

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

Andrew Hsiao^{1,1,2}, Isabelle Darvaux-Hubert^{1,1}, Dominique Hicks^{1,1,3}, Emilie Joux^{1,2}, Sarah De Freitas^{1,2}, Emeline Dracos^{1,2}, Jeanne Lizé^{1,2}, Julien Perrichet¹, Dominique Baas^{*1}, Owen Randlett^{*1,1,@}

¹ Laboratoire MeLiS, Université Claude Bernard Lyon 1 - CNRS UMR5284 - Inserm U1314, Institut NeuroMyoGène, Faculté de Médecine et de Pharmacie, 8 avenue Rockefeller, 69008 Lyon, France

² International Master in Life Sciences, Université Claude Bernard Lyon 1, France

³ Master of Biology Program, École normale supérieure de Lyon, France

¹equal contribution

^{*}equal contribution

¹⁰ @correspondence: owen.randlett@univ-lyon1.fr

Abstract

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

Introduction

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

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

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

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

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

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

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

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

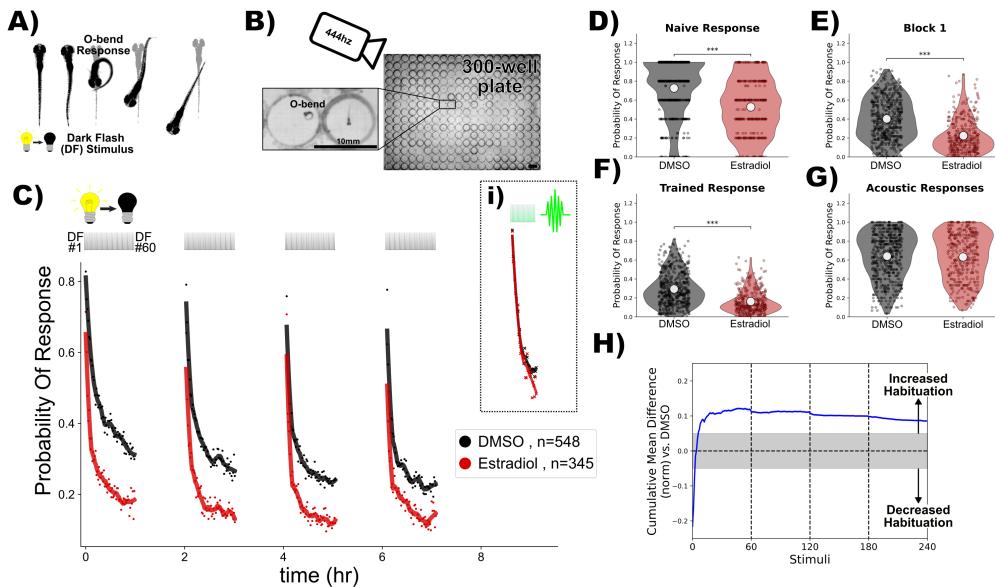


Figure 1. Estradiol increases habituation learning.

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

Estradiol increases visual habituation learning

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

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

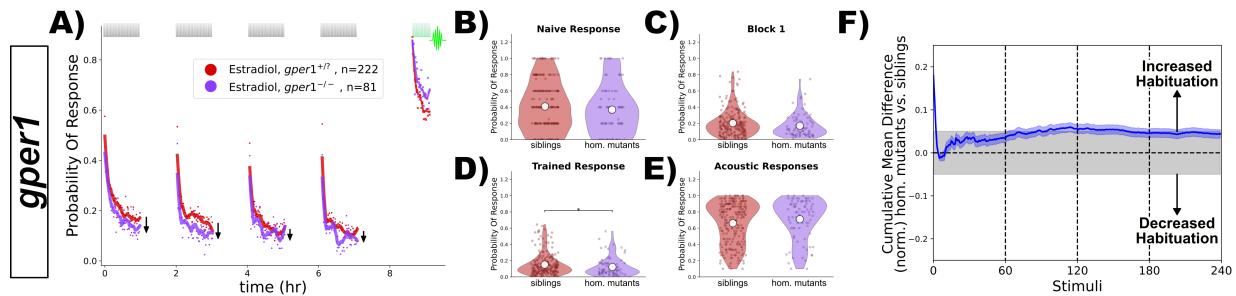


Figure 2. *gper1* mutants do not show habituation deficits after treatment with estradiol.

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.

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

102 Gper1 is dispensable for estradiol-promoted habituation learning

103 The effects of estradiol that we have observed occur very rapidly – larvae are only pretreated with estradiol for
 104 $\approx 25\text{min}$ -1hr before the first DF. This is the time necessary to set the apparatus and begin the experiment. Since
 105 the nuclear hormone receptors are thought to primarily exert their effects via transcriptional alterations, this
 106 necessitates a delay in their signaling. For this reason, we first hypothesized that membrane-initiated signaling
 107 through Gper1 was the most likely mechanism.

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

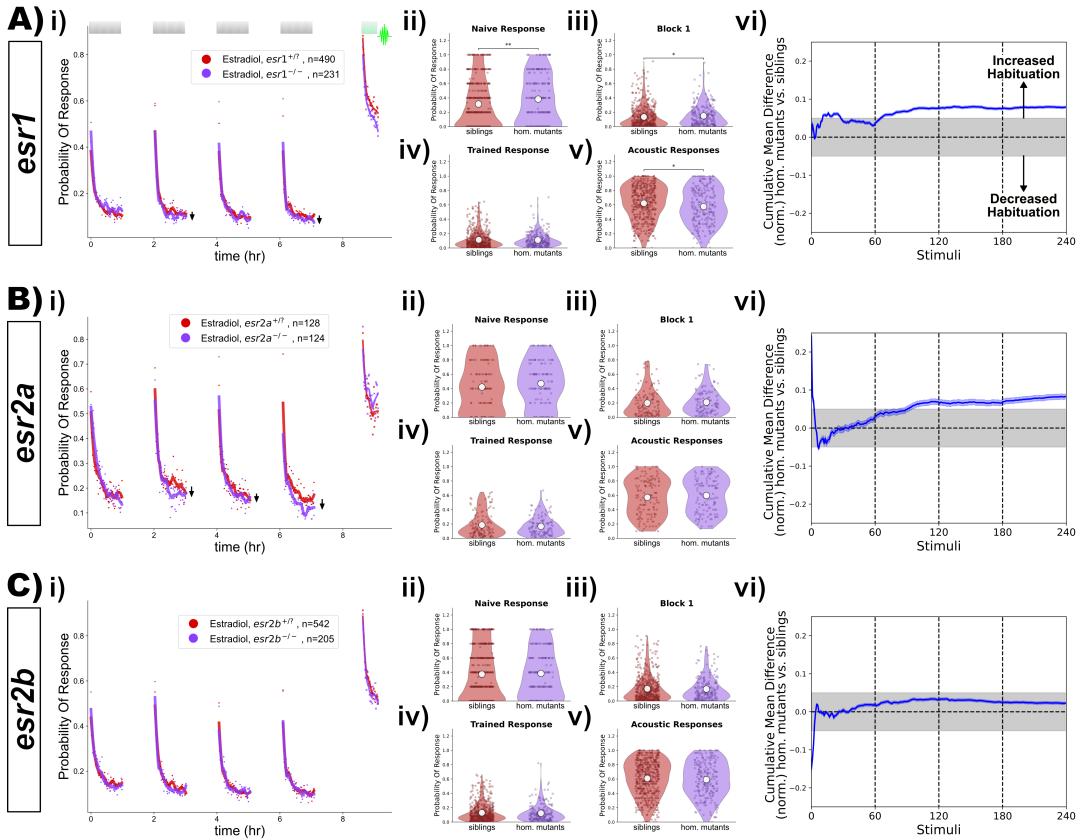


Figure 3. esr1, esr2a and esr2b mutants do not show habituation deficits after treatment with estradiol.

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

119 Nuclear ERs are dispensable for estradiol-promoted habituation learning

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

129 Nuclear ERs are simultaneously dispensable for estradiol-promoted habituation 130 learning

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

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

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

149 Gper1 and nuclear ERs are simultaneously dispensable for estradiol-promoted 150 habituation learning

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

158 Despite the fact that we analyzed the behaviour of 1152 larvae and successfully genotyping all 4 ER genes
159 in 373 individuals (after >4600 genotyping PCRs), we were only able to identify a single quadruple mutant
160 larva lacking all known ERs (*Figure 5E*). This is likely simply due to the limitations of combinatorial Mendelian
161 inheritance, and the fact that *esr1* and *esr2a* are linked on chromosome 20. While it is dubious to conclude much
162 from an n = 1 experiment, we find it remarkable that this larva exhibits the strongest increased habituation of

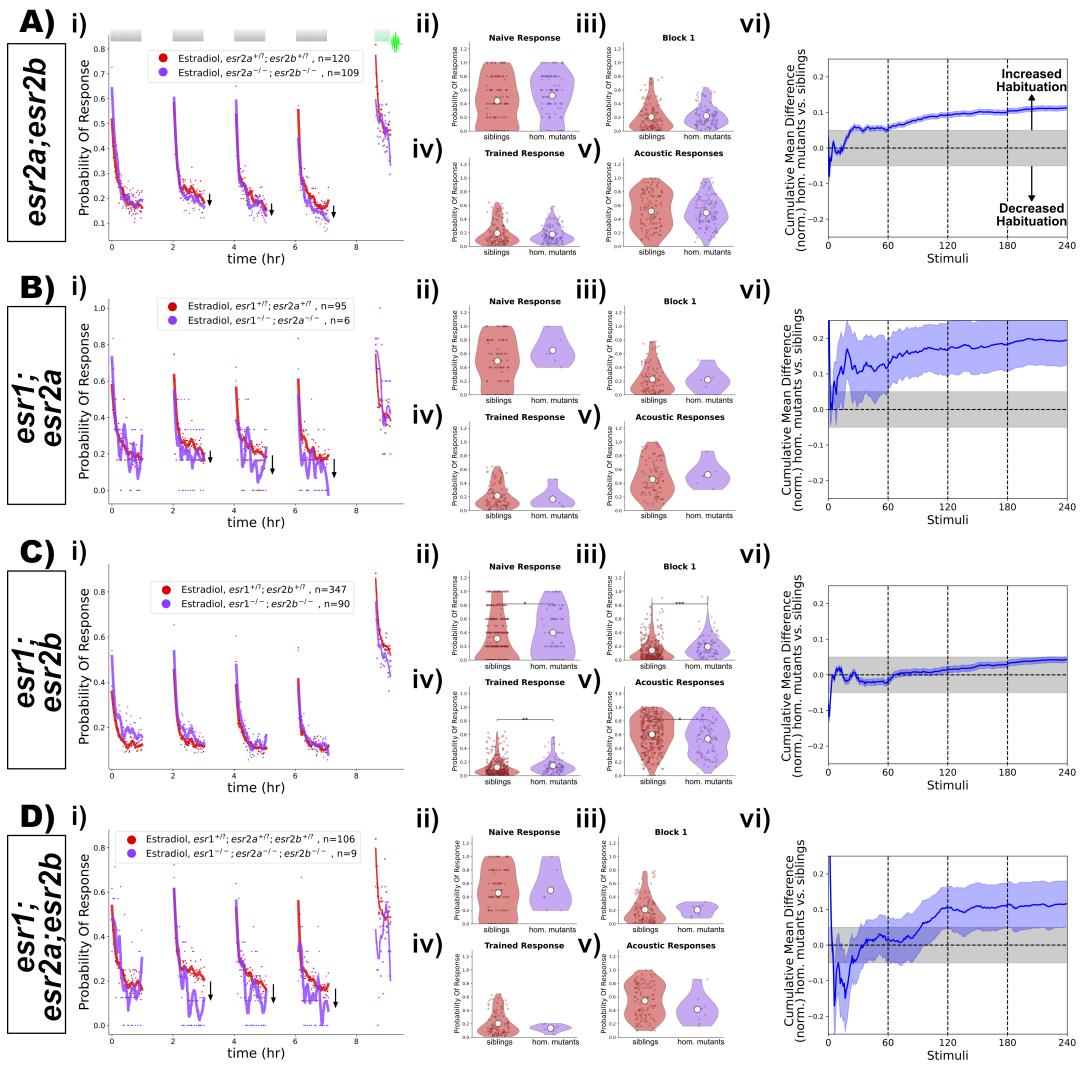


Figure 4. Double and triple mutant combinations of *esr1*, *esr2a* and *esr2b* do not show habituation deficits after treatment with estradiol.

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.

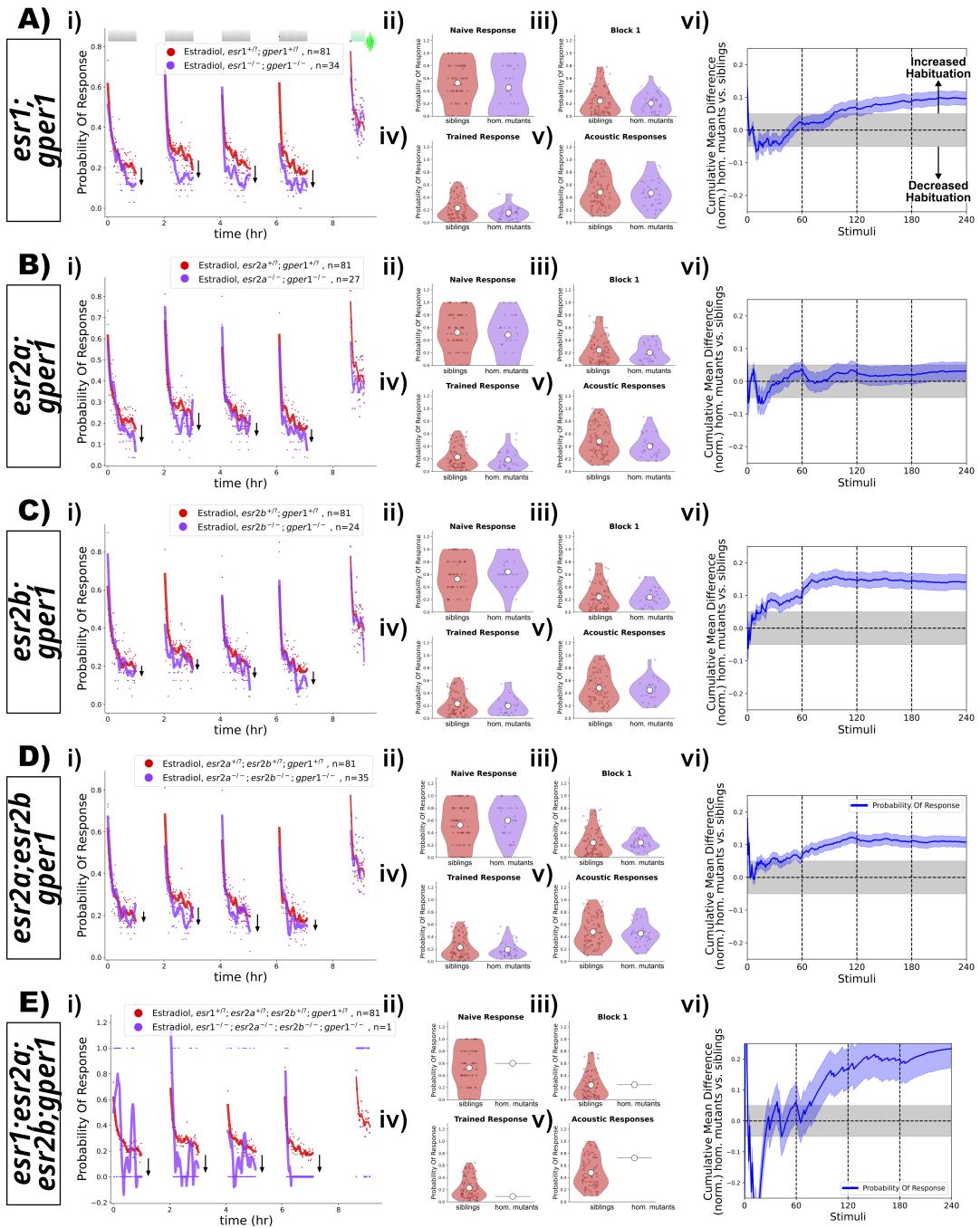


Figure 5. Double, triple and quadruple mutant combinations of *esr1*, *esr2a*, *esr2b*, and *gper1* do not show habituation deficits after treatment with estradiol.

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**.

163 all of our experiments, with suppression of responses (**Figure 5Ei**), and strong positive deviation in the CMD plot
164 (**Figure 5Evi**).

165 Discussion

166 **The promotion of habituation learning by estradiol is mediated by an unidentified 167 target**

168 Our experiments indicate that Gper1, ER1, ER2a and ER2b do not mediate the positive effects of estradiol on
169 habituation learning. As this is fundamentally a negative result, it is difficult to conclusively demonstrate this
170 beyond any doubt. One major caveat relates to the actual functional nature of the mutant alleles that we have
171 used. These are all Cas9-generated small deletions resulting in frameshift mutations that lead to early stop
172 codons, and are thus predicted null/knockout lines. Despite this genetic confidence, it is always possible that
173 residual activity could still remain, perhaps via alternate splicing or alternate start codons. For this reason, we
174 were originally unsure if we would publish these results at all. However, we recognize that this bias against
175 publishing negative results is bad for science. Negative results from well-designed and executed experiments are
176 of value for the community and making this knowledge public is our duty as responsible scientists (**Mlinarić et al.,
177 2017**). These high-throughput neurogenetic experiments are also a massive amount of work – raising, testing and
178 genotyping thousands of zebrafish larvae. This was undertaken primarily by technicians and Master's students,
179 all of whom deserve recognition, irrespective of the perceived "impact" of the outcome of their experiments.

180 While the possibility of "residual activity" in our mutants is a clear limitation of our approach, we argue that
181 this alternative interpretation is very unlikely. The *esr1uab118* and *esr2buab127* alleles both exhibited a lack of estradiol
182 responsiveness in other tissues (**Romano et al., 2017**), and *esr2buab127* mutants are female sterile/subfertile
183 (D. Gorelick, personal communication), indicating a non-functional receptor. Similarly, *gper1uab102* mutants show
184 a lack of estradiol responsiveness in heart rate modulation (**Romano et al., 2017**). Interestingly this was only
185 observed in maternal-zygotic mutants. While it seems unlikely that sufficient maternal mRNA/protein for Gper1
186 could persist in 5dpf larvae, we can formally rule this out with our current datasets. The *esr2auab134* mutants
187 have no previously published phenotype, and so we do not have an independent positive control for the nature
188 of this allele. However, the best evidence we have against the "residual activity" hypothesis is that we actually
189 found phenotypes in our assays for *esr1uab118*, *esr2auab134*, and *gper1uab102* mutants. These phenotypes are just
190 of the unexpected sign, where mutants show increased habituation (discussed below).

191 **Potential mechanisms for estradiol-promoted habituation learning**

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

199 What might this unidentified target be? Various leads exist in the literature. One hypothesis posits the
200 existence of an unidentified "Gq-mER" (Gq-coupled membrane estrogen receptor) (**Qiu et al., 2006; Vail and
201 Roepke, 2019**), and therefore estradiol may signal via additional GPCRs beyond Gper1. Another possibility is an
202 interaction between estradiol and other membrane receptors, for example: the Voltage-Gated Sodium Channel
203 Nav1.2 (**Sula et al., 2021; Treviño and Gorelick, 2021**), transient receptor potential (TRP) channels (**Payrits et al.,
204 2017; Ramírez-Barrantes et al., 2020**), or various other ion channels (**Kow and Pfaff, 2016**). The robust nature of

205 our "non-canonical" but clearly estradiol-dependent phenotype, combined with the high-throughput nature of
206 our behavioural assays, could be an ideal assay for future screening efforts to attempt to identify novel estradiol
207 target(s).

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

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

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

223 **Conclusion**

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

237 **Materials and Methods**

238 **Animals**

239 All experiments were performed on larval zebrafish at 5 days post fertilization (dpf), raised at a density of ≈ 1
240 larvae/mL of E3 media supplemented with 0.02% HEPES pH 7.2. Larvae were raised in a 14:10h light/dark
241 cycle at 28-29°C. Adult zebrafish were housed, cared for, and bred at the following zebrafish facilities: Plateau
242 de Recherche Expérimentale en Criblage In Vivo (PRECI, SFR Biosciences, Lyon) and the Animalerie Zebrafish
243 Rockefeller (AZR, SFR Santé Lyon Est, Lyon). Adult zebrafish used to generate larvae were housed in accordance
244 with regulations of the PRECI and AZR facilities, which are regulated by an internal animal wellbeing committee,
245 and were approved by the animal welfare committee (comité d'éthique en expérimentation animale de la Région

²⁴⁶ Rhône-Alpes: CECCAPP, Agreement # C693870602). Behaviour experiments were performed at the 5dpf stage,
²⁴⁷ and are thus not subject to ethical review, but these procedures do not harm the larvae.

²⁴⁸ Mutant lines were obtained from D. Gorelick's lab, and were of the following alleles:

²⁴⁹ *esr1^{uab118}* is a 4bp deletion (ZDB-ALT-180420-2), yielding a predicted null frameshift/stop mutation, confirmed
²⁵⁰ by a lack of estradiol responsiveness in the heart as assayed by *Tg(5xERE:GFP)^{c262}* expression (**Romano et al.,**
²⁵¹ **2017**).

²⁵² *esr2a^{uab134}* is a 2bp deletion (ZDB-ALT-180420-3), yielding a predicted null frameshift/stop mutation (**Romano**
²⁵³ **et al., 2017**)

²⁵⁴ *esr2b^{uab127}* is a 4bp deletion (ZDB-ALT-180420-4), yielding a predicted null frameshift/stop mutation, con-
²⁵⁵ firmed by a lack of estradiol responsiveness in the liver as assayed by *Tg(5xERE:GFP)^{c262}* expression (**Romano**
²⁵⁶ **et al., 2017**).

²⁵⁷ *gper1^{uab102}* is a 133bp deletion (ZDB-ALT-180420-1), yielding a predicted null frameshift/stop mutation,
²⁵⁸ confirmed by a lack of estradiol responsiveness in heart beating rate in maternal-zygotic mutants (**Romano et al.,**
²⁵⁹ **2017**).

²⁶⁰ Genotyping

²⁶¹ *esr1^{uab118}* was genotyped by PCR using the forward/reverse primer pair:

²⁶² GCTGGTCACCTTGAATGCTT/TGAGATGTGAGAGATGACTAGGA with a T_m of 58°C yielding a 381 bp PCR product
²⁶³ that was digested with the restriction enzyme ApeKI. The mutant product is not digested, and the wild type has
²⁶⁴ two bands at 177 and 204 bp.

²⁶⁵ *esr2a^{uab134}* was genotypes by PCR using the forward/reverse primer pair:

²⁶⁶ CTTCAGCTGCAGGAAGTGG/AAAGTCGGCTTAGCGACTG with a T_m of 58°C yielding a 236 bp PCR product that
²⁶⁷ was digested with the restriction enzyme MboI. The mutant product is not digested, and the wild type has two
²⁶⁸ bands at 180 and 56 bp

²⁶⁹ *esr2b^{uab127}* was genotypes by PCR using the forward/reverse primer pair:

²⁷⁰ TGGGCCTGAGATGCAGTAGT/GTGTGTGCTTGGCCTCCTC with a T_m of 60°C yielding a 431 bp PCR product that
²⁷¹ was digested with the restriction enzyme Mbil. The mutant product is digested into two bands of 150 and 281
²⁷² bp and the wild type into 3 bands of 78, 150 and 198 bp.

²⁷³ *gper1^{uab102}* was genotypes by PCR using the forward/reverse primer pair:

²⁷⁴ ATGGAGGAGCAGACTACCAATGTG/CCATCCAGATGAGGCTGCAA with a T_m of 60°C yielding a mutant product of
²⁷⁵ 372bp and a wild type product of 505 bp.

²⁷⁶ Pharmacology

²⁷⁷ β -Estradiol (Sigma E2758, here referred to as "estradiol") was dissolved in dimethyl sulfoxide (DMSO) and stored
²⁷⁸ at -20°C. Larvae were treated with estradiol immediately before the behavioural assay by pipetting 10-30uL of
²⁷⁹ 10x solution directly into the behavioural wells, always with a final concentration of 0.1% DMSO in E3.

²⁸⁰ Habituation behaviour testing

²⁸¹ Larval behavior was evaluated in 300-well plates using an updated version of the experimental setup previously
²⁸² described (**Randlett et al., 2019; Lamiré et al., 2023**). Briefly, 300-well plates were custom made using laser-cut
²⁸³ acrylic sheets where each well measures 8 mm in diameter and 6 mm in depth, corresponding to an approximate
²⁸⁴ water volume of 300 μ L. These plates are suspended under a water bath held at 31°C, acting as a heated lid to
²⁸⁵ minimize condensation and maintain a 29°C water temperature within each well. Behavioral recordings were
²⁸⁶ made using a Mikrotron CXP-4 camera running at 444hz in conjunction with a Silicon Software frame grabber
²⁸⁷ (Marathon ACX-QP, Basler), illuminated by IR LEDs (TSHF5410, digikey.com). Visual stimuli were presented using
²⁸⁸ a rectangular array of 155 WS2813 RGB LEDs (144LED/M, aliexpress.com). For the DF stimulus, the LEDs were

289 briefly switched off (1 s), then linearly returned to the original brightness over a 20 s interval. Vibration/Tap
290 stimuli were administered using a solenoid (ROB-10391, Sparkfun). This behavioral paradigm was designed to
291 be symmetrical: each 1 hr block of stimulation was followed by 1 hr of rest. During these rest periods, the camera
292 was moves using a stepper motor controlled linear actuator (Hanpose HPV4, 500cm), which moved the camera
293 between two plates, allowing us to screen up to 600 fish per experiment across two 300-well plates.

294 Control of the apparatus (RGB LEDs, solenoid, camera linear actuator) was implemented via a Raspberry
295 Pi Pico microcontroller running CircuitPython (<https://circuitpython.org/>) (code : `code.py`) and custom Python
296 software which handled the camera acquisition via the **Python wrapper of the Silicon Software Framegrabber**
297 **SDK**, triggered stimuli via the Raspberry Pi Pico, and tracked the head and tail coordinates of the larvae across the
298 300-wells at a baseline framerate of between 20-30hz (code : `Run_BigRig2.py`). When a stimulus is delivered (DF
299 or Vibration/Tap), a 1-second "Burst" video is recorded at the full frame rate as a Tiff file, from which the head
300 and tail coordinates are subsequently tracked offline (code : `TrackBurst_BigRig.py`). Larval zebrafish tracking
301 was done via background subtraction and morphological operations implemented using multiple open-source
302 packages, including: OpenCV (*Bradski, 2000*), scikit-image (*Van der Walt et al., 2014*), NumPy (*Harris et al., 2020*),
303 SciPy (*Virtanen et al., 2020*), and Numba (*Lam et al., 2015*).

304 Data analysis

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

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

327 Data Availability

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

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336

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