# Estradiol increases visual habituation learning independently of the canonical estrogen receptors

**Andrew Hsiao** [!,1,2], **Isabelle Darvaux-Hubert** [!,1], **Dominique Hicks** [1,3], **Emilie Joux** [1,2], **Sarah De Freitas** [1,2], **Emeline Dracos** [1,2], **Jeanne Litze** [1,2], **Dominique Baas** [*,1], **Owen Randlett** [*,@,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
[!]equal contribution
[*]equal contribution
[@]correspondence: owen.randlett@univ-lyon1.fr

## Abstract

Habituating to the constant stimuli in the environment is a critical adaptive learning process conserved across species. We use the 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 reduced responses. In an attempt to identify the receptor(s) mediating these effects we used established mutant lines with expected null alleles for the known estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gper1*). Our experiments failed to identify a receptor required for the effects of Estradiol on habituation learning. Surprisingly, nuclear-receptor mutants showed increased habituation relative to sibling controls when treated with estradiol, indicating that activation of these receptors has paradoxical inhibitory effects on habituation learning. These experiments confirm that Estradiol is a potent modulator of learning in the vertebrate brain, but suggest that these effects occur independently of the classical estrogen receptor-mediated signaling pathways, which may in fact act to inhibit learning performance in this paradigm.

## Introduction

A primary task of the brain is to learn from ongoing experiences and adjust behavior accordingly. This often involves sharpening attention and behavioural resources toward salient cues while tuning out irrelevant background stimuli. For instance, it may be critical to recognize the alarm calls of a nearby animal, whereas continually registering a steady hum from distant traffic is far less important. The capacity to reduce responses to repetitive, non-essential stimuli is known as habituation, a phenomenon widely considered one of the simplest forms of learning and memory (***Rankin et al., 2009***).

We have been studying 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***). In this analysis, we emphasize long-term habituation as a practical model for examining the fundamental processes that shape neural circuit plasticity. 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 the Estrogen Receptors (ERs), since we identified multiple estradiol analogs (ethinyl estradiol, estradiol valerate, and hexestrol), which strongly increased habituation learning when applied at 5-10µM doses in the water (*Lamiré et al., 2023*). 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 gender-affirming therapy.

Estrogen signaling in learning and memory, any data about receptors.

In this project we aimed to identify the relevant estrogen receptor mediating the effects of estradiol analogs on habituation learning by undertaking a classical analysis of genetic knockout alleles. Not only did we fail to identify a mutant (or combination of mutants) that lead to estradiol insensitivity, leading us to conclude that these receptors act to inhibit habituation learning, rather than mediating the habituation-promoting effects of Estradiols in the zebrafish brain. estrogne receptor mutants that show increased sensitivity to estradiol in the habituation paradigm.

# Materials and Methods

## Animal Ethics Statement

Adult zebrafish used to generate larvae were housed in accordance with PRCI facility approved by the animal welfare committee (comité d'éthique en expérimentation animale de la Région Rhône-Alpes: CECCAPP, Agreement # C693870602). Behaviour experiments were performed at the 5dpf stage, and are thus not subject to ethical review, but these procedures do not harm the larvae.

## Animals

All experiments were performed on larval zebrafish at 5 days post fertilization (dpf), raised at a density of ≈1 larvae/mL of E3 media supplemented with 0.02% HEPES pH 7.2. Larvae were raised in a 14:10h light/dark cycle at 28-29°C. Adult zebrafish were housed, cared for, and bred at the Lyon PRECI zebrafish facility. Mutant lines were obtained from D. Gorelick's lab, and were of the following alleles:

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

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

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

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

## Genotyping

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

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

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

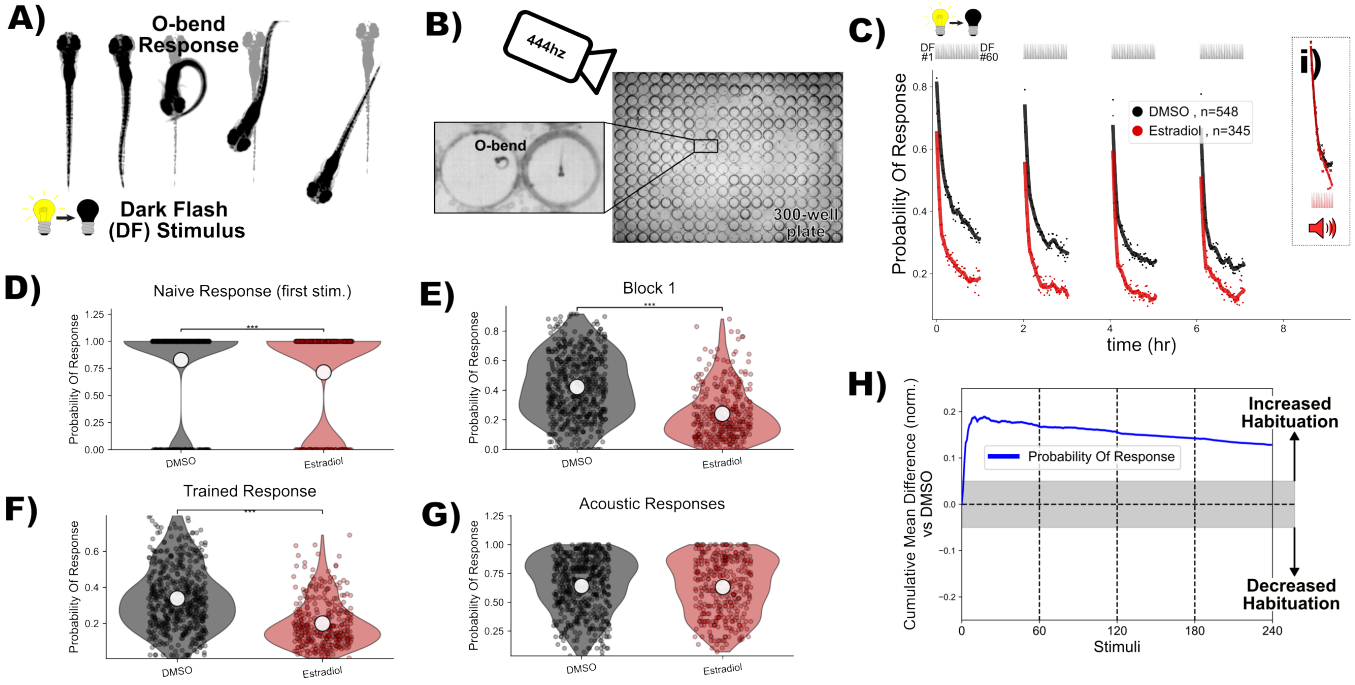


**Figure 1.** Estradiol increases habituation learning.
**A)** In response to a dark flash (DF), larval zebrafish perform a large turning maneuver termed an O-bend response.
**B)** High-throughput setup for recording and quantify O-bend responsiveness using a high-speed camera recording at 444hz observing larvae in 300-well plates.
**C)** 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 acoustic stimuli are delivered at 1-minute intervals (**i**). Each dot is the probability of response to one flash. 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 DF stimulus (binary metric); **E)** the mean response to the remaining DF stimuli in the Block 1 (DFs 2:60); **F)** all DFs during the training period (DFs 2:240); **G)** the 30 acoustic stimuli delivered with a tap from a solenoid on the 300-well plate platform.
**H)** Cumulative plot of the differences in habituation rate for the response components. These plots display the cumulative average differences in the mean response after normalization to the naive response. Estradiol treatment is compared to DMSO controls. Difference from 0 reflects divergence in response across the 240 dark-flash stimuli in the 4 training blocks, with positive values reflecting increased habituation. The widths of the lines are bootstrapped 99.5% confidence intervals. The gray boxed region reflects the expected non-significant effect size (***Randlett et al., 2019***).
Treatment groups are: Estradiol = 10μM estradiol treatment (n = 345 fish); DMSO = 0.1% DMSO vehicle controls (n = 548 fish)

---

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

### Pharmacology

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

## Results and Discussion

### Estradiol increases habituation learning

In response to a sudden global dimming event, which we refer to as a Dark Flash (DF), larval zebrafish execute an "O-bend" maneuver, characterized by a deep "O"-shaped bend, resulting in a high-amplitude turn (***Burgess and Granato, 2007***). 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 (***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 1***A,B, ***Randlett et al., 2019***; ***Lamiré et al., 2023***). When stimulated with DFs repeated at 1-minute intervals in repeated 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 dark-flash 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 β-Estradiol (estradiol), which is the major natural estrogen in humans. A 10μM dose of 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 across stimuli (***Figure 1***C-H). Consistent with what we previously observed after hexestrol and ethinyl estradiol treatment (***Lamiré et al., 2023***), there is a modest reduction in the initial responsiveness of the estradiol-treated larvae to the first DF stimulus (***Figure 1***D), but the major effect is observed during the training phase (***Figure 1***C,E,F), as is revealed by the consistent positive deviation in the cumulative mean difference plot (***Figure 1***H, ***Randlett et al., 2019***). Importantly, the responsiveness of the larvae to acoustic stimuli delivered after the DF training is indistinguishable from controls (***Figure 1***Ci, G), indicating that estradiol does not affect global arousal levels but rather has specific effects on habituation learning.

**Nuclear Estrogen receptors are not required for the effects of estradiol on habituation learning**

We hypothesized that the effects of estradiol on habituation would occur via agonism of one or multiple Estrogen Receptors.

## Funding

This work was supported by funding from the ATIP-Avenir program of the CNRS and Inserm, a Fondation Fyssen research grant, and the IDEX-Impulsion initiative of the University of Lyon.

## Data Availability

Software and analysis code is available here: https://github.com/owenrandlett/2025_HabEstrogen. Datasets are available here: .

## References

**Barros LA**, Tufik S, Andersen ML. The role of progesterone in memory: an overview of three decades. Neurosci Biobehav Rev. 2015 Feb; 49:193–204.

**Burgess HA**, Granato M. Sensorimotor Gating in Larval Zebrafish. The Journal of Neuroscience. 2007 May; 27(18):4984–4994. https://doi.org/10.1523/jneurosci.0615-07.2007, doi: 10.1523/jneurosci.0615-07.2007.

**Dillon TS**, Fox LC, Han C, Linster C. 17$\beta$-estradiol enhances memory duration in the main olfactory bulb in CD-1 mice. Behav Neurosci. 2013 Dec; 127(6):923–931.

**El-Sherif Y**, Tesoriero J, Hogan MV, Wieraszko A. Melatonin regulates neuronal plasticity in the hippocampus. J Neurosci Res. 2003 May; 72(4):454–460.

**Jilg A**, Bechstein P, Saade A, Dick M, Li TX, Tosini G, Rami A, Zemmar A, Stehle JH. Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency. J Pineal Res. 2019 Apr; 66(3):e12553.

**Lamiré LA**, Haesemeyer M, Engert F, Granato M, Randlett O. Functional and pharmacological analyses of visual habituation learning in larval zebrafish. Elife. 2023 Dec; 12.

**Naderi M**, Salahinejad A, Attaran A, Niyogi S, Chivers DP. Rapid effects of estradiol and its receptor agonists on object recognition and object placement in adult male zebrafish. Behav Brain Res. 2020 Apr; 384(112514):112514.

**Nilsson S**, Gustafsson JA. Biological role of estrogen and estrogen receptors. Crit Rev Biochem Mol Biol. 2002; 37(1):1–28.

**Randlett O**, Haesemeyer M, Forkin G, Shoenhard H, Schier AF, Engert F, Granato M. Distributed plasticity drives visual habituation learning in larval zebrafish. Curr Biol. 2019 Apr; 29(8):1337–1345.e4.

**Rankin CH**, Abrams T, Barry RJ, Bhatnagar S, Clayton DF, Colombo J, Coppola G, Geyer MA, Glanzman DL, Marsland S, McSweeney FK, Wilson DA, Wu CF, Thompson RF. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. Neurobiol Learn Mem. 2009 Sep; 92(2):135–138.

**Rawashdeh O**, de Borsetti NH, Roman G, Cahill GM. Melatonin suppresses nighttime memory formation in zebrafish. Science. 2007 Nov; 318(5853):1144–1146.

**Romano SN**, Edwards HE, Souder JP, Ryan KJ, Cui X, Gorelick DA. G protein-coupled estrogen receptor regulates embryonic heart rate in zebrafish. PLoS Genet. 2017 Oct; 13(10):e1007069.

**Wolman MA**, Jain RA, Liss L, Granato M. Chemical modulation of memory formation in larval zebrafish. Proceedings of the National Academy of Sciences. 2011 Sep; 108(37):15468–15473. https://pnas.org/doi/full/10.1073/pnas.1107156108, doi: 10.1073/pnas.1107156108.