Neural and sensory basis of homing behavior in the invasive cane toad, *Rhinella marina*

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Abstract

The behavioral, sensory, and neural bases of vertebrate navigation are primarily described in mammals and birds. However, we know much less about navigational abilities and mechanisms of vertebrates that move on smaller scales, such as amphibians. To address this knowledge gap, we conducted an extensive field study on navigation in the cane toad, *Rhinella marina*. First, we performed a translocation experiment to describe how invasive toads in Hawaiʻi navigate home following displacements of up to one kilometer. Next, we tested the effect of olfactory and magnetosensory manipulations on homing, as these senses are most commonly associated with amphibian navigation. We found that neither ablation alone prevents homing, suggesting that toad navigation is multimodal. Finally, we tested the hypothesis that the medial pallium, the amphibian homolog to the hippocampus, is involved in homing. By comparing neural activity across homing and non-homing toads, we found evidence supporting the involvement of the medial pallium, lateral pallium, and septum in navigation, suggesting a conservation of neural structures supporting navigation across vertebrates. Our study lays the foundation to understand the behavioral, sensory, and neural bases of navigation in amphibians and to further characterize the evolution of behavior and neural structures in vertebrates.

Introduction

The ability to process spatial information is fundamental to survival for many animals, who must navigate through their environment to locate shelter, forage, care for young, and more. Much of our understanding of spatial navigation comes from research in mammals, where the hippocampus has been recognized as the neural center for spatial representations [1–3], alongside other regions like the and entorhinal cortex [4,5]. Recent efforts have characterized similar functions of homologous hippocampal brain regions in birds [6,7], fish [8,9] and reptiles [10], suggesting conservation of the neural architectures supporting this critical behavior. However, the neural basis of amphibian spatial behavior remains understudied, especially in the context of navigation in complex natural environments. As amphibians represent the major evolutionary transition from aquatic to terrestrial habitats in the vertebrate lineage, filling this gap will provide a more complete understanding of the neural representations of spatial cognition and their evolution across vertebrates.

Research across many amphibian species suggests a wide-spread ability to navigate to fixed sites important for reproduction. For example, many temperate-region amphibians migrate to breeding sites on scales up to several kilometers, such as some salamanders (including *Taricha rivularis* and *Plethodon jordani*)[11–13] and pond breeding toads (*Bufo bufo*, *B. japonicus*) [14,15]. Some neotropical poison frog species, such as *Oophaga sylvatica*, *Dendrobates tinctorius*, and *Allobates femoralis*, move with high spatial precision within a few hundred meters to transport tadpoles to water pools [16–18]. A variety of sensory modalities facilitate these movements, with olfaction [12,14,19] and magnetoreception [20–22] being the most often implicated in migratory amphibians. In one relevant example, Sinsch (1987) demonstrated that the European common toad (*Bufo bufo*) relies on both olfaction and magnetoreception to orient towards its breeding pond [14]. Impressive navigation abilities have also been demonstrated in amphibians that do not have fixed reproduction sites, such as the

cane toad, *Rhinella marina* [23]. However, the sensory modalities used for navigation back to terrestrial home sites, rather than to water bodies has rarely been investigated. More research is needed on amphibian navigation behaviors outside of reproduction and what sensory modalities are important, which will provide a more complete picture of vertebrate perception and sensory integration, and how these lead to successful homing.

The cane toad is native to Central and South America and was introduced globally, including in Hawai‘i and Australia. In its invasive front in Australia, the cane toad can disperse and travel long distances [24,25], sometimes moving 200-meters per night to new locations [26], creating difficulties for species management. However, other than on the Australian invasive front, cane toads are relatively sedentary, rarely move more than 200 meters, and often return to the same diurnal shelters [23,27–29]. We previously showed that toads in their native French Guiana are able to return home accurately from a full kilometer, a distance exceeding their routine movements [23]. These navigation abilities and the toad’s extreme prevalence as a devastating invasive species make it an ideal model for studying homing behavior and probing unanswered questions regarding the neural and sensory basis of homing.

In this study, we conduct a field experiment to test cane toad navigational ability, quantify neural activity associated with navigational outcomes, and test which sensory systems are required for navigation. We hypothesized that the medial pallium (the amphibian hippocampal homolog [30–32]) is involved in navigation and predicted that homing toads would have higher neural activity in this brain region compared to non-homing and non-translocated toads. We next hypothesized that olfaction and magnetoreception guide homing behavior, given their functionality in other bufonids [14,15,33], and predicted that olfactory or magnetoreception ablations would disrupt the homing ability or accuracy. Our study identifies behavioral characteristics involved in homing, brain regions that are more active during navigation, and provides future directions on sensory system research in cane toad navigation.

Materials and Methods

(a) Study sites

Experiments occurred at four sites on the island of O‘ahu in Hawai‘i. We sampled at two sites on the southern, leeward side of the island (West Loch Golf Course: 21° 22' 04.7" N 158° 01' 31.5" W; Hawai‘i Prince Golf Course: 21° 18' 41.5" N 158° 00' 32.7" W) and two sites on the northern, windward side of the island (Waimea Valley: 21° 38' 04.4" N 158° 03' 14.6” W; Turtle Bay Golf Course: 21° 42' 07.5" N 157° 59' 44.6” W). Waimea Valley was a forested area while the other three sites were golf courses (Figure 1B).

(b) Animals

Toads were located visually at night (sunset was approximately 19:00 and sunrise approximately 06:30), as cane toads are nocturnal and generally do not exhibit diurnal movements. We photographed the dorsum for re-identification purposes, measured snout-vent-length (SVL) with a tape measure, and measured mass with a portable scale (KUBEI-790, KUBEI, China). Eighty-two toads (40 males, 42 females) were tagged and tracked for at least three days between 18 February and 12 May 2022 across the four sites. Their mean mass was 223.8 g (sd +/- 98.6) and mean SVL was 12.4 cm (+/- 1.6). Toad size was not evenly distributed between sites (Mass: Kruskal-Wallis, $\chi^2(3)= 19.687$, $p = 0.0002$; SVL: ANOVA, $df = 3$, $F = 14.67$, $p = 1.15e-07$). Sex was determined by size and male release call, and confirmed by gonad identification upon dissection. Females were significantly larger than males (Mass: Wilcoxon, $W = 1315.5$, $p = 1.05e-05$; SVL: t-test, $t = 4.44$, $df = 77.41$, $p = 2.99e-05$). Unless otherwise stated, “size” refers to SVL hereafter.

(c) Tagging and baseline tracking

Radio tagging and subsequent tracking was executed similarly to the method we previously described [23]. Briefly, radio tags (BD2, Holohil Systems Ltd, Carp, ON, Canada) were attached to the toad's waists using belts made of silicone tubing and thread (Figure 1A). The tagging process lasted 14.2 min on average (+/- 7.8) after which toads were released. A GPS point was taken in the ArcGIS Field Maps app (ESRI, Redlands, CA, USA) using a Bad Elf GPS Pro+ gps device (Bad Elf, West Hartford, CT USA) connected by Bluetooth to an iPhone (Apple, Cupertino, CA, USA). Following release, toads were located once in the evening prior to their active, nocturnal period and at least two times each night (between sunset and sunrise). Toads were tracked for natural baseline movement information (termed 'baseline' hereafter) between three and 28 days (5.701 +/- 4.04 days) prior to translocation or euthanasia.

(d) Translocation

Translocations occurred during the night generally between 20:00 and 22:00. Sixty-two toads were translocated. Toads were caught and their waists examined to ensure no abrasion from the waist belt. Toads were placed into an enclosed net cage and walked in an indirect route for 30-60 minutes to a distance approximately 500- or 1000-meters from the baseline position. At the release site, the distance from the toad's nearest baseline point was confirmed on a GPS-connected handheld device, the net cage was left at the release point for 5 minutes, and the toad was gently released from the cage and allowed to move freely.

Following release, toads were relocated approximately once per hour during the nighttime, beginning at 20:00 and continuing until tagged animals had stopped moving (06:40 at the latest). On subsequent days, toads were relocated once in the early evening to determine location before nocturnal movements began. If a toad was not active at night (e.g. not moving or making small, multidirectional movements), the frequency of observations was reduced to every 2-3 hours while tracking moving toads.

147 Tracking ended once the animal reached a point within 200-meters of one of its recorded
148 baseline positions or 72 hours had passed after translocation. Homing toads (captured within
149 200-meters of a baseline point) were euthanized and tissues were collected for downstream
150 analyses (described below) within 30 minutes of crossing the 200-meter threshold. Non-homing
151 toads were sacrificed after 20:00 wherever they were at that time. Two toads in the homing
152 group were unintentionally caught before they crossed the 200-meter threshold (B8 at 233-m
153 and D5 at 218-m) and two more toads were caught and euthanized approximately 300-m from
154 home after encountering physical obstacles following large homeward movements (C8 and F5) ,
155 but were included in the homing condition (both for considering behavior and neuronal activity).
156 One toad moved outside of the study area and was euthanized because it became impossible
157 to track; its behavior and brain data were not included in analysis.

158 (e) Sensory Ablations

159 A subset of translocated toads (n = 43) received magnetoreception or olfactory ablations
160 to test the roles of these sensory systems in homing. In each group, we had experimental (i.e.
161 ablated) animals, as well as control animals receiving sham ablation treatments. Toads were
162 tracked for at least three days prior to ablation, and then were tracked for at least 48 hours post-
163 ablation before translocation to evaluate any effects of the ablation procedures on space use.
164 In the olfactory ablation group (n = 11), toads were captured and lightly anesthetized
165 through immersion in an MS-222 bath (1.4 g/L) for ~5 min. We performed olfactory ablations
166 through zinc sulfate ($ZnSO_4$) treatment, which temporarily damages the olfactory epithelium in
167 juvenile and adult amphibians [34,35] and other vertebrates [36,37]. Once animals were
168 anesthetized and not responding to toe pinches, they were held upside-down and each nostril
169 was washed with 1% $ZnSO_4$ dissolved in amphibian ringer solution (NaCl, KCl, $CaCl_2 \cdot 6H_2O$ and
170 distilled water; Electron Microscopy Services, Hatfield, PA 19440) applied in five 200 µl doses
171 through the internal nare with a P200 pipette. Any $ZnSO_4$ that spilled into the animal's mouth

172 was sucked up with the pipette. Following administration of $ZnSO_4$, the toad's mouth was
173 washed several times with amphibian ringer solution. Animals recovered in a net cage until fully
174 mobile (usually 20-30 minutes), after which they were released at the capture point. Animals
175 experiencing sham ablations (n = 11) underwent an identical procedure, but their nostrils were
176 washed with amphibian ringer solution without $ZnSO_4$.

177 Magnetoreception ablations were carried out by attaching a small Nickel plated
178 Neodymium magnet (N50, 10 mm x 6 mm x 2 mm, ~1 g; Super Magnet Man, Pelham, Alabama,
179 USA) with cyanoacrylate based adhesive (EM-150, Starbond, Torrance, CA, USA) to the skin
180 over the skulls of toads (n = 11), similarly to previous studies in anurans [14,33,38]. Toads were
181 captured, magnets applied, and toads were kept in a net cage for ~30 minutes before release.
182 Sham ablation animals had a brass rectangle (12.7 mm x 6 mm x 1.6 mm, ~ 1 g; Metals Depot,
183 Winchester, Kentucky, USA) attached in place of a magnet (n = 10). Two animals lost their
184 magnets following translocation, so their behavior and brain measurements were not included in
185 the magnetoreception analysis.

186 (f) Tissue collection

187 Toads were euthanized at one of three timepoints: (a) the completion of at least three
188 days of baseline tracking if the individual was being used as a baseline comparison for non-
189 homing and homing translocated toads (baseline, n = 12), (b) three days post-translocation if
190 the toad failed to return within 200-meters of its baseline location (non-homing, n = 27), (c) or
191 once the toad reached a point 200-meters from a previously observed baseline location
192 (homing, n = 33).

193 Toads were euthanized through MS-222 injection (0.7% solution, 300 mg/kg body
194 weight) followed by cervical transection with sharpened hedge shears. Brains were immediately
195 dissected, placed in 1 mL of 4% paraformaldehyde (PFA), and kept on ice until they could be
196 placed into a 4℃ refrigerator. Brains were fixed overnight in PFA, washed three times in 1x PBS

197 and then placed into 1x PBS with 0.02% sodium azide, or 1x PBS if transportation to the
198 laboratory site would happen in two days. Brains were kept in 1x PBS with sodium azide < 1
199 week and two days prior to travel to the laboratory, the brains were transferred to 1 mL of 30%
200 sucrose dissolved in 1x PBS for cryoprotection. Once the brains sank in sucrose, they were
201 transported on ice to a laboratory at the University of Hawai'i at Manoa, where they were
202 embedded in Tissue-Tek OCT Compound (Electron Microscopy Sciences) and frozen on dry ice
203 before being stored at -80℃. We collected 73 brains (61 translocated and 12 baseline). One
204 homing toad lost its tag within 200-meters of its home area, so its brain was not collected.

205 (g) Immunohistochemistry

206 Brains were sectioned on a cryostat in 20 μm slices and thaw-mounted onto slides in
207 four series. Slides dried at room temperature for ~48 hours before being transferred to -80℃ for
208 storage prior to staining. We used immunohistochemistry to detect Phospho-S6 Ribosomal
209 Protein (pS6) as a proxy for neural activity [39] as previously described by our laboratory
210 [40,41], except with a 1:300 primary antibody dilution (full protocol is presented in SM1). pS6 is
211 a reliable activity marker in amphibians [42] and other animals [39]. Of the 73 collected brains,
212 two were discarded due to poor tissue quality and four were discarded for being collected at the
213 wrong time point, leaving 67 for analysis.

214 (h) Microscopy and Cell Counting

215 Brains were imaged on a Leica DM6B microscope with brightfield capability at 20x
216 magnification. One hemisphere was selected to image for each brain. Nine sections along the
217 AP axis were chosen based on anatomical landmarks: three from the anterior of the
218 telencephalon (posterior to the olfactory bulb), three from the medial portion, and three from the
219 posterior of the telencephalon (anterior to the ventral hypothalamus) (Figure S1). ImageJ (NIH,
220 Bethesda, MD, USA) was used to count pS6-positive cells in each of five brain regions: medial

221 pallium (Mp), lateral pallium (Lp), dorsal pallium (Dp), septum (Sep) and the striatum (Stri) given
222 their role in spatial behavior in toads [43–45] and other vertebrates [3,46,47]. Delineation of
223 brain regions was based on Sotelo et al. (2016), which defined pallial regions by Westhoff and
224 Roth (2002) and ventral regions by Moreno and González (2004)[30,43,48]. ImageJ was used
225 to calculate the surface area of each region. Densities of pS6-positive neurons for each region
226 were calculated by dividing the number of pS6-positive cells by the area.
227 For toads in the olfaction ablation experiment, we used ImageJ to count active cells in a
228 300 μm by 300 μm box positioned at the lateral edge of the granule cell layer in the olfactory
229 bulb (Figure S2) to measure the effect of the olfactory ablation in comparison to the sham
230 ablation.

231 (i) Data analysis

232 (i) Toad movement data

233 GPS points were visualized in ArcGIS online and ArcGIS Pro 2 (ESRI). Spatial and
234 temporal attributes were calculated using the package 'adehabitatLT' [49] in R Studio (v
235 2023.09.1+494, Posit Software, PBC, Sunnyvale, CA) running R (v 4.3.1, R Foundation for
236 Statistical Computing, Vienna, Austria). We calculated several descriptors of toad movement
237 and homing: cumulative movement, daily movement, movement range, straightness index,
238 homing duration/speed, and active homing duration/speed (details of descriptor calculations in
239 SM2).
240 To estimate the similarity of a toad's home range before and after ablation treatment, we
241 plotted minimum convex polygons in ArcGIS Pro encapsulating all location points pre-ablation
242 and calculated the proportion of post-ablation relocations that fell inside the polygon.
243 Outliers were values more than three standard deviations from the mean and were
244 removed prior to statistical comparisons and visualization. Normality was verified using the
245 Shapiro-Wilks Test. Toad size (SVL) was compared between sexes using a Student's t-test and

between field sites by ANOVA with a post-hoc Tukey's test. Comparisons of mass, movement range, and homing duration/speed between field sites were done with Kruskal-Wallis tests with a post-hoc Dunn's test. Wilcoxon Rank Sum Tests were used to compare mass and movement range between sexes. It was also used to compare the effects of translocation distance on homing descriptors (straightness, duration, speed, and time to move 100 meters homeward), as well as differences between sham and ablated individuals in home area pre/post manipulation, homing duration, and cumulative movement. A Paired Wilcoxon test was used to compare movement before and after ablations for sensory manipulated toads. Pearson's Chi-Squared tests were used to compare binary homing success between translocation distances and sham/ablated individuals. The effect of toad size on homing duration was modeled with a linear model using the 'lm' function in the 'stats' R package [50] and with a linear mixed-effects model using the 'lme4' R package [51], where duration was the response variable, size was the fixed effect, and field site was the random effect.

We used the 'circular' package in R [52] to analyze the directionality of movement angles between homing and non-homing toads. The "ltraj" objects generated using the 'adehabitatLT' package provide absolute angles (i.e. compass direction) and relative angles (i.e. the change in angle from the previous movement) for each movement segment. In our comparisons, we calculated the circular mean of all absolute angles of movement for each toad and normalized with respect to the home direction. We used the Rayleigh test of circular uniformity to test whether mean absolute angles differed from the home direction in homing and non-homing toads. We also compared the distribution of mean angles directly between homing and non-homing animals using a MANOVA based on trigonometric functions [53] using 'manova' functions in the 'stats' R package [50].

(ii) Cell Count Data

We used generalized linear mixed models ('glmmTMB' in R [54]) to test for differences in brain activity and its relation with behavior. Models used a negative binomial distribution and best model fit was confirmed using the 'DHARma' package in R [55]. Homing condition, brain region, and their interaction were the main effects predicting the number of pS6-positive cells. Toad ID was included as a random factor and brain region area was included as an offset variable to account for brain area size. We modeled differences in brain activity due to ablations separately using the same model parameters as above except that ablation was also included as a main effect. Post hoc pairwise contrasts (between homing condition groups and ablation treatments) were calculated with estimated marginal means using the "emmeans" package in R [52].

To test if brain activity was related to either straightness or duration of homing, we performed linear mixed effects models using the "lme4" package, where pS6 count was the response variable and the homing descriptor (straightness or duration), brain region, and their interaction were fixed effects. Toad ID was a random effect and brain region area was an offset variable in the model. Linear mixed effects models were also performed within count data specific to each brain region, in which the response variable was pS6 counts within a given region, the homing descriptor was the fixed effect, Toad ID was a random effect, and region area was an offset variable.

Results

Toads generally exhibit site fidelity

Prior to translocation, toads exhibited a mean movement range of 130.19 m (s.d. 138.75 m), mean cumulative movement of 539.12 m (s.d. 745.45), and mean daily movement of 110.48 m (s.d. 157.20). Movement range varied significantly between sites (Kruskal-Wallis, $\chi^2(3)$ =

13.82, p = 0.0032), with toads at the Waimea Valley forest site exhibiting smaller ranges than toads at golf courses (Figure 1B). There was no difference in movement range between males and females (Wilcoxon, W = 783, p = 0.96). All three statistical outliers were males. Two of these individuals moved between two water sources more than 500-meters apart (Figure S3), apparently for mating, as we observed mating calls from the destination ponds. Size did not have a significant effect on range.

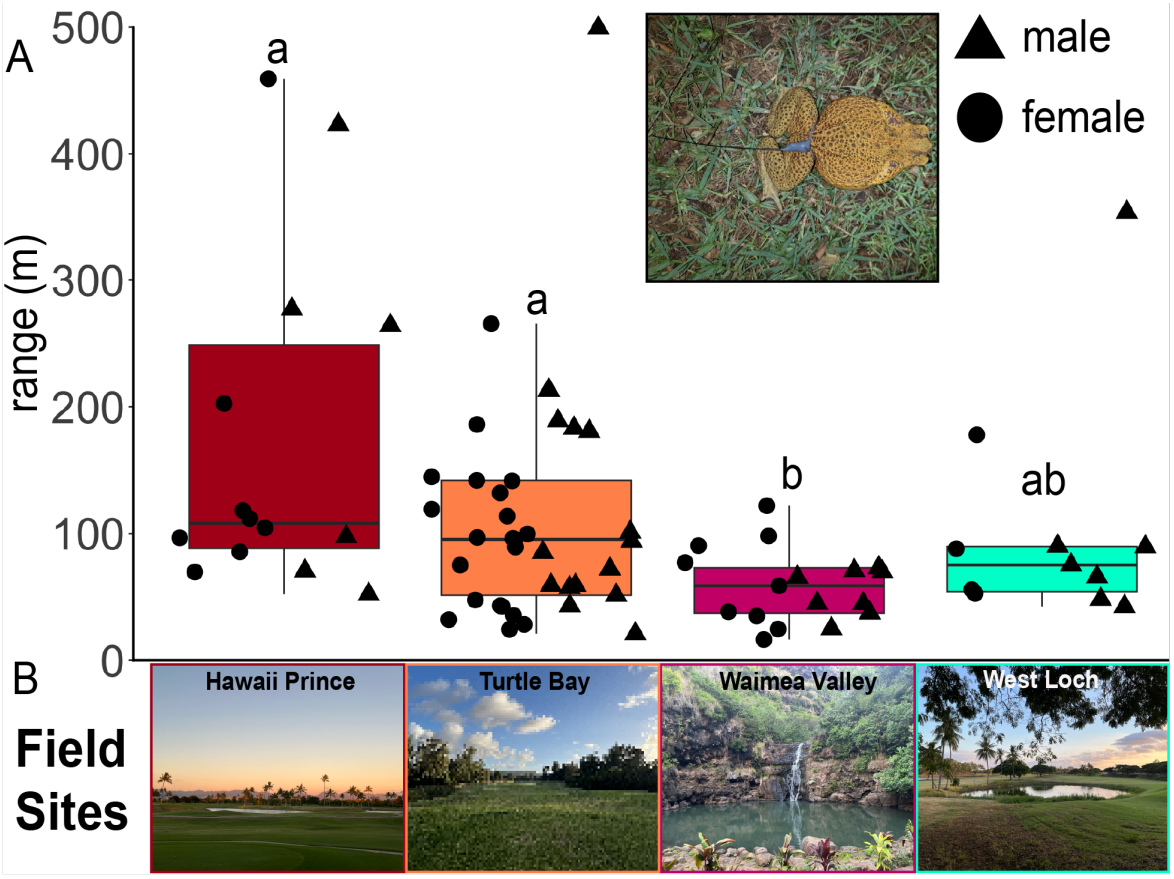

Figure 1. Movement range varies across locations. (A) Observed movement range of cane toads (in meters) at each tracking site. Letters represent significant differences between sites (Kruskal-Wallis, $\chi^2(3) = 13.82$, $p = 0.0032$). Shapes represent sex (▲= male, ●= female). Inset shows toad with radio tag. Letters represent significant differences from post-hoc Dunn's Test (with Benjamini-Hochberg adjustment) **(B)** Photographs depicting typical landscapes at each tracking site.

Toads exhibited long-distance homing

Out of 62 translocated tods, 34 exhibited homing behavior (Figure 2A-D). Toads returned home in 3.27 to 77.48 hours (25.50 +/- 22.23) with a mean straightness of 0.81 (+/- 0.16). Considering all toads (including those in ablation experiments), 19 out of 31 toads returned home from 500-meters and 15 out of 30 toads returned from 1000-meters. There was no effect of translocation distance on homing success (Pearson's Chi-squared, $\chi^2_{(1)} = 0.40$, $p = 0.53$). Distance had no effect on homing duration (Wilcoxon, $W = 147.5$, $p = 0.99$) (Figure S4A) and toads translocated 1000-meters had a faster homing speed (Wilcoxon, $W = 72$, $p = 0.014$; Figure S4B). A linear mixed-effects model accounting for the unequal distribution of size across field sites showed no significant effect of size on homing duration ($t = -0.51$, $df = 30.52$, $p = 0.61$; Figure S5).

Homing speed and duration varied significantly between sites, with toads at Waimea Valley homing slower than toads at Hawaii Prince and West Loch (Speed: Kruskal-Wallis, $\chi^2(3)= 10.52$, $p = 0.015$; Duration: Kruskal-Wallis, $\chi^2(3)= 12.38$, $p = 0.0062$; Figure 2E). Toads often did not immediately move homeward, so we measured an "active homing speed" using only movement and time after the first 100-meters of homeward movement. Active homing speed and duration did not vary between sites (Speed: Kruskal-Wallis, $\chi^2(3) = 5.37$, $p = 0.15$; Duration: Kruskal-Wallis, $\chi^2(3)= 0.66$, $p = 0.88$), suggesting that the main differences in homing time are due to stationary periods prior to homeward movements (Figure 2F). On average, the time to move at least 100-meters homeward was 64% of the total homing duration.

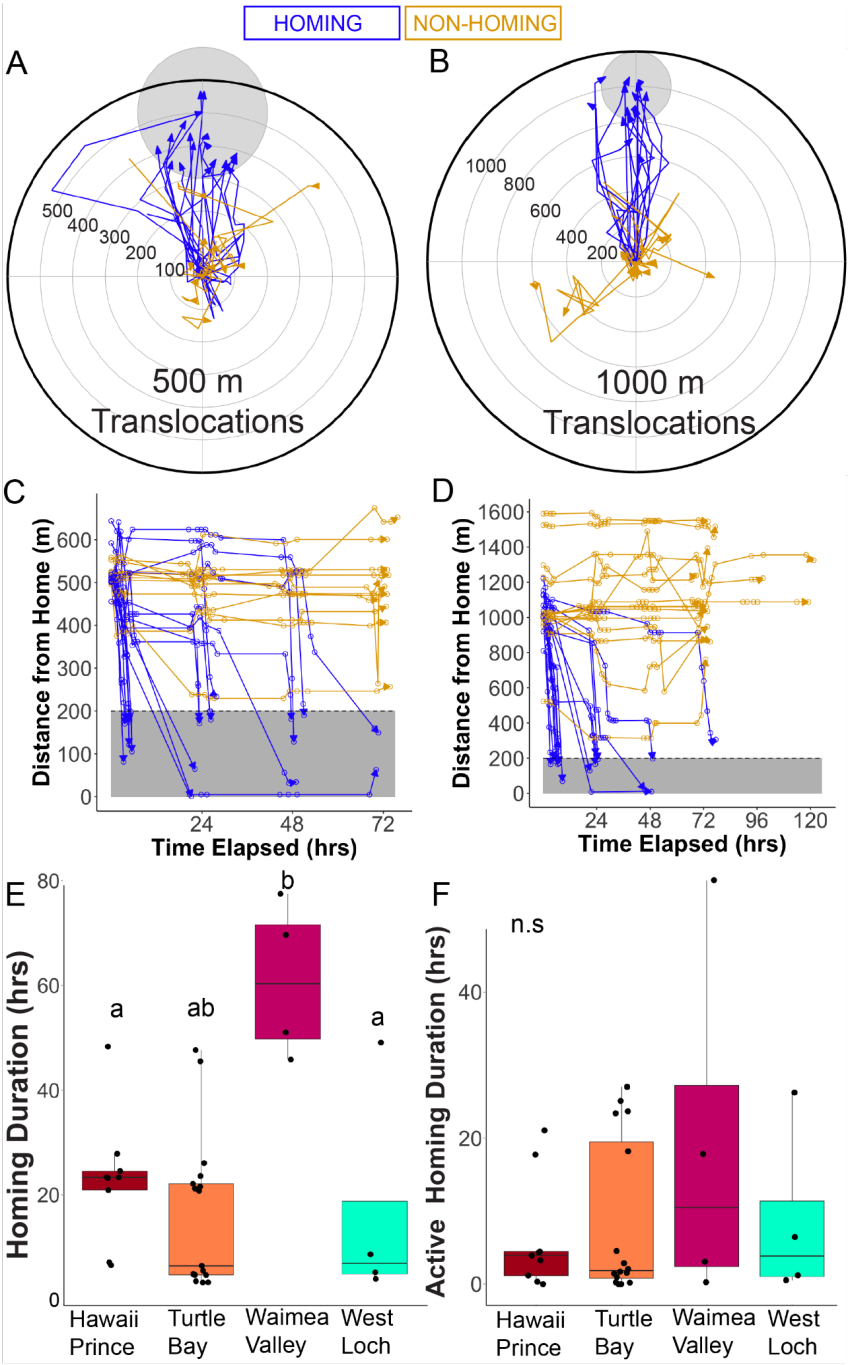

Figure 2 Toads exhibit long-distance homing. Normalized trajectories of toads translocated **(A)** 500-m and **(B)** 1000-m. Plot centers represent translocation release points and the gray circles at the top represent the 200-m buffer threshold around baseline points. 19/31 toads returned from 500-m and 15/30 from 1000-m. Distance to home over time for toads translocated **(C)** 500-m and **(D)** 1000-m. Gray rectangle at the bottom of the plot represents the 200-m buffer threshold around baseline points **(E)** Duration of homing differs between the experimental sites (Kruskal-Wallis, $\chi^2(3)= 12.38$, $p = 0.0062$) Letters represent significant differences from post-hoc Dunn's Test (with Benjamini-Hochberg adjustment). **(F)** Active homing time shows no difference between locations (Kruskal-Wallis, $\chi^2(3)= 0.66$, $p = 0.88$).

Twenty-seven of the 62 translocated toads did not exhibit homing behavior. Cumulative movement of non-homing toads averaged 547.07 m (+/- 488.5) and the mean minimum distance from home reached was 719.3 m (+/- 359.5). Mean movement angles of homing toads showed a significant orientation towards the home direction (Rayleigh $p = 0.024$) and non-homing toads did not (Rayleigh $p = 0.83$). However, there was only a marginal difference in distribution of angles of movement between homing and non-homing toads (MANOVA, Pilliai = 0.092, $F(2,58) = 2.95$, $p = 0.06$; Figure S6).

Pallial and septal brain regions show increased neural activity in homing toads

Given the variability in homing performance, we next asked whether neural activity in brain regions is correlated with homing success by quantifying the number of pS6-positive cells in five brain regions (Figure 3, Table S1). We first asked whether toads, regardless of sensory treatment, showed differences in brain activity due to homing behavior. We found a significant effect of homing behavior on neural activity in a brain region specific manner (brain region x homing condition: $\chi^2(8) = 103.742$, $p < 2.2e-16$) (Table 1). In the medial pallium and the septum, we found that homing animals had more pS6-positive neurons than both non-homing and baseline toads (Mp: Homing-Baseline- $z = -3.65$, $p = 0.0008$; Homing-Non-homing- $z = 4.18$, $p = 0.0001$. Sep: Homing-Baseline - $z = -3.71$, $p = 0.0006$; Homing-Non-homing - $z = 4.25$, $p = 0.0001$). In the lateral pallium, we also found differences in the number of pS6-positive neurons between Homing and Non-homing toads ($z = 2.77$, $p = 0.015$) and marginal differences between Homing and Baseline toads ($z = -2.34$, $p = 0.051$). Neither the dorsal pallium nor the striatum showed significant differences in the number of pS6-positive cells associated with homing and there were no differences between Non-homing and Baseline toads in any brain region.

We next asked if neural activity was correlated with path straightness and homing duration. There were no differences in brain activity across brain regions due to path straightness (Tables S2 and S3). However, the medial pallium ($p = 0.0098$) and septum ($p = 0.0034$) showed significant differences in activity related to duration of homing compared to other brain regions (Table S4) and duration showed a significant effect of homing on septal neuronal activity ($t = -2.59$, $df = 27.02$, $p = 0.015$) (Table S5).

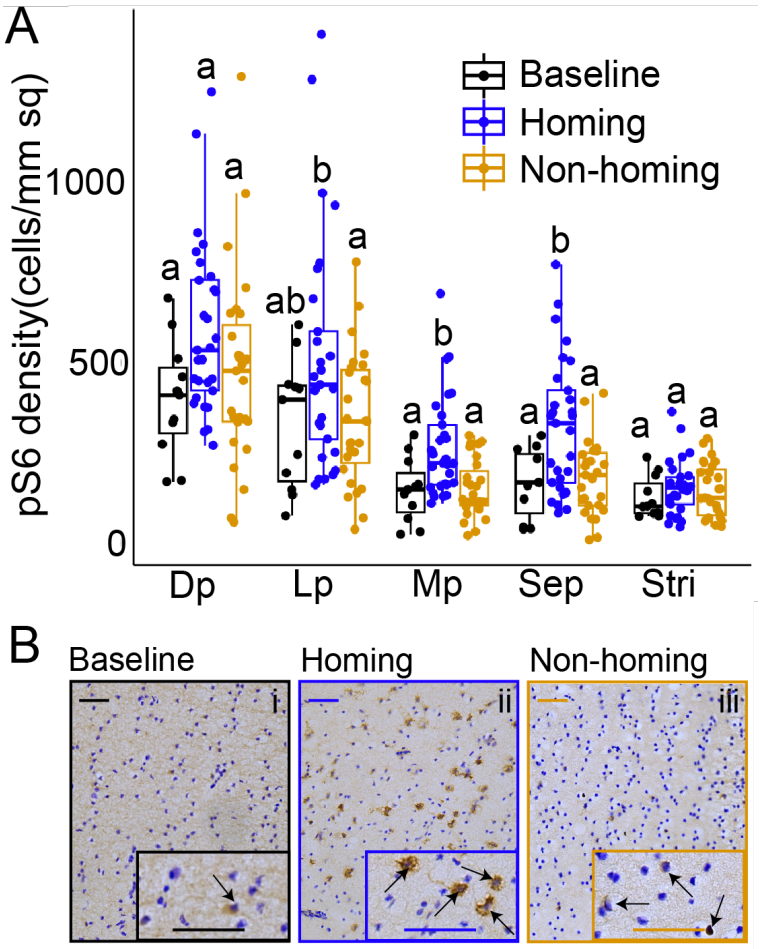

Figure 3. Pallial regions and the septum are more active due to homing behavior. (A) pS6-positive cells per mm^2 averaged per individual for each brain region across homing conditions. Brain activity within brain regions varied significantly due to homing conditions ($\chi^2(8) = 103.742$, $p < 2.2e-16$). Letters near box plots represent significant pairwise differences within brain regions based on a mixed model with cell count as a response factor. Abbreviations: Dp, dorsal pallium; Lp, lateral pallium; Mp, medial pallium; Sep, septum; Stri, striatum. **(B)** Representative examples of stained Mp brain tissue from each homing group. Scale bars represent 50 μm. In inset, arrows indicate pS6-positive cells.

We next restricted our analysis to toads without sensory system manipulations (Figure S7, Tables S6 and S7) and found that differences in pS6-positive cells between Homing and Baseline individuals in the medial and lateral pallium still hold (Mp: Homing-Baseline- z = -3.553, p = 0.0011; Lp: Homing-Baseline- z = -3.019, p = 0.0072). The differences in the number of pS6-positive cells between Homing and Baseline and Non-homing animals in the septum were also consistent (Sep: Homing-Baseline- z = -3.508, p = 0.0013; Homing-Non-homing- z = 3.005, p = 0.0075). Similar to the full sample, there were no differences between groups in the dorsal pallium and striatum. In contrast to the full dataset, there were no differences between Homing and Non-homing animals in the medial and lateral pallium. The differences in the results are likely due to sample size, as the non-ablated subset includes only 28 out of 67 animals (11 Baseline, 10 Homing, and 7 Non-homing).

Table 1. Summary of main statistical effects from generalized linear mixed model for differences in brain activity

	χ^2	df	p
Homing Condition	13.075	2	0.001448
Brain Region	2529.991	4	< 2.2e-16
Homing Condition x Brain Region	103.742	8	<2.2e-16

Olfactory ablation influences the homing path

We next asked whether ablation of olfaction led to a disruption in spatial behavior and navigation, as has been demonstrated in other amphibians [13–15]. There was no significant difference in movement range pre- and post-ablation for sham (Paired-Wilcoxon, V = 38, p = 0.70) or ablated animals (Paired Wilcoxon V = 36, p = 0.83). However, when comparing the space occupied pre- and post-ablation, ablated toads showed greater divergence from their pre-ablation area than sham animals (Wilcoxon, W = 340, p = 0.021), suggesting that loss of

395 olfaction might disrupt routine movements and space use. There were no significant differences
396 of homing success ($\chi^2(1) = 0.73$, $p = 0.39$) or duration (Wilcoxon, $W = 23$, $p = 0.42$) between
397 sham and ablated groups (ablated homing success = 45.5%, sham homing success
398 63.6%)(Figure 4A). After translocation, ablated toads moved more than sham toads when
399 considering Homing and Non-homing toads together (Wilcoxon, $W = 93$, $p = 0.034$) (Figure 4B).
400 Though the sample is too small for statistical comparison, ablated toads that homed showed
401 more cumulative movement (831.90 m +/- 288.07) than sham toads that homed (629.79 m +/-
402 334.19).
403 We next tested whether olfactory ablations influenced brain activity during homing.
404 Ablated toads had significantly less pS6-positive cells in their olfactory bulbs than sham toads,
405 confirming reduced olfactory function from chemical ablation (Wilcoxon, $W = 93.5$, $p =$
406 0.0074)(Figure 4C). While there was a significant interaction effect of ablation and brain region
407 on pS6 count ($\chi^2(4) = 19.23$, $p = 0.0071$)(Table S8), the magnitude of the effect was insufficient
408 to detect significant pairwise differences in region-specific activity between ablated and sham
409 groups (Table S9, Figure S8).

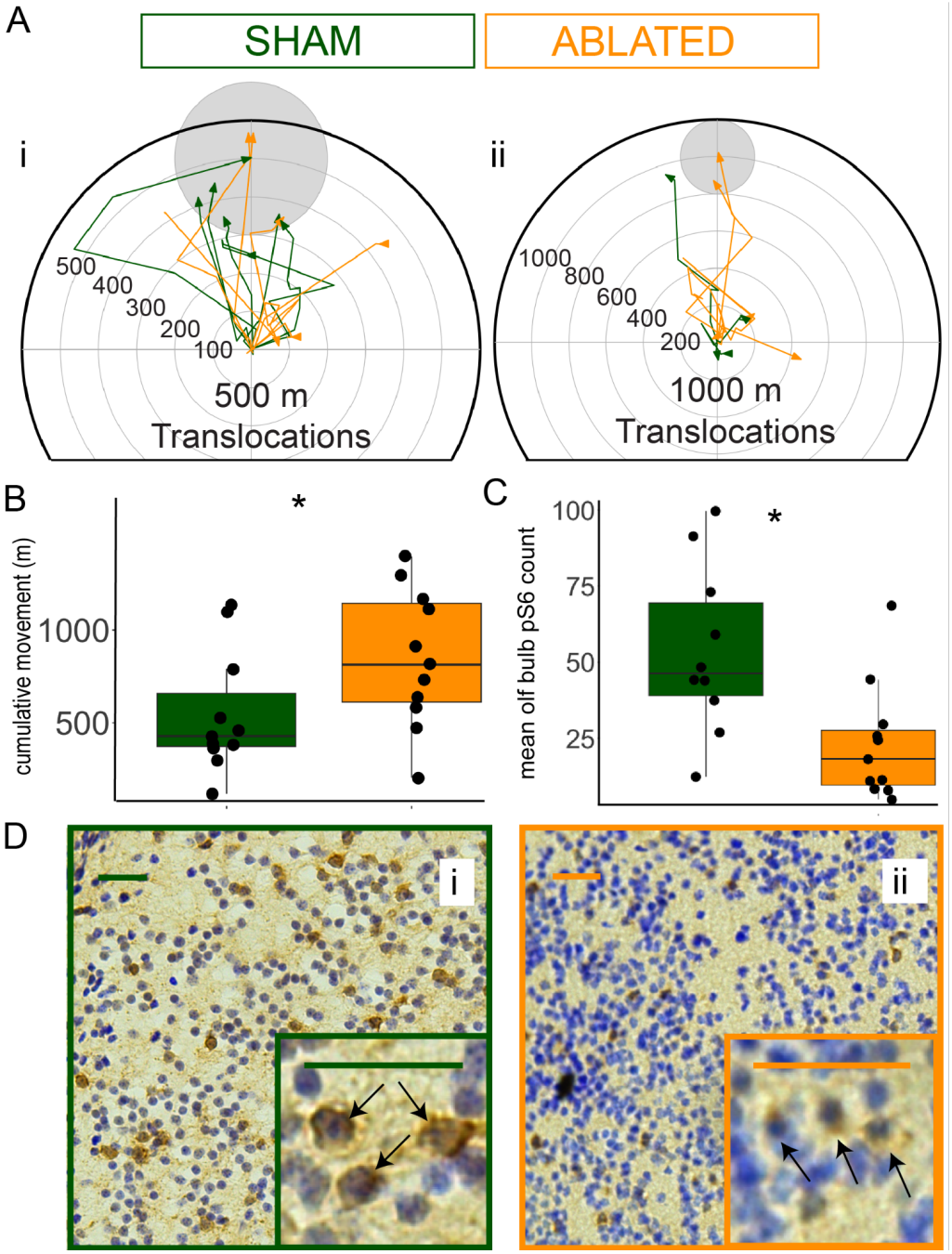

Figure 4 Olfactory ablation did not prevent homing but may affect trajectories. **(A)**Trajectories of toads in the olfactory ablation experiment translocated (i) 500-meters and (ii) 1000-meters. **(B)** Ablated toads exhibited higher cumulative movement post- translocation than sham toads (Wilcoxon, W = 93, p = 0.036). **(C)** Sham toads exhibited more pS6-positive cells in the olfactory bulb than ablated animals (Wilcoxon, W = 93.5, p = 0.0074). Each point represents the mean pS6 count across three sections of olfactory bulb in the same toad. **(D)** Representative areas of olfactory bulb (i) sham and (ii) ablated animals. Scale bars represent 25 µm. In inset, arrows indicate pS6-positive cells.

Magnetoreception ablations do not affect homing

Magnetoreception is a sensory modality linked to homing in several amphibians, so we tested the hypothesis that cane toads also use magnetoreception for homing. Movement range was significantly lower in both sham (Paired Wilcoxon, $V = 56$, $p = 0.042$) and ablated (Paired Wilcoxon, $V = 62$, $p = 0.0068$) animals (Figure 5C). There was no significant difference when comparing similarity in positions pre- and post-ablation (Wilcoxon, $W = 236$, $p = 0.90$). There were no significant differences of homing success ($\chi^2(1) = 1.27$, $p = 0.26$) or duration (Wilcoxon, $W = 5$, $p = 0.55$) between sham and ablated groups (ablated homing success = 55.6%, sham homing success = 30.0%) (Figure 5A). There was no difference in cumulative movement post-translocation between sham and ablated animals (Wilcoxon, $W = 55$, $p = 0.44$)(Figure 5B). While there was a significant interaction effect of ablation and brain region on pS6 count ($\chi^2(4) = 14.22$ $p = 0.0066$) (Table S10), the effect was insufficient to detect significant pairwise differences in activity in any region between ablated and sham groups (Figure S9, Table S11).

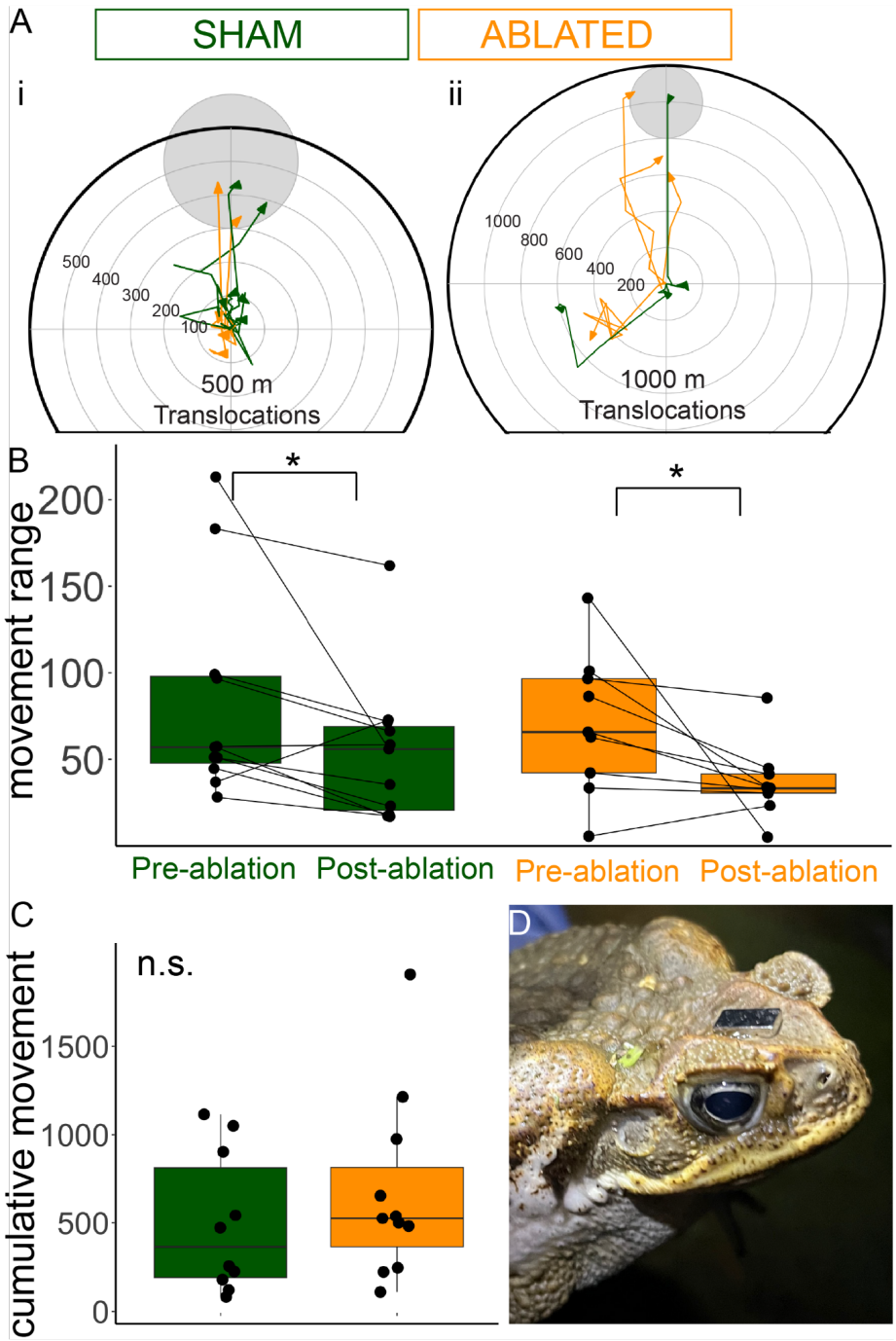

Figure 5 Magnetoreception ablation did not affect homing. (A) Trajectories of toads in the magnetoreception ablation experiment translocated (i) 500 meters and (ii) 1000 meters. **(B)** There was no difference in cumulative movement between sham and ablated toads. **(C)** Movement range varied significantly between pre- and post- ablation periods in both sham (Paired Wilcoxon, V = 56, p = 0.042) and ablated (Paired Wilcoxon, V = 62, p = 0.0068) toads. **(D)** Example of a toad with a magnet.

Discussion

In the current study, we examined the neural correlates and sensory basis of toad homing behavior. On a behavioral level, our results demonstrate an impressive map-like navigation ability in cane toads that did not lessen with increasing distance. On the neural level, we found that areas associated with navigation in mammals and birds, such as medial pallium and the septum, show increased activity in homing toads. Contrary to our predictions, we found that neither olfactory nor magnetic ablation alone disrupted homing, suggesting that this behavior is multimodal or based on other sensory modalities. These results contribute to filling in gaps in the understanding of amphibian spatial behaviors and neural architecture of vertebrate spatial cognition.

(a) Homing in toads

We found that toads displayed robust navigation from one kilometer. We observed that toads would often remain relatively stationary near their release site for some time before moving home. In other studies, direct homing following a stationary period has been observed in amphibians, including dendrobatid poison frogs [17,46], where homing abilities have been assumed to be adaptations to the spatial challenges of parental care. However, the homing abilities of cane toads suggest that refined navigation may not be reserved for species with specialized reproductive behaviors but is instead more widely present across amphibians. While our translocations exceeded the scale of routine movements of most toads, observations of occasional longer distance movements further suggest the presence of unexplored spatial abilities. Toad homing is even more impressive when considering their habitat and type of locomotion. Well-studied species capable of long distance navigation, such as pigeons, bats, and sea turtles, move through three-dimensions [57–59], allowing them to travel efficiently and overcome obstacles. That toads, confined to the ground and moving through obstructed

464 landscapes, including water-crossing, demonstrate direct navigation on the scale of a kilometer
465 reveals a remarkable capacity for spatial accuracy.

466 (b) Conserved role of pallial brain regions in spatial tasks

467 Our study contributes to the accumulating evidence that medial pallium is functionally
468 homologous to the mammalian hippocampus. The hippocampus has long been studied as a
469 center of navigation-related activity, as it holds neurons encoding place information [1,3] and is
470 required in the consolidation of spatial memory [2]. In birds, hippocampal lesions impair homing
471 in pigeons [57] and caching behavior is associated with larger hippocampal regions [60]. Fish
472 and turtles require homologous regions to solve a spatial task [10]. In amphibians, the medial
473 pallium has been identified as a region with similar developmental origins [31], but different
474 topography compared to amniotes [32]. Numerous neuronal activation and lesion studies in *R.*
475 *arenarum* suggest that the medial pallium is used in spatial tasks involving environmental
476 geometry [43] and feature cues [44]. The medial pallium is also shown to have increased activity
477 in tadpole-transporting poison frogs, a parental care task that requires high spatial precision
478 [41]. Our work corroborates this accumulating evidence that the medial pallium is involved in the
479 regulation of spatial behaviors in amphibians.

480 In our experiment, the lateral pallium showed increased activity in homing animals, while
481 the dorsal pallium did not. The role of these brain regions is not well studied in amphibians, but
482 both regions were shown to have increases in activity in a geometry based task in *R. arenarum*
483 [43]. Specifically, the lateral pallium showed increased activation when a visual cue was present
484 in the maze [43]. Anatomically, the pallial regions are interconnected, where the dorsal pallium
485 receives input from the lateral pallium and sends input to both the lateral and medial palliums,
486 and the lateral pallium connects to the medial pallium through the dorsal association bundle
487 [31]. Molecular profiling in salamanders suggests that the dorsal pallium resembles the
488 entorhinal cortex, another mammalian region involved in spatial coding [61]. The lateral pallium

also receives olfactory projections from the lateral olfactory tract [31], but we saw no evidence of ablation-related effects. Our experiment suggests distinct functionality may be expected from these regions in relation to navigation and more work is needed to understand specific functions associated with each area.

Our results showed increased activity in the septum in homing animals and increasing activity with faster homing. The medial septum has been implicated as an important regulator of spatial tasks in rodents, integrating the function of multiple circuits important to path integration [62]. In mammals, the lateral septum is also innervated by the hippocampus and holds neurons that exhibit reward-related place fields [63,64]. The role of the septum in navigation has not been as well studied outside of mammals. In amphibians, dendrites from the region connect with the medial pallium [31]. In *R. arenarum,* the septum showed no difference in activity in various conditions in a geometry based arena [35], but the medial septum did show heightened activity when animals located goal locations in relation to mating calls [45]. Further study in different navigation scenarios will be necessary to determine the septum's spatial function.

The striatum did not exhibit a difference based on homing conditions in our study. In mammals, the dorsal striatum has been implicated in memory and may be an integral part of neural circuitry that allows for the integration of sensory information in goal-directed spatial tasks [46]. For example, rats with dorsal striatum ablations could not perform a "reinforced stimulus response task" that required association of a maze arm food with light cues [65]. Interestingly, *R. arenarum* showed no differential activity of the striatum when completing a maze with a visual cue [43]. Future investigations of differences in region-associated spatial functions in amphibians and other vertebrates may pinpoint evolutionary changes in neural architecture.

One important caveat of our experiment is the relationship of the behavior and the timing of the endpoint. Homing animals were often stationary before moving back in a straight line, suggesting that the animals gain their bearings during this time. The snapshot of brain activity

515 captured by our pS6 immunohistochemistry does not account for this period, but for brain
516 activity happening during active homing. In future experiments, it would be useful to compare
517 this brain activity to that of the pre-homing period. Furthermore, we cannot conclude brain
518 regions with higher neural activity are required for homing behavior, as surgeries for brain
519 region-specific lesions in field conditions are very difficult. Future studies that incorporate
520 regional ablations or laboratory electrophysiology experiments would elucidate the specific roles
521 of these regions in amphibian navigation.

522 (c) Use of sensory systems in homing

523 Olfaction and magnetoreception have been implicated as important senses in navigation
524 studies in many amphibians [12–15,20,22,33,66] and other vertebrates [67,68]. Ablation
525 experiments strongly suggested that *B. bufo* relies on these senses to orient towards breeding
526 ponds [14], while *B. japonicus* seemed to rely solely on olfaction [15]. In a variety of salamander
527 species (*P. jordani*, *P. cinerus*, and *Notophthalmus viridescens*), both magnetoreception and
528 olfaction are used to orient and return to breeding streams [12,13,20,21]. However, in our study,
529 ablations did not disrupt *R. marina* homing. If either is used in cane toad navigation, they likely
530 work in combination with other senses or can compensate for each other.

531 Olfaction is used in multiple ways in navigation. Birds use odors distributed by wind to
532 determine goal locations [67]. Mammals sample odor information through sniffing to gain
533 information about their environment [68]. For amphibians, cues can come directly from aquatic
534 navigation goals, as seems to be the case in other toad species [14,15] and poison frogs [19].
535 Some salamanders may smell pheromones left by themselves or conspecifics [12]. Although we
536 did not observe a reduced success in homing with olfactory ablations, ablated toads showed
537 less similarity in space occupied post-ablation and moved more post-translocation than sham
538 toads. These results suggest that olfactory cues may help toads move in more direct paths and
539 are important for recognizing home range.

Magnetic orientation has been demonstrated in several amphibians [20,69,70], including other bufonids [14,71], but we found no evidence that magnetic field disruption impacts long-distance homing in cane toads. Other vertebrates, such as pigeons, sea turtles, and cetaceans exhibit impressive, long range navigation using magnetic cues [58,72,73]. However, much of the functional understanding of magnetoreception comes from salamanders [20,22,69]. Amphibian magnetoreception can be light dependent [21] and can be used not only as a compass, but also as a map sense (similarly to sea turtles and birds), even though their movements are on much smaller scales [74–76]. The effect of magnetoreception ablation on homing in other toad species is variable: *B. bufo* showed a less accuracy in orientation following translocation [14], while *B. japonicus* showed no response to ablation [15]. We cannot discount magnetoreception in *R. marina* on the basis of the current experiment, as the sham group performed poorly, with only 30% of toads returning home. We used methodology previously deployed in other amphibians [14,38], and performed our sham ablations by attaching brass bars in the same manner as the magnets. Both ablated and sham groups moved less following ablation, suggesting that the ablation potentially had an unintended consequence on behavior, possibly through physically covering the pineal gland, which contains photoreceptors [77] that may play a role in magnetic compass orientation.

Other senses may play a role in homing behavior. For example, both male and female cane toads exhibit phonotaxis to male display calls [78]. However, at our field sites, we observed toads distributed over several ponds and calling not always occurring when toads were homing, making the use of auditory cues unlikely. Vision may also play a role and is used by toads and poison frogs in laboratory arenas [43,79]. However, it is unlikely to be the primary sense involved in homing, as the visual information toads receive (ground-level, at night) is very limited in relation to the scale of the translocation distance. In addition, migratory amphibians with visual ablations are able to return home [13,14]. However, vision may be used to sense celestial cues, which provide compass information to some amphibians [66,80]. The field of

amphibian navigation would benefit from further study of the functionality of many possible modalities.

Conclusion

Our work combines intensive field-based telemetry with laboratory analysis of brain activity to study the neural and sensory basis of navigation. We demonstrate that cane toads (a) are capable of long-distance navigation to specific sites, (b) exhibit a potentially conserved function of the pallial and septal regions in relation to spatial behavior, and (c) likely have a multimodal basis of navigation. In the future, lesion experiments are needed to identify the specific function of pallium brain regions in navigation. In addition, further lab-based studies incorporating electrophysiology may characterize how neurons encode spatial information in amphibians. The current work provides a foundation upon which to begin further examinations of the neural and sensory basis of amphibian navigation through the integration of field and laboratory data.

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Data Availability

All data is included in supplementary materials.

Author contributions

Conceptualization: D.A.S., A.P., L.A.O; Methodology: D.A.S.; Formal analysis: D.A.S.; Investigation: D.A.S., D.P.M., C.G.; Resources: L.A.O.; Data curation: D.A.S., D.P.M., C.G; Writing – original draft: D.A.S.; Writing – review and editing: D.A.S., L.A.O., A.P., D.P.M., C.G.; Visualization: D.A.S.; Supervision: L.A.O.; Project administration: L.A.O.; Funding acquisition: D.A.S., L.A.O.

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Competing interests

The authors declare no competing or financial interests.

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