

¹ Estradiol promotes habituation learning via ² an unidentified target, bypassing the ³ suppressive effects of the established ⁴ Estrogen Receptors

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¹⁰ Abstract

¹¹ Habituating to the constant stimuli in the environment is a critical learning process conserved across species. We
¹² use a larval zebrafish visual response to sudden darkness as a model for studying habituation learning, where
¹³ zebrafish reduce their responses to repeated stimulations. In this paradigm, treatment with estradiol strongly
¹⁴ increases learning rate, resulting in more strongly suppressed responses. We used knockout mutant lines for the
¹⁵ Estrogen Receptors (*esr1*, *esr2a*, *esr2b*, *gper1*) in an attempt to identify the receptor(s) mediating these effects. These
¹⁶ experiments failed to identify a necessary receptor (or combination of receptors). Surprisingly, *esr1*, *esr2a*, and *gper1*
¹⁷ mutants showed weak but consistent increases in habituation, indicating these receptors suppress habituation
¹⁸ learning. These experiments demonstrate that estradiol is a complex modulator of learning in our model, where the
¹⁹ learning-promoting effects are mediated by an unidentified estradiol target, and the classical Estrogen Receptors
²⁰ act in competition to subtly suppress learning.

21

²² Introduction

²³ A primary function of the brain is to learn from experiences and adjust behavior in response. One aspect of learned
²⁴ behaviour involves sharpening attention and behavioural resources toward salient cues by ignoring irrelevant
²⁵ background stimuli. For instance, it may be critical to recognize the alarm calls of a nearby animal, whereas
²⁶ continually registering the steady hum from distant traffic is far less important. The capacity to reduce responses
²⁷ to repetitive, non-essential stimuli is known as habituation, which is considered the simplest form of learning and
²⁸ memory (*Rankin et al., 2009*).

²⁹ We study a paradigm for long-term habituation where larval zebrafish reduce their responsiveness to sudden
³⁰ pulses of whole-field darkness, or dark flashes (DFs) (*Wolman et al., 2011; Randlett et al., 2019; Lamiré et al., 2023*).
³¹ We recently reported that multiple hormonal signaling pathways show strong modulation of habituation learning

32 performance, including melatonin, progesterone, and estrogen (*Lamiré et al., 2023*). The ability of these signaling
33 pathways to modulate learning is consistent with previous results in other systems and paradigms (*Nilsson and*
34 *Gustafsson, 2002; Naderi et al., 2020; Dillon et al., 2013; Rawashdeh et al., 2007; Jilg et al., 2019; El-Sherif et al.,*
35 *2003; Barros et al., 2015*), and may be an important mechanism to shift learning and memory performance or
36 strategies based on biological rhythms or external fluctuations like seasons, weather or the day/night cycle.

37 In this project we have focused on estrogen signaling. We identified multiple estradiol analogs which strongly
38 increased habituation learning when bath applied at 5-10 μ M doses (ethinyl estradiol, estradiol valerate, and hexe-
39 strol, *Lamiré et al., 2023*). 17 β -estradiol (here referred to as estradiol) is the most potent and biologically active form
40 of estrogen, and is used in a variety of clinical contexts including contraception, hormone replacement therapy, and
41 feminizing hormone therapy (*Kuhl, 2005; Unger, 2016; Farkas et al., 2022*).

42 Our discovery of a role for estradiol in promoting habituation learning is not surprising, as it has well-documented
43 effects on other learning and memory processes (*Frick, 2015*). This has been most extensively characterized in the
44 hippocampus, where estradiol promotes behavioural performance and the cellular/circuit hallmarks of hippocam-
45 pal plasticity, including Long-term potentiation (LTP) and modulation of dendritic spine density (*Iqbal et al., 2024;*
46 *Luine, 2014; Finney et al., 2020; Nilsson and Gustafsson, 2002*). While the role of estradiol in habituation is less well
47 explored, it has previously been shown to increase memory retention for olfactory habituation in mice (*Dillon et al.,*
48 *2013*), indicating it plays conserved roles in plasticity regulation across paradigms.

49 Estradiol signals via two established classes of Estrogen Receptors (ERs): the ligand-activated transcription fac-
50 tors ER α , and ER β , and the seven-transmembrane G-protein coupled receptor Gper1. ER α/β are thought to mediate
51 the long-term "genomic" effects of estrogens through transcriptional activation of target genes, and thus are typi-
52 cally termed nuclear ERs. Estradiol also has acute effects on physiology – often called "non-genomic", or "membrane-
53 initiated". These acute effects are thought to be mediated largely by the G protein coupled receptor Gper1, which
54 signals via multiple G-proteins, and potentially epidermal growth factor (EGF) receptor transactivation (*Prossnitz*
55 *and Barton, 2023; Revankar et al., 2005; Filardo et al., 2000*). In this way, Gper1 signalling impacts multiple core
56 second-messenger systems, including: adenylyl cyclase, ERK, PI3K-Akt, and nitric oxide synthase. There is evidence
57 from receptor-specific pharmacology and genetic knockout experiments in mice for a role of all of these receptors
58 in hippocampal plasticity (*Finney et al., 2020; Koitmäe et al., 2023; Briz et al., 2015*).

59 Pharmacological experiments in adult zebrafish indicate that nuclear ERs are involved in the consolidation of
60 object recognition memory, while Gper1 is involved in the consolidation of object placement memory (*Naderi et al.,*
61 *2020*). Therefore, both classes of receptors are good candidates for plasticity regulation in zebrafish. However, we
62 are unaware of any previous studies using genetic knockout lines to test for the function of zebrafish ERs in learning
63 or memory.

64 In this project we aimed to identify the relevant ER(s) mediating the effects of estradiol on habituation using
65 genetic knockout alleles. Zebrafish have single gene encoding ER α (ER1, *esr1*) and Gper1 (*gper1*), and two homologs
66 of ER β : ER2a (*esr2a*) and ER2b (*esr2a*) (*Romano et al., 2017; Menuet et al., 2002*). We found that none of these
67 mutants were insensitive to estradiol's effects, indicating that estradiol acts in this context via an alternative receptor
68 or pathway. Surprisingly, our experiments found that mutants for *esr1*, *esr2a*, and *gper1* actually habituate more
69 than their sibling controls. While the effect size is small and behavioural-genetic experiments can be variable, these
70 data indicate that these ERs actually act to inhibit habituation learning, rather than mediating the habituation-
71 promoting effects of estradiol that we observe pharmacologically.

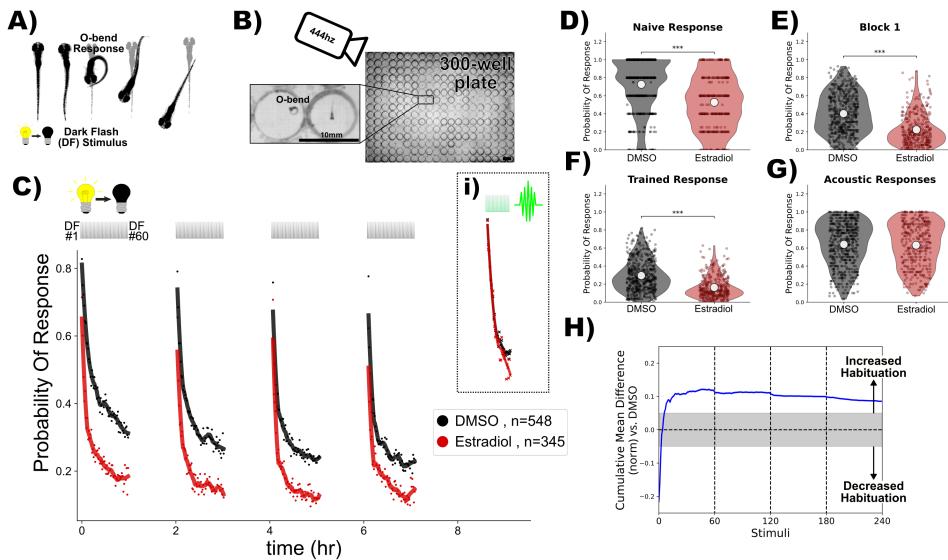


Figure 1. Estradiol increases habituation learning.

A) In response to a dark flash (DF), larval zebrafish perform a large turning manoeuvre termed an "O-bend" response.

B) High-throughput setup for recording and quantifying responsiveness using a high-speed camera recording at 444hz observing larvae in 300-well plates. Scale bar = 10mm.

C) Treatment with estradiol (red) results in more rapid and profound decreases in the probability of response to DF stimuli during habituation training relative to DMSO vehicle controls (black). DF stimuli are delivered at 1-minute intervals, in 4 blocks of 60 stimuli, separated by 1hr of rest (from 0:00-7:00). 1.5 hours later a block of 30 vibration stimuli are delivered at 1-minute intervals (i). Each dot is the probability of response to one DF. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2).

D-G) Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U test, *** = $p < 0.001$. D) the naive response to the first 5 DF stimuli; E) the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); F) the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); G) the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.

H) Cumulative mean difference (CMD) plot quantifying relative habituation performance after estradiol treatment. These plots display the cumulative average differences in the mean response across larvae of the treatment group (estradiol) relative to the control group (DMSO). Difference from 0 reflect a divergence in the change in responsiveness across the 240 DF stimuli in the 4 training blocks, with positive values reflecting increased habituation. The widths of the line is a bootstrapped 99.5% confidence intervals. The gray boxed region reflects the expected non-significant effect size (Randlett et al., 2019).

Treatment groups are: Estradiol = 10 μ M estradiol treatment ($n = 345$ fish); DMSO = 0.1% DMSO vehicle controls ($n = 548$ fish)

Results

73 Estradiol increases visual habituation learning

74 In response to a sudden global darkening stimulus, which we refer to as a dark flash (DF), larval zebrafish execute
 75 an "O-bend" maneuver, characterized by a deep "O"-shaped bend and a high-amplitude turn (Burgess and
 76 Granato, 2007, Figure 1a). Habituation learning manifests as a progressive reduction in response to repeated
 77 stimuli, and this learning can be retained for seconds/minutes, or hours/days for short-, and long-term habituation,
 78 respectively (Rankin et al., 2009). We use high-speed cameras, machine-vision analysis, and 300-well plates to
 79 quantify habituation across large populations of larvae to identify molecular/genetic mechanisms of long-term
 80 habituation(Figure 1A,B, Randlett et al., 2019; Lamiré et al., 2023). When stimulated with DFs repeated at 1-minute
 81 intervals in blocks of 60 stimuli, larval zebrafish exhibit long-term habituation, reducing not only the probability of
 82 executing a response, but also modulating the latency and other kinematic aspects of the response (Randlett et al.,
 83 2019).

84 Our previous small-molecule screening experiments identified multiple synthetic Estrogen Receptor agonists
 85 as positive modulators of DF habituation learning, including ethinyl estradiol, estradiol valerate, and hexestrol
 86 (Lamiré et al., 2023). The major effect we observed was a stronger decrease in the probability of executing a O-
 87 bend response during the training/learning blocks. We have confirmed and extended these results using estradiol,

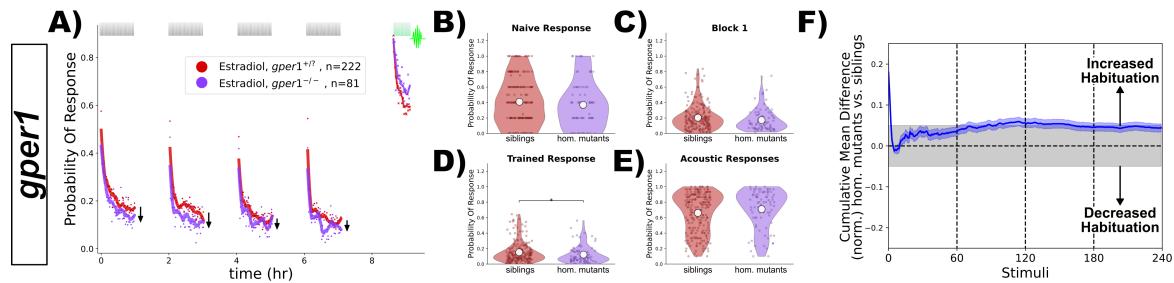


Figure 2. *gper1* mutants do not show habituation deficits after treatment with estradiol.

A) Homozygous *gper1*^{-/-} mutants ($n = 81$ fish, purple) do not show impaired habituation relative to sibling controls (*gper1*^{+/+}) and *gper1*^{+/+} ($n = 222$ fish, red). Rather, there is a slight suppression of responsiveness in the mutant group (arrows), indicating weakly increased habituation. Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2).

B-E) No significant differences are observed in the responsiveness distributions for the naive response to the first 5 DF stimuli (B), during the first training block (C), or the vibration response (E), while a subtle but statistically significant decrease in responsiveness is observed in the trained response (D). Statistical significance was calculated using Mann-Whitney U test, * = $p < 0.05$.

F) Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls, consistent with slightly increased habituation rate in mutant larvae.

which is the major natural estrogen in vertebrates.

An acute dose of 10 μ M estradiol potently increases habituation learning, which is observable when the response probability of the population of estradiol-treated larvae is compared with DMSO-treated vehicle controls (Figure 1C-H). Consistent with our previous experiments (Lamiré et al., 2023), there is a reduction in the naive responsiveness of the estradiol-treated larvae to the first DF stimuli (Figure 1D), but the major effect is observed during the training phase (Figure 1C,E,F), as is revealed by the consistent positive deviation in the cumulative mean difference (CMD) plots that are normalized to the naive response level in order to quantify response suppression indicative of habituation performance (Figure 1H, Randlett et al., 2019). Importantly, the responsiveness of the larvae to vibration stimuli delivered after the DF stimulation (Figure 1Ci), which elicit c-bend escape responses (Kimmel et al., 1974), is indistinguishable from controls (Figure 1G). From this we conclude that estradiol does not affect global arousal levels but rather has specific effects on habituation learning.

Gper1 is dispensable for estradiol-promoted habituation learning

The effects of estradiol that we have observed occur very rapidly – larvae are only pretreated with estradiol for ≈ 25 min-1 hr before the first DF. This is the time necessary to set the apparatus and begin the experiment. Since the nuclear hormone receptors are thought to primarily exert their effects via transcriptional alterations, this necessitates a delay in their signaling. For this reason, we first hypothesized that membrane-initiated signaling through Gper1 was the most likely mechanism.

To test this we used a knockout allele *gper1*^{uab102} (Romano et al., 2017), and generated larvae from *gper1*^{uab102} heterozygous or homo/heterozygous crosses to generate clutches of larvae of mixed genotypes. Larvae were treated with estradiol during habituation, and were subsequently genotyped. We reasoned that if *gper1* is required for the effect of estradiol on habituation, mutants would be insensitive to estradiol and habituate significantly less than sibling controls. Contrary to this hypothesis, we found that *gper1* mutants showed no deficits in habituation (Figure 2). Remarkably, rather than observing the anticipated inhibition of habituation, *gper1* mutants appeared to habituate slightly more than controls, with the responsiveness level slightly but consistently below the sibling controls across stimuli (Figure 2A). This is further supported by a weak but statistically significant decrease in the responsiveness of the larvae during the training period (Figure 2D), and a deviation towards positive values in the CMD plot (Figure 2F). From these experiments we conclude that Gper1 agonism does not promote habituation learning, but rather may act to suppress it.

116 Nuclear ERs are dispensable for estradiol-promoted habituation learning

117 Since we found that *gper1* was unnecessary for the habituating-promoting effects of estradiol, we next focused
118 on the three nuclear receptors in the zebrafish genome: *esr1*, *esr2a* and *esr2a*. Using the same strategy as for
119 *gper1*, we analyzed previously established knockout mutants (*esr2a^{uab134}*, *esr2a^{uab134}*, and *esr2b^{uab127}*), looking for
120 a mutant with insensitivity to estradiol. However, we failed to identify any deficits in habituation (*Figure 3*). To our
121 surprise, we again found that both *esr1* and *esr2a* mutants showed subtle increases in habituation (*Figure 3Avi,Bvi*),
122 similar in magnitude to what we had seen for *gper1* mutants (*Figure 2F*). From these data we conclude that none
123 of the nuclear ERs are required for the effects of estradiol on promoting habituation. As for Gper1, ER1 and ER2a
124 show a weak inhibitory effect on habituation, indicating that they also act to suppress habituation learning.

125 Nuclear ERs are simultaneously dispensable for estradiol-promoted habituation learning

126 While our experiments demonstrated that *esr1*, *esr2a* and *esr2a* mutants remain sensitive to estradiol, it is plausible
127 that they could act in a redundant fashion to mediate the effects of estradiol on habituation, perhaps via co-
128 expression in a critical cell type. To test for this possibility, we generated combinations of mutants by crossing
129 individual lines together (*Figure 4*). A likely scenario could be that the two ER β paralogs, ER2a and ER2b, act
130 redundantly. However, we found that double mutants for *esr2a;esr2b* did not show habituation deficits (*Figure 4A*).
131 Neither did we observe suppression of habituation in double mutants for *esr1;esr2a* (*Figure 4B*), or *esr1;esr2b*
132 (*Figure 4C*). Finally, we tested triple mutants for all three nuclear ERs (*esr1;esr2a;esr2b*), but again failed to identify
133 suppressions in habituation (*Figure 4D*).

134 We note that we did see a statistically "significant" increase in DF responsiveness in the *esr1;esr2b* double mutants
135 (*Figure 4Cii-iv*). This is attributable to a small increase in naive responsiveness, and did not result from
136 habituation deficits according to our normalized CMD analysis (*Figure 4Cvi*). Considering that we did not observe
137 any increased responsiveness in the triple mutants (*Figure 4D*), we conclude that the observed increased respon-
138 siveness in the double mutants is likely a result of biological noise, that only rose to statistical "significance" due to
139 the very large number of larvae tested in our high-throughput experiments.

140 Consistent with the paradoxical effect of increased habituation in *esr1* and *esr2a* single mutants (*Figure 3A,B*), we
141 again observed that double and triple mutants containing these genes also showed a slight increase in habituation
142 (with the exception of the *esr1;esr2b* double mutants). This adds further support to the model in which ER1 and
143 ER2b act to suppress learning in this context, rather than promote it.

144 Gper1 and nuclear ERs are simultaneously dispensable for estradiol-promoted habitua- 145 tion learning

146 While it is unclear how the GPCR Gper1 might act redundantly with the nuclear ERs, we nevertheless decided to
147 test this possibility by combining the *gper1* and the *esr1*, *esr2a*, and *esr2b* mutations (*Figure 5*). As with the previous
148 iterations of this experiment, we did not find combinations of mutants with suppressed habituation (*Figure 5*).
149 Consistent with our model of (*gper1*, *esr1* and *esr2a*) having inhibitory affects on learning, we again found that most
150 of these combinations of mutants showed evidence of increased habituation (*Figure 5i,vi*). While the responsiveness
151 distributions did not show significant differences (*Figure 5ii-iv*), the normalized CMD plots consistently showed
152 positive deviations, which reflect increased habituation (*Figure 5vi*).

153 Despite the fact that we analyzed the behaviour of 1152 larvae and successfully genotyping all 4 ER genes
154 in 373 individuals (after >4600 genotyping PCRs), we were only able to identify a single quadruple mutant larva
155 lacking all known ERs (*Figure 5E*). This is likely simply due to the limitations of combinatorial Mendelian inheritance,
156 and the fact that *esr1* and *esr2a* are linked on chromosome 20. While it is dubious to conclude much from an
157 n = 1 experiment, we find it remarkable that this larva exhibits the strongest increased habituation of all of our
158 experiments, with suppression of responses (*Figure 5Ei*), and strong positive deviation in the CMD plot (*Figure 5Ev*).

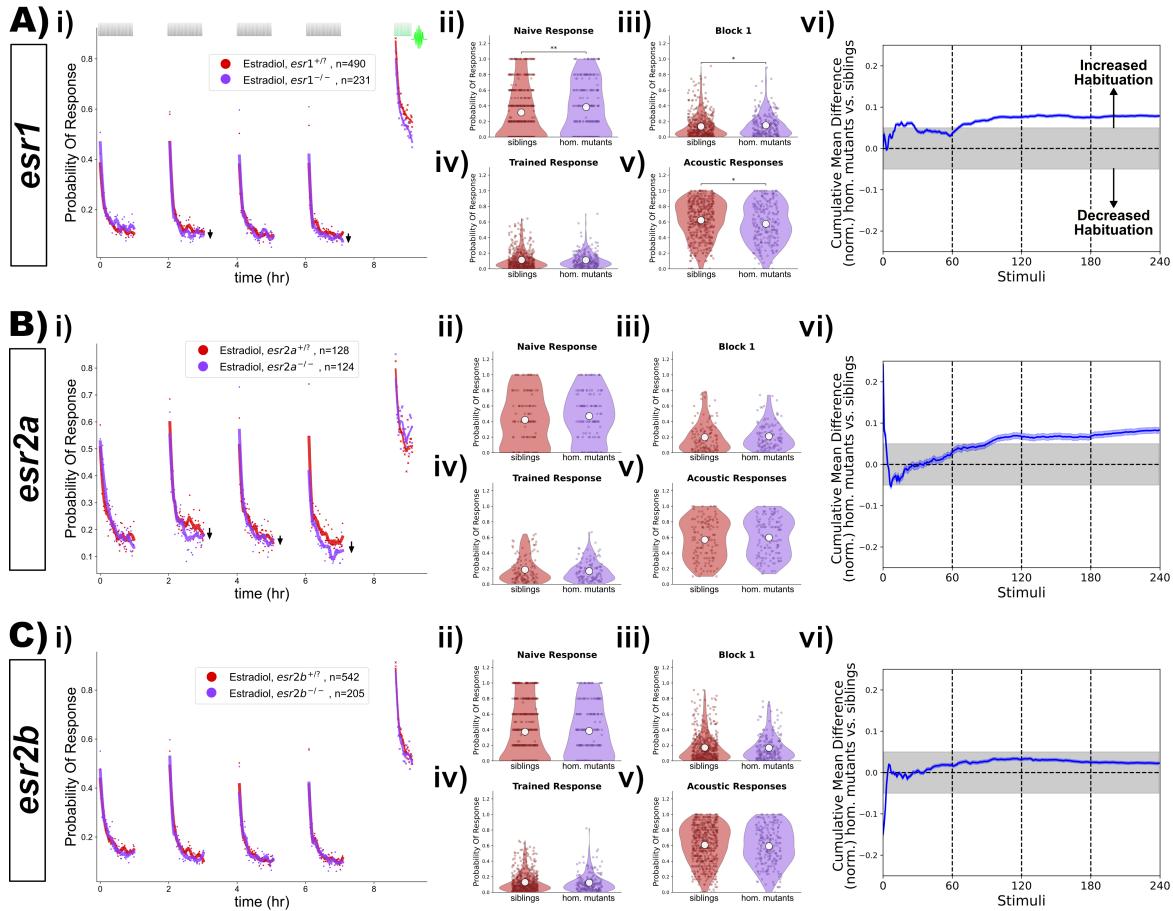


Figure 3. *esr1*, *esr2a* and *esr2b* mutants do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1^{-/-}* mutants (n = 231 fish) do not show impaired habituation relative to sibling controls (*esr1^{+/+}*) and *esr1^{+/+}* (n = 490 fish).

B) Homozygous *esr2a^{-/-}* mutants (n = 214 fish) do not show impaired habituation relative to sibling controls (*esr2a^{+/+}* and *esr2a^{+/+}*, n = 128 fish).

C) Homozygous *esr2b^{-/-}* mutants (n = 205 fish) do not show impaired habituation relative to sibling controls (*esr2b^{+/+}* and *esr2b^{+/+}*, n = 542 fish).

i)-vi) For each lettered section:

i) Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2). Suppression of responsiveness is indicated by arrows, potentially reflecting increased habituation.

ii-v) Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U test, * = p < 0.05, ** = p < 0.01. ii) the naive response to the first 5 DF stimuli; iii) the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); iv) the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); v) the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.

vi) Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls

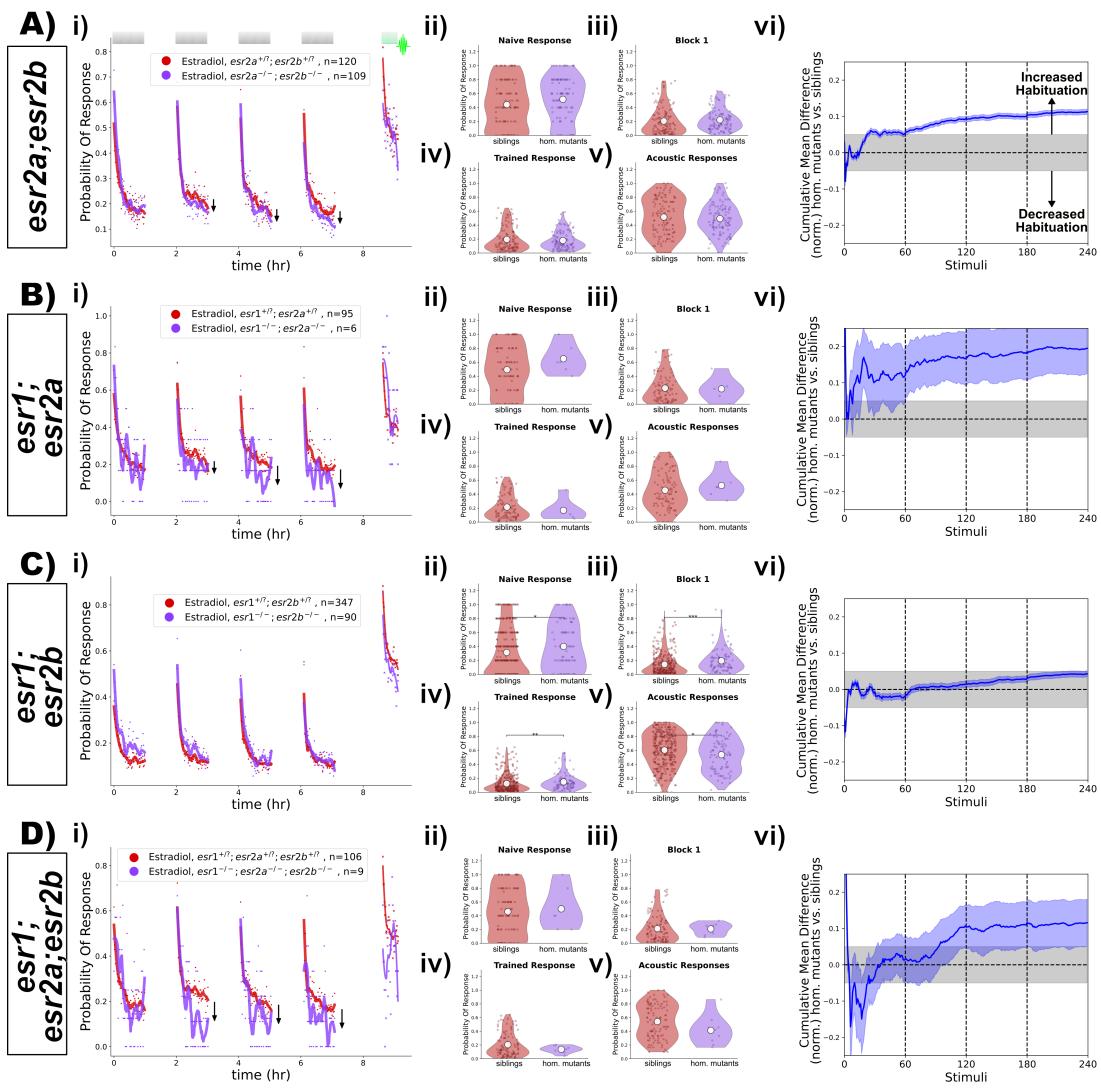


Figure 4. Double and triple mutant combinations of *esr1*, *esr2a* and *esr2b* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr2a^(-/-);esr2b^(-/-)* double mutants (n = 109 fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (^(+/-)/^(+/-), n = 120 fish).

B) Homozygous *esr1^(-/-);esr2a^(-/-)* double mutants (n = 6 fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (^(+/-)/^(+/-), n = 95 fish).

C) Homozygous *esr1^(-/-);esr2b^(-/-)* double mutants (n = 90 fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (^(+/-)/^(+/-), n = 347 fish).

D) Homozygous *esr1^(-/-);esr2a^(-/-);esr2b^(-/-)* triple mutants (n = 9 fish) do not show impaired habituation relative to triple heterozygous or homozygous sibling controls (^(+/-)/^(+/-)/^(+/-), n = 106 fish).

i)-vi) For each lettered section:

i) Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2).

ii-v) Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U test, * = p < 0.05, ** = p < 0.01. ii) the naive response to the first 5 DF stimuli; iii) the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); iv) the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); v) the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.

vi) Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls.

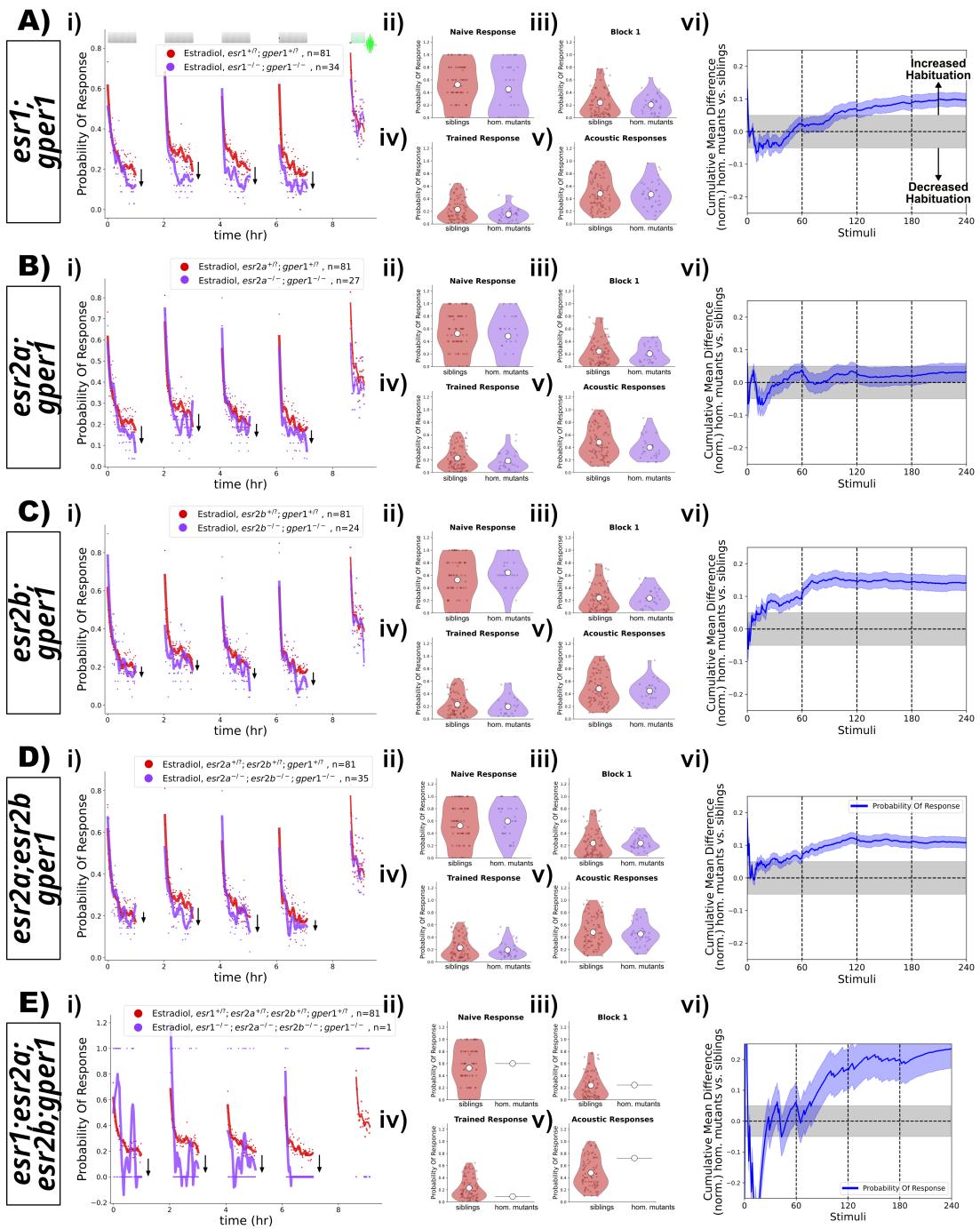


Figure 5. Double, triple and quadruple mutant combinations of *esr1*, *esr2a*, *esr2b*, and *gper1* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1^(-/-);gper1^(-/-)* double mutants ($n = 34$ fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($n = 81$ fish, red). **B)** Homozygous *esr2a^(-/-);gper1^(-/-)* double mutants ($n = 27$ fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($n = 81$ fish, red). **C)** Homozygous *esr2b^(-/-);gper1^(-/-)* double mutants ($n = 24$ fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($n = 81$ fish, red). **D)** Homozygous *esr2a^(-/-);esr2b^(-/-);gper1^(-/-)* triple mutants ($n = 35$ fish, purple) do not show impaired habituation relative to heterozygous or homozygous sibling controls ($n = 81$ fish, red). **E)** A single homozygous *esr1^(-/-);esr2a^(-/-);esr2b^(-/-);gper1^(-/-)* quadruple mutant ($n = 1$ fish, purple) does not appear to show impaired habituation relative to heterozygous or homozygous sibling controls ($n = 81$ fish, red).

i-vi) For each lettered section: **i)** Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2). **ii-v)** Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U test, * = $p < 0.05$, ** = $p < 0.01$. **ii)** the naive response to the first 5 DF stimuli; **iii)** the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); **iv)** the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); **v)** the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform. **vi)** Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls. As mutant larvae were all derived from the same experiments, the sibling control data (red) is the same in **A-E**.

159 Discussion

160 The promotion of habituation learning by estradiol is mediated by an unidentified target

161 Our experiments indicate that Gper1, ER1, ER2a and ER2b do not mediate the positive effects of estradiol on
162 habituation learning. As this is fundamentally a negative result, it is difficult to conclusively demonstrate this
163 beyond any doubt. One major caveat relates to the actual functional nature of the mutant alleles that we have
164 used. These are all Cas9-generated small deletions resulting in frameshift mutations that lead to early stop codons,
165 and are thus predicted null/knockout lines. Despite this genetic confidence, it is always possible that residual
166 activity could still remain, perhaps via alternate splicing or alternate start codons. For this reason, we were originally
167 unsure if we would publish these results at all. However, we recognize that this bias against publishing negative
168 results is bad for science. Negative results from well-designed and executed experiments are of value for the
169 community and making this knowledge public is our duty as responsible scientists (*Mlinarić et al., 2017*). These
170 high-throughput neurogenetic experiments are also a massive amount of work – raising, testing and genotyping
171 thousands of zebrafish larvae. This was undertaken primarily by technicians and Master's students, all of whom
172 deserve recognition, irrespective of the perceived "impact" of the outcome of their experiments.

173 While the possibility of "residual activity" in our mutants is a clear limitation of our approach, we argue that this
174 alternative interpretation is very unlikely. The *esr1^{uab118}* and *esr2b^{uab127}* alleles both exhibited a lack of estradiol
175 responsiveness in other tissues (*Romano et al., 2017*), and *esr2b^{uab127}* mutants are female sterile/subfertile (D.
176 Gorelick, personal communication), indicating a non-functional receptor. Similarly, *gper1^{uab102}* mutants show a lack
177 of estradiol responsiveness in heart rate modulation (*Romano et al., 2017*). Interestingly this was only observed in
178 maternal-zygotic mutants. While it seems unlikely that sufficient maternal mRNA/protein for Gper1 could persist
179 in 5dpf larvae, we can formally rule this out with our current datasets. The *esr2a^{uab134}* mutants have no previously
180 published phenotype, and so we do not have an independent positive control for the nature of this allele. However,
181 the best evidence we have against the "residual activity" hypothesis is that we actually found phenotypes in our
182 assays for *esr1^{uab118}*, *esr2a^{uab134}*, and *gper1^{uab102}* mutants. These phenotypes are just of the unexpected sign, where
183 mutants show increased habituation (discussed below).

184 Potential mechanisms for estradiol-promoted habituation learning

185 We have concluded that the lack of habituation deficits in our mutants is due to the presence of an alternative re-
186 ceptor or pathway that mediates the learning-promoting effects of estradiol. In support of this model, a very recent
187 study of estradiol-induced thrombosis came to the same conclusion, namely that an unidentified target mediates
188 this process in larval zebrafish (*Yu et al., 2024*). Importantly, these thrombosis experiments were performed on
189 different knockout alleles which are full genetic deletions, in which "residual activity" is not plausible. Together with
190 our observations, these data suggest that a novel estradiol target exists which has multiple critical functions (at
191 least in zebrafish).

192 What might this unidentified target be? Various leads exist in the literature. One hypothesis posits the existence
193 of an unidentified "Gq-mER" (Gq-coupled membrane estrogen receptor) (*Qiu et al., 2006; Vail and Roepke, 2019*),
194 and therefore estradiol may signal via additional GPCRs beyond Gper1. Another possibility is an interaction between
195 estradiol and other membrane receptors, for example: the Voltage-Gated Sodium Channel Nav1.2 (*Sula et al., 2021;*
196 *Treviño and Gorelick, 2021*), transient receptor potential (TRP) channels (*Payrits et al., 2017; Ramírez-Barrantes*
197 *et al., 2020*), or various other ion channels (*Kow and Pfaff, 2016*). The robust nature of our "non-canonical" but
198 clearly estradiol-dependent phenotype, combined with the high-throughput nature of our behavioural assays, could
199 be an ideal assay for future screening efforts to attempt to identify novel estradiol target(s).

200 **Multiple Estrogen Receptors act to suppress habituation learning.**

201 While we were surprised to find that the classical ERs do not promote habituation, we were shocked to find clear
202 evidence of the opposite! We found that *esr1^{uab118}*, *esr2a^{uab134}*, and *gper1^{uab102}* mutants habituate more than their
203 sibling controls (**Figure 1**, **Figure 2**), consistent with a role for these ERs in inhibiting habituation. While effect sizes of
204 these magnitudes border on those that are easily dismissible as "noise", they were not only observed in the single
205 mutants experiments, but also in the double and triple mutant combinations of these alleles (**Figure 3-Figure 5**), pro-
206 viding good evidence that they are biologically meaningful effects. In fact, these multi-mutants generally exhibited
207 larger effect sizes, consistent with an additive interaction.

208 Untangling the mechanisms of ER1-, ER2a- and Gper1-dependent suppression of habituation will require con-
209 siderable further work. The additive interaction we observed genetically indicates that the ERs act cooperatively to
210 suppress habituation learning. All three receptors are expressed in the larval zebrafish brain (**Thisse and Thisse,**
211 **2008; Romano et al., 2017**), but whether they are acting in the same or different cell types awaits characterization.
212 The study of this inhibitory pathway may be challenging since it opposes the major learning-promoting effect of
213 estradiol, and therefore may be more straightforward to study after the identification and deletion of the estradiol
214 target that promotes habituation.

215 **Conclusion**

216 What began as a straightforward study to identify the receptor(s) that mediate the habituation-promoting effects
217 of estradiol has instead led us to a surprising and paradoxical result; canonical ERs do regulate habituation, but
218 are suppressive and act in opposition to the habituation-promoting effects of estradiol. This fits with the general
219 theme of our studies of this habituation paradigm – we find increasing complexity and contradiction within this
220 "simple" learning process the deeper we look. This began with our detailed observations of behaviour, leading us
221 to conclude that habituation results from a distributed plasticity processes that adapts different aspects of behavior
222 independently (**Randlett et al., 2019**). We believe that this property underlies our subsequent discoveries of phar-
223 macological and genetic manipulations that can result in either specific changes in specific aspects of habituation
224 (but not others), or even opposing effects, where a single manipulation can simultaneously increase and decrease
225 habituation, depending on which component of behavior is measured (**Randlett et al., 2019; Lamiré et al., 2023**).
226 This complexity appears to be a fundamental property of habituation (**McDiarmid et al., 2019**), and that the study of
227 habituation will likely continue to surprise us, hopefully leading to unexpected insights into the nature of plasticity
228 underlying learning and memory.

229 **Materials and Methods**

230 **Animals**

231 All experiments were performed on larval zebrafish at 5 days post fertilization (dpf), raised at a density of ≈1
232 larvae/mL of E3 media supplemented with 0.02% HEPES pH 7.2. Larvae were raised in a 14:10h light/dark cycle at
233 28-29°C. Adult zebrafish were housed, cared for, and bred at the following zebrafish facilities: Plateau de Recherche
234 Expérimentale en Criblage In Vivo (PRECI, SFR Biosciences, Lyon) and the Animalerie Zebrafish Rockefeller (AZR, SFR
235 Santé Lyon Est, Lyon). Adult zebrafish used to generate larvae were housed in accordance with regulations of the
236 PRECI and AZR facilities, which are regulated by an internal animal wellbeing committee, and were approved by
237 the animal welfare committee (comité d'éthique en expérimentation animale de la Région Rhône-Alpes: CECCAPP,
238 Agreement # C693870602). Behaviour experiments were performed at the 5dpf stage, and are thus not subject to
239 ethical review, but these procedures do not harm the larvae.

240 Mutant lines were obtained from D. Gorelick's lab, and were of the following alleles:

241 $esr1^{uab118}$ is a 4bp deletion (ZDB-ALT-180420-2), yielding a predicted null frameshift/stop mutation, confirmed
242 by a lack of estradiol responsiveness in the heart as assayed by $Tg(5xERE:GFP)^{c262}$ expression (**Romano et al., 2017**).
243 $esr2a^{uab134}$ is a 2bp deletion (ZDB-ALT-180420-3), yielding a predicted null frameshift/stop mutation (**Romano**
244 **et al., 2017**)
245 $esr2b^{uab127}$ is a 4bp deletion (ZDB-ALT-180420-4), yielding a predicted null frameshift/stop mutation, confirmed
246 by a lack of estradiol responsiveness in the liver as assayed by $Tg(5xERE:GFP)^{c262}$ expression (**Romano et al., 2017**).
247 $gper1^{uab102}$ is a 133bp deletion (ZDB-ALT-180420-1), yielding a predicted null frameshift/stop mutation, con-
248 firmed by a lack of estradiol responsiveness in heart beating rate in maternal-zygotic mutants (**Romano et al., 2017**).

249 **Genotyping**

250 $esr1^{uab118}$ was genotyped by PCR using the forward/reverse primer pair:
251 GCTGGTCACCTTGAATGCTT/TGAGATGTGAGAGATGACTAGGA with a T_m of 58°C yielding a 381 bp PCR product that
252 was digested with the restriction enzyme ApeKI. The mutant product is not digested, and the wild type has two
253 bands at 177 and 204 bp.

254 $esr2a^{uab134}$ was genotypes by PCR using the forward/reverse primer pair:
255 CTTCAGCTGCAGGAAGTGGAAAGTCGGGCTTAGCGACTG with a T_m of 58°C yielding a 236 bp PCR product that was
256 digested with the restriction enzyme MboI. The mutant product is not digested, and the wild type has two bands at
257 180 and 56 bp

258 $esr2b^{uab127}$ was genotypes by PCR using the forward/reverse primer pair:
259 TGGGCCTGAGATGCAGTAGT/GTGTGTGTTGGCCTCCTC with a T_m of 60°C yielding a 431 bp PCR product that was
260 digested with the restriction enzyme Mbil. The mutant product is digested into two bands of 150 and 281 bp and
261 the wild type into 3 bands of 78, 150 and 198 bp.

262 $gper1^{uab102}$ was genotypes by PCR using the forward/reverse primer pair:
263 ATGGAGGAGCAGACTACCAATGTG/CCATCCAGATGAGGCTGCAA with a T_m of 60°C yielding a mutant product of 372bp
264 and a wild type product of 505 bp.

265 **Pharmacology**

266 β -Estradiol (Sigma E2758, here referred to as "estradiol") was dissolved in dimethyl sulfoxide (DMSO) and stored
267 at -20°C. Larvae were treated with estradiol immediately before the behavioural assay by pipetting 10-30uL of 10x
268 solution directly into the behavioural wells, always with a final concentration of 0.1% DMSO in E3.

269 **Habituation behaviour testing**

270 Larval behavior was evaluated in 300-well plates using an updated version of the experimental setup previously
271 described (**Randlett et al., 2019; Lamiré et al., 2023**). Briefly, 300-well plates were custom made using laser-cut
272 acrylic sheets where each well measures 8 mm in diameter and 6 mm in depth, corresponding to an approximate
273 water volume of 300 μ L. These plates are suspended under a water bath held at 31°C, acting as a heated lid to
274 minimize condensation and maintain a 29°C water temperature within each well. Behavioral recordings were made
275 using a Mikrotron CXP-4 camera running at 444hz in conjunction with a Silicon Software frame grabber (Marathon
276 ACX-QP, Basler), illuminated by IR LEDs (TSHF5410, digikey.com). Visual stimuli were presented using a rectangular
277 array of 155 WS2813 RGB LEDs (144LED/M, aliexpress.com). For the DF stimulus, the LEDs were briefly switched off
278 (1 s), then linearly returned to the original brightness over a 20 s interval. Vibration/Tap stimuli were administered
279 using a solenoid (ROB-10391, Sparkfun). This behavioral paradigm was designed to be symmetrical: each 1 hr block
280 of stimulation was followed by 1 hr of rest. During these rest periods, the camera was moves using a stepper motor
281 controlled linear actuator (Hanpose HPV4, 500cm), which moved the camera between two plates, allowing us to
282 screen up to 600 fish per experiment across two 300-well plates.

283 Control of the apparatus (RGB LEDs, solenoid, camera linear actuator) was implemented via a Raspberry Pi Pico
284 microcontroller running CircuitPython (<https://circuitpython.org/>) (code : [code.py](#)) and custom Python software
285 which handled the camera acquisition via the [Python wrapper of the Silicon Software Framegrabber SDK](#), triggered
286 stimuli via the Raspberry Pi Pico, and tracked the head and tail coordinates of the larvae across the 300-wells at a
287 baseline framerate of between 20-30hz (code : [Run_BigRig2.py](#)). When a stimulus is delivered (DF or Vibration/Tap),
288 a 1-second "Burst" video is recorded at the full frame rate as a Tiff file, from which the head and tail coordinates
289 are subsequently tracked offline (code : [TrackBurst_BigRig.py](#)). Larval zebrafish tracking was done via background
290 subtraction and morphological operations implemented using multiple open-source packages, including: OpenCV
291 ([Bradski, 2000](#)), scikit-image ([Van der Walt et al., 2014](#)), NumPy ([Harris et al., 2020](#)), SciPy ([Virtanen et al., 2020](#)),
292 and Numba ([Lam et al., 2015](#)).

293 Data analysis

294 Data was analyzed in Python using custom written analysis scripts (code : [Analyze_EsrHab.py](#)). Responses to DFs
295 and vibration/taps were identified as movement events that had a cumulative tail bend angle greater than 3 radian
296 (O-bend)and 1 radian (C-bend), respectively. Data was analyzed using multiple open-source packages, including:
297 NumPy ([Harris et al., 2020](#)), SciPy ([Virtanen et al., 2020](#)),Numba ([Lam et al., 2015](#)) and Pandas ([Wes McKinney, 2010](#)).
298 Data was plotted using Matplotlib ([Hunter, 2007](#)) and seaborn ([Waskom, 2021](#)). Statistical "significance" between
299 the distributions was tested using the Mann-Whitney U test implemented in Scipy ([Virtanen et al., 2020](#)).

300 The cumulative difference plots to assess changes in habituation performance for the treatments were calcu-
301 lated as previously ([Randlett et al., 2019](#)), where we first calculated the average response across larvae for each
302 group for each DF. This generated a mean vector for each group. These two vectors were normalized by dividing
303 them by the naive response (mean response to the first 5 DFs), and then the treatment group was subtracted
304 from the control group, yielding a "mean difference" vector between stimulus and controls at each flash. From
305 this mean difference vector we calculated the cumulative mean distribution using Numpy's 'nancumsum' function
306 divided by the number of stimuli experienced, or the index of the vector. To generate statistical confidence in
307 these distributions, we bootstrapped 2000 replicates, and calculated the 99.5% confidence intervals using SciPy's
308 'stats.norm.interval' function. The assumption of this analysis is that if the two groups are habituating similarly, then
309 the "mean difference" vector will exhibit a noise distribution centered at a mean of 0, and thus the cumulative mean
310 distribution would remain near 0. Treatments that affect habituation will show strong increasing or decreasing
311 cumulative mean distributions, reflecting increased or decreased habituation performance throughout training,
312 respectively. We use an empirically defined threshold of ± 0.05 as the statistically meaningful effect size in this
313 analysis, as is reflected in the shaded gray regions in the plots ([Randlett et al., 2019](#)).

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317 Data Availability

318 Software and analysis code is available here: https://github.com/owenrandlett/2025_HabEstrogen. All datasets
319 used in these analyses are available here: [HabEstrogen_Datasets](#).

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