Integrative and Comparative Biology

Integrative and Comparative Biology, pp. 1–17
doi:10.1093/icb/icaa032

Society for Integrative and Comparative Biology

SYMPOSIUM

Cognitive Phenotype and Differential Gene Expression in a Hippocampal Homologue in Two Species of Frog

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From the symposium "Integrative Comparative Cognition: Can Neurobiology and Neurogenomics Inform Comparative Analyses of Cognitive Phenotype?" presented at the annual meeting of the Society for Integrative and Comparative Biology January 3–7, 2020 at Austin, Texas.

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Synopsis The complexity of an animal's interaction with its physical and/or social environment is thought to be associated with behavioral flexibility and cognitive phenotype, though we know little about this relationship in amphibians. We examined differences in cognitive phenotype in two species of frog with divergent natural histories. The green-and-black poison frog (*Dendrobates auratus*) is diurnal, displays enduring social interactions, and uses spatially distributed resources during parental care. Túngara frogs (*Physalaemus=Engystomops pustulosus*) are nocturnal, express only fleeting social interactions, and use ephemeral puddles to breed in a lek-type mating system. Comparing performance in identical discrimination tasks, we find that *D. auratus* made fewer errors when learning and displayed greater behavioral flexibility in reversal learning tasks than túngara frogs. Further, túngara frogs preferred to learn beacons that can be used in direct guidance whereas *D. auratus* preferred position cues that could be used to spatially orient relative to the goal. Behavioral flexibility and spatial cognition are associated with hippocampal function in mammals. Accordingly, we examined differential gene expression in the medial pallium, the amphibian homolog of the hippocampus. Our preliminary data indicate that genes related to learning and memory, synaptic plasticity, and neurogenesis were upregulated in *D. auratus*, while genes related to apoptosis were upregulated in túngara frogs, suggesting that these cellular processes could contribute to the differences in behavioral flexibility and spatial learning we observed between poison frogs and túngara frogs.

Introduction

Variation in animal cognition is generally associated with the complexity of the physical and/or social environments with which the animals cope (Godfrey-Smith 2002; de Waal and Tyack 2003). For example, spatial learning ability and navigational strategy are correlated with environmentally imposed navigational challenges that are required for survival and reproduction (Brodbeck 1994; Clayton and Krebs 1994; MacDonald 1997; Lavenex et al. 1998; Day et al. 1999a; Pravosudov and Clayton 2002). Likewise, complex social and physical environments are associated with higher levels of behavioral flexibility, which reflect how efficiently animals adapt their behavior to changes in the environment (Bond et al. 2007; Amici et al. 2008). While not all apparent differences in animal cognition can (or should) be attributed to adaptations to ecology, there is increasing evidence that selection can act on an animal's ability to learn and remember information in a flexible manner (Maille and Schradin 2016; Chen et al. 2019; Shaw et al. 2019; Sonnenberg et al. 2019). Further, species differences in spatial learning and behavioral flexibility are associated

with differences in the structure and function of the hippocampus (O'Keefe and Nadel 1978; Krebs et al. 1989; Day et al. 1999a).

In adopting terrestrial reproduction, poison frogs (family Dendrobatidae) have evolved complex interactions with their physical and social environments (Vági et al. 2019). They are diurnal, territorial, and engage in complex navigation in support of their parental care (Brown 2013). Females lay clutches on the leaf litter within the male's territory and the male guards and hydrates the fertilized eggs until they hatch. While poison frogs are terrestrial, their tadpoles are not. Thus, the parents must transfer newly hatched tadpoles from the leaf litter to a source of water where the tadpoles can complete development. Once tadpoles hatch, parents navigate directly to tadpole deposition sites without exploration (Beck et al. 2017), indicating that they are utilizing memory for site locations, although the cues they use to do so are unknown. For many species, tadpole deposition sites are an ephemeral and highly distributed resource. The ability to remember tadpole deposition sites has been attributed to spatial memory (Pašukonis et al. 2016) and we recently provided direct evidence that *Dendrobates auratus* readily learn, unlearn, and relearn spatial cues in a flexible manner (Liu et al. 2016; Liu et al. 2019). Further, the amphibian homolog of the hippocampus is active during the expression of parental care (Fischer et al. 2019) and is required for aspects of learning and memory (Bingman and Muzio 2017). In addition to these complex navigational challenges, poison frogs engage in enduring social interactions beyond those with territorial neighbors. For example, *D. auratus* females display mate guarding and deceptive courtship (Summers 2014) and *Ranitomeya imitator* is monogamous with ongoing care of the tadpoles that depends on interaction between the parents over the course of months (Brown et al. 2010).

To examine whether the ability of poison frogs to flexibly learn visual cues generalizes to species that lack complex spatial and social demands on cognition, we compared the performance of green-and-black poison frogs (*D. auratus)* to túngara frogs in the same mazes and training protocols. Túngara frogs (*Physalaemus=Engystomops pustulosus*) are nocturnal and breed opportunistically in temporary puddles, requiring only fleeting social interactions (Ryan 1985). The parents make a foam nest but provide no ongoing parental care. We chose to compare *D. auratus* with túngara frogs because túngara frogs are distributed in the same habitats as *D. auratus* and are similar in body size, allowing us to use the same maze and motivator for the two species, but túngara frogs do not exhibit the complex navigational feats, nor do they engage in the types of social interactions, for which poison frogs are known. To compare learning ability and behavioral flexibility between *D. auratus* and túngara frogs, we used a simple discrimination task using a two-arm maze in which we rewarded choice of the correct arm with access to a shelter and return to the home cage. We provided visual cues that were closely associated with the goal that animals could use as beacons in direct guidance and/or position cues on the maze walls that animals could use to spatially orient themselves relative to the goal. In a first experiment, beacons and position cues were both available during training. In a follow-up experiment, only position cues were provided. In a third experiment, we conducted a pilot study of differential gene expression of the medial pallium, the amphibian homolog of the hippocampus, of *D. auratus* and túngara frogs to explore whether such a contrast could be fruitful in identifying candidate genes that contribute to species differences in the ability to learn and remember visual cues in a flexible manner.

Experiment 1: Beacons and position cues available

In an earlier study, we found that female túngara frogs were successful at using visual cues as beacons in direct guidance to solve a two-arm maze constructed from painted bricks (Fig. 1A and B; Liu and Burmeister 2017). Using the same maze and procedure, in this study, we tested the ability of *D. auratus* to solve this task and compared their performance to that of the female túngara frogs reported in Liu and Burmeister (2017). While we previously reported our results from túngara frogs (Liu and Burmeister 2017), the two species were tested in the same apparatus by the same researchers and at similar times. Since male túngara frogs failed to learn this task, we restricted our comparison in this study to female túngara frogs. However, the sex difference observed in Experiment 1 is context dependent, as it is not observed in other training procedures (see Experiment 2; Ventura et al. 2019).

Materials and methods

Animals

We acquired our male ($n=5$) and female ($n=6$) poison frogs from Indoor Ecosystems, LLC (Whitehouse, OH) and compared them to the seven female túngara frogs originally published in Liu and Burmeister (2017). Both species were captive bred

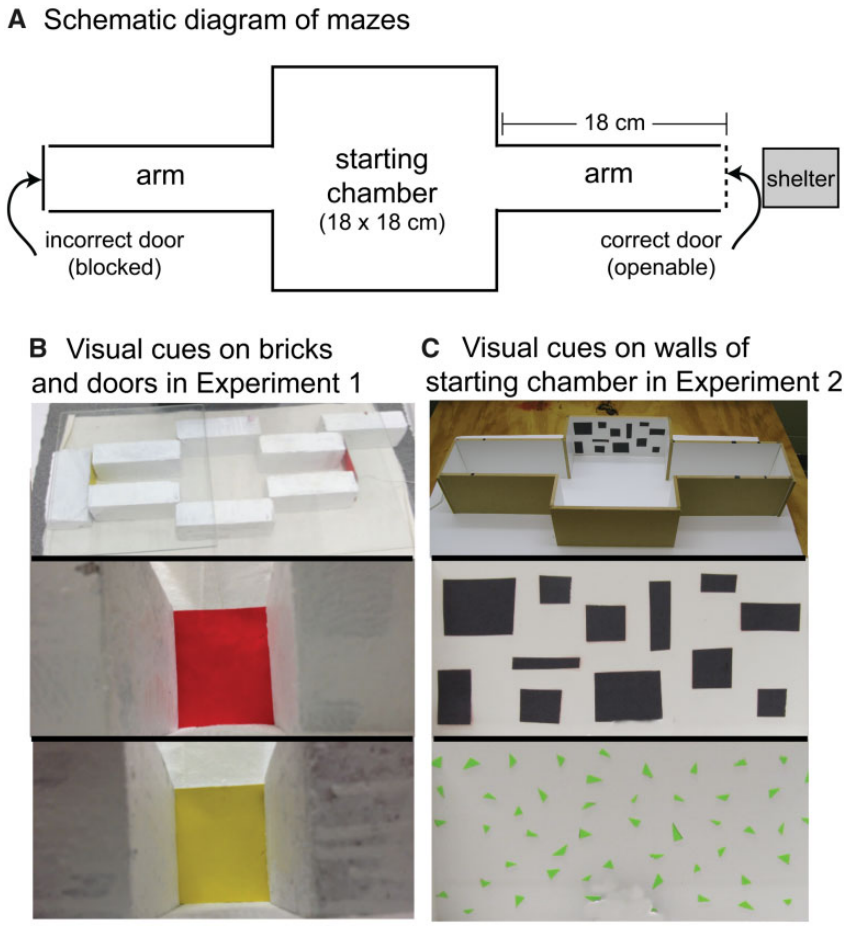

Fig. 1 Maze and cues used to test learning and flexibility in a two-option discrimination task. In the task, frogs were released in a starting chamber and could choose one of two arms to locate an exit leading to a shelter and return to the home cage (**A**). In Experiment 1, the maze was constructed of painted bricks and frogs could use cues on the bricks and/or on the doors leading out of the maze to choose the correct exit (**B**). In Experiment 2, the maze was constructed of uniform fiberboard and cues were only provided on the walls of the starting chamber (**C**).

for one or two generations. All experimental animals were sexually mature and naïve to any experiment at the time tested. We maintained the animals under conditions that approximated their natural habitat: 25°C, 80% relative humidity (RH), 12:12 light:dark cycle (lights on at 07:00 h). We fed them fruit flies that were dusted with calcium and vitamins three times per week. The University of North Carolina's Institution for Animal Use and Care Committee approved all procedures (protocol 14-026).

Apparatus

We used a two-arm maze composed of six bricks that were painted white (Fig. 1A and B; Liu and Burmeister 2017). Because bricks are not smooth, irregularities on the walls of the maze provided visual cues that could be used to spatially orient to the goal (i.e., position cues; Fig. 1B). The maze consists of a central starting chamber and two arms. We blocked the exits at the end of the arms with a red or yellow poster board to serve as doors that could act as beacons in direct guidance. We blocked the incorrect door from behind with a brick, which was not visible to the frog in the maze. We attached a string to the reverse side of the correct door, enabling us to open it. We rewarded the correct arm of the maze by associating it with access to a shelter and return to the home cage. The red door was always associated with the same maze arm and the same place in the room; thus, the beacon (colored doors), position cues (irregularities on bricks), and potential place cues were confounded.

To motivate the frogs to locate the correct exit, we created a bright, hot (37°C), and dry (10% RH) environment inside the maze. To maintain the maze temperature, we placed a heater along one longer side of the arena (providing an additional cue). To prevent the frogs from escaping, we covered the maze with glass. We covered the floor of the maze with absorbent paper that we replaced every other

day. We surrounded the maze with a 1.4 m-high white curtain in order to block other visual cues in the room.

Acclimation

Before training began, we acclimated the frogs to the arena in two trials over 2 days. During acclimation, we removed the colored doors, leaving both channels open. We released the frog in the middle of the starting chamber, with the frog oriented perpendicular to the arms leading out of the maze. The direction of release orientation in the first acclimation trial was determined arbitrarily and was switched (facing opposite wall) for the second acclimation trial. Once each frog exited the maze, we returned it to its home cage.

Acquisition

We closed the exits of the maze by placing a yellow and a red door at the end of each maze arm (Fig. 1A and B). During acquisition, the red door (correct door) could be opened, leading to the shelter, while the yellow one was blocked. We trained the frogs in two trials per day for nine successive days with an inter-trial interval of at least 1 h. In the first trial of the day, we placed the frog in the starting chamber oriented perpendicularly to the two arms, with the direction determined arbitrarily, and then alternated their orientation 180° for the second trial of that day in order to prevent them from solving the task by remembering turning direction. As in Liu and Burmeister (2017), frogs were given 3 min to locate to door and we defined the trial as successful if the frog knocked down the correct door directly, touched the correct door, or sat very close (<0.5 cm). In the latter case, we pulled the string to open the door. If the frogs failed to complete the task after 3 min, we defined it as an unsuccessful trial. Then we kept them for up to one more minute in the maze to motivate them in future trials. If they still could not get to the correct door, we opened the door and allowed them to exit. In all cases, we returned the frogs to their home cage upon exiting the maze.

Probe trials and reversal learning

Because door color (a beacon) was confounded with visual irregularities on the walls of the maze that could be used as position cues, we used probe trials to determine which cues were used to navigate to the door that was rewarded during training (i.e., door color, maze walls, or some other place cue). The first probe trial (both species) took place on Day 10 (after 9 days of acquisition) and tested the role of the beacons (door color) during learning by switching the locations of the doors. During each 3-min probe trial, we blocked both doors and released frogs perpendicular to the maze arms with randomly determined orientation for each frog. For both species, the first probe trial was followed by reversal learning. During reversal learning, we used the same maze and procedure as acquisition, except that the red door was blocked while the yellow door could be opened to lead to the exit from the maze. Hence, it required the frogs to reverse the associations they had learned during acquisition.

The results from the first probe trial indicated that the poison frogs did not use the door color to find the maze exit. Therefore, following reversal learning, we re-trained the same poison frogs for 6 days until they reached a success rate as high as that reached during the original 9 days of acquisition. Next, we conducted a probe trial in which we rotated the maze walls 180° (but not the doors or heater) and found that they searched for the maze exit in the location now indicated by the position cues on the maze walls.

Data analysis and statistics

We quantified behaviors from video recordings. We used success rate (mean number of successful trials per day) as a measure of performance across days. We used a repeated measures analysis of variance (ANOVA; species × day) to examine species differences in success rate (after arcsine transformation) in acquisition and reversal. We defined a learning criterion as five successful trials out of six and used a *t*-test to compare species in the number of trials to criterion. In addition to examining species differences, we also tested for a sex difference in acquisition and reversal of poison frogs using repeated measures ANOVA (sex × day). For probe trials, we quantified the duration (seconds) that the frogs spent in each maze arm as a measure of preference. To compare species, we then used a two-way ANOVA to examine the interaction between species and arm on time in the probe trial. For individual probe trials, we used a paired *t*-test to determine if the frogs expressed a preference for one arm of the maze.

In addition, to assess whether general species differences in activity or speed of movement might contribute to differences in acquisition, we divided the number of times individuals entered each zone of the maze (center chamber and two arms) by trial duration (latency to find the exit) and used a

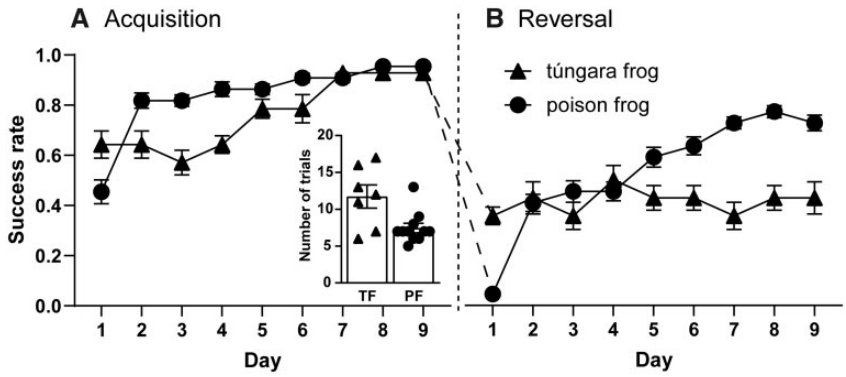

Fig. 2 Performance in a two-choice discrimination task when beacons and position cues were available (Experiment 1). During initial acquisition of the task (**A**), poison frogs had a steeper learning curve and reached the learning criterion earlier than túngara frogs (inset; $t_{16} = 2.9$, $P = 0.011$). During reversal learning in which the reward contingencies were reversed compared to acquisition (**B**), poison frogs were able to learn the new association but túngara frogs were not (species × day: $F_{8,128} = 2.24$, $P = 0.028$). TF, túngara frog; PF, poison frog.

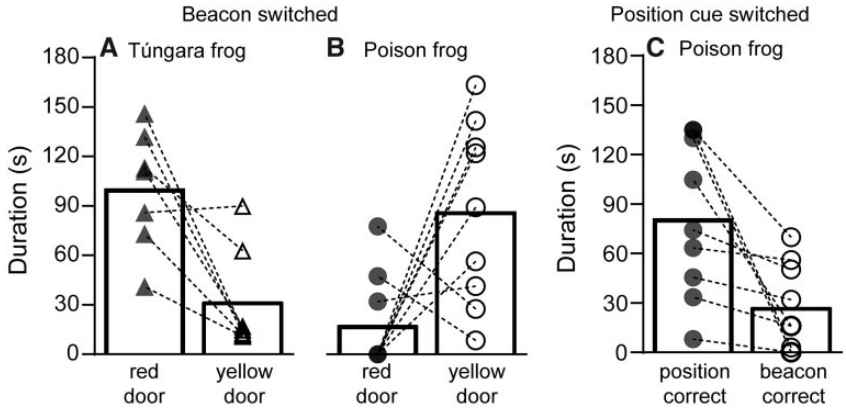

Fig. 3 Probe trials showed that túngara frogs and poison frogs used different cues when learning the discrimination task when beacons and position cues were available (Experiment 1). When the beacons (door color) were switched, túngara frogs searched in the arm that was now indicated by the red door (A), which had been rewarded during training, while poison frogs continued to search in the originally rewarded arm (now cued by the yellow door; **B**; species × arm, $F_{1,14} = 16.2$, $P = 0.001$). When the maze doors were left in their original locations but the position cues on the walls of the maze were rotated, poison frogs searched in the arm of the maze that was associated with the spatial cues during training (**C**; $t_8 = 3.14$, $P = 0.014$).

repeated measures ANOVA to test whether activity varied between species across days (species × day).

Results

The two species showed similar learning curves during acquisition (species × day: $F_{8,128} = 1.04$, $P = 0.41$; Fig. 2A), although poison frogs reached the learning criterion in fewer trials ($t_{16} = 2.9$, $P = 0.011$; Fig. 2A). Species did not differ in movement speed (day × species: $F_{8,128} = 0.46$, $P = 0.88$; species: $F_{1,16} = 0.69$, $P = 0.42$), suggesting that the species difference in acquisition were not simply a reflection of differences in activity or exploratory behavior. In addition, we found that the two species differed in their ability to learn the reversal task (species × day: $F_{8,128} = 2.243$, $P = 0.028$; Fig. 2B). While poison frogs successfully learned reversal by showing increasing success rate ($F_{8,80} = 6.46$, $P < 0.001$; linear trend: $F_{1,10} = 51.0$, $P < 0.001$), female túngara frogs failed to reverse their learned associations ($F_{8,48} = 0.6$, $P = 0.78$). In contrast to this species difference, we found no evidence that male and female *D. auratus* differed in their ability to acquire (sex: $F_{1,9} = 2.46$, $P = 0.15$; day × sex: $F_{8,72} = 1.63$, $P = 0.13$) or reverse (sex: $F_{1,9} = 1.1$, $P = 0.33$; day × sex: $F_{8,72} = 0.75$, $P = 0.65$) the learned associations.

Túngara frogs and poison frogs used different cues when learning the discrimination task (species × arm, $F_{1,14} = 16.2$, $P = 0.001$; Fig. 3A and B). While female túngara frogs learned to find the maze exit by following the door color ($t_6 = 3.7$, $P = 0.010$; Fig. 3A), there was little evidence that the poison

frogs, as a group, used the door color to find the exit ($t_{10} = 2.0$, $P = 0.07$; Fig. 3B). However, in the probe trial in which the maze was rotated 180° but the doors remained in their original locations, the poison frogs disregarded door color and spent more time in the arm that was associated with the position cues on the maze walls during training ($t_8 = 3.1$, $P = 0.014$; Fig. 3C).

Experiment 2: Position cues provided

In Experiment 1, we found that the poison frogs used the visual cues on the bricks of the maze rather than the provided beacons (door color) so we designed a maze that gave us better control over the position cues within the maze. This maze was constructed from uniform fiberboard; we provided visual cues on the walls of the starting chamber that could be used to spatially orient to the goal but no cues in the maze arms or on the door exits (Fig. 1A and C; Liu et al. 2016). We previously reported that the poison frogs used these position cues to learn to find the maze exit and could flexibly reverse their associations (Liu et al. 2016). Using the same procedure and maze, here we report results from túngara frogs that were simultaneously tested alongside the poison frogs from Liu et al. (2016). While we previously reported our results from *D. auratus* (Liu et al. 2016), both species were tested in the same apparatus by the same researchers at the same time.

The procedure was the same as Experiment 1 with a few differences, as described below: (1) we released the frogs in the start box in random orientation, (2) we used three trials per day, and (3) we trained individuals to a learning criterion (rather than a set number of days) to better assess reversal learning. In addition, we measured and analyzed errors during learning and reversal to better understand species differences in performance.

Materials and methods

Animals and acclimation

We compared 7 túngara frogs (3 male, 4 female) to the 10 *D. auratus* (4 male, 6 female) that were previously reported in Liu et al. (2016). Both species were captive bred for one or two generations.

Before training, we acclimated the frogs to the maze in two trials approximately 24 h apart. During acclimation, both doors were open. Unlike in Experiment 1, we released the frogs in the starting chamber from a small, overturned pot with a cardboard floor that was rotated during transfer from the home cage, resulting in an unpredictable orientation of the frog at the start of each trial. All frogs appeared highly motivated to leave the maze and successfully exited within 2 min.

Acquisition, probe trials, and reversal

For the initial learning trials (acquisition), we arbitrarily determined which door was correct. We trained the frogs with three trials per day with an inter-trial interval of 60–80 min. Once an individual reached the learning criterion (see below), we conducted a probe trial the next day. Methods for the probe trials were similar to Experiment 1: we blocked both doors during the 3-min probe and quantified time spent in each arm. In the first probe trial, we moved the walls of the starting chamber to the opposite side. We refer to the two arms as spatial-correct, which was the correct side indicated by the cues in the starting chamber, and original-correct, which was the correct arm during acquisition. Following the probe trial, we reversed the association so that the previously unrewarded arm was now rewarded. If an individual failed to improve during reversal, we trained it for twice the number of trials as during acquisition.

Because the results of the first probe trial indicated that the túngara frogs failed to use the position cues in the starting chamber, we conducted two additional probe trials following reversal. First, we retrained all túngara frogs to the acquisition task. Then we repeated the first probe trial (walls of starting chamber switched to the opposite sides) to confirm the results of the first probe (data not shown). Following another 3-day inter-probe training session, we conducted a probe trial in which the entire maze (starting chamber, arms, doors) was rotated 180° with respect to the room.

Data analysis and statistics

We operationally defined a learning criterion in order to determine when an individual's performance demonstrated sufficient evidence of learning, as follows. We used the outcomes on the first day of training (i.e., in naïve frogs) to estimate the random probability of performing a successful trial without error, which was 17%. We, therefore, defined our learning criterion as seven successful trials without error in nine sequential trials (77.8%), a percentage that differs significantly from the performance of naïve frogs (i.e., 17% vs. 77.8%; $P = 1.5 \times 10^{-4}$). Thus, our primary measure of learning is at the individual level. For direct comparison between species, we also recorded the number of trials to reach criterion for each individual.

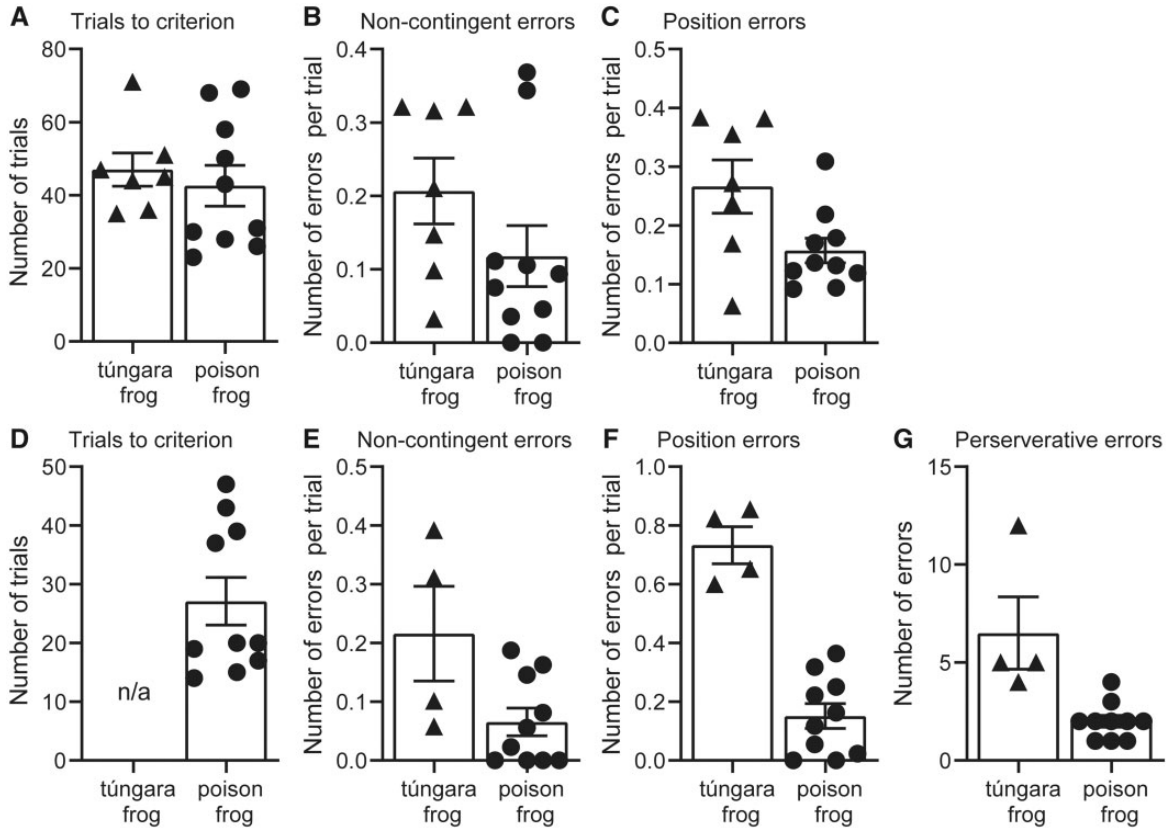

Fig. 4 Performance in a two-choice discrimination task when only position cues were provided (Experiment 2). Túngara frogs and poison frogs reached the learning criterion at similar rates during acquisition (**A**; $t_{15} = 0.57$, $P = 0.58$). During acquisition, while non-contingent error rates were similar (**B**; $t_{15} = 1.4$, $P = 0.18$), túngara frogs committed significantly more position errors than poison frogs (**C**; $t_{15} = 2.4$, $P = 0.029$). During reversal, poison frogs learned the new association in about half the trials compared to acquisition, while none of the túngara frogs reached the learning criterion in spite of being trained for twice the number of trials as acquisition (**D**). During reversal training, túngara frogs committed more non-contingent (**E**; $t_{12} = 2.46$, $P = 0.03$), position (**F**; $t_{12} = 7.47$, $P < 0.00001$), and perseverative errors (**G**; $t_{12} = 3.8$, $P = 0.003$).

In addition, we analyzed position errors, non-contingent errors, and perseverative errors as described in Liu et al. (2016). We defined position errors as cases in which a frog advanced half the length of the incorrect arm; frogs could commit multiple position errors within a single trial. We defined noncontingent errors as cases in which the frogs failed to approach either door. This error may reflect lack of an understanding that the task is to approach a door in order to exit or a lack of motivation to complete the task. During reversal learning, we also assessed perseverative errors, defined as the number of position errors before the first success after the start of reversal training. Perseverative errors reflect poor extinction (i.e., the lack of inhibition of previously learned responses; Mackintosh et al. 1968). Extinction is a critical step in learning a reversal task because an animal must inhibit previously learned responses in order to learn new associations.

Position errors and non-contingent errors were quantified in each individual in both training sessions as sum of session error divided by number of session trials, as each individual was trained for different numbers of trial. We then used *t*-tests to compare position errors, non-contingent errors, and perseverative errors between túngara frogs and poison frogs. For probe trials, we quantified the time frogs spent in each maze arm as a measure of preference. We used a paired *t*-test to determine whether the frogs preferred to stay in the maze arm that was associated with particular cues and ANOVA to test whether species differed in preference (species × arm).

Results

Túngara frogs and poison frogs acquired the task in similar number of trials ($t_{15} = 0.57$, $P = 0.58$; Fig. 4A). The two species had similar numbers of non-contingent errors ($t_{15} = 1.4$, $P = 0.18$; Fig. 4B) indicating that there were no major differences in exploratory behavior, motivation to complete the

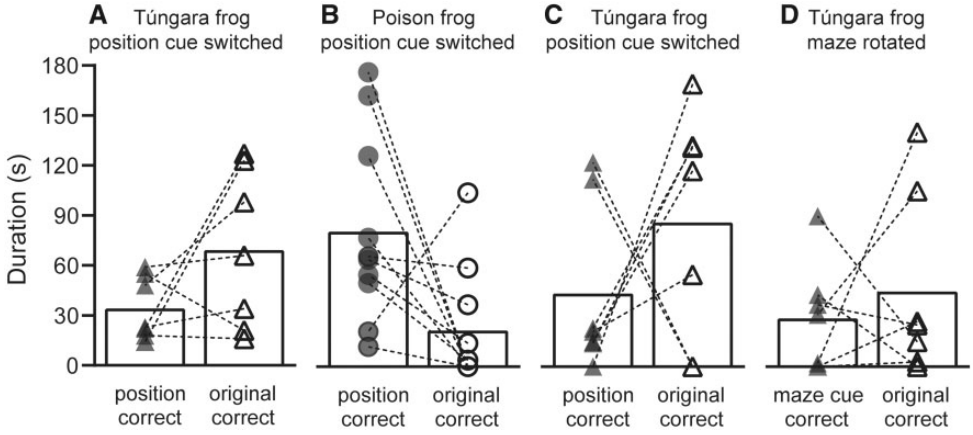

Fig. 5 Probe trials showed that túngara frogs and poison frogs used different cues when learning the discrimination task when only position cues were provided (Experiment 2). When the position cues on the walls of the starting chamber were switched to opposite sides, túngara frogs continued to search in the arm of the maze that was rewarded during acquisition (**A**) while poison frogs searched in the arm of the maze indicated by the new location of the position cues (**B**; species × arm: $F_{1,15} = 7.43$, $P = 0.016$). After reversal learning and retraining in the acquisition tasks, túngara frogs, as a group, continued to disregard the position cues when searching for the exit (**C**; $t_6 = 0.97$, $P = 0.37$), although two individuals now preferred the maze arm indicated by the position cues. To try to determine whether túngara frogs were using unintentional local cues on the maze, we rotated the entire maze (including position cues), but the results were ambiguous (**D**; $t_6 = 0.56$, $P = 0.58$).

task, or in learning that the goal of the task was to find an exit. However, poison frogs committed significantly fewer position errors during acquisition than túngara frogs ($t_{15} = 2.4$, $P = 0.029$; Fig. 4C), suggesting that poison frogs are better learners under these conditions.

During reversal learning, all poison frogs succeeded in learning the new association and they did so in about half the number of trials it took them to initially acquire the task, while none of the túngara frogs successfully reversed (Fig. 4D). Because we trained each túngara frog for twice the number of trials as it took them to reach the learning criterion during acquisition, we infer that the túngara frogs were unable to reverse their learned associations under these training conditions. During reversal, a difference in non-contingent errors emerged between the two species ($t_{12} = 2.46$, $P = 0.03$; Fig. 4E). As in acquisition, we found that during reversal, poison frogs committed fewer position errors per trial than túngara frogs ($t_{12} = 7.47$, $P < 0.00001$; Fig. 4F). Finally, túngara frogs committed significantly more perseverative errors ($t_{12} = 3.8$, $P = 0.003$; Fig. 4G), indicating that an inability to inhibit previously learned responses contributed to a failure to learn the new reward contingencies.

The probe trial in which the maze walls were switched to the opposite sides of the starting chamber showed that the two species utilized different cues when acquiring the task (species × arm: $F_{1,15} = 7.43$, $P = 0.016$; Fig. 5A and B). The túngara frogs continued to search for the exit in the originally-correct maze arm ($t_6 = 0.97$, $P = 0.37$; Fig. 5A) while the poison frogs, as a group, searched preferentially in the maze arm indicated by the position cues ($t_9 = 3.2$, $P = 0.011$; Fig. 5B). After reversal learning and retraining in the acquisition tasks, túngara frogs, as a group, continued to disregard the position cues when searching for the exit ($t_6 = 0.97$, $P = 0.37$; Fig. 5C), although two out of seven individuals now preferred the maze arm indicated by the position cues. The fact that two individual túngara frogs developed a preference for the arm associated with the position cues suggests that the difference in cue use we observed in these experiments reflect cue preferences, rather than an ability (or inability) to use a particular type of cue. The results of the probe trial in which the entire maze was rotated were ambiguous ($t_6 = 0.56$, $P = 0.58$; Fig. 5D), failing to confirm that the túngara frogs were using local cues in the maze to find the exit.

Experiment 3: Medial pallium gene expression

The behavioral results in these discrimination tasks suggest that, compared to túngara frogs, poison frogs are more adept at learning (i.e., learn faster and/or with fewer errors), prefer to learn position cues over beacons, and have greater behavioral flexibility. To assess whether baseline differences in medial pallium gene expression could identify candidate genes underlying these cognitive differences, we used RNAseq

to compare medial pallium gene expression in naive frogs of the two species. For this initial study, we assessed baseline differential gene expression in naive frogs, rather than changes in gene expression during learning, because our goal is to generate hypotheses regarding constitutive differences in medial pallium that could contribute to innate differences in learning potential.

Materials and methods

Sample preparation and RNA-Seq

We used eight experimentally naïve poison frogs (four male and four female) and túngara frogs (four male and four female). To collect tissue, we transferred individuals to the laboratory in their home tanks and allowed them to acclimate for 30 min. We then decapitated frogs without anesthesia, dissected the crania, and immersed in Tissue-Tek Optimal Cutting Temperature (O.C.T) compound (Sakura Finetek USA, Inc. Torrance, CA) before freezing in liquid nitrogen. All tissue samples were collected between 10:00 and 11:00 am. We sectioned brains at 200–300 μm and used a 350 μm diameter tissue punch to isolate the medial pallium which we preserved in TRIzol Reagent (Invitrogen) for ribonucleicacid (RNA) extraction. To generate sufficient RNA, we pooled tissue samples of four individuals of the same sex to produce two biological replicates per species (one male and one female), which results in an averaging of gene expression within the pool. RNA concentrations ranged from 21 to 31 μg/mL in 30 μL and RNA integrity numbers (RINs) were higher than eight. We created complementary DNA libraries using Invitrogen SuperScript II Reverse Transcriptase kits, performed library preparation using Illumina TruSeq (Illumina, Inc., San Diego, CA) and sequenced the transcriptomes on an Illumina HiSeq 2000 platform with 50 bp paired end reads.

Transcriptome assembly and reciprocal blast

We filtered sequences with quality control criterion (quality cut-off = 20; minimal percentage = 90%) through Galaxy version 15.03 (Goecks et al. 2010) and used Trinity (v 2.0.6) to assemble, de novo, reference transcriptomes for each species (Haas et al. 2013). In order to identify homologous transcripts between the two species, we ran a reciprocal BLAST search using the two assembled transcriptomes with an *e*-value threshold of 1^{-10}. A match was only recognized when two contigs from different assembled transcriptomes listed each other as the best hit (reciprocal best hits).

Annotation, contigs assembly, and gene expression

We blasted the sequences of contigs identified in both species against *Xenopus tropicalis* protein sequences as the reference genome with an *e*-value threshold of 1^{-10} for identifying a match. Assembled transcripts that matched the same protein sequence were treated as parts of the same gene. To build contiguous gene models, overlapping transcripts (exons or isoforms) mapping to the same *Xenopus* homolog were assembled as one gene according to their corresponding positions on the reference genome. The contiguous gene models from the two species were compared again using the results of the blast search against the *Xenopus* reference genome (secondary-assembled transcriptome). For each gene, we trimmed off non-homologous sequence that did not share the same fragments with *Xenopus* reference genome. The remaining trimmed version of each gene was reassembled in both species for downstream analysis (trimmed transcriptome). We then used the trimmed and secondarily-assembled transcriptomes as references to call the expression levels of each gene, using the Burrows–Wheeler Alignment tool (Li and Durbin 2009). We then transformed the rough expression values to reads per kilobase of transcript per million mapped reads to normalize expression level based on contig/gene length and the amount of RNA in the samples (Mortazavi et al. 2008).

Differential expression analysis

We used the nbinom test in the R Bioconductor package, DESeq2, to compare the expression levels of each reciprocally identified gene between the poison frog and the túngara frog (Love et al. 2014). We then used Benjamini–Hochberg procedure to adjust the *P* values for multiple comparisons (Benjamini and Hochberg 1995). Given the small sample size (two for each species), we set the threshold for significant evidence for differential expression as an adjusted $P < 0.05$ and a five-fold change (corresponding to log(2)-fold change of 2.2). We express the log(2)-fold change results as poison frog relative to túngara frog; that is, positive values represent increased expression in poison frog and negative values represent decreased expression.

Using the *Xenopus* orthologs, we matched the differentially expressed genes with their human homologs using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang et al. 2009) and bioDBnet (Mudunuri et al. 2009). We then imported the upregulated genes of each species to DAVID for a gene ontology (GO) enrichment

Table 1 GO enrichment analysis of upregulated genes in túngara frog

GO term	Percent observed	Percent expected	Fold enrichment	*P* value
Mitochondrion	17.188	5.426	3.168	0.002
Acetylation	29.688	16.686	1.779	0.013
Region of interest: Beta-galactoside binding	3.125	0.035	89.567	0.022
Transferase	17.188	8.100	2.122	0.029
GO:0044822~poly(A) RNA binding	15.789	6.921	2.281	0.038
hsa01100:Metabolic pathways	35.714	17.771	2.010	0.038
hsa00920:Sulfur metabolism	7.143	0.145	49.357	0.038
SM00276:GLECT	8.696	0.189	46.092	0.041
SM00908:SM00908	8.696	0.189	46.092	0.041
Lysosome	6.250	1.235	5.061	0.043
Lipid metabolism	7.813	2.100	3.720	0.043
GO:0055114~oxidation-reduction process	10.526	3.515	2.995	0.046
GO:0071257~cellular response to electrical stimulus	3.509	0.089	39.268	0.049

We defined percent observed as the number of upregulated túngara frog genes associated with the GO term divided by the number of all upregulated túngara frog genes times 100. We defined percent expected as the number of all genes in the GO term divided by the number of all genes times 100.

analysis with a threshold of Benjamini–Hochberg adjusted $P < 0.05$ for inclusion. Although the preferred method is to use species-specific gene background for enrichment analysis, here we applied the default human background genes in DAVID database. Given the fact that fundamental functions of genes are conserved in vertebrates, the distribution across GO terms should also be conserved (Ovcharenko et al. 2005). Therefore, using human genes as background should not affect result of enrichment analysis. The expression levels of genes that belong to learning-associated GO terms (i.e., learning and memory, synaptic plasticity, neurogenesis, and apoptosis) were compared between species.

Raw sequence data are available via NCBI (project accession number PRJNA626021).

Results

When the raw reads from RNA-Seq were trimmed for quality control, >98% reads were retained and resulted in 53,160,202 and 53,813,117 reads for túngara frog and poison frog, respectively. De novo assembly of the transcriptomes returned 76,742 and 102,174 transcripts (RNA-contigs which included isoforms) in the túngara frog and the poison frog, respectively. Alignment rate, which reflects the proportion of reads involving assembly, was about 70.8% and 72.1% in túngara frog and poison frog, respectively. The túngara frog and the poison frog had 55,265 contigs that matched with each other. In these matched contigs, 18,976 of the túngara frog and 28,939 of the poison frog contigs matched with a specific *Xenopus* protein in the blast search. In this step, contigs—which could be exons or isoforms—were merged for the second time to form the gene models (a.k.a. "secondarily-assembled transcriptomes") that were used in downstream analysis. The secondarily-assembled transcriptomes had 11,156 and 12,386 transcripts (genes) in the túngara frog and the poison frog, respectively. Finally, of the genes in the secondarily-assembled transcriptomes, we found that 9566 genes were expressed in both species. Of these genes, 87 were upregulated in the túngara frog, while 143 were upregulated in the poison frog. However, 964 túngara frog and 1987 poison frog assembled transcripts did not match any contig of the other species. Conservatively, we removed these "orphan" transcripts from analysis as we could not confidently associate them with a known gene function, or prove conclusively based on our current data and the absence of a reference genome, that they were truly missing in the other species.

DAVID and bioDBnet matched 64 (of 87) and 121 (of 143) of the differentially expressed transcripts of the túngara frog and poison frog, respectively, to human homologs. The results of the enrichment analysis are shown in Tables 1 and 2 for the two species. Upregulated genes were mainly enriched for the category of metal binding and transcription in the túngara frog, while they were enriched for axon extension in the poison frog. When we used learning-associated GO terms to

Table 2 GO enrichment analysis of upregulated genes in *D. auratus*

GO term	Percent observed	Percent expected	Fold enrichment	*P* value
Alternative splicing	75.207	51.507	1.460	1.04×10^{-7}
Splice variant	59.504	38.678	1.538	4.20×10^{-6}
Disease mutation	27.273	12.344	2.209	1.58×10^{-5}
Acetylation	31.405	16.686	1.882	9.05×10^{-5}
GO:0030424~axon	6.195	1.291	4.798	0.003
Nucleotide-binding	17.355	8.688	1.998	0.003
Metal-binding	28.099	17.683	1.589	0.005
Phosphoprotein	52.066	40.111	1.298	0.007
Mental retardation	5.785	1.434	4.034	0.008
Metal ion-binding site: Zinc 1	3.306	0.374	8.843	0.010
Metal ion-binding site: Zinc 2	3.306	0.379	8.727	0.011
Cytoskeleton	11.570	5.475	2.113	0.014
GO:0031965~nuclear membrane	5.310	1.286	4.130	0.015
GO:0006611~protein export from nucleus	2.804	0.179	15.689	0.015
RNA-binding	8.264	3.238	2.552	0.016
Mutagenesis site	18.182	10.921	1.665	0.020
GO:0005829~cytosol	27.434	18.663	1.470	0.022
Neurodegeneration	4.959	1.391	3.566	0.026
GO:0007190~activation of adenylate cyclase activity	2.804	0.238	11.767	0.026
Coiled coil	22.314	14.800	1.508	0.027
GO:0030819~positive regulation of cAMP biosynthetic process	2.804	0.256	10.946	0.030
GO:0031175~neuron projection development	3.738	0.637	5.865	0.030
Zinc	18.182	11.430	1.591	0.031
Sodium transport	3.306	0.569	5.811	0.031
ATP-binding	12.397	6.763	1.833	0.032
Sodium	3.306	0.603	5.483	0.036
Epilepsy	3.306	0.613	5.396	0.038
GO:0009267~cellular response to starvation	2.804	0.292	9.605	0.038
Compositionally biased region: Poly-pro	5.785	2.098	2.757	0.041
GO:0030659~cytoplasmic vesicle membrane	3.540	0.692	5.114	0.043
GO:0005938~cell cortex	3.540	0.698	5.073	0.044
Compositionally biased region: Lys-rich	3.306	0.653	5.063	0.044
GO:0042802~identical protein binding	8.411	3.770	2.231	0.047
GO:0005634~nucleus	38.053	29.832	1.276	0.049
Nucleus	33.058	25.447	1.299	0.050
GO:0005049~nuclear export signal receptor activity	1.869	0.049	38.114	0.051
Cell projection	7.438	3.408	2.182	0.053
GO:0030529~intracellular ribonucleoprotein complex	3.540	0.764	4.635	0.054
Protein biosynthesis	3.306	0.739	4.473	0.060

We defined percent observed as the number of upregulated túngara frog genes associated with the GO term divided by the number of all upregulated túngara frog genes times 100. We defined percent expected as the number of all genes in the GO term divided by the number of all genes times 100.

categorize these differentially expressed genes, we found that all of the 18 genes associated with learning and memory, all of the 18 genes related to synaptic plasticity, and 20 out of the 23 genes related to neurogenesis were upregulated in the poison frog. In contrast, 20 out of the 26 genes related to apoptosis and all of the 14 genes that negatively regulate biochemical synthesis and metabolism were

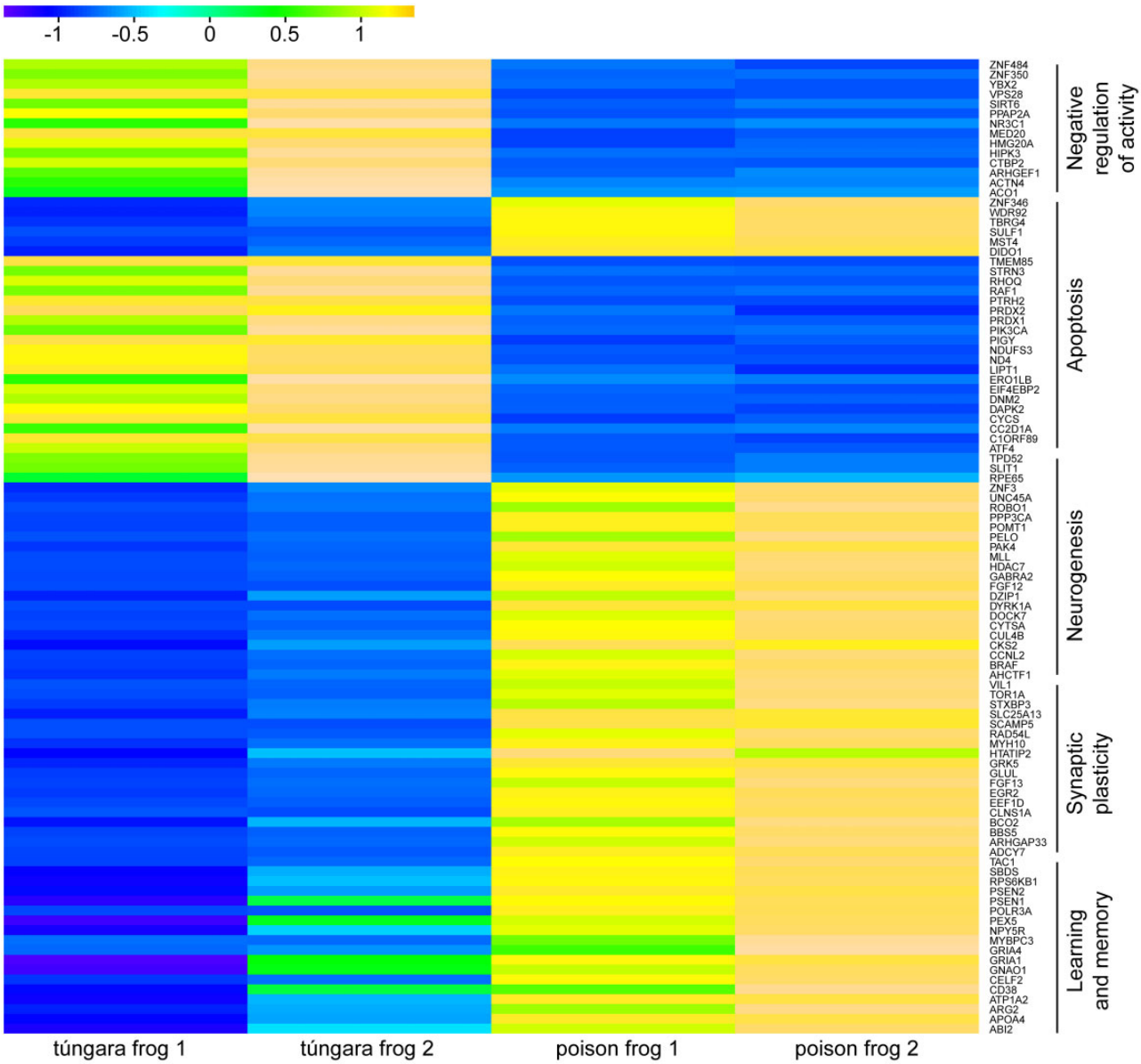

Fig. 6 Genes showing consistent patterns of differential expression between the two species of frog with different cognitive phenotypes. Using GO terms to categorize genes, we showed that among the transcripts with putative homologs to humans there is a strong enrichment for up-regulated genes associated with learning and brain development in poison frogs. This pattern is consistent with our observation that poison frogs show strong learning phenotypes, especially learning reversal.

downregulated in the poison frog (Fig. 6). The other half of the differentially expressed genes was unknown function and could not be categorized.

The candidate genes identified by our GO analysis include genes with causal roles in synaptic plasticity, neurogenesis, and cognition in mammals. For example, candidate genes linked to synaptic plasticity included BBS5 (log(2) fold change = 3.84, adjusted $P = 4.24 \times 10^{-15}$) and SULF1 (log(2) fold change = 5.90, adjusted $P = 6.74 \times 10^{-06}$), which are associated with dendritic growth and spine density (Haq et al. 2019), SLIT1 (log(2) fold change = −2.61, adjusted $P = 2.52 \times 10^{-10}$), which regulates axon growth (Skutella and Nitsch 2001), and ATF4 (log(2) fold change = −4.39, adjusted $P = 4.85 \times 10^{-17}$), which is associated with synaptic maintenance and memory (Wei et al. 2015). Candidate genes implicated in neurogenesis included CUL4B (log(2) fold change = 2.95, adjusted $P = 9.18 \times 10^{-07}$), which is associated with interneuron number (Chen et al. 2012; Liu et al. 2012), and FGF12 (log(2) fold change = 5.51, adjusted $P = 0.0004$) and FGF13 (log(2) fold change = 3.17, adjusted $P = 0.0011$), which regulate neurogenesis and neural

migration in both mammals and frogs (Wu et al. 2012; Zhang et al. 2012; Nishimoto and Nishida 2007) and are linked to learning and memory (Wu et al. 2012). Candidate genes linked to hippocampal degeneration and cognitive decline in mammals included PSEN1 (log(2) fold change = 2.33, adjusted $P = 0.0346$) and PSEN2 (log(2) fold change = 2.41, adjusted $P = 0.0016$), which are linked to neurogenesis (Delabio et al. 2014), and APOA4 (log(2) fold change = 2.38, adjusted $P = 0.0009$), which is linked to brain metabolism (Goldberg et al. 1990) and is required for normal expression of spatial memory (Cui et al. 2011). Finally, we identified candidate genes that regulate biochemical synthesis and metabolism including SIRT6 (log(2) fold change = −3.55, adjusted $P = 5.22 \times 10^{-10}$), which inhibits protein synthesis pathways and impairs memory formation (Yin et al. 2016).

Discussion

Using two-choice discrimination tasks, we compared cognitive phenotype of two species of frog that differ in natural history to investigate the relationship between evolutionary ecology and cognition in amphibians. We found that the green-and-black poison frog (*D. auratus*), which is diurnal and experiences greater social and spatial complexity, preferred position cues when learning, learned faster with fewer errors, and had greater behavioral flexibility compared to the túngara frog (*P. pustulosus*), which shares similar habitat with the poison frog but interacts with the physical and social environment in less complex ways. Using a pilot RNA-seq experiment, we generated hypotheses regarding baseline differences in the medial pallium that could contribute to species differences in learning ability. We found that differentially expressed genes associated with learning, synaptic plasticity, and neurogenesis were up-regulated in poison frog, while genes associated with apoptosis and negative regulation of cellular activity were up-regulated in túngara frog.

Cue preference

The types of information that animals use to navigate must be suited to their natural history. For example, when tested in the dark without visual cues, a burrowing rodent is more adept at response learning (i.e., remembering turns) compared to a non-burrowing species (Bruck et al. 2017). The great tit, a non-food storing species, is more adept at observational learning of cache locations than are the food-storing marsh tits (Urhan et al. 2017). Likewise, when we contrasted the diurnal, territorial, and parental poison frog *D. auratus* with the nocturnal, lek-breeding túngara frog, we find that the types of information the species depended on to navigate in a maze were different. The fact that túngara frogs prefer to use beacons that are physically associated with a goal makes sense considering that, in the nocturnal environment, visual cues that are physically distant from a goal are likely of little use for orientation.

In contrast, poison frogs used position cues to find the goal, presumably by associating the direction from the cue to the goal, an element of spatial learning (Mackintosh 2002). While a two-arm maze does not allow us to directly test whether the poison frogs were using the spatial relationships among the visual cues and the goal, our previous results provide unequivocal evidence that they are capable of doing so (Liu et al. 2019). A preference for distributed visual cues makes sense for the diurnal poison frogs that must remember territorial boundaries and make use of spatially dispersed resources for parental care, as such cues allow for more flexible navigation. Imagine, for example, a heavy storm fundamentally changing the microhabitat on the forest floor, eliminating or rearranging local cues. Yet some portion of distal cues, such as trees and bushes will remain intact. In this way, poison frogs are similar to food-storing birds that depend more on spatial cues rather than local cues associated with a remembered target (Brodbeck and Shettleworth 1995).

Learning and flexibility

Learning ability and behavioral flexibility allow animals to respond to complex physical and social environments in adaptive ways and they have been tied to ecological demands on social and spatial cognition (Godfrey-Smith 2002; Dunbar and Shultz 2007). For example, lizards that actively forage for distributed prey items are more successful in a reversal task than a sit-and-wait predator (Day et al. 1999b). We found that while túngara frogs and poison frogs were both capable of solving the two-arm discrimination task, poison frogs did so more quickly (Experiment 1) and with fewer errors (Experiment 2) than túngara frogs. The error analyses suggest that the poison frogs outperformed the túngara frogs due to faster correction of position errors rather than to higher familiarity with the maze or higher levels of motivation, which are linked to non-contingency errors. But perhaps more dramatically, the poison frogs clearly outperformed the túngara frogs in reversal learning, typically considered an assessment of the ability to adapt behavior to meet changing environmental demands. The túngara frogs failed to reverse

in either iteration of the task; in contrast, poison frogs improve their performance during reversal and are capable of serial reversal learning in which reward contingencies are sequentially reversed (Liu et al. 2016). One reason for the failure of túngara frogs in reversal learning was their relatively higher rates of preservative errors, a reflection of an inability to inhibit previously learned responses (Mackintosh et al. 1968). Thus, like pinyon jays (Bond et al. 2007), poison frogs show greater behavioral flexibility during reversal learning compared to species with lower levels of social complexity. Broadly, we conclude that, under the present conditions, poison frogs are better learners and have greater levels of behavioral flexibility than túngara frogs. How robust the species differences are, and their relationship with aspects of the species' natural history requires additional study.

Medial pallium transcriptome

Spatial cue learning and behavioral flexibility are both associated with the hippocampus in mammals and birds (Morris et al. 1982; Day 2003; Seeger et al. 2004). For example, food-storing birds, which outperform non-storers in spatial learning, have larger hippocampal sizes (Krebs et al. 1989). The higher volume of the hippocampus in food-storing birds has been partly attributed to a higher rate of neurogenesis in adults (Pravosudov and Smulders 2010; Sherry and Hoshooley 2010). Learning, especially long-term memory formation, relies on dendrite growth and neurogenesis (Aimone et al. 2006). Hence, a higher neurogenesis rates in the hippocampus could be associated with better spatial learning ability (Deng et al. 2010). The converse of neurogenesis is apoptosis, which has been negatively associated with spatial learning ability in mountain chickadees (Pravosudov et al. 2013). Consistent with these findings, our preliminary data suggest that neurogenesis-associated genes are more highly expressed, and apoptosis-associated genes are less highly expressed, in the medial pallium of poison frog compared to the túngara frog.

Synaptic plasticity is defined as the ability to modify synaptic strength due to changes in neural activity and is an essential mechanism underlying learning. We found that all differentially expressed genes associated with synaptic plasticity were upregulated in poison frogs. Similar results were found in chickadees, in that a population that showed better spatial memory upregulated most synaptic plasticity-related genes compared to a population with poorer spatial memory (Pravosudov et al. 2013). In addition, we found that genes that negatively regulate cellular activity were downregulated in the poison frogs, indicating higher levels of protein synthesis, steroid synthesis, and cholesterol and fatty acid metabolism. Because protein synthesis in the hippocampus is critical in long-term memory formation (Davis and Squire 1984) and bilayer lipid membranes, cholesterol, and fatty acids are important material in neurogenesis (Koudinov and Koudinova 2001; Das 2003), our results indicate that better learning abilities in the poison frog may also be associated with a higher level of hippocampal biosynthesis and metabolism.

In addition to identifying cellular processes that may contribute to species differences in medial pallium function, we found that the relative direction of expression (up- or down-regulated) across candidate genes was consistent with predictions based on the genes' function in mammals. These parallels suggest that, while our analysis is limited (see below), it has revealed a pattern convergent evolution of the cellular processes of spatial memory in mammals and poison frogs in spite of significant divergences between the mammalian hippocampus and the amphibian medial pallium. This raises the potential for using transcriptomes of the hippocampus or its homologs as biomarkers for cognitive phenotype in a broad range of vertebrates. The poison frog family, with species variation in navigational demands during parental care, may provide an opportunity to test the potential of transcriptomics to predict cognitive phenotype at the level of species.

Because RNA isolated from the medial pallium of individuals was not sufficient for sequencing at the time we performed this experiment, we had to pool RNA extracted from multiple individuals, reducing our biological replicates. To minimize the potential for false-positives under these conditions, we used a strict criterion (i.e., fold-fold change) to call differentially expressed genes; in the future, confirmatory studies using orthogonal approaches such as quantitative PCR or *in situ* hybridization will be required to follow up on particular genes. Another constraint on our transcriptome dataset was that the two samples from each species were from different sexes (i.e., we lacked replication of sex within a species). As such, any gene with strongly sexually dimorphic expression would not be identified as differentially expressed between the species. It is worth noting, however, that to date, we have detected only modest cognitive differences between male and female túngara frogs (Ventura et al. 2019) and none between male and female *D. auratus* (Experiment 1 and unpublished data). Nonetheless, the differentially

expressed genes we identified likely only represent genes that are similarly expressed between males and females and we may be missing some genes of interest. Additionally, neutral evolution of gene expression could also contribute to differences among species (Khaitovich et al. 2005). That said, the parallels across taxa noted above are hard to explain via a neutral evolutionary model and suggest that our approach is detecting a non-random signal. Thus, while our differential gene expression analysis provides a basis for future investigations of the neural mechanisms of cognitive phenotype in frogs, our small sample size and lack of replication requires that our conclusions remain cautious, particularly with regard to individual candidate genes, until additional studies can be completed.

Conclusions

We found that the complexity of interactions with the physical and social environment predicted aspects of cognitive phenotype in two species of frog. The species also differed in expression of genes related to neurogenesis, synaptic plasticity, neuronal apoptosis, and negative regulation of biosynthesis and metabolism in the medial pallium. While the amphibian medial pallium is the unequivocal homolog of the amniote hippocampus (Butler and Hodos 1996), it lacks the trilaminar organization that characterizes the hippocampus (Striedter 2016). Further, the medial pallium receives sensory input primarily from the thalamus or olfactory bulb (Northcutt and Ronan 1992; Roth and Westhoff 1999) rather than receiving substantial indirect sensory projections through sensory cortex (or cortical homologs) as in amniotes (Striedter 2016). Although functional information about the medial pallium is scant, preliminary data suggest that, unlike the mammalian hippocampus (Morris et al. 1982), the medial pallium is required for spatial learning, learning to use beacons in direct guidance, and response learning (i.e., navigation based on turn direction at a choice point; Bingman and Muzio 2017). In spite of these apparent differences between the structure and function of the medial pallium and the amniote hippocampus, our results suggest that convergent evolution of rapid and flexible spatial cue learning in poison frogs may have been accompanied by selection for the same cellular processes that contribute to these cognitive abilities in birds and mammals. Following additional functional studies to confirm our preliminary gene expression results, our findings could provide evidence of conserved mechanisms underlying the evolution of the hippocampus and associated cognition in all tetrapods.

Acknowledgments

Funds to conduct the research were provided by University of North Carolina. The Society for Integrative and Comparative Biology and the Company of Biologists generously funded our symposium presentation.

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