1 **Embryonic exposure to artificial light at night impairs learning abilities**

2 **and their covariance with behavioural traits in teleost fish**

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14   **Abstract**

15   The natural light cycle has profound effects on animals' cognitive systems. Its

16   alteration due to human activities, such as artificial light at night (ALAN), affects the

17   biodiversity of mammalian and avian species by impairing their cognitive functions. The

18   impact of ALAN on cognition, however, has not been investigated in aquatic species, in spite

19   of the common occurrence of this pollution along water bodies. We exposed eggs of a teleost

20   fish (the zebrafish *Danio rerio*) to ALAN and, upon hatching, we measured larvae' cognitive

21   abilities with a habituation learning paradigm. Both control and ALAN-exposed larvae

22   showed habituation learning, but the latter learned significantly slower, suggesting that under

23   ALAN conditions, fish require many more events to acquire ecologically relevant

24   information. We also found that individuals' learning performance significantly covaried

25   with two behavioural traits in the control zebrafish, but ALAN disrupted one of these

26   relationships. Additionally, ALAN resulted in an average increase in larval activity. Our

27   results showed that both fish's cognitive abilities and related individual differences are

28   negatively impacted by light pollution, even after a short exposure in the embryonic stage.

29

30   **Keywords:** ALAN; cognitive plasticity; cognitive variation; fish cognition; personality;

31   zebrafish

Introduction

Artificial light at night (ALAN) is recognised as a one of the most pervasive sources of pollution on Earth, with multiple reported negative effects on animals' biology [1-3]. Among these, substantial literature indicates that humans display cognitive impairments due to ALAN [4-6]. Similar effects have been reported for laboratory rodent models [7] and even more severe ones for wild avian populations, in which ALAN-mediated alterations in hormonal homeostasis hamper cognitive functioning [8-11; but see: 12]. Considering the often-reported influence of individuals' cognitive abilities on fitness [e.g., 13], these records suggest that ALAN might impact biodiversity via cognitive alteration.

As a significant proportion of human settlings are related to water bodies, aquatic animals are also affected by ALAN [14-17]. It is estimated that 22% of coastal areas worldwide are affected by light pollution [15] and linear freshwater bodies such as rivers and canals are often entirely affected due to streetlights [17]. Therefore, we can expect ALAN to impact aquatic species' cognition, as well. Previous reports found effects of ALAN on teleost fish behaviours such as activity, boldness, and preference for environments with different illumination [18-20]. Yet, the potential impact of ALAN on fish cognition remains unknown. To fill this gap, we experimentally investigated whether ALAN exposure affects cognitive abilities in a teleost fish.

We treated fish during their embryonic stages, which are intuitively more susceptible to ALAN due to the impossibility to actively avoid it and the sensitivity of the developing nervous system. We used the zebrafish *Danio rerio* as our study species, which is particularly useful to investigate cognitive abilities and their plasticity during early development [21,22] and is considered a general fish model [23,24]. After exposure to ALAN or control treatment, we assessed zebrafish cognitive abilities with a habituation learning assay [25]. We also measured two behavioural traits (activity and startle response). Multiple studies in fish have

57 reported that individuals' cognitive traits covary with behavioural traits [26,27]. It has been

58 hypothesised that covariations with personality might help maintain cognitive variation [28].

59 Additionally, these covariations might result from local adaptation to ecological conditions,

60 as suggested by different patterns shown by populations from distinct habitats [29,30].

61 Considering that similar relationships between traits are affected by environmental stressors

62 [31,32], it is important to consider the covariation between cognition and behaviour to fully

63 understand the potential impact of ALAN.

64

65 **Materials and methods**

66 (a) Experimental treatments

67   Groups of 25 wild-type embryos obtained with a standard breeding protocol (ESM,

68 S1) were randomly assigned to one of 12 Petri dishes (Ø = 9 cm) within 2 h from the

69 spawning. The Petri dishes with the subjects were maintained under either ALAN (N = 6) or

70 control condition (N = 6; details in ESM, S1) until testing. In both conditions, a white LED

71 strip (0.38 W/m^2; 570 lux) provided illumination 12 h per day (6:00-18:00 h). In the ALAN

72 condition, a single LED (0.008 W/m^2; 1.5 lux) was turned on during the night phase (18:00-

73 6:00 h), obtaining a night illumination comparable to that affecting aquatic species in urban

74 rivers [33,34]. The eggs began to hatch the third day post fertilisation (3 dpf). In contrast with

75 other species [35,36], we did not find ALAN effects on zebrafish eggs survival and hatching

76 (ESM, S1). At 4 dpf, hatched larvae underwent testing for behavioural and cognitive traits.

77

78 (b) Behavioural assays

79   The two behavioural traits (activity and startle response) were analysed before the

80 cognitive testing. The experimenter collected available larvae (90 from the ALAN treatment

81 and 85 from the control treatment; total N = 176 larvae; replicate N = 6). Larvae were moved

individually into the wells of a 48-wells culture plate (N = 4 plates overall). To assess the

behavioural activity, a tracking system recorded the distance moved by each subject for each

minute of testing, starting immediately after inserting the plate, for a total time of 60 minutes.

The second behavioural trait, the startle response, was measured after the behavioural

activity measure ended. The larvae were exposed to a sudden vibrational stimulation that

typically elicits an immediate startle response (i.e., increased activity; [37]). We measured the

startle response as the distance moved in the second after the stimulation, and we also

recorded the occurrence of unresponsive larvae (distance moved = 0). Details are provided in

ESM, S1.

(c) Habituation learning test

The subjects underwent a habituation learning assay based on the reduction of the

startled response elicited by repeated vibrational stimulations [25,37; ESM, S1]. This test

allows the earliest cognitive assessment in zebrafish. It has been shown to be sensitive to

alterations in learning due to pollutants even at 4 dpf [38], and can predict cognitive abilities

of older larvae (ESM, S1). The assay consisted of administering 25 additional stimulations

following the stimulation for the startle test. The stimulations were always separated by a 1-

second interval. Using the distance moved by each subject after each stimulation, we

calculated an index of activity reduction between each subsequent stimulation and the first

[38,39]. This index provided a measure of habituation learning as the reduction in each

individual's response to the repeated stimulation. Lower values of the index indicated greater

habituation learning performance.

Results

(a) ALAN increased activity

Activity was analysed in three temporal blocks according to the observed trend (Figure 1a). The activity peak in the first minute was not affected by the treatment (Linear Mixed-Effects Model, LMM: $\chi^2_1 = 1.404$, P = 0.236). In the following phase (minutes 2-11) of increasing activity (main effect of time: $\chi^2_1 = 50.933$, P < 0.001), the effect of the treatment approached the threshold of statistical significance ($\chi^2_1 = 3.708$, P = 0.054), while the interaction between treatment and time was not significant ($\chi^2_1 = 0.496$, P = 0.481). In the last phase (minutes 12-60), characterised by decreasing activity trend ($\chi^2_1 = 307.259$, P < 0.001), the main effect of treatment was not significant ($\chi^2_1 = 2.658$, P = 0.103). However, in the last phase, a significant interaction between treatment and time indicated a transitory higher activity of larvae from the ALAN treatment group ($\chi^2_1 = 21.052$, P < 0.001).

In the startle test, the proportion of individuals that responded did not significantly vary between the treatments (ALAN treatment: 0.85; control treatment: 0.78; Generalised Linear Mixed-Effects Model, GLMM: $\chi^2_1 = 1.806$, P = 0.179). In the responding subjects, the intensity of the startle did not differ between the treatments (LMM: $\chi^2_1 = 0.695$, P = 0.403; Figure 1b).

(b) ALAN impaired learning abilities

Overall, the habituation index significantly decreased across the stimulations, as expected due to learning (stimulation: LMM: $\chi^2_1 = 168.894$, P < 0.001; Figure 1c). Compared to the ALAN group, the control group showed a lower habituation index (treatment: $\chi^2_1 = 4.621$, P = 0.032; Figure 1c) and a greater response reduction across stimulations (treatment × stimulation interaction: $\chi^2_1 = 29.605$, P < 0.001; Figure 1c), both effects indicating greater habituation learning in the control group (Figure 1c).

131 (c) ALAN affected covariations between behaviour and cognition

132 In the control group, the habituation learning index was significantly correlated with

133 the behavioural activity (Kendall's T = 0.185, P = 0.032; Figure 2a) and the startle response

134 (Kendall's T = 0.178, P = 0.039; Figure 2b). In the ALAN-exposed larvae, habituation

135 learning significantly correlated with activity (Kendall's T = 0.210, P = 0.008; Figure 2c), but

136 not with startle response (Kendall's T = 0.006, P = 0.942; Figure 2d).

137

138 **Discussion**

139 ALAN pollution affects a significant proportion of aquatic habitats [14-17],

140 potentially exposing fish to the cognitive impairments associated with altered light-dark

141 cycles described for land vertebrates [4,7,8]. We demonstrated that fish larvae from

142 experimental populations exposed to ALAN exhibited reduced habituation learning. The

143 impairment was evident as a slower reduction of the startle response after repeated

144 stimulation [40]. For instance, in the second stimulation, the control group responded

145 approximately 75% less compared to the first stimulation, whereas the ALAN group only

146 showed a 25% response decrease. The performance of the two experimental groups became

147 similar only after nine stimulations. While our laboratory test is difficult to directly relate to a

148 natural situation, the treatment effect is not trivial: individuals exposed to ALAN require

149 many more events to acquire relevant information. Habituation learning has been associated

150 with several activities important for fitness, including antipredator responses [41,42], social

151 relationships [43], and human-wildlife interactions [44,45]. The impairment due to ALAN

152 might impact fitness in wild fish populations. Reduced habituation might prevent learning to

153 discern predator from non-predator species [46], ultimately explaining the increased

154 predation suffered by juvenile marine fish exposed to ALAN [19,47]. ALAN might also

affect other cognitive abilities, calling for investigations on adult fish, which can undergo more complex tests.

A second finding of our experiment involved the two behavioural traits and their relationship with cognition. In line with a study on the rockfish *Girella laevifrons* [48], zebrafish exposed to ALAN were more active than control subjects. This effect has been attributed to the loss of behavioural rhythmicity derived from night illumination [48]. Conversely, under ALAN, guppies, *Poecilia reticulata*, showed no significant activity alterations [18], and bluegill, *Lepomis macrochirus* showed activity reduction [49], suggesting a species-specific effect on behavioural activity. These interspecific differences might also be due to different responses to the assay. In our zebrafish, the effect on activity was mediated by time, being initially more marked and reducing after approximately 30 minutes. Therefore, we may have measured acclimation to the novel environment rather than plain activity. Regarding the second behavioural trait examined, the startle response, we detected no ALAN-related effects.

Critically, we found significant correlations between the two behavioural traits and individuals' learning performance in the control group, whereas in the ALAN group, we detected only one of these covariations. Relationships between cognition and behaviour have been increasingly reported in the literature [26,27], and are likely part of a more extended set of covariations involving physiological and life-history traits [50]. An earlier study has reported ALAN-driven disruption of the relationship between behaviour and metabolism in hermit crabs [51]. While the evolutionary significance of the covariation between cognition and behaviour is not fully understood, other covariations have been linked to fundamental life-history trade-offs [52,53], suggesting that the same may occur for the cognition-behaviour relationships. Under this scenario, when a stressor such as ALAN disrupts the covariation, it may alter how individuals optimise their investment across various traits.

Moreover, this intraspecific variability might be related to local adaptation [e.g., 29]. If this possibility will be confirmed by future studies, fitness effects of covariance disruption due to ALAN will deserve attention in wild fish populations. The scenario might be gloomier if we postulate that this, as well as other covariations between traits, could also be altered by additional, co-occurring anthropogenic stressors [32,54,55]. Lastly, the covariance between behaviour and learning may be involved in mechanisms of invasive species success [56], suggesting the importance of studying ALAN effects in this context.

Our focus on the embryonic stage has led to further insights into the impacts of ALAN on teleost fishes. First, we can conclude that even a short exposure to ALAN might induce phenotypic changes, at least during the earlier development. Due to behavioural and cognitive alterations, fish hatching from eggs laid in habitats affected by ALAN could suffer negative consequences from their first day of life, often the onset of a critical period for survival. Second, the observed effects were directly caused by individuals' experience without contribution of parental effect, which remain nevertheless interesting to investigate as reported for various cognitive functions [57,58]. An unanswered question is whether ALAN-mediated alterations persist for the entire life. Studies with various approaches suggest that stressors can determine long-lasting cognitive plasticity in fish [59,60]. If this will be confirmed for ALAN, its impacts could carry over into later life stages, even if later life stages move to habitats without light pollution. The adult fish brain, however, is probably the most plastic among vertebrates [61] and we cannot exclude continuous plasticity in response to changing light conditions.

Overall, this study highlighted the negative effects of early life ALAN exposure on fish cognition and its covariation with behavioural traits. Our findings advocate for investigation on other fish species in their early ontogenetic stages, as studies in zebrafish have been often predictive for other freshwater and marine teleosts [23,24]. Similarly,

205 cognitive impairments from embryonic ALAN exposure might occur in other aquatic taxa

206 such as invertebrates and amphibians.

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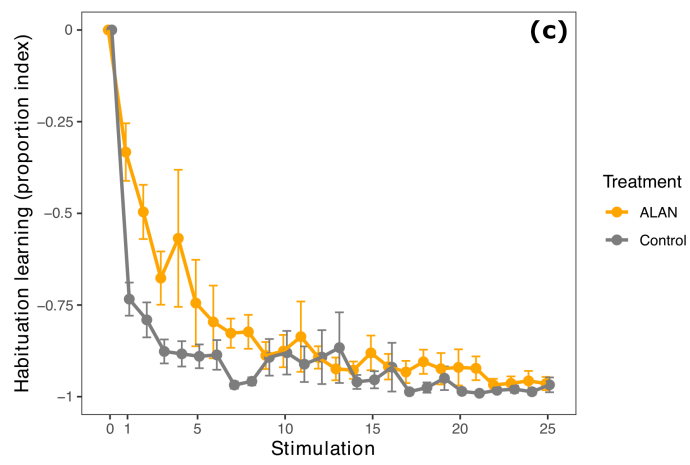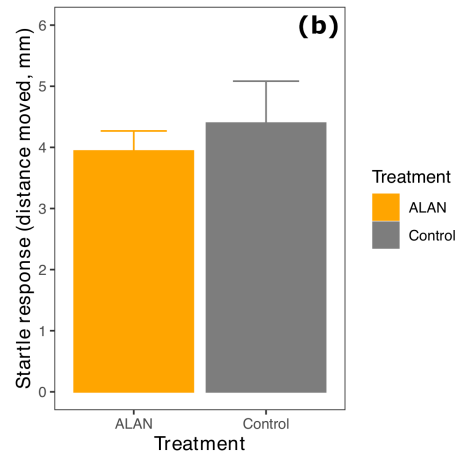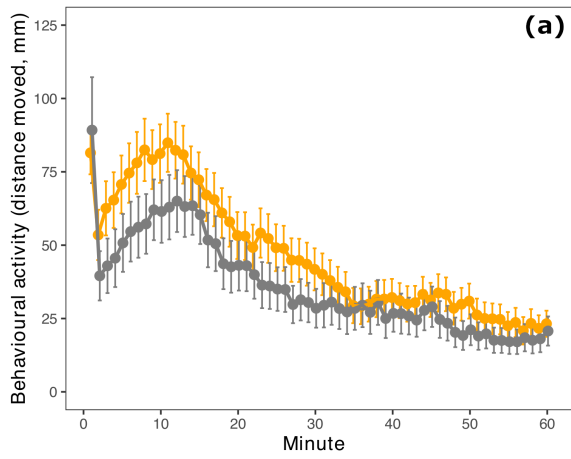454 with behavioural traits in teleost fish. Figshare. (doi:)

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Figure captions

Figure 1. Behavioural and cognitive alterations due to the ALAN. (a) Activity measured as distance moved; (b) index of first response to the stimulation; (c) habituation learning index. Data points and bars represent means and error bars represent standard errors; the stimulation '0' in panel b represents the index of the initial reference startle.
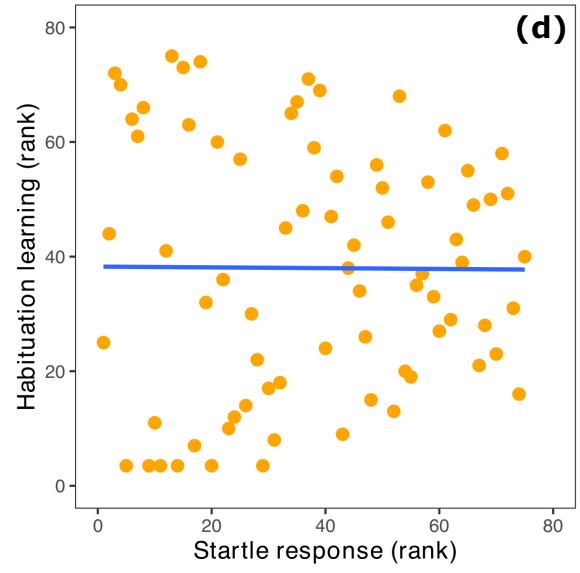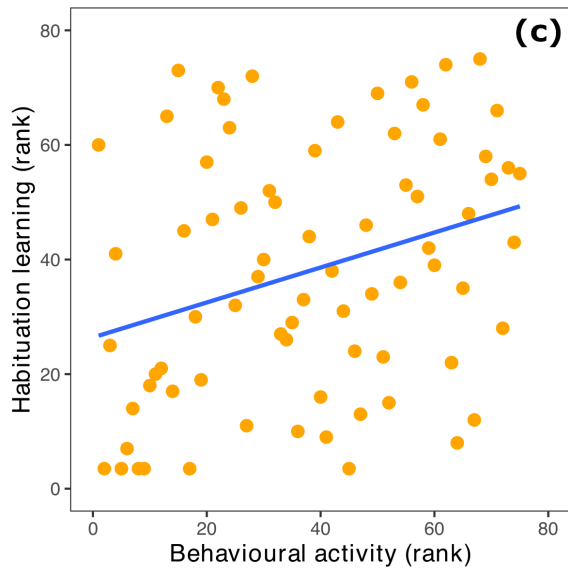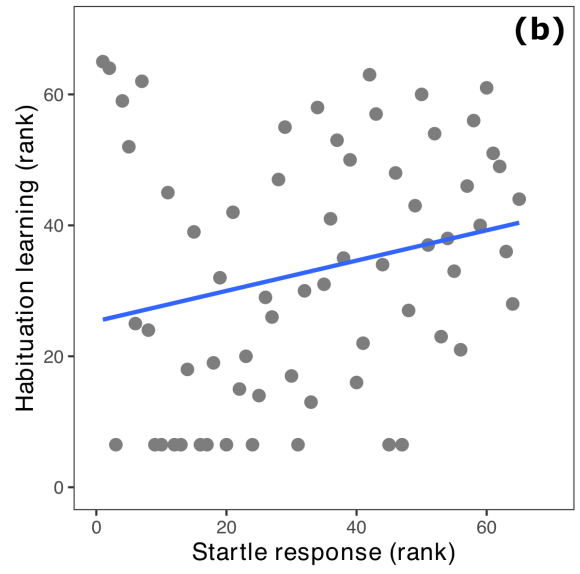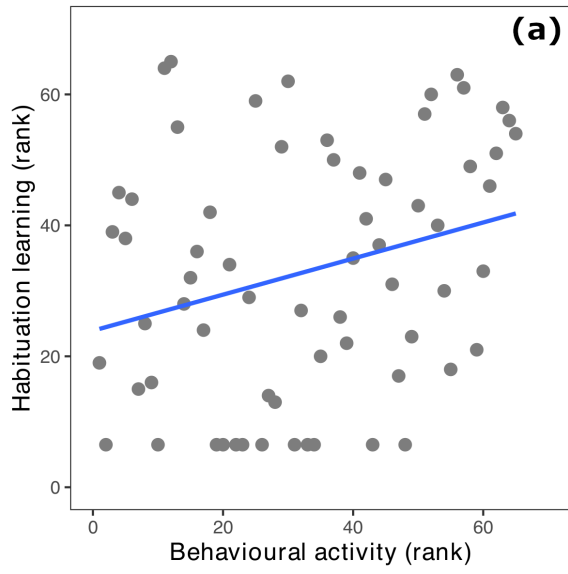

Figure 2. Covariation between the cognitive trait (habituation learning) and the two behavioural traits. Scatterplots of habituation learning versus (a) behavioural activity and (b) startle response in control zebrafish; scatterplots of habituation learning versus (c) behavioural activity and (d) startle response in ALAN-exposed zebrafish. Lines represent predicted values from linear regression displayed for illustrative purposes.

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