Scientific, Waltham, MA, USA). Sections were counterstained with 4′,6-diamidino-2-
487 phenylindole (DAPI) to identify cell nuclei. Fluorescent images were acquired with a confocal laser
488 scanning microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany) with the following
489 excitation/emission wavelengths: 405/410–480 nm (DAPI), 488/495–545 nm (fluorescein), and 552/620–

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490 700 nm (Alexa Fluor 555).

491 To calculate the percentage of neurons co-expressing *ara* and *arb*, at least 40 *ara*- and/or *arb*-expressing
492 neurons from at least four different sections of each brain nucleus per fish were photographed and analyzed
493 for overlapping expression of *ara* and *arb*.

494

495 **Statistical analysis.** Data for continuous variables were expressed as mean ± standard error of the mean
496 (SEM), with individual data points shown as dots. Categorical variables were expressed as percentages.
497 Behavioral time-series data were analyzed using Kaplan-Meier plots with the inclusion of fish that did not
498 exhibit the given act within the test period, in accordance with Jahn-Eimermacher et al. (57).

499 All statistics were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Comparisons
500 between two groups for continuous variables were performed using the unpaired two-tailed Student's *t* test.
501 Welch's correction was applied if the F-test indicated that the variance between group was significantly
502 different. Continuous variables in more than two groups were analyzed using one-way analysis of variance
503 (ANOVA), followed by Dunnett's post hoc test. If the Brown-Forsythe test indicated a significant difference
504 in variance across groups, the data were instead analyzed using the non-parametric Kruskal-Wallis test
505 followed by Dunn's post hoc test. Differences between Kaplan-Meier curves were tested for significance
506 using Gehan-Breslow-Wilcoxon test. A one-sample *t* test was used to test the null hypothesis that there
507 would be no difference between the time spent on the stimulus female side and that spent on the male/empty
508 side. The Pearson correlation coefficient was used to assess whether there was a linear correlation between
509 the frequency of aggressive acts and that of wrapping attempts. Comparisons of categorical variables were
510 performed using Fisher's exact test. All data points were included in the analyses and no outliers were
511 defined.

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513

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659 # Figures

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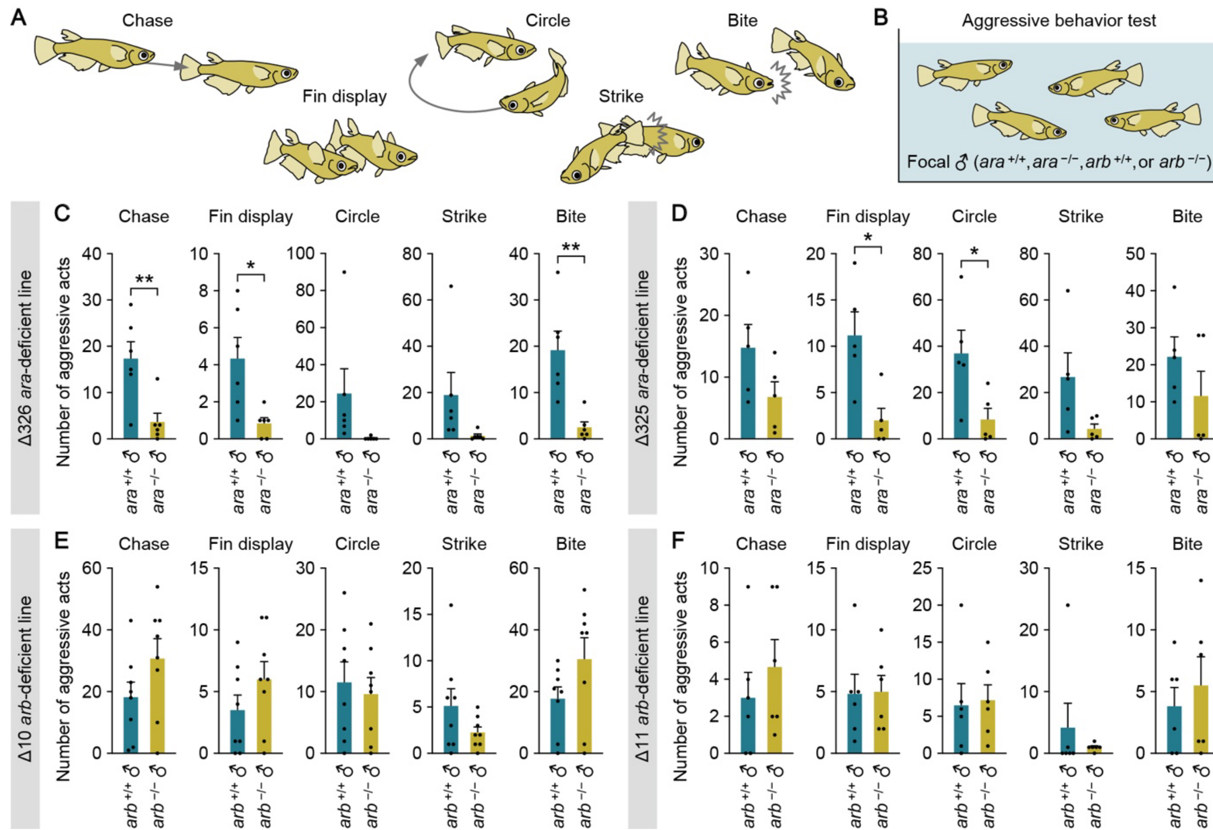

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663 **Fig. 1 Androgen/Ara signaling facilitates male aggression toward other males.** (**A**) Schematic
664 of the five types of aggressive act. (**B**) Set-up for testing aggressive behavior among grouped males. (**C** and
665 **D**) Total number of each aggressive act observed among *ara*$^{+/+}$ males and among *ara*$^{-/-}$ males in the tank.
666 Results from both Δ326 (n = 6 per genotype; C) and Δ325 (n = 5 per genotype; D) *ara*-deficient lines are
667 shown. (**E** and **F**) Total number of each aggressive act observed among *arb*$^{+/+}$ males and among *arb*$^{-/-}$ males
668 in the tank. Results from both Δ10 (n = 8 per genotype; E) and Δ11 (n = 6 per genotype; F) *arb*-deficient
669 lines are shown. Statistical differences were calculated by unpaired *t* test, with Welch's correction where
670 appropriate (C–F). Error bars represent SEM. *$P < 0.05$, **$P < 0.01$.

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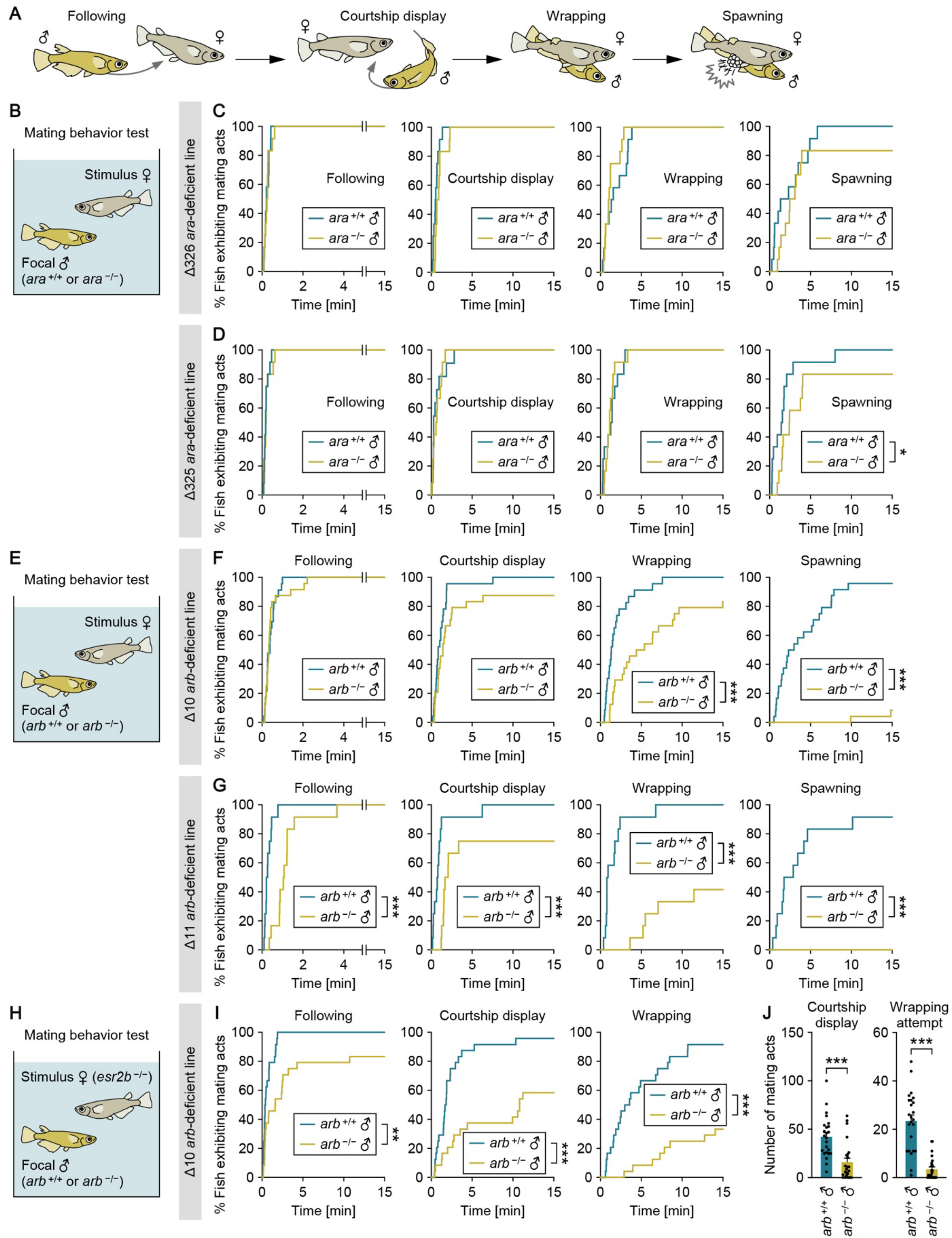

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673 **Fig. 2 Androgen/Arb signaling facilitates male mating with females.** (**A**) Schematic of a sequence

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674 of male-typical mating acts. (**B**) Set-up for testing the mating behavior of *ara*$^{+/+}$ and *ara*$^{-/-}$ males. (**C** and **D**)
675 Latency of *ara*$^{+/+}$ and *ara*$^{-/-}$ males to initiate each mating act toward the stimulus female. Results from both
676 Δ326 (n = 12 per genotype; C) and Δ325 (n = 12 per genotype; D) *ara*-deficient lines are shown. (**E**) Set-
677 up for testing the mating behavior of *arb*$^{+/+}$ and *arb*$^{-/-}$ males. (**F** and **G**) Latency of *arb*$^{+/+}$ and *arb*$^{-/-}$ males
678 to initiate each mating act toward the stimulus female. Results from both Δ10 (n = 23, and 24 for *arb*$^{+/+}$ and
679 *arb*$^{-/-}$, respectively; F) and Δ11 (n = 12 for each genotype; G) *arb*-deficient lines are shown. (**H**) Set-up for
680 the additional mating behavior test using an *esr2b*-deficient female as the stimulus. (**I**) Latency of the focal
681 *arb*$^{+/+}$ and *arb*$^{-/-}$ males (Δ10 line; n = 24 per genotype) to initiate each mating act. (**J**) Number of each
682 mating act performed. Statistical differences were calculated by Gehan-Breslow-Wilcoxon test (C, D, F, G,
683 and I) and unpaired *t* test (J). Error bars represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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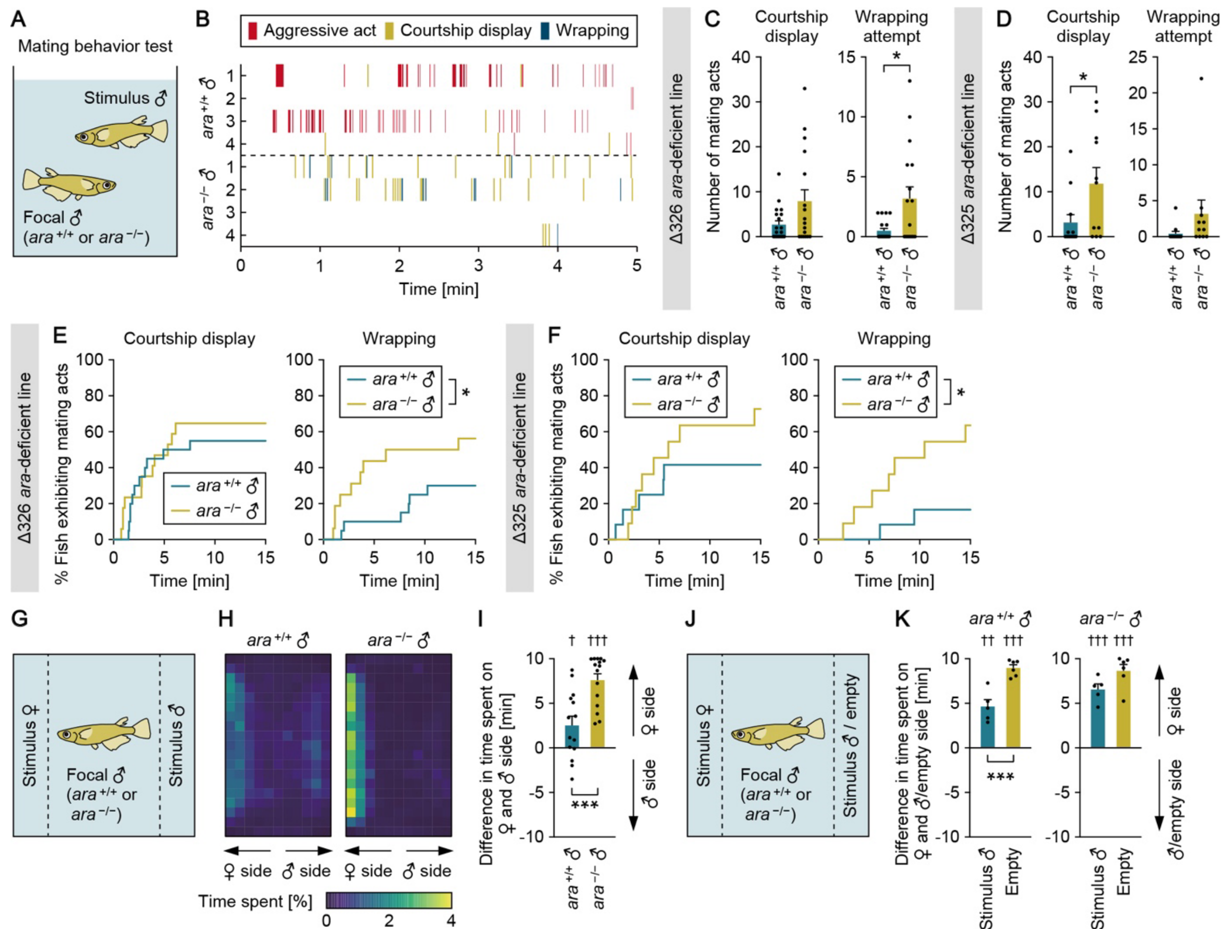

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686 **Fig. 3 *ara*-deficient males frequently attempt to mate with other males.** (**A**) Set-up for testing
687 the mating behavior of $ara^{+/+}$ and $ara^{-/-}$ males toward other males. (**B**) Raster plots of behavioral responses
688 of males to the stimulus male. Data from four representative males of each genotype ($ara^{+/+}$ and $ara^{-/-}$; Δ326
689 line) are shown. (**C** and **D**) Number of each mating act performed by $ara^{+/+}$ and $ara^{-/-}$ males toward the
690 stimulus male. Results from both Δ326 (n = 20 and 17 for $ara^{+/+}$ and $ara^{-/-}$, respectively; C) and Δ325 (n =
691 12 and 11 for $ara^{+/+}$ and $ara^{-/-}$, respectively; D) *ara*-deficient lines are shown. (**E** and **F**) Latency of males
692 to initiate each mating act. Results from both Δ326 (E) and Δ325 (F) lines are shown. (**G**) Set-up for the
693 three-chamber test to assess the sex discrimination ability of $ara^{+/+}$ and $ara^{-/-}$ males. (**H**) Heat maps
694 depicting the time spent by the focal males (Δ326 line; n = 14 and 15 for $ara^{+/+}$ and $ara^{-/-}$, respectively) in
695 each location of the test chamber. (**I**) Difference in time spent by the focal males on the stimulus female
696 versus the stimulus male side. (**J**) Set-up for the additional three-chamber test to assess the influence of
697 removing the stimulus male. (**K**) Difference in time spent by the focal $ara^{+/+}$ and $ara^{-/-}$ males (Δ326 line)
698 on the stimulus female versus the stimulus male (n = 5) or empty (n = 6) side. Statistical differences were
699 calculated by unpaired *t* test, with Welch's correction where appropriate (C, D, and comparisons between

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700 genotypes in I and K), Gehan-Breslow-Wilcoxon test (E and F), and one-sample *t* test (comparisons against
701 the null hypothesis of no difference in I and K). Error bars represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P <$
702 0.001 between genotypes. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ against the null hypothesis.

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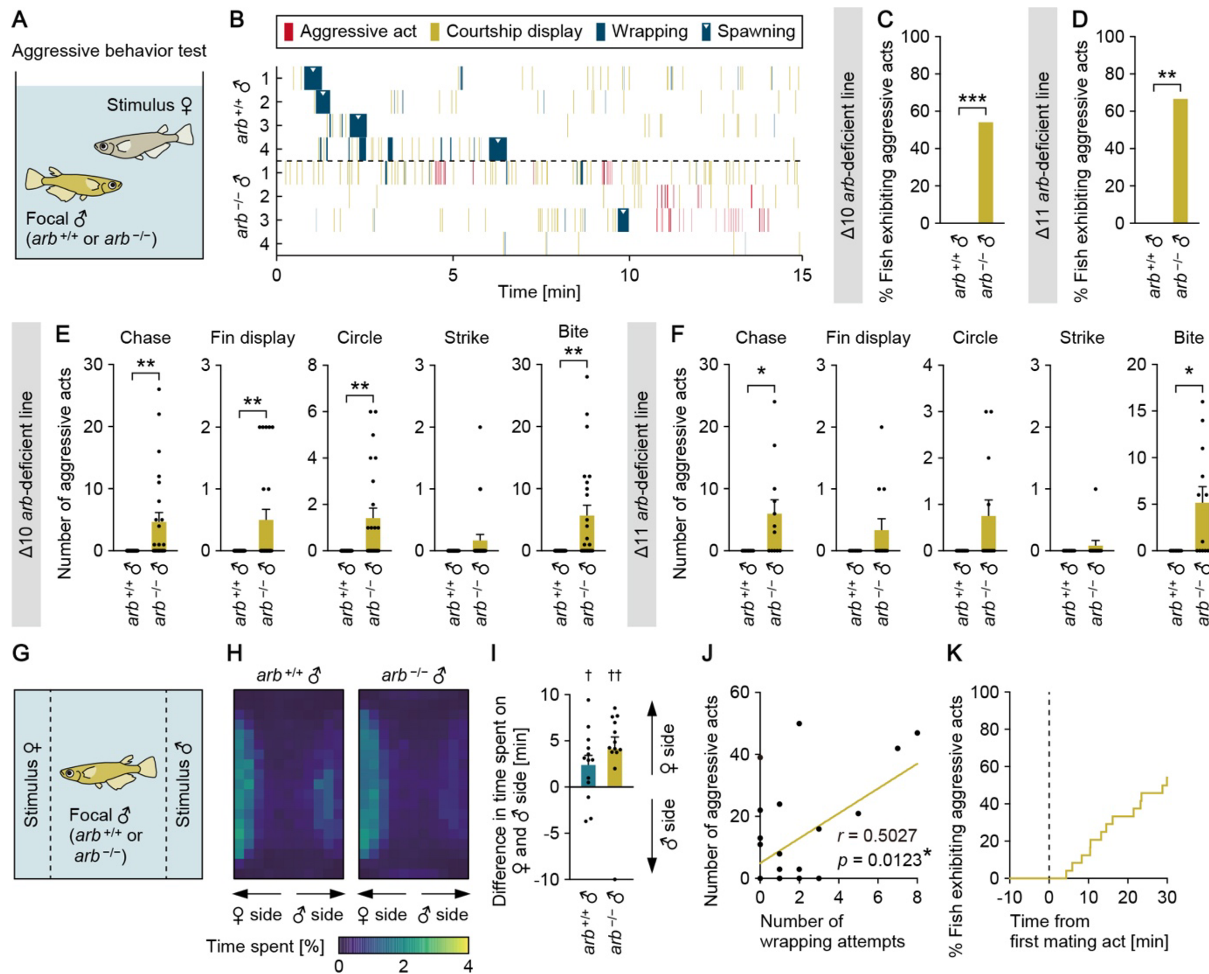

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705 **Fig. 4 *arb*-deficient males attack females.** (**A**) Set-up for testing the aggressive behavior of $arb^{+/+}$ and
706 $arb^{-/-}$ males toward females. (**B**) Raster plots of behavioral responses of males to the stimulus female. Data
707 from four representative males of each genotype ($arb^{+/+}$ and $arb^{-/-}$; Δ10 line) are shown. (**C** and **D**)
708 Percentage of $arb^{+/+}$ and $arb^{-/-}$ males exhibiting any aggressive act toward the stimulus female. Results from
709 both Δ10 (n = 23 and 24 for $arb^{+/+}$ and $arb^{-/-}$, respectively; C) and Δ11 (n = 12 for each genotype; D) *arb*-
710 deficient lines are shown. (**E** and **F**) Number of each aggressive act performed. Results from both Δ10 (E)
711 and Δ11 (F) lines are shown. (**G**) Set-up for the three-chamber test to assess the sex discrimination ability
712 of $arb^{+/+}$ and $arb^{-/-}$ males. (**H**) Heat maps depicting the time spent by the focal males (Δ10 line; n = 13 per
713 genotype) in each location of the test chamber. (**I**) Difference in time spent by the focal males on the stimulus
714 female versus the stimulus male side. (**J**) Scatter plot of the number of aggressive acts and wrapping attempts
715 performed by $arb^{-/-}$ males (Δ10 line; n = 24) toward the stimulus female. Each dot represents one focal
716 male; the unbroken line represents the regression line. (**K**) Latency from the first mating act to the initiation
717 of aggressive acts. Statistical differences were calculated by Fisher's exact test (C and D), unpaired *t* test,

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718 with Welch's correction where appropriate (E, F, and comparisons between genotypes in I), one-sample t
719 test (comparisons against the null hypothesis of no difference in I), and pairwise Pearson correlation (J).
720 Error bars represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between genotypes. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$
721 against the null hypothesis.

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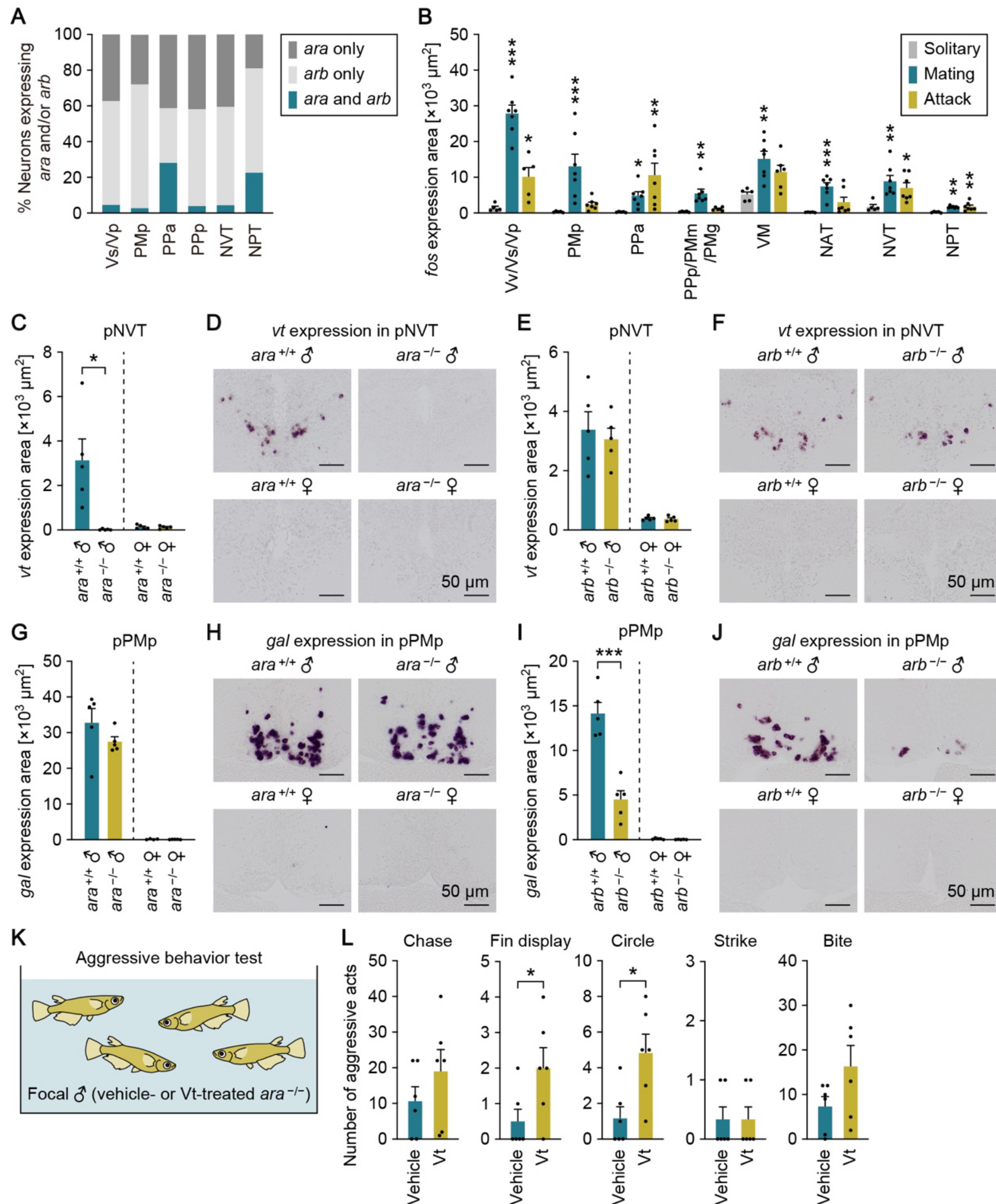

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724 **Fig. 5 Ara and Arb are expressed in different neurons and stimulate different behaviorally**

725 **relevant genes.** (**A**) Percentage of neurons expressing *ara* only, *arb* only, or both in each brain nucleus

726 of wild-type males. (**B**) Total area of *fos* expression signal in brain nuclei (where *ara* and/or *arb* are

727 expressed) of wild-type solitary males (n = 5), males that mated with females (n = 7), and males that attacked

728 other males (n = 7 except for Vv/Vs/Vp, PPp/PMm/PMg, and VM, where n = 5, 6, and 6, respectively). (**C**

729 and **D**) Total area (C) and representative images (D) of *vt* expression signal in the pNVT of males and

730 females of $ara^{+/+}$ and $ara^{-/-}$ fish (Δ326 line; n = 5 per sex per genotype). (**E** and **F**) Total area (E) and

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731 representative images (F) of *vt* expression signal in the pNVT of males and females of $arb^{+/+}$ and $arb^{-/-}$ fish
732 (Δ10 line; n = 5 per sex per genotype). (**G** and **H**) Total area (G) and representative images (H) of *gal*
733 expression signal in the pPMp of males and females of $ara^{+/+}$ and $ara^{-/-}$ fish (Δ326 line; n = 5 per sex per
734 genotype, except n = 4 for $ara^{+/+}$ females). (**I** and **J**) Total area (I) and representative images (J) of *gal*
735 expression signal in the pPMp of males and females of $arb^{+/+}$ and $arb^{-/-}$ fish (Δ10 line; n = 5 per sex per
736 genotype). (**K**) Set-up for testing aggressive behavior among $ara^{-/-}$ males treated with vehicle alone or Vt
737 peptide. (**L**) Total number of each aggressive act observed among $ara^{-/-}$ males in the tank (Δ326 line; n = 6
738 per treatment). All scale bars are 50 µm. For abbreviations of brain nuclei, see Table S1. Statistical
739 differences were calculated by Dunnet's or Dunn's post hoc test (B) and unpaired *t* test, with Welch's
740 correction where appropriate (C, E, G, I, and L). Error bars represent SEM. $*P < 0.05$, $**P < 0.01$, $***P <$
741 0.001.