



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Functional inhibition of deep brain non-visual opsins facilitates acute long day induction of reproductive recrudescence in male Japanese quail

Citation for published version:

Perez, J, Tolla, E, Bishop, V, Foster, RG, Peirson, SN, Dunn, I, Meddle, S & Stevenson, TJ 2023, 'Functional inhibition of deep brain non-visual opsins facilitates acute long day induction of reproductive recrudescence in male Japanese quail : Deep brain photoreceptors in birds ', *Hormones and Behavior*, vol. 148, 105298. <https://doi.org/10.1016/j.yhbeh.2022.105298>

Digital Object Identifier (DOI):

[10.1016/j.yhbeh.2022.105298](https://doi.org/10.1016/j.yhbeh.2022.105298)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Hormones and Behavior

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 Running title: Deep brain photoreceptors in birds
2
3
4
5

6 **Title: Functional inhibition of deep brain non-visual opsins facilitates acute long day**
7 **induction of reproductive recrudescence in male Japanese quail.**
8
9

10
11 Authors: Jonathan H. Pérez*¹, Elisabetta Tolla², Valerie R. Bishop³, Russell G. Foster⁴, Stuart N.
12 Peirson⁴, Ian C. Dunn³, Simone L. Meddle³, Tyler J. Stevenson².
13

14 Affiliation: 1. Biology Department, The University of South Alabama, Mobile Alabama, 36688,
15 USA.
16 2. Institute of Biodiversity, Animal Health and Comparative Medicine, University of
17 Glasgow, Glasgow G61 1QH, Scotland.
18 3. The Roslin Institute, The Royal (Dick) School of Veterinary Studies, University of
19 Edinburgh, Midlothian EH25 9RG, Scotland.
20 4. Sir Jules Thorn Sleep and Circadian Neuroscience Institute (SCNi) and Nuffield
21 Laboratory of Ophthalmology, Nuffield Department of Clinical Neurosciences,
22 Dorothy Crowfoot Hodgkin Building, University of Oxford, South Parks Road,
23 Oxford OX1 3QU.
24
25
26

27 *Corresponding author

28 Biology Department
29 The University of South Alabama
30 Mobile, AL 36688
31 5871 USA Dr
32 Email: jhperez@southalabama.edu
33 Phone: 251-460-6331
34
35

36 Disclaimer: The authors have nothing to disclose.
37
38
39

40 **Key words:** photoreceptor, VA opsin, neuropsin, OPN5, seasonality, reproduction, RNA
41 interference, viral vector
42

43 **Highlights**

- 44 • VA opsin and neuropsin RNAi silencing impact photoinduced activation of the
45 reproductive axis in quail
- 46 • Effects of RNAi silencing of VA opsin and Opn5 variably impact reproductive axis
47 activity
- 48 • Non-visual opsin photoreceptors appear to exert their effect at multiple points in the
49 regulation of the reproductive axis.

50

51 **Abstract**

52 For nearly a century, we have known that brain photoreceptors regulate avian seasonal biology.
53 Two photopigments, vertebrate ancient opsin (VA) and neuropsin (OPN5), provide a possible
54 molecular substrate for this photoreceptor pathways. VA fulfills many criteria for providing light
55 input to the reproductive response, but a functional link has yet to be demonstrated. This study
56 examined the role of VA and OPN5 in the avian photoperiodic response of Japanese quail
57 (*Coturnix japonica*). Non-breeding male quail were housed under short days (6L:18D) and
58 received an intracerebroventricular infusion of adeno-associated viral vectors with shRNAi that
59 selectively inhibited either VA or OPN5. An empty viral vector acted as a control. Quail were
60 then photostimulated (16L:8D) to stimulate gonadal growth. Two long days significantly
61 increased pituitary thyrotrophin-stimulating hormone β -subunit (*TSH β*) and luteinizing hormone
62 β -subunit (*LH β*) mRNA of VA shRNAi treated quail compared to controls. Furthermore, at one
63 week there was a significant increase, compared to controls, in both hypothalamic gonadotrophin
64 releasing hormone-I (*GnRH-I*) mRNA and paired testicular mass in VA shRNAi birds. Opn5
65 shRNAi facilitated the photoinduced increase in *TSH β* mRNA at 2 days, but no other differences
66 were identified compared to controls. Contrary to our expectations, the silencing of deep brain
67 photoreceptors enhances the response of the reproductive axis to photostimulation rather than
68 preventing it. In addition, we show that VA opsin plays a dominant role in the light-dependent
69 neuroendocrine control of seasonal reproduction in birds. Together our findings suggest the
70 photoperiodic response involves at least twoAfter 7 days of photostimulation of VA opsin either
71 directly co-expressed in GnRH-I neurons or indirectly (via disinhibition from VA neurons)
72 resulted in higher GnRH expression and increased LH β and FSH β expression photoreceptor
73 types and populations working together with VA opsin playing a dominant role.

75 **Introduction**

76 Annual changes in photoperiod, are the primary predictive cue regulating seasonal
77 reproduction in many vertebrates (Dawson et al., 2001; Rowan, 1925). The photoperiodic
78 response is a well characterized cascade of neuroendocrine changes that transition birds from a
79 short day (SD) non-breeding to a long day (LD) reproductive state (Follett and Pearce-Kelly,
80 1991; MacDougall-Shackleton et al., 2009). Exposure to a single day of photoperiod exceeding
81 13 hours of light stimulates thyrotrophs in the pars tuberalis to release *TSH β* , inducing a
82 reciprocal switch in deiodinase gene expression in the MBH (Nakao et al., 2008). Continued
83 long-term LD exposure maintains high GnRH-I synthesis in the preoptic area (POA) and permits
84 GnRH-I release, to stimulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH)
85 release from the anterior pituitary gland.

86 In mammals, a discrete population of retinal ganglion cells, expressing the non-visual
87 photoreceptor melanopsin (OPN4), are critical for transduction of seasonal light information
88 (Foster et al., 2020; Hankins et al., 2008). However, in most non-mammalian species, light-
89 driven changes in seasonal physiology occur via extra-retinal photoreceptors (Menaker et al.,
90 1970; Menaker and Keatts, 1968; Pérez et al., 2019). Targeted illumination studies have
91 localized the photoreceptors for avian reproduction to the medial basal hypothalamus (MBH)
92 (Benoit and Ott, 1944; Oliver and Baylé, 1975). The action spectrum (λ_{\max}) for reproductive
93 photostimulation in Japanese quail (*Coturnix japonica*) established the involvement of an
94 opsin/vitamin-A based photopigment with a maximum spectral response of ~492 nm (Foster et
95 al., 1985; Foster and Follett, 1985). To date, three photoreceptor opsins have been characterized
96 and localized within the hypothalamus of birds: vertebrate ancient opsin (VA; Halford et al.,
97 2009), neuropsin (OPN5; Nakane et al., 2014) and melanopsin (OPN4; Chaurasia et al., 2005).

98 VA is expressed in the preoptic area (POA) and mediobasal regions of the hypothalamus
99 (Halford et al., 2009). Crucially, VA cells exhibit an absorption spectrum closely matching the
100 reproduction spectra maximum for reproductive physiology (~490nm; Davies et al., 2012) and
101 are co-localize with gonadotropin-releasing hormone I (GnRH-I) expressing cells (Halford et al.,
102 2009) providing a link between light detection and activation of the reproductive axis (García-
103 Fernández et al., 2015). However, a functional role for VA in the photoperiodic response has yet
104 to be demonstrated. OPN4 is expressed in the pre-mammillary nucleus of turkeys (*Meleagris*
105 *gallopavo*) (Kang et al., 2010), with very low OPN4 expression recently reported in the quail
106 infundibular hypothalamic region (Nakane et al., 2019). However, the lack of strong mediobasal
107 localization suggests that OPN4 is not likely to be the primary photoreceptor for reproduction in
108 birds (Peirson and Foster, 2006). Conversely, histological analyses have demonstrated that
109 OPN5-expressing cells are localized to the periventricular organ (PVO) within the mediobasal
110 hypothalamus. OPN5 cells project towards the pars tuberalis, a region involved in coordinating
111 GnRH-I release (Nakane et al., 2014), yet OPN5 has an absorption spectrum (420 nm);
112 considerably lower than the reported maxima of ~492nm for photoperiodic induction. Functional
113 studies have shown that RNA inhibition of OPN5 alters thyrotrophin-stimulated hormone β -
114 subunit (*TSH β*), a key gene in the photostimulation of reproduction, expression in canaries
115 (*Serinus canaria*) (Stevenson and Ball, 2012), red-headed bunting (*Emberiza bruniceps*)
116 (Majumdar et al., 2014) and Japanese quail (Nakane et al., 2014). But OPN5 has not been linked
117 directly to other upstream components of the reproductive axis.

118 The three main components of the avian reproductive response are: i) deep brain
119 photoreceptor(s), ii) a circadian clock (Follett and Sharp, 1969) and iii) GnRH-I synthesis and
120 secretion (Stevenson et al., 2012). To date, only OPN5 has been functionally implicated in the

121 acute photoinduced regulation of *TSH β* . No study has examined the functional role of VA, nor
122 the long-term role of any photoreceptor. To address this deficit in knowledge, the current study
123 aimed to establish any functional roles that VA and OPN5 opsins may play in the photoperiodic
124 regulation of reproduction in the Japanese quail. Short-hairpin RNA (shRNA) constructs
125 packaged in adeno-associated virus (AAV) were used to test the hypothesis that VA and/or
126 OPN5 are necessary for i) the short-term photoinduction of *TSH β* , *GnRH-I*, *LH β -subunit* and
127 *FSH β -subunit (FSH β)* mRNA expression, and ii) are involved in the development and
128 maintenance of photoinduced reproduction.

129 **Materials and Methods**

130 **Ethical approvals**

131 Animal procedures were approved by the Roslin Institute Animal Ethics and Welfare
132 Review Board at the University of Edinburgh and were performed under Home Office approval
133 (PPL P61FA9171). The experiments were designed in accordance with the Animal Research
134 Reporting of In Vivo Experiments (ARRIVE) guidelines and National Centre for the
135 Replacement, Refinement and Reduction of Animals in Research.

136

137 **Adeno-associated viral vector, shRNA design and cellular** 138 **expression**

139
140 Custom adeno-associated virus serotype 2 vectors containing shRNA templates targeting
141 either OPN5 (*vOPN5*) or VA (*vVA*) were produced by Virovek (Hayward, CA). shRNAi
142 contained a hair-pin loop insert (TCAAGAG); both *vVA* and *vOPN5* targeted exon 4 (S1 Table
143 and Fig. 1A). To identify any potential off-target effects, Blastn search was conducted, no
144 sequences with high homology (>90%) were identified against the shRNAi. The low homology

145 aligned sequences identified by Blastn are not associated with regulation of the photoperiodic
146 response and nor expressed in the mediobasal hypothalamus, giving confidence that RNA
147 interference induced by the shRNAi constructs are highly specific for VA and OPN5.

148 shRNAi were expressed under a constitutively active CMV promoter in an expression
149 cassette, which also contains green fluorescence protein (GFP) to facilitate infected cell
150 identification (Fig.1B). The CMV expression cassette was selected based on pilot tests using
151 primary cell culture to confirm the capability of AAV2 to successfully transfect and be expressed
152 in quail nervous tissue (Fig. 1).

153

154 **Animals**

155 All studies used adult (>12-week-old) male Japanese quail (*Coturnix japonica*) hatched
156 and reared at the National Avian Research Facility, University of Edinburgh, Scotland, UK until
157 at least 3 months of age. Birds were provided food and water *ad libitum*. Following rearing, birds
158 were transferred to short days (6L:18D; Lights on at 07:00) for at least 8 weeks to ensure all
159 birds were in a photosensitive, non-breeding condition (Follett and Pearce-Kelly, 1991).

160

161 **Pilot testing of AAV2 vectors**

162 To determine suitability of the AAV2 vector construct, primary cell culture testing using
163 neural explants from embryonic quail were conducted. The basal portion of a day 10 quail
164 embryo brain (10 days of incubation; quail hatch at E18) was explanted and homogenized to
165 generate a primary cell culture for testing. Explants were transferred to ice cold phosphate
166 buffered saline (PBS) and then cultured in sterile 24 well culture plates with 500 μ l of incubation
167 media overnight at 37°C with 5% CO₂. Incubation media contained Dulbecco's Modified Eagle

168 Medium (DMEM; Fisher Scientific) base with 1% Penstrep and 10% fetal bovine serum (Fisher
169 Scientific). Following overnight incubation 400 μ l of incubation media was removed and
170 replaced with 600 μ l of fresh incubation media containing 4 μ l, 2 μ l, 1 μ l or 0.5 μ l of Virovek's
171 AAV2-CMV vector containing only GFP (Vector Labs, Hayward CA.; 2-2.5E+13 vg/100 μ l).
172 Incubation media was refreshed daily and explants were cultured for five days prior to
173 fluorescent imaging. GFP presence or absence was determined by fluorescence microscopy
174 (Zeiss Axiovert 25 and 100 fluorescent microscopes with AxioCam 503c cameras running Zeiss
175 Zen software; Figure S1C). Both 4 μ l and 2 μ l doses of vector produced strong detectable signal
176 distinctly localized to individual cells, indicating reliable transfection.

177

178 **Stereotaxic intracerebroventricular injection of shRNA-AAV2** 179 **vectors**

180

181 AAV2 vectors were delivered via stereotaxic intracerebroventricular (ICV) injection into
182 the third ventricle (3V) of the medial basal hypothalamus (MBH). Injection coordinates were
183 refined from estimates taken from the adult quail brain atlas (Baylé et al., 1974) using test
184 injections of India ink into fresh quail cadavers. Following test injections, brains were removed,
185 frozen on dry ice and coronally sectioned on a cryostat at 50 μ M. Sections were mounted to glass
186 microscope slides for rapid visualization via light microscopy. This process was repeated
187 iteratively to optimize the anatomical localization of injections directly into the MBH. The final
188 coordinates used were determined with respect to the bursa as X = 0.0 mm, y = 3.8 mm, and z = -
189 6.3 mm then pulled up to -6.0 mm for injection.

190 All experimental ICV injections were performed following standard protocols developed
191 in consultation with the Named Veterinary Surgeons using aseptic technique. Birds were

192 anesthetized with isoflurane (4-5%) administered by facemask with 1.5 L/min of O₂, following
193 induction of anesthesia Isoflurane was reduced to ~2%, monitored and adjusted continually by
194 the anesthetist to maintain desired anesthetic depth. Animals then received subcutaneous
195 injections of meloxicam (0.5 mg/kg) and butorphanol (1.5 mg/kg) as analgesia prior to
196 positioning in the stereotaxic frame (Kopf Instruments). Feathers were plucked and the surgical
197 site cleaned with Hibiscrub. A small ~1 cm incision was made by scalpel to expose the skull.
198 The tip of the injection syringe was aligned to the bursa and zeroed then moved to the injection
199 coordinates and a small mark placed with a pencil prior to clearing the syringe. A dental ball mill
200 drill bit (size #4; WPI) was used to drill a small hole (~ 1-2 mm in diameter) through the skull.
201 The syringe was then realigned and coordinates verified prior to insertion of the needle to target
202 coordinates. A 10 ul Hamilton syringe fitted with a sterilized 28-gauge microfillers (WPI
203 MF28G-5; 97mm, 0.35mm OD, 0.25mm ID) trimmed to half-length were used for injections.
204 Birds were injected with 1 µL of AAV2 at ~2.34E+13 vg/ml containing: blank cassette shRNAi
205 (control, CV), shRNAi vOPN5 or shRNAi vVA. The surgical incision was closed either by
206 veterinary adhesive (VetBond 3M) or sutures. Animals recovered in an isolated box before
207 transfer to a communal recovery pen, equipped with food, water and a heat lamp. The
208 photoperiod was lengthened by one-hour (7L:17D) to facilitate recovery.

209

210 **Study 1: The effect of acute photoinduction and shRNAi on the** 211 **hypothalamic-pituitary-gonadal axis**

212 To establish a non-breeding baseline of target gene expression, as well as determine any
213 direct effects of the AAV2 vector, a subset of birds (n = 5) were injected with the CV vector
214 whilst kept on SD (7L:17D) for 2 weeks and then culled. Birds were closely monitored during

215 this period for signs of any adverse effects. All birds remained healthy and the main experiment
216 commenced.

217 To determine the effects of opsin silencing on the initial photoinduction of the
218 reproductive axis birds were injected with CV (n = 5), *vOPN5* (n = 5) or *vVA* (n = 8) held on
219 short days SD (7L:17D; lights on 07:00) for 2 weeks and then transferred to 16L:8D for 2 days
220 or 7 days (CV n= 6, *vOPN5* n = 9, *vVA* n= 7) prior to collection. Blood samples ($\leq 250 \mu\text{l}$) were
221 collected by venipuncture of the wing vein using a 26-gauge needle and collected from the
222 surface using heparinized microcapillary tubes. Blood was collected from all birds 2 days prior
223 to photostimulation and then again at cull (2 or 7 days). In total across treatments and time points
224 45 quail were used in the experiment (CV n=16, *vOPN5* n=14, *vVA* n=15). Plasma was
225 separated by centrifuging the microcapillary tubes at 10,000 g for 5 minutes, aspirated and then
226 stored at -20°C until assay for testosterone. Birds were euthanized by cervical dislocation
227 followed by rapid decapitation between the hours of 10:00 and 12:00. This early phase of the
228 light-dark cycle was selected due the increased levels of gonadotropins identified previously
229 (Meddle and Follett, 1997, 1995). Brain and pituitary stalk tissues were rapidly dissected and
230 fresh frozen on dry ice. Both testes were dissected and weighed on an analytical balance (Sartoris
231 model A200S) to the nearest 0.1g. All tissues were stored at -80°C .

232

233 **Study 2: The effect of chronic photostimulation and shRNAi on the** 234 **photoperiodic response**

235 To determine the effects of long-term opsin silencing a second study was conduct by
236 transferring birds from SD to LD for 28 days, a period well-established to stimulate maximal
237 gonadal growth (Follett and Pearce-Kelly, 1991). Birds were pseudo-randomly administered with
238 ICV shRNAi containing either i) CV (n= 8), or treatment groups that consisted of ii) *vVA* opsin

239 shRNAi (n= 9), or iii) *vOPN5* shRNAi (n= 9), then kept on a photoperiod of 7L:17D for 2 weeks
240 and then photostimulated (16L:8D). Blood samples ($\leq 250 \mu\text{l}$) were collected as described above
241 2 days pre-photostimulation and then at 7, and 28 days following photostimulation. Blood was
242 centrifuged and plasma aspirated as above and stored at -20°C until assay. Tissues were
243 dissected and stored as described above.

244

245 **Brain sectioning and AAV GFP histological localization**

246 Brains from the second study were coronally sectioned at $30 \mu\text{m}$, mounted onto polysine
247 slides (Fisher 10219280) and cover slipped with VECTASHIELD Antifade Mounting Media
248 with DAPI (Vector Labs, Burlingame, CA USA). Slides were examined under fluorescence
249 microscopy to confirm localization of GFP signal to the MBH (Fig. 1). Birds where injections
250 were found to be off target, or there was no sign of injection were excluded from subsequent
251 analyses (see below).

252

253 **Western Blot assays of hypothalamic extracts for VA and OPN5**

254 Polyclonal antibodies against VA and OPN5 were custom made by Cambridge Research
255 Biochemicals, Inc. (see Supplemental Table 3 for target sequences) in rabbit hosts with sub-
256 fractions of collected sera purified via affinity chromatography. Antibody sequence target were
257 selected based on previously published specificity for VA (Halford et al., 2009) and OPN5
258 (Nakane et al., 2010). BLASTp analyses confirmed that the OPN5 sequence has 100% sequence
259 homology with the predicted OPN5 target and non-specific hits were $<80\%$ homology with

260 <80% coverage. For VA there was 100% identity and 100% coverage of the antibody sequence
261 for the Japanese quail sequence, confirming the high homology. Furthermore, there was low non-
262 specificity for off-target sequences with <80% identity and <80% coverage.

263 To confirm reduced VA and OPN5 protein expression, western blots were conducted on
264 hypothalamic protein extracts. The hypothalami were dissected (as described above) and
265 homogenized in 700 μ l of 100 mM Tris-HCL buffer with 4% w/v SDS and protease inhibitors
266 (Halt™ Protease Inhibitor Cocktail, EDTA-free, Thermo Fisher Scientific). Samples were then
267 centrifuged at 20,000 x g for 20 minutes at 4°C. Supernatant was collected and stored at -80°C.
268 Total protein concentration for all samples was determined using 1 μ l of supernatant using a
269 BCA Protein Assay (Pierce™ BCA Protein Assay Kit) per manufacturer's instructions.
270 Supernatant volume for use in western blots was then standardized to add 10 μ g of protein to
271 each well by diluting with water. 20 μ l of diluted sample was combined with 10 μ l of LDS
272 buffer (NuPAGE™ LDS Sample Buffer, Thermo Fisher Scientific) then incubated at 98°C for 2
273 minutes prior to loading. Samples were loaded onto 4-12% Bis-Tris pre-cast gels (NuPAGE™,
274 Thermo Fisher Scientific), 10 μ l per sample and were loaded onto two separate gels that were
275 run in parallel in the same gel tank. Treatment groups were spread across gels. Gels were run at
276 90V or 5 minutes to ensure even entry of samples into the gel, then at 175V for 1 hour. One
277 duplicate gel was then immediately incubated in 40 ml of OptiBlue protein stain for 1 hour, to
278 quantify total protein loading, on an orbital shaker. The second gel was then processed for
279 western blot transfer. Protein was transferred to a PDVF membrane (iBlot™ Transfer Stacks,
280 PVDF, regular size, Thermo Fisher Scientific) using the iBlot 2 system. Membranes were then
281 washed in 1X PBS 5 times for 5 minutes before being blocked for 30 minutes in Odyssey
282 blocking buffer in 50 ml falcon tubes. Blocking buffer was discarded and replaced with 5 ml of

283 primary antibody solution (5 ml Odyssey buffer, 1 AB, 0.1% Tween 20). Following primary
284 antibody incubation, samples were rinsed in PBS 6 times for 5 minutes. Incubation with
285 secondary antibody was performed using IRDye 680RD Goat Anti-Rabbit antibody (LI-COR) at
286 1:10,000 in 5 ml Odyssey Blocking Buffer with 0.1% Tween 20 before being incubated in
287 secondary antibody for 90 minutes at room temperature. Membranes were then rinsed with PBS
288 6 times for 5 minutes prior to imaging. Membranes were imaged on a LI-COR Odyssey imager
289 using Image Studio software (Image Studio™ LI-COR). Western blots were imaged at 3.5
290 intensity, medium image quality at 169 µm resolution. Total protein gels were rinsed in distilled
291 water and then imaged on the 700 nm channel at lowest image quality, Intensity 3, 169 µm
292 resolution.

293

294 **Enzyme-linked immunosorbent assay (ELISA) for testosterone**

295 To measure plasma testosterone levels, the Parameter™ Testosterone Assay (R&D
296 Systems, Bio-Techne) was used according to manufacturer's instructions. Samples were assayed
297 in duplicate using 50 µl plasma and absorbance was measured at 450 nm and 570 nm using a
298 microplate reader (LT-4500, Labtech). For each well, the value from 570 nm was subtracted
299 from the value for 450 nm to obtain a normalized fluorescent measurement. The sensitivity of the
300 assay ranged from 0.012-0.041 µg/ml. Cross-reactivity of the assay was <0.1% for androsterone,
301 estradiol, prednisolone and progesterone. Two assays were conducted, the intra-assay coefficient
302 of variation was 7.7% and 9.4%. The inter-assay coefficient of variation was 13%.

303

304 **RNA isolation and cDNA synthesis**

305 Hypothalami were dissected from the first study using well characterized
306 neuroanatomical landmarks (Baylé et al., 1974; Pérez et al., 2020). Hypothalami were then
307 homogenized in 700 µl of 100 mM Tris-HCL buffer with 4% w/v SDS and protease inhibitors
308 (Halt™ Protease Inhibitor Cocktail, EDTA-free, Thermo Fisher Scientific). Homogenized
309 samples were then centrifuged to pellet debris and the supernatant was then transferred to clean
310 microcentrifuge tubes for protein analysis (S1 File). Hypothalamic pellets and pituitary glands
311 were homogenized using 1 ml of TRIzol (Thermo Fisher Scientific) using a Kinematica Polytron
312 PT1200E handheld homogenizer (Thermo Fisher scientific). Following a 5minute room
313 temperature incubation, 200 µl of chloroform was added to each sample and vortexed to mix.
314 Samples were then incubated for 3 minutes at room temperature, then centrifuged at 12,000 g for
315 15 minutes at 4°C. The upper aqueous phase was pipetted into a fresh microcentrifuge tube. 500
316 µl of isopropanol was added and incubated for 10 minutes. Tubes were centrifuged again at
317 12,000 g for 15 minutes at 4°C. Supernatant was discarded and the pellet re-suspended in 1 ml of
318 75% ethanol. Samples were centrifuged at 7,500 g for 5 minutes at 4°C and supernatant
319 discarded. The remaining pellet containing the RNA was re-suspended in 30 µl of RNase-free
320 water. Nucleic acid quality (260/280) and concentration was determined by Nanodrop (Thermo
321 Fisher Scientific). 2 µg of RNA was reverse transcribed using a Precision nanoScript2 Reverse
322 Transcription kit (Primerdesign Ltd) following the manufacturer's instructions and cDNA was
323 stored at -20°C until quantification.

324

325 **Real-time quantitative PCR (qPCR)**

326 qPCRs were performed on a Stratagene Mx3000 Real Time PCR machine in 20 µl
327 reactions. For each well, the qPCR mix consisted of 5 µl cDNA template, 10 µl SYBR green

328 (Primerdesign Ltd), 0.5 μ l (300nM) forward primer, 0.5 μ l (300nM) reverse primer and 4 μ l
329 RNase-free H₂O. Samples were run in duplicate in a 96-well plate format under the following
330 conditions: i) denaturing at 95°C for 5 min., then 39 cycles of ii) 95°C for 10 s, iii) 30 s at
331 annealing temperature dependent on primer (See Table 2), and finally iv) an extension step of
332 72°C for 30 s. Melt curves were analyzed to ensure the specificity of each reaction. PCR
333 Miner (Zhao and Fernald, 2005) was used to determine reaction efficiencies and
334 quantification cycle (Ct). Fold expression was measured in relation to
335 the average Ct for two reference genes (*GAPDH* and *β ACTIN*) and calculated using 2-
336 ($\Delta\Delta$ Ct). The average Ct obtained from short day CV treatment group was used as the second
337 delta value in order to identify photoinduced changes in transcript levels.

338

339 **Exclusion criteria**

340 Some quail were excluded from statistical analyses based on both a priori exclusion
341 criteria and based on outlier testing during statistical analysis. For the first study, one short day
342 control bird, two CV and 3 *vOPN5* shRNAi treated birds were removed because testicular mass
343 values were indicative of breaking the non-breeding state (i.e. 1.4 - 3.2g) while still on short
344 days. Opsin silencing was confirmed by both Western Blot and qPCR to confirm high efficiency
345 of AAV2 vector delivery to MBH (S1G and S1H Fig). Two *vOPN5* and two *vVA* treated birds
346 were excluded from analyses due to mRNA expression values being similar to control treated
347 birds, as a consequence of off-target viral injections. One *vOPN5* bird was excluded from the
348 western blot validations because of a technical error in gel loading. In the second study birds
349 were screened for inclusion by presence of GFP signal in the MBH, taking advantage of the

350 AAV2 vector's GFP expression (independent of shRNA. One CV and one *vOPN5* were excluded
351 based on lack of GFP expression in the MBH. Raw data and R code are available in S2 File.

352

353 **Statistical analyses**

354 All statistical analyses were conducted in R v 4.0.2 (R Core Development Team, 2020)
355 using the following packages: car (Fox and Weisberg, 2019), emmeans (Lenth et al., 2018), grid
356 (Murrell, 2005), ggthemes (Arnold, 2017), ggpubr (Kassambara, 2019), and tidyverse (Wickham
357 et al., 2019). Paired testes mass was analyzed by linear model. Model assumptions were checked
358 by graphic visualization of residual outputs using the plot() function to confirm model fit.
359 Initially, RNA expression data failed to fit linear model assumptions even when log transformed,
360 therefore, these data were subsequently analyzed by generalized linear model using a gamma
361 distribution with a log link. Model fit was checked via visualization of model residuals; use of
362 gamma models improved model fit based on AIC. Main effects within the model were assessed
363 using a Likelihood Ratio Test.

364

365 **Results**

366 **Long day stimulation of testicular growth**

367 In order to determine the long-term functional role of VA and OPN5, SD photosensitive
368 birds received a single intracerebroventricular (ICV) injection of shRNAi constructs targeting
369 VA (*vVA*), OPN5 (*vOPN5*), or empty cassette (CV) and then after a two-week recovery, were
370 exposed to stimulatory photoperiods for either 2, 7 or 28 days. Testes mass was significantly
371 heavier 7 days post photostimulation in all birds (Fig 2A; $F_{1,37}=14.69$, $p<0.001$, partial $\eta^2 =$

372 0.562). There was no detectable interaction effect for days post photostimulation and ICV
373 injection ($F_{2,37}=2.66$, $p=0.083$, partial $\eta^2 = 0.126$). However, based on the loss of statistical power
374 (<0.8) due to imbalance in sample sizes across treatments, and following visual inspection of
375 plotted data, opted to investigate photoinduced increases in testes mass at 2, 7, and 28 days post-
376 photostimulation separately as well.

377

378 We found that 2 days following photostimulation there was no effect of shRNAi
379 silencing on testes mass ($F_{2,13}=0.35$, $p=0.713$, partial $\eta^2 = 0.05$). After 7 days of
380 photostimulation, shRNAi silencing resulted in a significant increase in testes mass ($F_{2,19}=4.31$,
381 $p=0.029$, partial $\eta^2 = 0.312$), with *vVA* injected birds having heavier testes compared to control
382 (CV) birds ($t=-2.78$, $p=0.031$, Cohen's $d = 1.50$) suggesting that *vVA* treatment facilitated the
383 photoperiod induced growth of testes. There was no difference detected at 7 days between
384 *vOPN5* and CV birds ($t=-0.80$, $p=0.706$, Cohen's $d = 0.49$) not *vVA* and *vOPN5* birds ($t=-2.23$, p
385 $= 0.092$, Cohen's $d = 1.03$). There was no significant difference in testes mass between birds that
386 received shRNAi against OPN5 or VA for 28 days compared to CV (Fig 2B; $F_{2,23}=0.28$,
387 $p=0.761$, partial $\eta^2 = 0.024$). These data indicate that shRNAi of VA facilitated the long day
388 photoinduced transition (i.e. day 7) to a fully photostimulated reproductive state, accelerating
389 gonadal growth, which was complete in all birds by 28 days regardless of treatment.

390

391 **Long days increased plasma testosterone concentrations**

392 As testosterone is the predominant hormone produced by the testes, we sought to identify
393 whether circulating concentrations would parallel long day induced increases in testes mass.
394 There was no significant effect of treatment on plasma testosterone following 2 days of LD (Fig

395 2C; LR $\chi^2=0.81$, $p=0.667$, partial $\eta^2 = -0.933$) nor an interaction of shRNAi injection and days
396 post-photostimulation (LR $\chi^2=0.64$, $p=0.726$, partial $\eta^2 = 0.438$). Testosterone significantly
397 increased from 2 to 7 days post-photoinduction (LR $\chi^2=104.70$, $p<0.001$, partial $\eta^2 = 0.938$).
398 Chronic silencing had no effect on plasma testosterone after 28 days (LR $\chi^2=0.52$, $p=0.77$,
399 partial $\eta^2 = 0.941$). The lack of an effect of shRNAi on plasma testosterone concentrations at 28
400 days suggests that VA and OPN5 do not regulate short-term, daily rhythms in reproductive
401 physiology and instead, confirms their role in the long-term photoperiodic response.

402

403 **Long days and shRNAi increased hypothalamic GnRH-I expression**

404 Next, we sought to determine the neuroendocrine mechanisms underlying the shRNAi
405 induced increase in testicular mass. Using quantitative PCR (qPCR) we examined hypothalamic
406 *GnRH-I* and *GnIH* expression. There was a significant interaction for hypothalamic *GnRH-I*
407 expression between injection and days post photoinduction (Fig 3A; LR $\chi^2=16.70$, $p<0.001$,
408 partial $\eta^2 = 0.996$) as well as a significant main effect of ICV injection (LR $\chi^2=10.59$, $p=0.005$,
409 partial $\eta^2 = 0.929$). Post hoc testing using Student's T distribution via the `summary.glm()`
410 function indicated a significant increase in hypothalamic *GnRH-I* expression in *vVA* birds at 7
411 days when compared to CV ($t_{18}=2.25$, $p=0.037$, Cohen's $d = 1.121$), no other differences were
412 detected. Conversely, *GnIH* expression did not change following either 2 or 7 LD (Fig 3B; LR
413 $\chi^2=2.81$, $p<0.094$, partial $\eta^2 = 0.261$). There was also no effect of ICV injection (LR $\chi^2=0.29$,
414 $p=0.867$, partial $\eta^2 = 0.232$) nor an interaction between injection and days post photoinduction
415 (Fig 3D; LR $\chi^2=0.99$, $p=0.610$, partial $\eta^2 = 0.853$). Taken together, these data indicate that VA
416 either directly (Halford et al., 2009) or indirectly is involved in regulating the photoperiod
417 induced increase in testicular mass via the master neuropeptide for reproduction, GnRH-I.

418

419 **Long days regulate anterior pituitary secretagogue expression**

420 Next, we investigated whether gonadotroph release from the pituitary gland was
421 regulated by shRNAi of VA and OPN5. Using qPCR, we assessed the photoperiod induced
422 change in *LHβ*, *FSHβ*, and *TSHβ* expression in birds after 2, 7 and 28 days. There were no
423 significant effects of ICV injection (LR $\chi^2 = 0.43$, $p = 0.807$, partial $\eta^2 < 0.001$), following LD
424 transfer, nor any interaction between injection and days of photostimulation (LR $\chi^2 = 1.55$, $p =$
425 0.460 , partial $\eta^2 = 0.447$). As the findings indicated increased variation in *LHβ* expression at 2
426 LD, the effect of shRNAi on 2- and 7 LD were examined separately. There was a significant
427 effect of ICV injection at 2 days (Fig 3C; LR $\chi^2=7.26$, $p=0.027$, partial $\eta^2 = 0.277$) with *vVA*
428 injected birds having higher *LHβ* mRNA expression compared to CV birds ($t=2.64$, $p=0.022$,
429 Cohen's $d = 0.283$) indicating that *vVA* increased *LHβ* expression. There was no difference
430 between CV and *vOPN5* birds ($t=0.72$, $p=0.487$, Cohen's $d = 0.100$). Chronic silencing had no
431 effect on *LHβ* expression (Fig 3D LR $\chi^2=2.56$, $p=0.279$, partial $\eta^2 = -4.11$). *vVA* and *vOPN5*
432 groups did not show a significant reduction in *FSHβ* expression at 2 days LD (Fig 3E; LR
433 $\chi^2=5.45$, $p=0.066$, partial $\eta^2 = 0.999$). No effect of injection on *FSHβ* expression was detected
434 following 7 days (LR $\chi^2 = 1.47$, $p = 0.481$, partial $\eta^2 = 0.951$) nor 28 days (Fig 3F; LR $\chi^2=2.16$,
435 $p=0.339$, partial $\eta^2 = 0.785$) of photostimulation.

436

437 There was no effect of ICV injection nor a significant interaction when *TSHβ* expression
438 was modelled over the entirety of the SD, 2 LD and 7 LD period. Due to the significant variation
439 at 2 days, treatment days were analyzed separately. Based on Cook's distance analysis of the
440 initial *TSHβ* glm model (at 2 days) residuals a single data point was identified as an outlier

441 (2.503 for bird 1703 CV at 2 days) and removed. Re-running of the analysis indicated that both
442 *vVA* and *vOPN5* significantly increased *TSHβ* expression at 2 days (Fig 3G; LR $\chi^2=16.81$,
443 $p<0.001$, partial $\eta^2 = 0.614$). Post hoc testing indicated a very weak effect of *vOPN5* ($t_{12}=2.19$,
444 $p=0.050$, Cohen's $d = 0.02$) and a moderate effect of *vVA* ($t_{12}=4.35$, $p<0.001$, Cohen's $d = 0.571$)
445 silencing, increasing *TSHβ* expression compared to controls. No effect of injection was detected
446 at either 7 LD (LR $\chi^2=1.08$, $p=0.584$, partial $\eta^2 = 0.619$) nor following 28 LD (Fig 3H; LR
447 $\chi^2=1.51$, $p=0.469$, partial $\eta^2 = 0.455$).

448

449 Discussion

450 We found that selective inhibition of VA opsin mRNA expression facilitated the
451 photoinduced increase of *TSHβ* and *LHβ* mRNA expression in quail. By 7 days, *GnRH-I* mRNA
452 levels and testes mass increased in *vVA* treated birds compared to controls. Birds treated with
453 shRNAi against OPN5 were only observed to show increased *TSHβ* expression during early
454 photostimulation (2 days). Silencing treatment inhibited both VA and OPN5 opsin expression
455 and reduced associated protein levels with the exception of a transient increase in VA opsin at 2
456 days of photostimulation. Overall, these represent the first causal evidence that VA opsin plays a
457 functional role in the light-dependent neuroendocrine control of seasonal reproduction in birds.
458 However, contrary to our a priori hypotheses, silencing of deep brain photoreceptors enhances
459 the response of the reproductive axis to photostimulation rather than preventing it. This
460 challenges our previous understanding of the functional role of deep brain photoreceptors in the
461 activation of the reproductive axis (Fig 4).

462

463 This study provides the first long-term functional investigation of the hypothalamic
464 photoreceptors underpinning seasonal reproduction in birds. Previous studies have focused on
465 the well-characterized first LD release model to examine the impact of short-term inhibition of
466 OPN5 on photoinduced changes in *TSH β* expression (Nakane et al., 2014; Stevenson and Ball,
467 2012), but have not explored long-term changes in neuroendocrine gene expression. In this
468 study, long-term inhibition of VA and OPN5 was achieved using adeno-associated viral
469 constructs (Nectow and Nestler, 2020). Using shRNAi, we are able to overcome the limitations
470 and potential detrimental impacts associated with use of systemic genetic knockouts (also not
471 currently available in quail) or lesions. By targeting both the early (i.e. 2-7 days) and late stages
472 (i.e. 28 days) of photostimulation our study aimed to monitor photoperiod induced changes at
473 genetic, physiological and morphological levels across time. Our findings expand our
474 understanding of the mechanisms regulating the avian photoperiodic response, identifying the
475 long-term impacts of VA and OPN5 inhibition on the neuroendocrine circuit that governs
476 testicular growth and function. However, as the complex differential response of the various
477 elements of the neuroendocrine cascade to silencing observed here highlights, transduction of
478 photic cues is a multi-modal process relying on multiple receptor types and likely multiple
479 receptor populations (Stevenson et al., 2022). These features make it challenging to isolate the
480 full role of even a single photoreceptor type in detail.

481

482 **VA as a multi-modal photoreceptor for the avian photoperiodic** 483 **response**

484

485 Our findings identified that *vVA* significantly increased both TSH β and LH β at 2 LD.
486 Subsequently, GnRH-I and paired testes mass were increased at 7 LD. Interestingly, VA opsin is

487 closely related to the visual opsins phylogenetically (Beaudry et al., 2017; Soni et al., 1998);
488 both are within the subfamily of photoreceptors that activate intracellular G α proteins by
489 catalyzing the exchange of GDP to GTP (Shichida and Matsuyama, 2009). Activation of
490 photoreceptors that couple the Gt subtype (i.e. rods, VA) generally decrease intracellular cGMP.
491 The second-messenger intracellular enzyme cyclic guanosine monophosphate (cGMP) has been
492 shown to bind to a 1.5kb enhancer motif upstream of the *GnRH-I* promoter repressing
493 transcription (Belsham et al., 1996). One potential link between light detection by VA opsin and
494 *GnRH-I* expression is via the direct action of cGMP and associated intracellular signaling
495 pathways. Immunohistochemical studies have demonstrated co-localization of VA and GnRH-I
496 in GnRH-I expressing neurons, in the anterior regions of the hypothalamus (García-Fernández et
497 al., 2015; Halford et al., 2009). Thus, we would expect tonic repression of *GnRH-I* expression in
498 these neurons that is released upon activation of VA opsin, based on the general mechanism of
499 action for opsin proteins. How then is it that silencing of the releaser, VA opsin, results in
500 upregulation of *TSH β* and *LH β mRNA* expression after 2 LD and particularly increased *GnRH-I*
501 expression and paired testes mass at 7 LD? The answer likely lies in the pathways that link VA
502 opsin to cGMP and *GnRH-I* transcription, and also the broad distribution of VA opsin within the
503 hypothalamus, where it has been identified in multiple nuclei (García-Fernández et al., 2015;
504 Halford et al., 2009). Co-expression of GnRH-I and VA opsin is limited primarily to anterior
505 nuclei: nucleus magnocellularis preopticus, nucleus anterior medialis hypothalamic, and the
506 nucleus supraopticus. Previous neuroanatomical analyses in European starling established that
507 the rostral preoptic area as the primary location of photoperiodic and gonadal hormone feedback
508 regulation on GnRH-I expression (Stevenson et al., 2009). However, there is also substantial VA
509 expression in the nucleus paraventricularis magnocellularis (both pars ventralis and medialis;

510 PVN) and the medial bed nucleus of stria terminalis with VA-ir positive fibers projecting into the
511 median eminence with an apparent interface with the PT (Halford et al., 2009). These two VA
512 opsin populations have the potential to independently regulate GnRH neuronal function and
513 GnRH-I release. Our ICV injections were targeted to the 3V in the medial basal hypothalamus to
514 optimize targeting of the PVN and ME, which contain both VA and OPN5 expression. Given the
515 targeting of our injections, the spread of GFP expression and the fact we still detected some level
516 of VA protein expression (Fig. 1) in our hypothalamic extracts, we posit that we were far more
517 effective at silencing MBH expression than anterior hypothalamic expression of VA opsin.
518 Based on this supposition and the anatomical localization of GnRH-I and VA opsin describe
519 above we suggest that VA acts in a modular manner to regulate different aspects of GnRH-
520 dependent control of reproductive physiology.

521 VA opsin neurons in the MBH project fibers through the ME to the PT allowing for direct
522 interaction with PT based thyrotrophs. Silencing of VA expression in these cells results in the
523 premature activation of these thyrotrophs resulting in the observed increase in *TSH β* expression
524 at 2D. Work in sheep has suggested anterograde signaling from the PT directly to the pars
525 distalis of the anterior pituitary with respect to seasonal regulation of prolactin (Lincoln, 2002).
526 Anterograde signaling from the PT to pars distalis gonadotrophs provides a plausible mechanism
527 for the observed 2D elevation of *LH β* in the vVA group. This model suggests some sort of
528 inhibition of PT thyrotrophs by this population of VA neurons that is released by removing the
529 opsin, likely requiring interaction with endogenous timing mechanisms. This population of VA
530 neurons is anatomically positioned to provide indirect regulation of the reproductive axis via the
531 canonical PT *TSH β* to *DIO2* pathway, potentially regulating GnRH release via modulation of
532 tanycyte endfeet interaction with the GnRH-I nerve terminal (Yamamura et al., 2004). At present

533 there is no clear mechanism by which this would occur in VA expressing neurons, but the
534 proposed model provides a framework to further investigate the molecular and microcircuit
535 mechanisms involved.

536 Conversely, the second major population of VA expressing neurons in the anterior
537 portion of the hypothalamus appears to be the GnRH-I neurons themselves, opening the potential
538 for direct regulation of GnRH-I by VA opsin. Based of our GFP localization data we suggest that
539 this population of VA opsin experienced reduced silencing and potentially remained functionally
540 intact. Thus, photostimulation of VA opsin expression in these neurons is expected to have the
541 effect of increasing GnRH-I expression by reducing cGMP as described above. Taken together
542 with the above findings a timeline of VA activity can be formulated, suggesting that the
543 photoinducible phase involves a VA -dependent increase in *TSH β* and the subsequent stimulation
544 of *LH β* within 2 days of LD transfer mediated by the MBH populations of VA opsin expressing
545 neurons. Simultaneously, VA opsin expressed in GnRH-I neurons modulates GnRH-I expression
546 to support sustained release of GnRH-I triggered by the TSH β induced conformational changes
547 in ventricular tanycytes. The enhancement of reproductive physiology induced by VA silencing,
548 supported by prolonged suppression of VA mRNA, suggests that MBH VA expressing cells may
549 be acting as a brake upon an otherwise active reproductive system, preventing its activation.
550 Under this model LD exposure results in the removal of inhibitory tone on pars tuberalis
551 thyrotrophs beginning the neuroendocrine cascade events leading to reproduction. However,
552 despite the consistent and reliable reduction in VA mRNA for several weeks we observed a
553 transient increase in VA opsin protein on day 2 of photostimulation (Fig. 1F). Increased VA
554 opsin protein may have resulted in a larger pool of receptors in the MBH to detect light at day 2.
555 Therefore our results may be alternatively explained by stimulatory activation of VA opsin cells

556 in the MBH early in the photoperiodic response that facilitated gonadal growth. However, under
557 this explanation we might expect a subsequent decrease in gonadal growth later in
558 photostimulation when VA opsin protein levels were reduced, which we do not see. Overall, the
559 data reported herein provides the first functional evidence that VA opsin is integral to the
560 photoperiodic response in birds. The precise mechanisms linking VA opsin to gonadotrophin
561 release remain to be resolved, though it is clear that VA opsin influences GnRH-I mediated
562 regulation of reproductive physiology on the scale of days to weeks.

563

564

565

566

567 **OPN5 as a mediator of the initial photoperiodic response**

568 The findings presented here also support a functional role for OPN5 in the initial light-
569 dependent activation of seasonal reproduction. The current experimental design sought to
570 identify the effects of OPN5 inhibition on pituitary secretagogue (i.e. *LHβ*, *FSHβ*) expression,
571 identified as occurring early on the second LD in quail (Meddle and Follett, 1997). Early
572 morning tissue collection limits comparisons with previous studies, which utilized later evening
573 collections (Nakane et al., 2014; Stevenson and Ball, 2012). However, *TSHβ* expression was
574 increased after 2 LD in *vOPN5* treated birds. These data are similar to reports from Border
575 canaries in which shRNAi against OPN5 resulted in higher *TSHβ* expression 14 hours after light
576 onset on the first LD (Stevenson and Ball, 2012). Interestingly, in the present study we do not
577 find a clear long-term effect of *vOPN5* treatment on the HPG axis, suggesting that light
578 activation of this photoreceptor has only a short-term effect on the photoperiodic response

579 limited to the first days following LD stimulation. Collectively, these data suggest that activation
580 of OPN5 expressing cells is likely critical for early light detection and initiation of short-term
581 effects on the avian photoperiodic response, but that longer term effects are dependent on input
582 from other photoreceptors such as VA opsin.

583

584 **Hierarchical organization of light detection by avian deep brain** 585 **photoreceptors**

586 In the quail hypothalamus, VA mRNA and protein expression is 2x and 20x higher than OPN5
587 respectively. In addition VA cells are identified in several nuclei and have immunoreactive fibers
588 extend into multiple hypothalamic regions, in addition to the median eminence (Halford et al.,
589 2009). The observed robust and long-term effects of *vVA* on multiple neuroendocrine substrates
590 presented here suggest a predominant role for VA in the avian photoperiodic response, though
591 whether via stimulatory pathways of removal of inhibitory tone remains to be confirmed.
592 Furthermore, the VA action spectrum has the closest match to the action spectrum for the quail
593 photoperiodic response and is the only photoreceptor to meet all the criteria set forth for the
594 avian photoperiod response (García-Fernández et al., 2015). Therefore, we propose that light
595 detection by VA expressing cells in the MBH is the primary mechanism for acute (1st week LD)
596 photoinduction of reproductive physiology. However, it is likely that other photoreceptors, such
597 as anterior hypothalamic VA opsin, OPN5 or perhaps even OPN4 provide additional input to
598 facilitate the activation of the HPG axis.

599

600 A synthesis of the present and past studies highlights the complexity of opsin regulation
601 of seasonal reproduction (Pérez et al., 2019). Combining our data with this past synthesis we
602 propose a model of seasonal photoreception in which OPN5 expressing ependymal cells and

603 MBH VA cells regulate the initial response to photostimulation during the first long day by
604 acting on *TSH β* expressing cells in the pars tuberalis to increase *TSH β* expression (Nakane et al.,
605 2014; Stevenson and Ball, 2012). Simultaneously, VA expressing neurons in the preoptic
606 area/mediobasal hypothalamus respond to light stimulation over several days to weeks,
607 stimulating an increase in GnRH-I expression necessary to support long term activation of the
608 reproductive axis (Fig 4). Under this paradigm, VA serves as a long-term regulator, maintaining
609 the response to light and leading ultimately to reproductive competence. Whether these pathways
610 act completely independently or interact either directly, or indirectly, remains unclear at present.
611 Further work is needed to test the model proposed above and determine whether the involvement
612 of multiple opsins represents evolutionary redundancy or is a mechanism for enabling increased
613 flexibility and control of reproductive timing.

614

615

616 **Acknowledgements**

617 The authors would like to acknowledge Sir Brian Follet for his input and advice on the initial
618 development of this project and associated funding proposals.

619

620 **References**

621 Arnold, J.B., 2017. ggthemes: Extra themes, scales and geoms for “ggplot2.” R package version
622 3.4. 0.

623 Baylé, J., Ramade, F., Oliver, J., 1974. Stereotaxic topography of the brain of the quail (*Coturnix*
624 *coturnix japonica*). *J. Physiol.* 68, 219–241.

625 Beaudry, F.E.G., Iwanicki, T.W., Mariluz, B.R.Z., Darnet, S., Brinkmann, H., Schneider, P.,
626 Taylor, J.S., 2017. The non-visual opsins: eighteen in the ancestor of vertebrates,
627 astonishing increase in ray-finned fish, and loss in amniotes. *J. Exp. Zool. Part B Mol. Dev.*
628 *Evol.* 328, 685–696. <https://doi.org/10.1002/jez.b.22773>

629 Belsham, D.D., Wetsel, W.C., Mellon, P.L., 1996. NMDA and nitric oxide act through the
630 cGMP signal transduction pathway to repress hypothalamic gonadotropin-releasing
631 hormone gene expression. *EMBO J.* 15, 538–547.

632 Benoit, J., Ott, L., 1944. *External and Internal Factors in Sexual Activity* 17.

633 Chaurasia, S.S., Rollag, M.D., Jiang, G., Hayes, W.P., Haque, R., Natesan, A., Zatz, M., Tosini,
634 G., Liu, C., Korf, H.W., Iuvone, P.M., Provencio, I., 2005. Molecular cloning, localization
635 and circadian expression of chicken melanopsin (Opn4): Differential regulation of
636 expression in pineal and retinal cell types. *J. Neurochem.* 92, 158–170.
637 <https://doi.org/10.1111/j.1471-4159.2004.02874.x>

638 Davies, W.I.L., Turton, M., Peirson, S.N., Follett, B.K., Halford, S., Garcia-Fernandez, J.M.,
639 Sharp, P.J., Hankins, M.W., Foster, R.G., 2012. Vertebrate ancient opsin photopigment
640 spectra and the avian photoperiodic response. *Biol. Lett.* 8, 291–294.
641 <https://doi.org/10.1098/rsbl.2011.0864>

642 Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in
643 birds. *J. Biol. Rhythms* 16, 365–380. <https://doi.org/10.1177/074873001129002079>

644 Follett, B.K., Pearce-Kelly, A.S., 1991. Photoperiodic induction in quail as a function of the
645 period of the light-dark cycle: implications for models of time measurement. *J. Biol.*
646 *Rhythms* 6, 331–341.

647 Follett, B.K., Sharp, P.J., 1969. Circadian rhythmicity in photoperiodically induced

648 gonadotrophin release and gonadal growth in the quail. *Nature* 223, 968–971.

649 Foster, R.G., Follett, B.K., 1985. The involvement of a rhodopsin-like photopigment in the
650 photoperiodic response of the Japanese quail. *J. Comp. Physiol. A* 157, 519–528.
651 <https://doi.org/10.1007/BF00615153>

652 Foster, R.G., Follett, B.K., Lythgoe, J.N., 1985. Rhodopsin-like sensitivity of extra-retinal
653 photoreceptors mediating the photoperiodic response in quail. *Nature* 313, 50–52.
654 <https://doi.org/10.1038/313050a0>

655 Foster, R.G., Hughes, S., Peirson, S.N., 2020. Circadian Photoentrainment in Mice and Humans.
656 *Biology (Basel)*. 9, 180.

657 Fox, J., Weisberg, S., 2019. *An R Companion to Applied Regression*, Third Edit. ed. Sage.

658 García-Fernández, J.M., Cernuda-Cernuda, R., Davies, W.I.L., Rodgers, J., Turton, M., Peirson,
659 S.N., Follett, B.K., Halford, S., Hughes, S., Hankins, M.W., Foster, R.G., 2015. The
660 hypothalamic photoreceptors regulating seasonal reproduction in birds: A prime role for VA
661 opsin. *Front. Neuroendocrinol.* 37, 13–28. <https://doi.org/10.1016/j.yfrne.2014.11.001>

662 Halford, S., Pires, S.S., Turton, M., Zheng, L., González-Menéndez, I., Davies, W.L., Peirson,
663 S.N., García-Fernández, J.M., Hankins, M.W., Foster, R.G., 2009. VA Opsin-Based
664 Photoreceptors in the Hypothalamus of Birds. *Curr. Biol.* 19, 1396–1402.
665 <https://doi.org/10.1016/j.cub.2009.06.066>

666 Hankins, M.W., Peirson, S.N., Foster, R.G., 2008. Melanopsin: an exciting photopigment.
667 *Trends Neurosci.* 31, 27–36.

668 Kang, S.W., Leclerc, B., Kosonsiriluk, S., Mauro, L.J., Iwasawa, A., El Halawani, M.E., 2010.
669 Melanopsin expression in dopamine-melatonin neurons of the premammillary nucleus of
670 the hypothalamus and seasonal reproduction in birds. *Neuroscience* 170, 200–213.

671 <https://doi.org/10.1016/j.neuroscience.2010.06.082>

672 Kassambara, A., 2019. ggpubr:“ggplot2” based publication ready plots. R package version 3.4.0.

673 Lenth, R., Singmann, H., Love, J., Buerkner, P., Herve, M., 2018. Emmeans: Estimated marginal
674 means, aka least-squares means. R Packag. version 1, 3.

675 Lincoln, G.A., 2002. Neuroendocrine regulation of seasonal gonadotrophin and prolactin
676 rhythms: lessons from the Soay ram model. *Reprod. Suppl.* 59, 131–147.

677 MacDougall-Shackleton, S.A., Stevenson, T.J., Watts, H.E., Pereyra, M.E., Hahn, T.P., 2009.
678 The evolution of photoperiod response systems and seasonal GnRH plasticity in birds.
679 *Integr. Comp. Biol.* 49, 580–589.

680 Majumdar, G., Yadav, G., Rani, S., Kumar, V., 2014. A photoperiodic molecular response in
681 migratory redheaded bunting exposed to a single long day. *Gen. Comp. Endocrinol.* 204,
682 104–113. <https://doi.org/10.1016/j.ygcen.2014.04.013>

683 Meddle, S.L., Follett, B.K., 1997. Photoperiodically Driven Changes in Fos Expression within
684 the Basal Tuberal Hypothalamus and Median Eminence of Japanese Quail.

685 Meddle, S.L., Follett, B.K., 1995. Photoperiodic activation of Fos-like immunoreactive protein in
686 neurones within the tuberal hypothalamus of Japanese quail. *J. Comp. Physiol. A* 176, 79–
687 89. <https://doi.org/10.1007/BF00197754>

688 Menaker, M., Keatts, H., 1968. Extraretinal Light Perception In The Sparrow, II. Photoperiodic
689 Stimulation of Testis Growth*. *Proc. Natl. Acad. Sci.* 60, 146–151.
690 <https://doi.org/10.1073/pnas.60.1.146>

691 Menaker, M., Roberts, R., Elliott, J., Underwood, H., 1970. Extraretinal Light Perception in the
692 Sparrow, III: The Eyes Do Not Participate in Photoperiodic Photoreception*.

693 Murrell, P., 2005. R Graphics. Chapman & Hall/CRC Press.

694 Nakane, Y., Ikegami, K., Ono, H., Yamamoto, N., Yoshida, S., Hirunagi, K., Ebihara, S., Kubo,
695 Y., Yoshimura, T., 2010. A mammalian neural tissue opsin (Opsin 5) is a deep brain
696 photoreceptor in birds. *Proc. Natl. Acad. Sci. U. S. A.* 107, 15264–15268.
697 <https://doi.org/10.1073/pnas.1006393107>

698 Nakane, Y., Shimmura, T., Abe, H., Yoshimura, T., 2014. Intrinsic photosensitivity of a deep
699 brain photoreceptor. *Curr. Biol.* 24, R596–R597. <https://doi.org/10.1016/j.cub.2014.05.038>

700 Nakane, Y., Shinomiya, A., Ota, W., Ikegami, K., Shimmura, T., Higashi, S.I., Kamei, Y.,
701 Yoshimura, T., 2019. Action spectrum for photoperiodic control of thyroid-stimulating
702 hormone in Japanese quail (*Coturnix japonica*). *PLoS One* 14, 1–15.
703 <https://doi.org/10.1371/journal.pone.0222106>

704 Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y.,
705 Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P.J., Iwasawa, A., Suzuki, Y.,
706 Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H.R., Yoshimura, T.,
707 2008. Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature* 452, 317–
708 322. <https://doi.org/10.1038/nature06738>

709 Nectow, A.R., Nestler, E.J., 2020. Viral tools for neuroscience. *Nat. Rev. Neurosci.* 21, 669–681.

710 Oliver, J., Baylé, J.D., 1975. Multiple Unit Activity Patterns of Neuronal Populations in
711 Gonadotropic Areas of the Quail Hypothalamus: Spontaneous and Photically-Induced
712 Firing. *Neuroendocrinology* 17, 175–188. <https://doi.org/10.1159/000122353>

713 Peirson, S., Foster, R.G., 2006. Melanopsin: another way of signaling light. *Neuron* 49, 331–339.

714 Pérez, J.H., Swanson, R.E., Lau, H.J., Cheah, J., Bishop, V.R., Snell, K.R.S., Reid, A.M.A.,
715 Meddle, S.L., Wingfield, J.C., Krause, J.S., 2020. Tissue-specific expression of 11 β -HSD
716 and its effects on plasma corticosterone during the stress response. *J. Exp. Biol.* 223.

717 <https://doi.org/10.1242/jeb.209346>

718 Pérez, J.H., Tolla, E., Dunn, I.C., Meddle, S.L., Stevenson, T.J., 2019. A Comparative
719 Perspective on Extra-retinal Photoreception. *Trends Endocrinol. Metab.* 30, 39–53.
720 <https://doi.org/10.1016/j.tem.2018.10.005>

721 Rowan, W., 1925. Relation of light to bird migration and developmental changes [3]. *Nature*
722 115, 494–495. <https://doi.org/10.1038/115494b0>

723 Shichida, Y., Matsuyama, T., 2009. Evolution of opsins and phototransduction. *Philos. Trans. R.*
724 *Soc. B Biol. Sci.* 364, 2881–2895. <https://doi.org/10.1098/rstb.2009.0051>

725 Soni, B.G., Philp, A.R., Foster, R.G., Knox, B.E., 1998. Novel retinal photoreceptors [3]. *Nature*
726 394, 27–28. <https://doi.org/10.1038/27794>

727 Stevenson, T.J., Ball, G.F., 2012. Disruption of neuropsin mRNA expression via RNA
728 interference facilitates the photoinduced increase in thyrotropin-stimulating subunit β in
729 birds. *Eur. J. Neurosci.* 36, 2859–2865. <https://doi.org/10.1111/j.1460-9568.2012.08209.x>

730 Stevenson, T.J., Bernard, D.J., Ball, G.F., 2009. Photoperiodic condition is associated with
731 region-specific expression of GNRH1 mRNA in the preoptic area of the male starling
732 (*Sturnus vulgaris*). *Biol. Reprod.* 81, 674–680.
733 <https://doi.org/10.1095/BIOLREPROD.109.076794>

734 Stevenson, T.J., Hahn, T.P., Macdougall-Shackleton, S.A., Ball, G.F., 2012. Gonadotropin-
735 releasing hormone plasticity: A comparative perspective. *Front. Neuroendocrinol.* 33, 287–
736 300. <https://doi.org/10.1016/j.yfrne.2012.09.001>

737 Stevenson, T.J., Liddle, T.A., Stewart, C., Marshall, C.J., Majumdar, G., 2022. Neural
738 programming of seasonal physiology in birds and mammals: A modular perspective. *Horm.*
739 *Behav.* 142. <https://doi.org/10.1016/j.yhbeh.2022.105153>

740 Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., Grolemond, G.,
741 Hayes, A., Henry, L., Hester, J., 2019. Welcome to the Tidyverse. *J. Open Source Softw.* 4,
742 1686.
743 Yamamura, T., Hirunagi, K., Ebihara, S., Yoshimura, T., 2004. Seasonal Morphological Changes
744 in the Neuro-Glial Interaction between Gonadotropin-Releasing Hormone Nerve Terminals
745 and Glial Endfeet in Japanese Quail. *Endocrinology* 145, 4264–4267.
746 <https://doi.org/10.1210/en.2004-0366>

747

748 **Statements and Declarations**

749

750 **Funding**

751 This work was supported by: The Leverhulme Trust [RPG-2016-392] to TJS, SLM and ICD,
752 Roslin Institute Strategic grant funding from the Biotechnology and Biological Sciences
753 Research Council, UK (BB/P013759/1) to SLM and ICD and The British Society for
754 Neuroendocrinology to JHP.

755

756 **Competing Interests**

757 The authors have no relevant competing financial or non-financial interests to disclose.

758

759 **Author Contributions**

760 TJS, SLM, ICD and RGF conceived of the initial study design. TJS, SLM and ICD secured and
761 provided the grant funding for the experiments described. JHP, TJS, SLM and ICD refined initial
762 experimental design. JHP carried out the experiments with SLM. JHP conducted all
763 morphometric data collection. JHP, ET, VRB, and TJS conducted the molecular lab work. TJS
764 and SNP designed the shRNAi sequences. RGF provided initial antibody stocks. JHP conducted
765 the statistical analysis and drafted the main manuscript. All authors contributed to revision and
766 editing of the draft manuscript prior to final submission

767

768 **Data Availability**

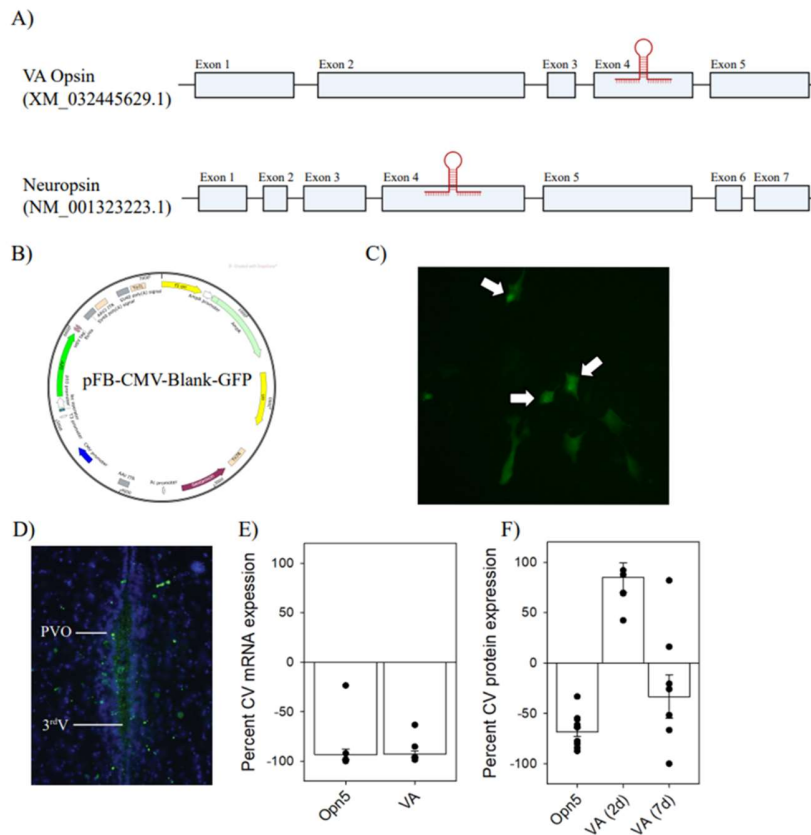
769 Data and analytic code (text format) for R analyses are provided in the supplementary files. For
770 the purpose of open access, the author has applied a CC BY public copyright license to any
771 Author Accepted Manuscript version arising from this submission.
772

773 **Ethics Approvals**

774 Animal procedures were approved by the Roslin Institute Animal Ethics and Welfare Review
775 Board at the University of Edinburgh and were performed under Home Office approval (PPL
776 P61FA9171). The experiments were designed in accordance with the Animal Research
777 Reporting of In Vivo Experiments (ARRIVE) guidelines and National Centre for the
778 Replacement, Refinement and Reduction of Animals in Research.
779

780

781 **Figures**

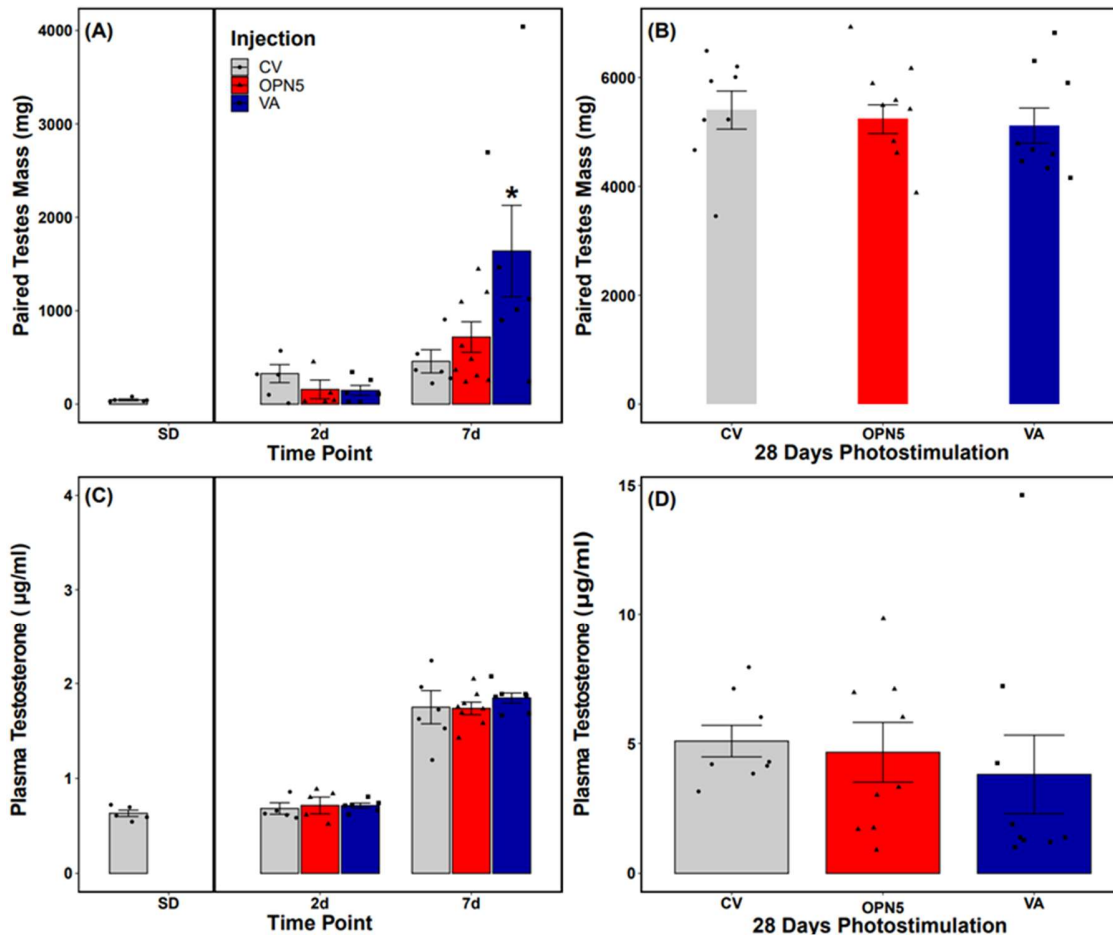


782

783

784 **Fig. 1. Specificity and effectiveness of photoreceptor RNA interference**

785 Specificity of RNA interference (RNAi) was achieved by designing probes that target Exon 4 in
 786 both VA opsin and Neuropsin (OPN5) sequences (A). Plasmids included a CMV promoter and
 787 green fluorescent protein (GFP). shRNAi vectors for VA opsin or OPN5 were inserted upstream
 788 to the CMV promoter, blank ‘control’ plasmid is shown (B). Primary cell culture of embryonic
 789 Japanese quail (E10) cells were used to confirm transduction capacity of AAV2 viral serotypes.
 790 Cells cultured with 2ul of AAV2 blank constructs showed robust transfection indicated by the
 791 white arrows (C). Representative photomicrograph of a coronal section through the mediobasal
 792 hypothalamus with the Periventricular organ (PVO) showing strong fluorescence. GFP
 793 expression was used to confirm the anatomical localization of intracerebroventricular injection of
 794 shRNAi constructs and presence of transfected cells 6-weeks after surgery (D). qPCR and
 795 western-blot assays were conducted to establish the effectiveness of shRNAi to reduced mRNA
 796 (E) and protein (F) expression for OPN5 and VA opsin. OPN5 mRNA and protein showed
 797 highly-effective reduction in photoreceptor expression. shRNAi against VA opsin induced a near
 798 100% reduction in expression. VA opsin protein levels are shown separately for 2 day and 7-day
 799 treatment groups to highlight the variation across conditions. Higher VA opsin protein levels
 800 observed in 2-day birds likely reflects increased translation of VA opsin mRNA reserves or a
 801 transient disruption in the homeostatic balance of photoreceptor levels.



802
803

804 **Fig 2. Effects of acute and chronic opsin silencing on opsin expression.**

805 Effects of acute (A) or 28-day chronic (B) shRNAi knockdown of vertebrate ancient opsin (VA)
806 and neuropsin (OPN5) compared to control AAV2 vector cassette (CV) on paired testes mass in
807 Japanese quail reveals VA knockdown leads to accelerated induction of testes growth after 7
808 days of photostimulation (16L:8D). Plasma testosterone, (µg/ml) measured by ELISA, was
809 unaffected by either acute (C) or 28-day chronic (D) opsin silencing, but increased with duration
810 of photostimulation from acute to chronic. Asterisk (*) indicate $p < 0.05$ significant differences
811 from control birds within the given time point. All values plotted as mean \pm sem.

812

813

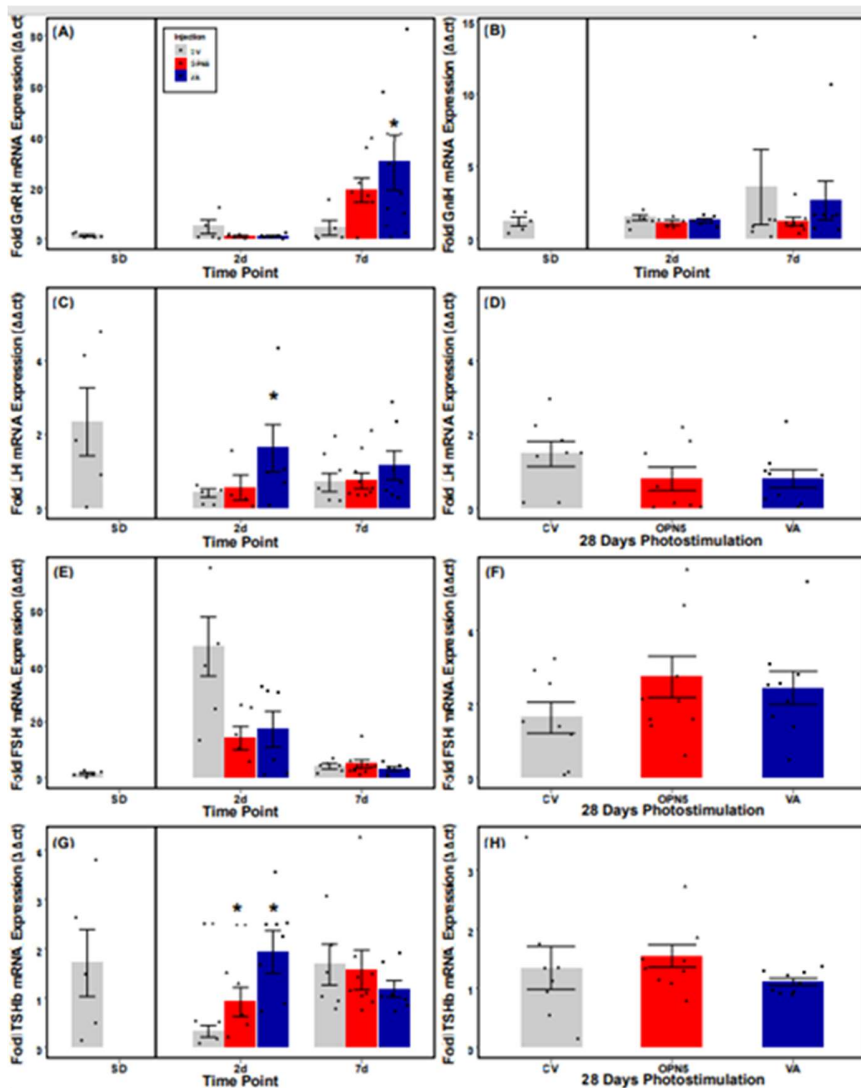
814

815

816

817

818



819

820

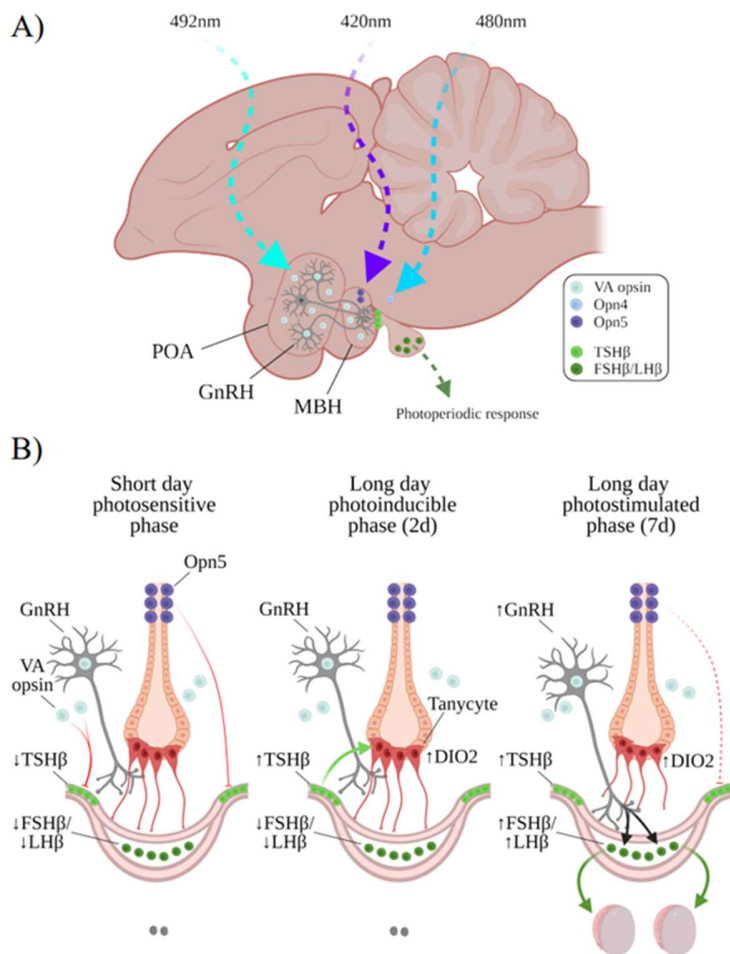
821 **Fig 3. Photoinduced neuroendocrine gene expression.**

822 Gene expression measured by RT-qPCR in control (CV) and vertebrate ancient opsin (VA) and
 823 neuropsin (OPN5) silenced quail for GnRH-I (A), GnIH (B), acute LH (C), 28-day chronic LH
 824 (D), acute FSH (E), 28-day chronic (F), acute TSHβ (G) and 28-day chronic TSHβ (H).

825 Knockdown treatment induced increased TSHβ expression during the early photoinducible phase
 826 (2D) in both OPN5 and VA silenced animals, but only VA opsin knockdown effected
 827 gonadotroph expression with increased LH expression at 2 days and subsequently increased
 828 GnRH-I expression at 7 days of photostimulation. Asterisk (*) indicate p < 0.05 significant
 829 differences from control birds within the given time point. All values plotted as mean ± sem.
 830 Removed outliers are shown as black points, except in panel C where an outlier, value 10.52, in
 831 the CV at 2 days was removed.

832

833



834

835

836 **Fig 4. Schematic representation of the photoperiodic control of the neuroendocrine axis in**
 837 **birds.** (A) Multiple wavelengths of light penetrate deep into the quail brain and are detected by
 838 at least three photoreceptors: vertebrate ancient opsin (VA opsin; λ 492), neuropsin (OPN5; λ 420)
 839 and melanopsin (OPN4; λ 480). VA opsin is widely distributed in the preoptic area (POA) and
 840 mediobasal hypothalamus (MBH). OPN5 is localized to the PVO in the MBH and OPN4 is
 841 sparsely distributed in the MBH. Gonadotropin-releasing hormone (GnRH-I) neurons project
 842 from the preoptic area into the MBH to stimulate the release of luteinizing hormone (LH) and
 843 follicle-stimulating hormone (FSH). (B) Short day birds maintain regressed gonads due to an
 844 inability of GnRH-I neurons to contact the basal lamina membrane that separates the median
 845 eminence from the pituitary gland. Photoinduction by long days activates VA opsin and OPN5
 846 cells located in the POA and mediobasal hypothalamus. Two- days light stimulation of VA opsin
 847 and OPN5 facilitated *TSH β* expression. After 7 days of photostimulation of VA opsin either
 848 directly co-expressed in GnRH-I neurons or indirectly (via disinhibition from VA neurons)
 849 resulted in higher *GnRH* expression. GnRH-I access to the basal lamina permits that ability to
 850 stimulate gonadotropin release from the pituitary and trigger gonadal growth.

851 **Supporting information**

852

853

854 **S1 Figure. 28 LD Testes histology and gene expression measured by qPCR.** Gene expression
855 metrics include Androgen Receptor (A), LH Receptor (B), and FSH Receptor (C). All gene
856 expression values presented as fold expression using $\Delta\Delta CT$ method as describe in main text with
857 GAPDH and β -Actin reference genes. Testes histology for Sox9 as a Sertoli cell marker (D),
858 total number of tubules (E), and Sox9 positive cell to tubule ratio (F). For each testes marker
859 treatment effect was analyzed by linear model. Line with “*” indicates a significant difference
860 between treatment and control group $p < 0.05$.

861

862 **S1 Table. AAV2 shRNA sequences used to suppress neuropsin and VA opsin expression**

863

864 **S2 Table. qPCR primers used for Japanese quail hypothalamus and annealing**
865 **temperatures.**

866

867 **S3 Table. Antibody target and sequence for immunohistochemistry and western blot**

868

869 **S2 File. R analytic code and data file.** The R code used for all analyses and figures presented in
870 main and supporting documents as well as raw data file used in the analysis presented in a PDF
871 format.

872