Adenylate kinase 1 overexpression increases locomotor activity in medaka fish

Michiyo Maruyama[1, 2], Yuko Furukawa[2], Masato Kinoshita[3], Atsushi Mukaiyama[4, 5], Shuji Akiyama[4, 5], Takashi Yoshimura[1, 2*]

[1]Laboratory of Animal Integrative Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.

[2]Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Nagoya 464-8601, Japan.

[3]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

[4]Research Center of Integrative Molecular System (CIMoS), Institute for Molecular Science, National Institute of Natural Sciences, Okazaki 444-8585, Japan.

[5]Department of Functional Molecular Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki 444-8585, Japan.

*Corresponding author

E-mail: takashiy@agr.nagoya-u.ac.jp (TY)

Abstract

Maintenance of the energy balance is indispensable for cell survival and function. Adenylate kinase (Ak) is a ubiquitous enzyme highly conserved among many organisms. Ak plays an essential role in energy regulation by maintaining adenine nucleotide homeostasis in cells. However, its role at the whole organism level, especially in animal behavior, remains unclear. Here, we established a model using medaka fish (*Oryzias latipes*) to examine the function of Ak in environmental adaptation. Medaka overexpressing the major Ak isoform Ak1 exhibited increased locomotor activity compared to that of the wild type. Interestingly, this increase was temperature dependent. Our findings suggest that cellular energy balance can modulate locomotor activity.

Introduction

Energy homeostasis is crucial for survival and the maintenance of cell function. Disruption of energy balance is suggested to be associated with many conditions, including obesity, heart failure, and neurodegeneration [1–4]. Adenylate kinase (Ak) is an essential enzyme found in nearly every organism because of its crucial role in cellular energy metabolism [4–7]. Ak catalyzes the nucleotide phosphoryl exchange reaction

(2ADP ↔ ATP + AMP) to maintain the adenine nucleotide balance and monitor energy consumption. Ak and its downstream adenine nucleotide signaling pathway have attracted attention because of their vital functions in many biological processes, such as the cell cycle, hormone secretion, and stress tolerance [5,7–11]. To date, nine isoforms of AK (AK1–AK9) have been identified and well characterized in humans [6]. AK1 is the major isoform of AK and is expressed in the cytosol of most human tissues, with particularly high levels in the brain, the heart, skeletal muscles, and erythrocytes. Several reports have demonstrated the importance of Ak1 in metabolic stress conditions [9–11]. Interestingly, it was recently reported that hyperactive rat strains exhibit increased *Ak1* expression [12]. To our knowledge, however, the role of Ak1 at the whole organism level, especially in animal behavior, remains unclear.

In this study, we sought to clarify the effect of *Ak1* on animal behavioral rhythms. The medaka (*Oryzias latipes*), a small freshwater fish, has recently emerged as a useful vertebrate model because of its small size and short generation time. Additionally, there are well-established methods for transgenic and genomic editing in medaka. Behavioral assays are also well established, for both medaka and zebrafish, but medaka can survive a wider range of water temperatures (4–40 °C) than zebrafish (20–32 °C), and they exhibit seasonal behavioral patterns [13–16]. Therefore, the medaka is an excellent model with

which to investigate adaptive strategies to environmental changes.

Here, we successfully established *Ak1*-overexpressing (*Ak1*-OE) medaka. Behavioral assays of *Ak1*-OE medaka larvae revealed a temperature-dependent increase in locomotor activity. These findings shed new light on the function of *Ak1*.

Materials and methods

Ethics statement for animal experiments

All animal studies were performed in accordance with the ARRIVE guidelines. All methods were conducted in compliance with the relevant guidelines and regulations and were approved by the Animal Experiment Committee of Nagoya University (approved number: AGR2020009).

Animals

Medaka fish (*Oryzias latipes*) were obtained from a local dealer (Fuji 3A Project, Nagoya, Japan). The fish were maintained in a housing system (MEITO system; Meito Suien, Nagoya, Japan) under a 14 h light/10 h dark photoperiod (lights on at 05:00 and off at 19:00) at a water temperature of 25 °C. They were fed twice a day with Hikari Labo 450

(KYORIN, Tokyo, Japan).

Gene expression analysis

Total RNA was purified using an RNeasy micro kit (Qiagen, Hilden, Germany) with DNase I. Extracted RNA was stored at –80 °C. Reverse transcription was performed on total RNA (200 ng) using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). RT-PCR was performed using TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan) and the following primers: *Ak1* forward (F): 5'-ACACTCACCTGTCTTCAGGC-3' and reverse (R): 5'-CTGTGTCCAGGGGTACAAGC-3', and *Actb* F: 5'-GATTCCCTTGAAACGAAAAGCC-3' and R: 5'-CAGGGCTGTTGAAAGTCTCAAAC-3'. Amplification was conducted at 94 °C for 2 min, followed by 30 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min. The PCR products were analyzed using a MultiNA microchip electrophoresis system (Shimadzu Corporation, Kyoto, Japan).

For real-time quantitative PCR (qPCR), 2 µL of the synthesized cDNA was mixed with SYBR Premix Ex Taq II (Takara Bio Inc.) and 0.4 µM primers (same as above) to a total volume of 20 µL. qPCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The *Actb* gene was used as an internal

96 control.

97

98 Construction of *Ak1*-OE plasmid

99 Total RNA was purified from embryos at 4 dpf using an RNeasy micro kit (Qiagen) with
100 DNase I. Reverse transcription of total RNA (200 ng) was performed using a ReverTra
101 Ace qPCR RT kit (Toyobo). The entire *Ak1* open reading frame (excluding the stop
102 codon) was amplified by PCR (98 °C for 2 min; 35 cycles of 98 °C for 10 s, 65 °C for 30
103 s, 72 °C for 1 min, and 72 °C for 5 min) using a Q5 Hot Start High-Fidelity 2× Master
104 Mix (New England Biolabs, Ipswich, MA, USA) and primers containing the restriction
105 sites of *Spe*I or *Bgl*II for subsequent subcloning (F: 5'-
106 CCACTAGTATGGCAGACAAAATCAAGGAC-3' and R: 5'-
107 CCAGATCTCTTCAGTGAATCAATAGCCTG-3').
108 The overexpression plasmid contained the Actin β (*Actb*) promoter followed by the first
109 noncoding exon and the first intron, a 3×FLAG tag, an internal ribosomal entry site
110 (IRES), GFP and SV40 polyA sequences in the *Not*I-*Not*I interval [17]. *Ak1* cDNA and
111 plasmids were digested with *Spe*I and *Bgl*II and purified using NucleoSpin Gel and PCR
112 Clean-up (Macherey-Nagel, Düren, Germany). Ligation was performed using Ligation
113 High Ver. 2 (Toyobo). The sequence of the constructs was confirmed by direct

sequencing.

Generation of *Ak1*-OE medaka

The *Not*I-*Not*I interval of the *Ak1*-OE plasmid was purified and diluted with sterilized water (final concentration: 20 ng/μL) and then microinjected into the cytoplasm of one-cell stage embryos (F0). F0 fish with mosaic GFP fluorescence were raised and crossed with WT fish to obtain heterozygous transgene carriers (F1). F1 fish with GFP fluorescence were crossed with the WT fish. Heterozygous F2 offspring were used for behavioral experiments.

Behavioral assays

F2 *Ak1*-OE and WT embryos were incubated under a 14L10D cycle at 25 °C until hatching. At the end of the light phase at 8 dpf, *Ak1*-OE larvae and their WT siblings were distributed among 48 round well plate (CELL STAR; Greiner Bio-One, Kremsmünster, Austria) each containing 1200 μL of water, with 1 larva per well. The plate was then installed in a DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands). At 19:00 8 dpf, the light was turned off and the larvae were acclimated for 10 h during the dark phase. Behavior was recorded from 05:00 9 dpf to 5:00 12 dpf (for 3 days) under

a 14 h light (300 lx) and 10 h dark (0 lx) photoperiod (lights on at 05:00 and off at 19:00) at a water temperature of 15 or 25 °C. Behavior was recorded at 3.75 frames per second and analyzed using the tracking software EthoVision XT (Noldus). Locomotor activity was calculated by measuring the "distance moved" every 10 min.

Statistical analysis

Data are presented as the mean ± SEM generated using the statistical software GraphPad Prism 9. Two-tailed Welch's *t*-test was used for comparisons between two groups. For comparisons among three or more groups, two-way ANOVA and post hoc tests were conducted using GraphPad Prism 9.

Results

Generation of *Ak1*-OE medaka

We first examined *Ak1* expression in various medaka tissues. Reverse transcription polymerase chain reaction (RT-PCR) results showed widespread expression (Fig 1A). To evaluate the effect of constitutively high *Ak1* expression, we used a construct that overexpressed *Ak1* in response to regulation of the *Actb* promoter (Fig 1B). This

150 construct enabled us to visualize transgene expression via green fluorescent protein (GFP),
151 which was linked to the 3' internal ribosome entry site (IRES) downstream of *Actb-Ak1*
152 [17].

153 To generate *Ak1*-OE medaka, the construct was microinjected into the cytoplasm
154 of one-cell stage embryos. Exhibiting chimeric GFP expression embryos were raised as
155 F0 medaka. These medaka were paired with the wild type to obtain heterozygous
156 transgene carriers (F1). GFP-expressing F1 medaka were then crossed with the wild type
157 to generate heterozygous F2 offspring (Figs 1C and D). Overexpression of *Ak1* in GFP-
158 positive F2 medaka was confirmed by quantitative RT-PCR (qPCR) (Fig 1E).

159

160 **Fig 1. Generation of *Ak1*-overexpressing medaka.** (A) Electrophoresis image of *Ak1*
161 and *Actb* RT-PCR products in various adult medaka tissues. B, Brain; E, Eye; O, Ovary;
162 L, Liver; S, Skin; N, No-template control. (B) Structure of *Ak1* overexpression construct.
163 The construct consisted of the medaka *Actb* regulatory region, *Ak1* cDNA (without the
164 stop codon), C-terminus 3× FLAG tag, internal ribosome entry site (IRES), green
165 fluorescent protein (GFP) open reading frame, and SV40 polyA signal. (C) Design of
166 genetic crosses for generating heterozygous *Ak1*-overexpressing (*Ak1*-OE) medaka and
167 their wild-type (WT) siblings. (D) Bright-field and fluorescence images of WT and *Ak1*-

168 OE embryos at 5 days post-fertilization (dpf). (E) *Ak1* expression level of larvae at 9 dpf.
169 Data represent the mean ± SEM, n = 4 each. WT expression level was set at 1. The *p*-
170 value was calculated using two-tailed Welch's *t*-test. Each dot represents an individual
171 value.
172

173 *Ak1*-OE larvae exhibited increased locomotor activity at 25 °C

174 The high-throughput behavioral tracking assay is well established in larvae of
175 zebrafish and medaka fish [14,15]. Therefore, we used this method to examine the
176 locomotor activity of medaka larvae. *Ak1*-OE embryos and their wild-type (WT) siblings
177 were raised in an incubator under a 14 h light/10 h dark (14L10D) cycle (lights on at
178 05:00 and off at 19:00) at 25 °C. At the end of the light phase at 8 days post-fertilization
179 (dpf), hatched larvae were placed in each well of a 48-well plate. The plate was then put
180 into a high-throughput behavior tracking system. Larvae were maintained under a
181 14L10D cycle at 25 °C. After 10 h of habituation, at the onset of the light phase at 9 dpf,
182 locomotor activity was tracked for three full days (Fig 2A). The tracking profiles showed
183 that both *Ak1*-OE and WT larvae were more active during the light phase than during the
184 dark phase (Fig 2B). Compared to that on the first day of the assay, the total distance
185 moved by the WT larvae significantly decreased on the third day (Fig 2C). As in previous

186 studies [14,15], larvae were not fed during the assay to avoid interrupting it. This may
187 have caused decreased energy, and hence, decreased activity, later in the experiment.
188 Notably, the average locomotor activity of the *Ak1*-OE larvae tended to be higher than
189 that of the WT larvae throughout the assay. This trend was particularly obvious on day 3,
190 at which point the total locomotor activity of the *Ak1*-OE larvae was significantly higher
191 than that of the WT larvae, both in the light and dark phases (Fig 2D).
192
193 **Figure 2. Locomotor activity of *Ak1*-overexpressing larvae at 25 °C.** (A) Structure of
194 the behavioral assay. Larvae (8 dpf) were placed into a photoperiod- and temperature-
195 controlled chamber (14L10D, 25 °C) at the end of the light phase. After 10 h of
196 habituation (Hab.), recording was started at the onset of the light phase at 9 dpf and
197 continued for 3 full days. (B) Mean locomotor activity of *Ak1*-overexpressing (*Ak1*-OE)
198 and wild-type (WT) larvae. (C) Total locomotor activity of *Ak1*-OE and WT larvae per
199 day. Data represent the mean ± SEM and were analyzed via two-way repeated measures
200 ANOVA. Effect of time, $F_{(1.386, 37.43)} = 11.45$, $p = 0.0006$; effect of overexpression, $F_{(1, 27)}$
201 $= 7.891$, $p = 0.0091$; effect of interaction, $F_{(2, 54)} = 0.2186$, $p = 0.8044$. *$p < 0.05$; n.s., not
202 significant (Dunnett's multiple comparisons test, vs. day 1). (D) Total locomotor activity
203 of *Ak1*-OE and WT larvae per day during the light phase (white) and dark phase (gray).

Data represent the mean ± SEM. The *p*-values (*$p < 0.05$) were calculated using two-tailed Welch's *t*-test. Each dot represents an individual value. (B–D) n = 15 (WT), n = 14 (*Ak1*-OE).

Ak1-OE larvae did not exhibit increased locomotor activity at 15 °C

Ambient temperature directly affects the metabolic rate of ectothermic animals. Therefore, we further examined the locomotor activity of *Ak1*-OE larvae at a lower temperature (15 °C), a condition associated with lower energy expenditure. After they were raised at 25 °C, at 8 dpf, larvae were placed in a tracking chamber and exposed to a 14L10D cycle at 15 °C (Fig 3A). In both the *Ak1*-OE and WT larvae, locomotor activity levels on days 2 and 3 were similar to those on day 1 (Figs 3B and C). This suggests that the larvae conserved their energy until the end of the experiment at the lower temperature. Unlike the results at 25 °C, there was no difference in locomotor activity between the *Ak1*-OE and WT larvae throughout the experiment (Fig 3D). This suggests that the increase in the locomotor activity of *Ak1*-OE larvae was temperature dependent.

Figure 3. Locomotor activity of *Ak1*-overexpressing larvae at 15 °C. (A) Structure of the behavioral assay. Larvae were maintained in a chamber under a 14L10D cycle at 15 °C. (B) Mean locomotor activity of *Ak1*-overexpressing (*Ak1*-OE) and wild-type (WT) larvae.

(C) Total locomotor activity of *Ak1*-OE and WT larvae per day. Data represent the mean ± SEM and were analyzed via two-way repeated measures ANOVA. Effect of time, $F_{(1.910, 68.74)} = 1.848$, $p = 0.1669$; effect of overexpression, $F_{(1, 36)} = 3.607\times10^{-5}$, $p = 0.9952$; effect of interaction, $F_{(2, 72)} = 0.2813$, $p = 0.7556$. n.s., not significant (Dunnett's multiple comparisons test, vs. day 1). (D) Total locomotor activity of *Ak1*-OE and WT larvae per day during the light phase (white) and dark phase (gray). Data represent the mean ± SEM. n.s., not significant (two-tailed Welch's *t*-test). Each dot represents an individual value. (B–D) n = 20 (WT), n = 18 (*Ak1*-OE).

Discussion

Ak are essential enzymes that play critical roles in metabolic monitoring and signaling of cells. Among the Ak family members, we focused on Ak1, the major cytosolic isoform of Ak. In the present study, in various medaka tissues, we observed *Ak1*

239 expression similar to that reported in other animals [6] (Fig 1A). Ak1 is highly conserved
240 among many organisms. Indeed, the alignment of human AK1 (NP_000467.1, 194 aa)
241 with medaka Ak1 (XP_004074790.1, 194 aa) exhibited 75% identical residues and 88%
242 similar residues (via the NCBI BLAST Needleman-Wunsch Global Align function).
243 To know the function of Ak1 at the whole organism level, we established an
244 *Ak1*-OE medaka line (Figs 1B–E). Behavioral assays of the medaka larvae revealed that
245 *Ak1*-OE larvae exhibited increased locomotor activity compared with that of the WT
246 larvae at 25 °C, and the difference between the *Ak1*-OE and WT larvae was most obvious
247 on the last day of the experiment (Fig 2D). *Ak1*-OE larvae maintained a higher activity
248 level throughout the entire experiment, whereas the total amount of locomotor activity of
249 the WT larvae significantly decreased on day 3 (Fig 2C). In contrast, throughout the
250 experiment at 15 °C, the locomotor activity of *Ak1*-OE larvae did not differ from that of
251 the WT larvae (Fig 3D). Several knockout studies have shown that *Ak1* deficiency can be
252 compensated for by other members of the Ak family under normal conditions, whereas
253 *Ak1* deficiency leads to failure of metabolic homeostasis under stress conditions
254 [10,11,18]. As our behavioral assays were conducted without feeding, the lack of energy
255 later in the experiment might have mimicked metabolic stress even at the moderate
256 temperature of 25 °C, and this could have caused the large difference in activity between

257 the *Ak1*-OE and WT larvae on the last day of the assay.

258 In summary, Ak1 is known to play crucial roles in cell function. Our results
259 indicate the importance of *Ak1* not only at the cellular level but also at the behavioral
260 level.

261

262

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270 Author contributions

271 M.M. and T.Y. designed the research. M.M. and Y.F. conducted the experiments
272 and analyzed the data. M.K., S.A. and A.M. provided new material and methods. M.M.
273 and T.Y. wrote the manuscript. All authors discussed the results and commented on the
274 manuscript.

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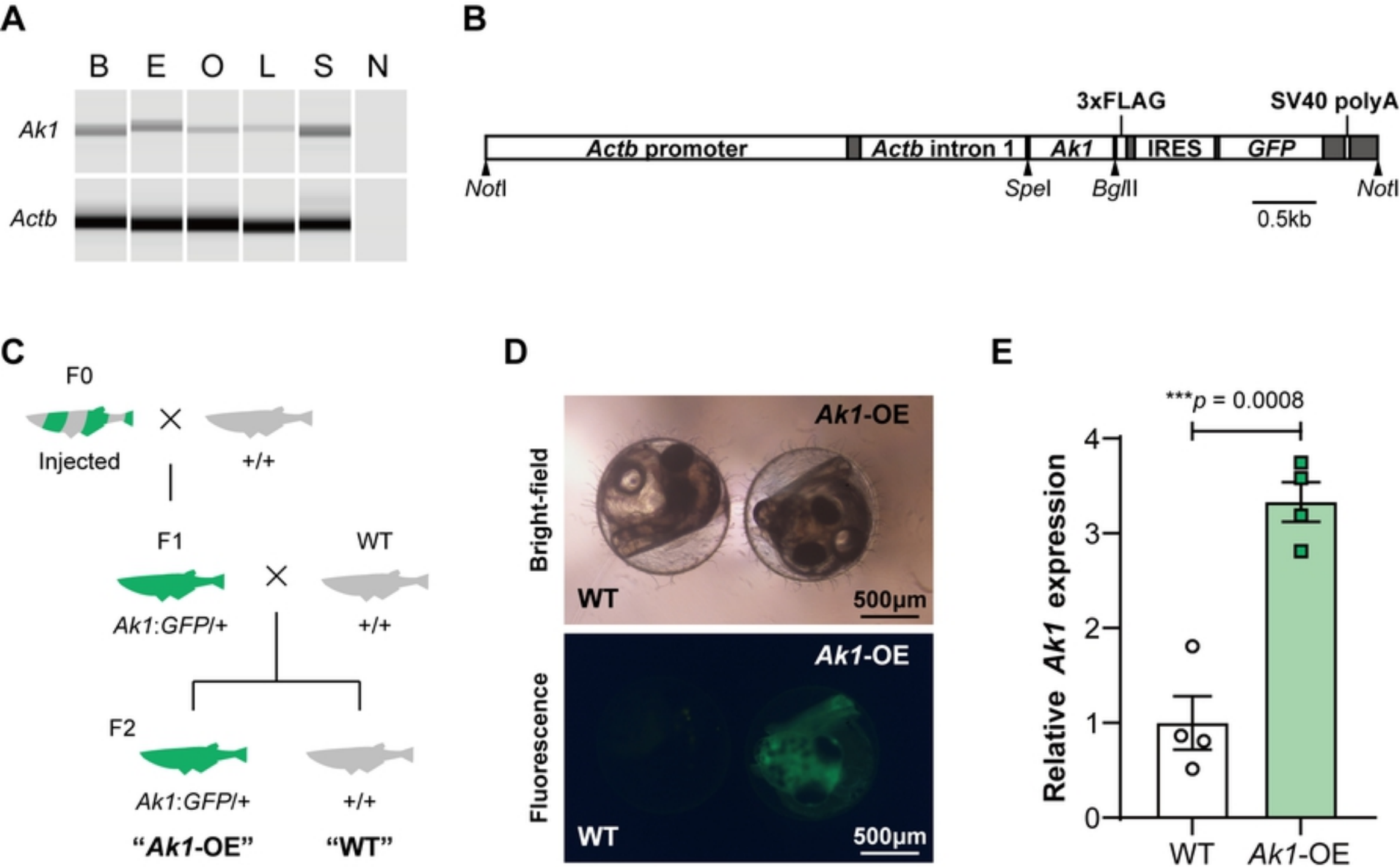
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B E O L S N
Ak1
Actb
B
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Ak1
IRES
GFP
NotI
SpeI
BglII
NotI
0.5kb
C
F0
Injected
+/+
F1
WT
Ak1:GFP/+
+/+
F2
Ak1:GFP/+
+/+
"Ak1-OE"
"WT"
D
Bright-field
Ak1-OE
WT
500μm
Fluorescence
Ak1-OE
WT
500μm
E
***p = 0.0008
Relative Ak1 expression
0
1
2
3
4
WT
Ak1-OE

Figure 1

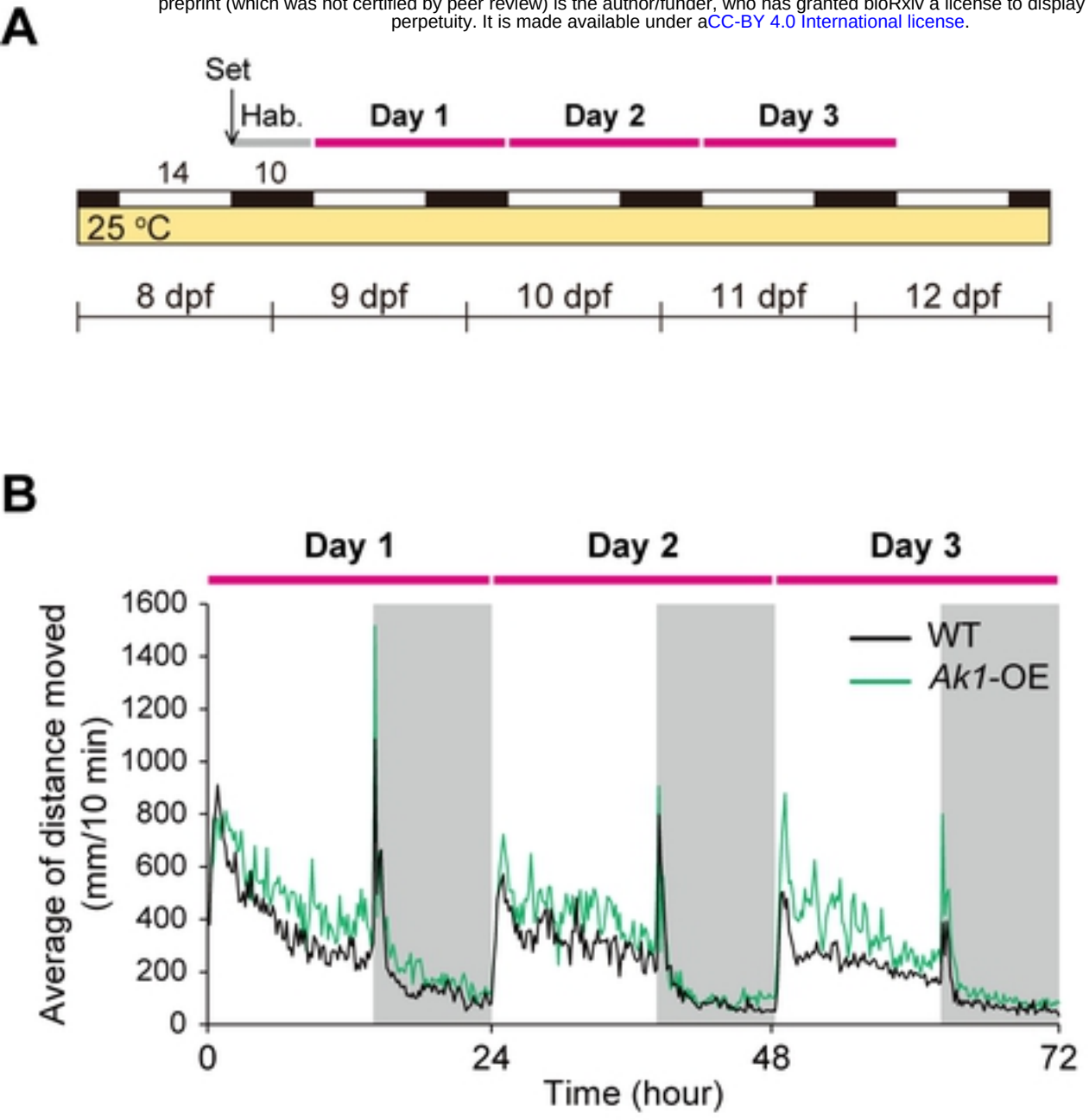
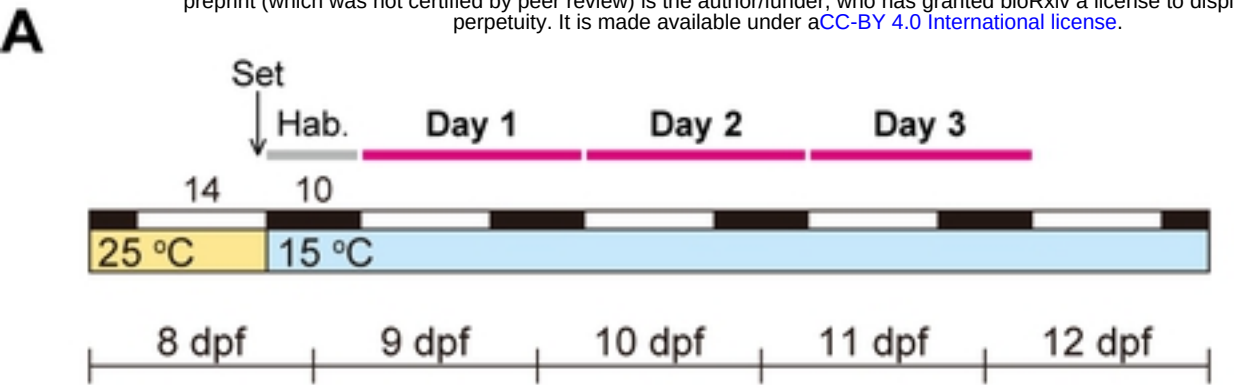
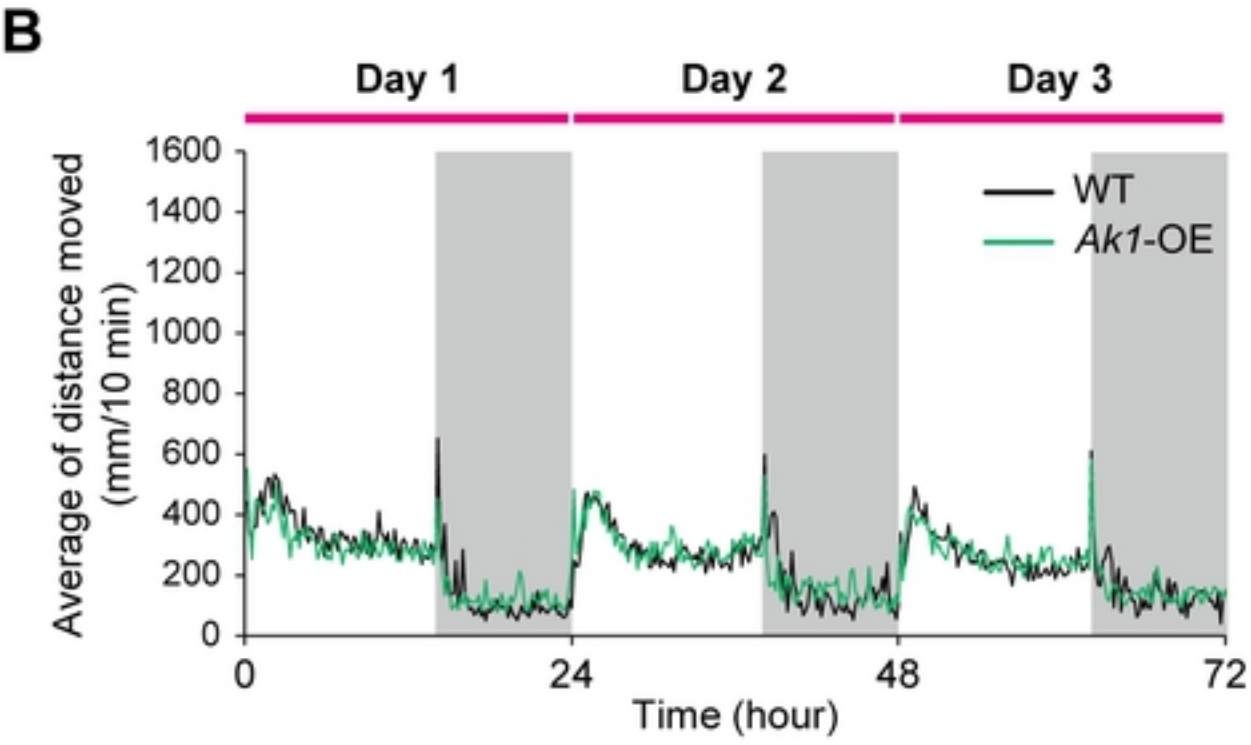
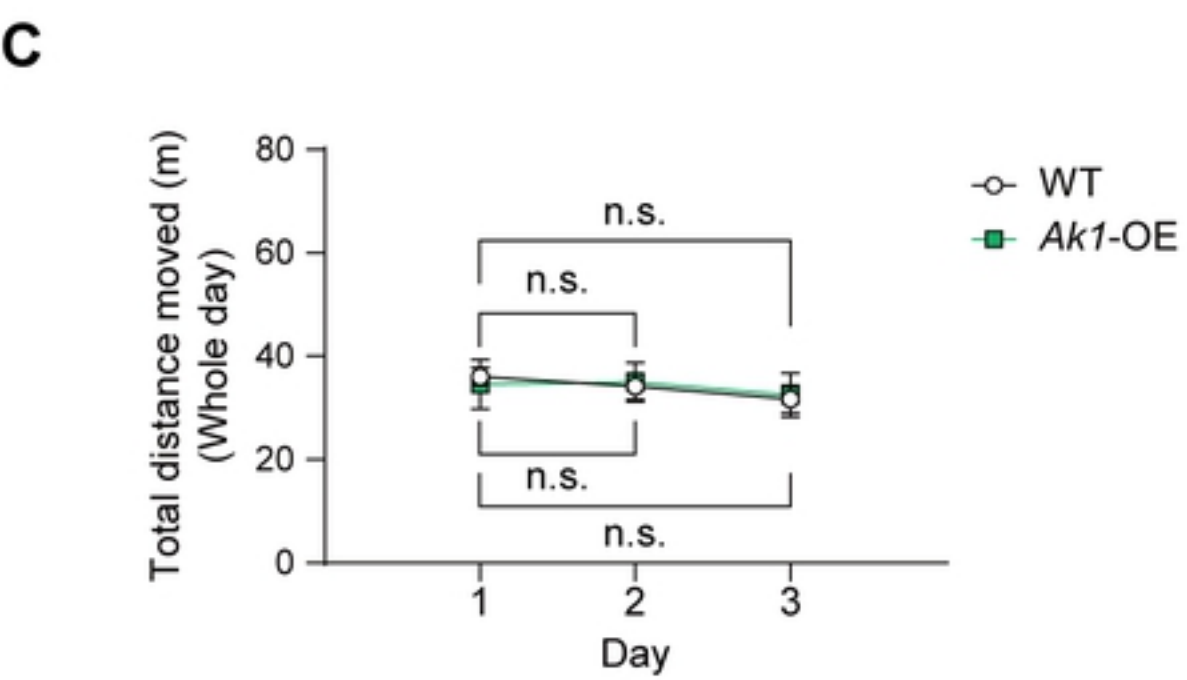


Figure 2







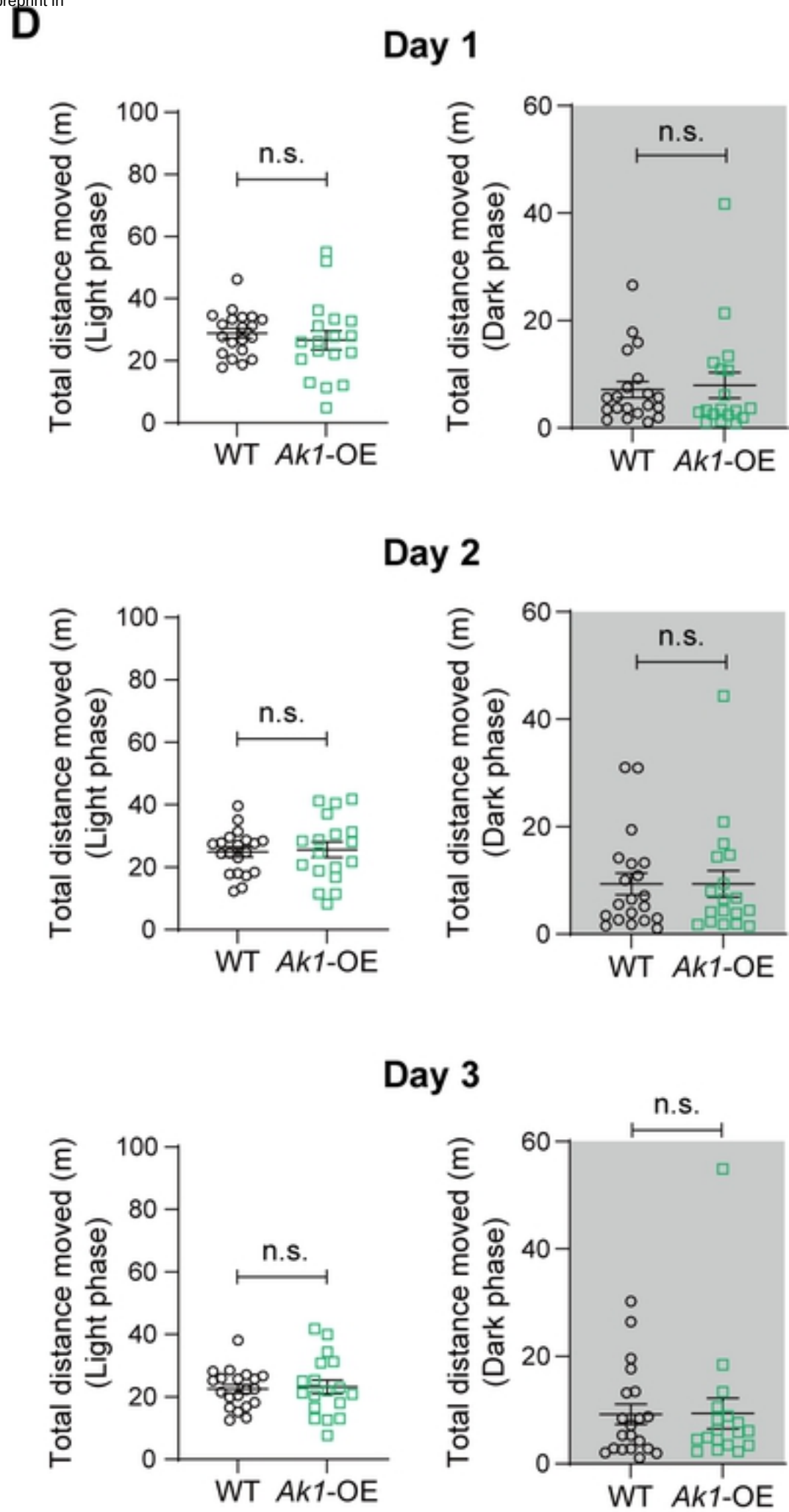


Figure 3