Artificial selection for large and small relative brain size in guppies (*Poecilia reticulata*) results in differences in cognitive ability

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Summary

Vertebrate brain size is remarkably variable at all taxonomic levels. Brains of mammals for example, range from 0.1 gram in small bats (*Chiroptera*) to about 8-9 kilos in Sperm whales (*Physeter macrocephalus*). But what does this variation in size really mean? The link between brain size and cognition is debated due to, for instance the difficulties of comparing cognitive ability in different species. A large number of comparative studies continue to provide information about correlations found both within and between species. The relative size of the brain is an example of a popular measurement that correlates with cognitive ability. But to date, no experimental studies have yielded any proof causality between relative brain size and cognitive ability. Here I used guppies selected for either large or small relative brain size to investigate differences in cognitive performance of a quantity discrimination task. The results from this experiment provide experimental evidence that relative brain size is important for cognitive ability, and that a difference in cognitive ability could be obtained already after two generations of selection experiments on relative brain size in a vertebrate.

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1. Introduction

Brain size has been found to be enormously variable among and within vertebrate species (Striedter 2005). The smallest brains are found in *agnathans*, and within this radiation the brain size of hagfish (*Myxini*) is two to three times larger than the brains of lampreys (*Petromyzontidae*) with the same body size. Among mammals, the largest brains in relation to body size is found in primates and cetaceans, while the smallest are found in non-placental mammals, for example marsupials (*Marsupialia*) and rodents (*Rodentia*) (Northcutt 2002). These variations in brain size are believed to be due to different selection pressures. One such selection pressure may be social living, also mating strategy, diet and habitat complexity have been found to play an important role (Dunbar 1998, Gonzalez-Voyer & Kolm 2010, Kolm *et al.* 2009 Pollen *et al.* 2007)

A huge variation across and within vertebrates is also found when it comes to cognitive ability. Cognitive ability refers to all mechanisms animals use to acquire, process, store and act on information from the environment (Shettleworth 2010). How variation in cognitive abilities is related to these variations in brain size is, however, still disputed (Healy & Rowe 2007, Chittka & Niven 2009).

A general hypothesis is that animals with larger brains possess higher cognitive abilities because of more neurons in, for example, the neocortex, which is the outermost layer of the cerebral cortex (Finlay & Darlington 1995). Based on a simple comparison between humans and shrews the premise seems to be correct. The brains of humans are larger than the brains of shrews, and humans are also considered to possess higher cognitive abilities. Moreover, Deaner *et al.* (2007) found that within non-human primates absolute brain size best predicted the variance in cognitive abilities. However, if a comparison is made between an elephant, which has a brain weight of approximate 4 kilos, and a human, which has an average brain weight of 1.35 kilos, the premise fails, since humans are generally assumed to possess higher cognitive abilities than elephants (Striedter 2005).

Something that has been a lot more investigated, and that seems to more strongly correlate with cognitive abilities is various quantifications of relative brain size, for instance the encephalization quotient (EQ). Across species, a larger brain most often comes with a larger body and by using a mathematical equation, a predicted brain size can be calculated. Following quantification of the actual brain size, a ratio between the predicted and the actual size can then be calculated for a group of organisms of interest. This ratio is known as the EQ and it indicates how much an observed brain mass deviates from the expected brain mass based on body size. When a comparison between mammals is made, it is shown that humans possess the highest EQ out of all mammals (Jerison 1973). This is based on the idea that a brain should be of a certain size to be able to process body-related information and the brain mass that exceeds this is thus used for cognitive abilities. This would mean that animals with small brains but high EQ would possess higher cognitive abilities than animals with larger brains and smaller EQ. When comparing across individuals of the same species, another form of relative brain size is often used, most often simply through using body size as a covariate in the analyses (*e.g.* Kolm *et al.* 2009).

A lot of comparative studies have been undertaken in the field of brain evolution and cognition. Some of these studies have been criticised (for review see Healy & Rowe 2007), but at the same time they have provided important information of correlations between various brain properties, cognition and ecology. But the next step forward is to investigate causations. Hence, proper experimental tests, for instance based on selection experiments followed by assays of cognitive ability, would be of great interest. Such approaches could accurately test many of the hypotheses regarding brain evolution and the link between brain morphology and cognitive ability.

In many animal species cognitive abilities have shown to be of great importance when it comes to survival and/or reproduction. One example is a recent laboratory experiment done with gerbils (*Gerbillinae*). When the gerbils were presented with a simulated predator, they could choose the best escape routes by taking into account relative distances to a safe refuge, barriers and their own location in relation to the shelter. Hence, gerbils that possess this ability to a wider extent would be better at escaping a predator and thereby increase their survival (Ellard & Eller 2009). Furthermore, animals may use landmarks to navigate their habitat to find food and shelter. Studies of three-spined stickleback have shown that they use different cues to navigate depending on which habitat they originally come from. To be able to use the best cues in a certain environment to, for example, be able to find food may maximize their food intake, and this is probably why these differences are present (Odling-Smee & Braithwaite 2003). Specific cognitive abilities, such as discriminating between quantities, can also be of great importance for various animals. Predators may for example choose which prey to hunt based on their own group size and the prey group size. By doing this, they can choose to attack the group and prey that would result in the best chance of successful capture and also highest profit (Scheel 1993). For prey species it might be more beneficial to join a larger group than a smaller one to avoid predation (Hamilton 1971). In recent years, the ability to discriminate between quantities has been found not only in higher vertebrates, but also lower ones, such as salamanders and fish (Bisazza *et al.* 2010, Krusche *et al.* 2010).

In this study, recently developed guppy lines with large and small brains were used to experimentally test if relative brain size matters for cognitive ability. Guppies are an ideal model organism for selection and cognition experiments of this kind. The females are live-bearers and after mating, females usually give birth to a clutch of up to 20 offspring within 4 weeks. Generation time can be as short as three months in the lab. The cognitive ability of fish has in recent years received more attention and the quantity discrimination found in guppies mentioned above is one example of this. Hence the guppy is also a suitable model organism for cognitive tests, and in this experiment their quantity discrimination ability was used. In order to investigate whether brain size matters for cognition an associative learning approach were used. Guppies were presented with two stimuli, one showing 4 objects and one showing 2 objects, and were taught to associate the former with a food reward. A test was then applied were no food was presented to investigate whether individuals would approach the stimuli with four objects, and thus had learnt to discriminate between the stimuli. Under the assumption that a larger brain leads to higher cognitive ability, I predicted that learning success would be higher in individuals from the large-brained lines.

2. Materials and Methods

2.1 Artificial selection

Guppies were obtained from Prof. Gunilla Rosenqvist's lab in Trondheim University, where fishes had been kept since 1998, but were originally imported from Trinidad. In the lab they were kept in large populations, with more than 500 individuals and were allowed to reproduce freely. From these larger populations four smaller populations were formed that each consisted of 50 males and 50 females. Juveniles from these populations were transferred to smaller tanks (4 L) in groups of up to 6 individuals. Males and females in these tanks were separated at first signs of maturity and reared separately until maturity, after which they were paired at random. Hence, these males and females formed the F0 generation. To be able to measure the brain weight of the parents they had to be sacrificed, which was done after each pair had produced two clutches. At the same time body size of the parents was also measured. When this was done, offspring from parents with the largest (top 25 % of the residuals of the brain size on body size) and smallest (bottom 25 % of the residuals of the brain size on body size) brains in relation to body size were paired to form the F1 generation. The experiment used three replicates for each treatment (three large-brained replicates, and three small-brained replicates). The offspring were kept together with up to five siblings in tanks, and again males were removed at first signs of maturity. After reaching maturity, offspring descending from large-brained parents were randomly paired together, and the same thing for offspring descending from parents with small brains. Full-sib pairs were avoided. The offspring from two generations of selections were then used in the cognition experiment. At this time, the fish from the large-brained lines had on average a 9 % larger relative brain size as compared to the small-brained lines ($p <$ 0.0001, Kotrschal *et al.* unpublished data).

2.2 Cognition experiment

2.2.1 General
Overall, 48 fishes were used for the experiments; 12 females and 12 males with large brains and 12 females and 12 males with small brains divided over the three replicates. These fishes were randomly and blindly chosen and placed in a tank, which was 40 x 15 x 15 cm large and had constant aeration. To minimize stress, which was shown to occur from a previous experiment when the fish was placed alone in a novel environment, a "friend" was also added to each tank (Niclas Kolm and Alexander Kotrschal personal communication). The friends were non-mature guppies and were changed at different times during the experiment when they approached sexual maturity and/or seemed to interfere with the behaviour of the experimental fish. On the short side of the tanks, cardboard was placed to prevent the fishes from seeing each other, which could otherwise bias the results. The bottom of each tank was covered with approximately 1 cm sand and the water level was kept at 8 cm. The knowledge of which fishes that belonged to large- and small-brained individuals was unknown to me during the experiment. Further, tanks were divided into different zones; a neutral zone, a half choice zone and a choice zone (Figure 1). The reason for the half choice zone was to provide an extra neutral zone for the fish which would lead to more accurate quantification of true choice in the experiment. The light regime was kept at 13 h light: 11 h dark. The temperature of the room was held constant at 25 - 26 degrees Celsius. All handling of the fish was made during the light period and during the dark period the tanks were covered with green nets to prevent the fishes from jumping out. Throughout the experiments *Sera Vipan* flake food was used to feed the fishes, the same food had been used in their previous tanks. The experiment lasted for 6 weeks from mid-March till mid-May 2012 and consisted of three different phases; a food-habituation phase, an association learning phase and an association trial phase.
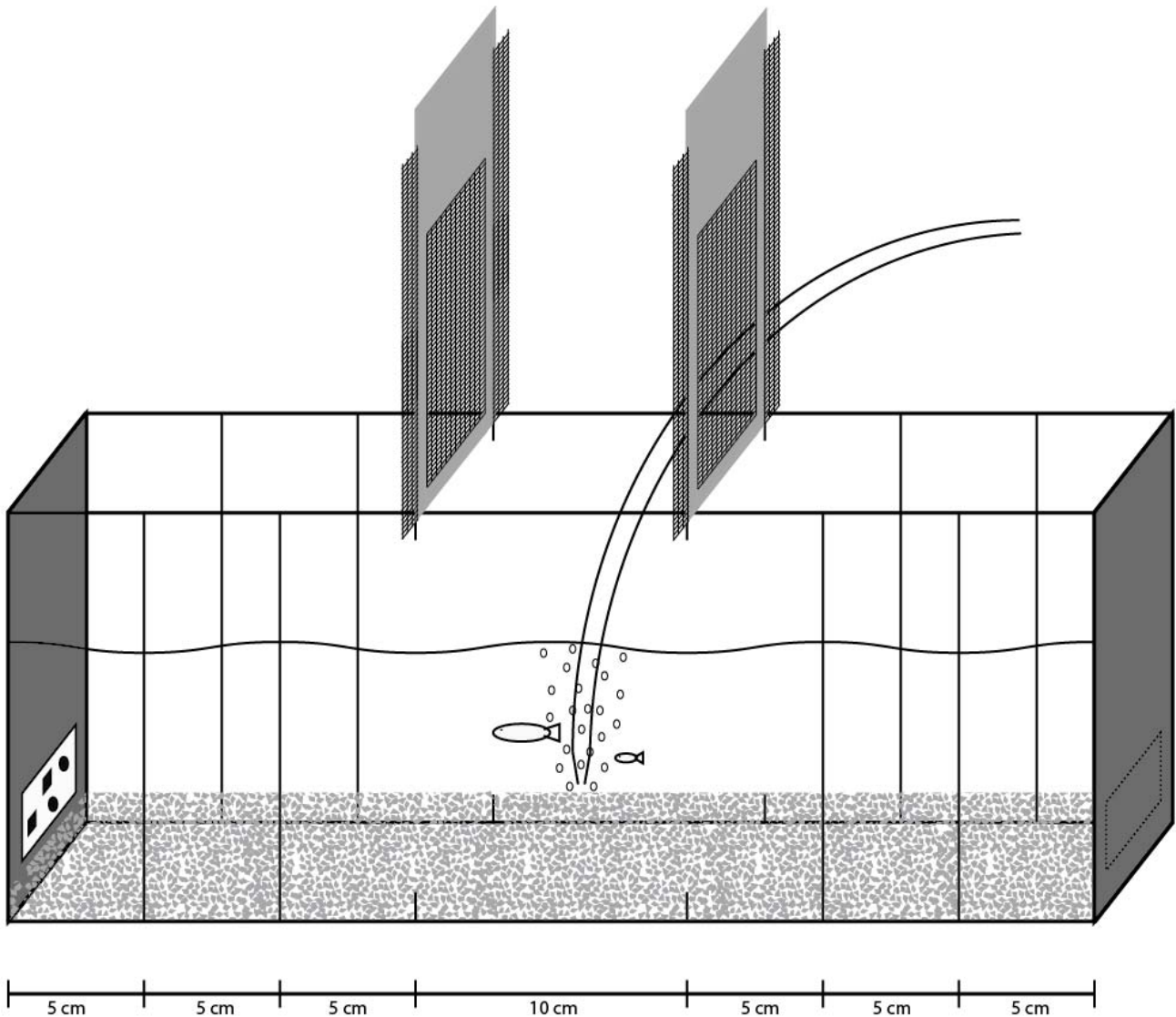
Figure 1. Tank set-up showing the different zones, stimulus, cardboard on the sides, portable walls and aeration.

2.2.2 Food-habituation

In order for the associative learning phase to work, assurance that the fish were eating had to be obtained. At the same time, the fish also got time to acclimatise to their new environment. During this phase food was randomly placed in one of the two choice zones on the short side of the tanks. Since the food consisted of flakes, the flakes were crushed into smaller pieces and then placed on the bottom of the tank. The fishes were then able to eat from the food without moving large pieces around in the tank. The fishes were allowed to feed and swim freely for 4 hours while being observed for 2 hours, after which tanks were cleaned using a siphon and sand and water was added again. This was done daily until at least 80 % of the fishes were observed eating the food for two consecutive days. Eating was recorded when the fishes took a nip at the food.

2.2.3 Association learning phase

In order to test memory and learning ability when it came to quantity discrimination in the association trial phase, a learning phase started the experiment. In this phase stimuli was presented randomly with respect to side (right or left) so the fish could learn to associate food with the stimuli. Stimuli consisted of 6 x 3 cm white cards with two or four black objects. To avoid any bias resulting from shape differences between stimuli, and to increase the generality of the experimental set up through the use of multiple shapes of stimuli, half of the objects on each card were circles and the

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other half were squares. In order to prevent the fish from using cumulative surface area as a cue when discriminating between quantities, which previously was shown to be done by mosquitofishes (Agrillo *et al.* 2009), the objects on both type of cards had the same cumulative surface area (1 cm^2). This resulted in that the individual objects on the cards consisting of four objects were half as large (0.25 cm^2) as the objects on the cards consisting of two objects (0.50 cm^2) (Figure 2). Adobe Illustrator was used to create 100 cards with two objects and 100 cards with 4 objects. The objects on the cards could be placed on eight different places, and by randomizing the placement a great variety of cards could be acquired that ensured that no bias in terms of the location of the objects would be introduced. 48 pairs of stimuli were randomly chosen for the first training, for the following training sessions the pairs of stimuli were sequentially moved one tank each time. Two portable walls, each consisting of a plastic frame and a hole covered with green see-through net, were used to keep the experiment fish and the friend in the neutral zone just prior to the training session (Figure 1). Meanwhile the stimuli were placed on the sides, and food was added to the tank in front of the stimuli consisting of four objects. Fishes were kept in the neutral zone for approximately 5 minutes before the training session started. The portable walls were then slowly removed, and I observed each focal individual for one minute to tell if the fish was eating or not. To maximize the effect of the associative learning, the fish was allowed to swim freely in the tanks and eat for 1.5 hours with the stimuli, and whether individuals fed or not were documented. This was done to be able to exclude fishes that did not eat during training from later analyses. When 1.5 hours had passed the food residues were cleaned out using a siphon. Two training sessions took place each day with a 5 hour break in between for each fish. Refilling water up to the 8 cm level was done after the second training. For three consecutive days training was preformed following a day of association trials and then it started all over again.
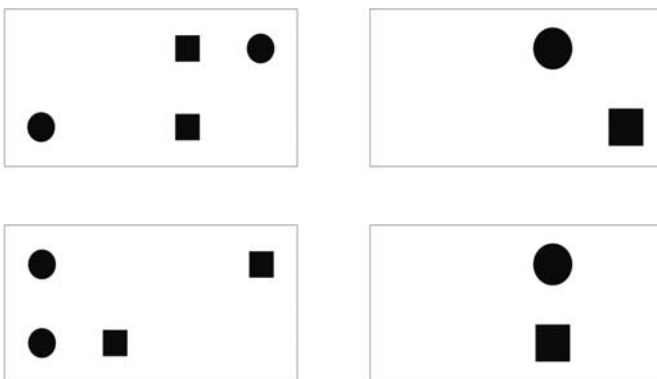

Figure 2. Two examples of stimuli pair used in the associative learning phase. The larger objects had an individual surface area of 0.5 cm^2, and the smaller objects an individual surface area of 0.25 cm^2 resulting in identical cumulative surface areas between the two stimuli.

2.2.4 Association trial phase
During the association trial phase, fishes were kept in the middle of the tank, again by using the portable walls. This time no food was added to the tank, but the stimuli were placed in the same way as in the training phase. The stimuli used in this phase were however slightly different than the learning phase. They consisted of squares or circles only and all the objects had the same area (0.375 cm^2) (Figure 3). The reason for this, was i) to ensure that the size of the objects was not used as a cue to discriminate between the stimuli and ii) to ensure that the number of objects and not the shape of the stimulus were used as a cue during this phase. Stimuli were randomly chosen from a pool of 200 (100 with only squares and 100 with only circles). If, for example, a stimulus with 4 squares was picked, then a matching stimulus with 2 squares was randomly selected. The fishes were kept in the neutral zone for 5 minutes, allowing them to acclimatize and at the same time see both of the stimuli. When the portable walls were slowly removed documentation of which full choice zone first entered by the fish was made. When the correct choice had been made the fish was

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rewarded with food. However, this time the food had been crushed and then mixed with some water. By doing this, a pipette could be used to gently add the food to the tank in front of the stimuli without scaring the fish. Maximum time allowed to make a choice was 5 minutes, after which food was added in front of the correct stimulus anyway. Just as in the training phase the fish was left to feed 1.5 hours after which the tank was cleaned out.
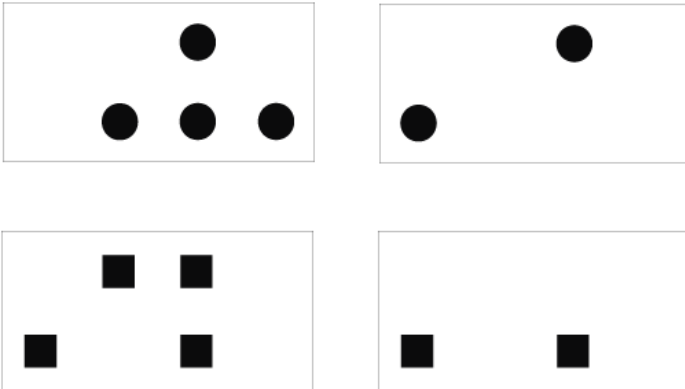

Figure 3. Two examples of stimuli pair used in the association trial phase. The individual objects had a surface area of 0.375 cm^2 each.

2.3 Statistical analyses

SPSS was used to analyse all data, and by first setting brain size, sex and replicates as fixed effects an analysis of participation among fishes could be done. In order to analyse number of correct choices made, a generalized linear model (GLM) was used. This was done because the data consisted of either a 1 (correct choice) or a 0 (wrong choice), and a normal distribution of the data could not be obtained. In addition, a GLM could 'weight' data points differently depending on number of participations.

3. Results

Females participated and made choices more often than males ($p = 0.012$, Figure 4). At the same time, when comparing participations further, no significant differences were found between brain size, replicates or the interaction of brain size and sex (Table 1).

Table 1. Analysis of participation differences.

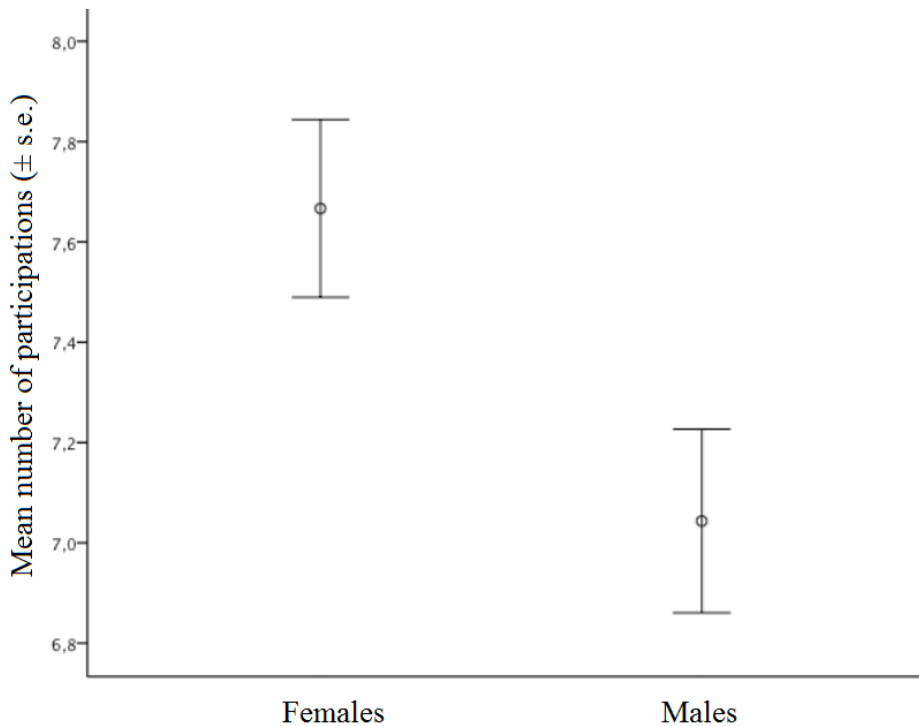| Variable		df	F	p-value
Brain size	Hypothesis	1	0.08	0.78
	Error	41		
Sex	Hypothesis	1	6.84	0.01
	Error	41		
Replicate	Hypothesis	2	1.72	0.19
	Error	41		
Brain size * Sex	Hypothesis	1	1.81	0.19
	Error	41		

Figure 4. Participation differences between males and females.

In my overall model, no effect of brain size was found ($p = 0.15$). Effects of sex and replicate were also shown to be non-significant (Table 2). However, there was a significant interaction found between brain size and sex on number of correct choices ($p = 0.020$). This interaction was caused by a strong significant effect of brain size on correct choices in females ($p = 0.006$), while no effect was found in males (Figure 5).

Table 2. Results from a generalized linear model, where analysing differences in correct number of choices.

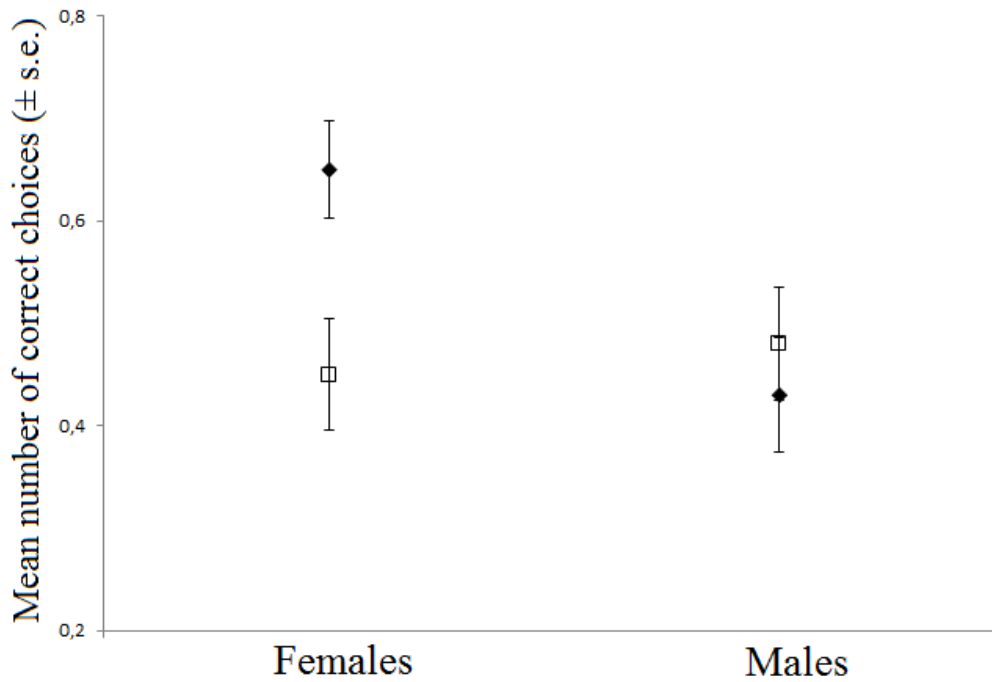| Variable	Wald Chi-Square	df	*p*-value
Brain size	2.12	1	0.15
Sex	3.47	1	0.06
Replicate	0.97	2	0.62
Brain size * sex	5.44	1	0.020

Figure 5. Mean number of correct choices for small- and large-brained females and males (Black diamonds = large brains, white squares = small brains). A significant difference between females with different relative brain size were found ($p = 0.006$), while no such difference was detected in males ($p = 0.535$).

4. Discussion

In this experiment females with larger brains were able to successfully discriminate 4 from 2 objects, while females with smaller brains were unable to do so. In males no differences in choice accuracy were found between small- and large-brained individuals. These findings provide experimental support that relative brain size is highly important for cognitive ability.

Comparative studies of the link between relative brain size and cognition have been criticised (Healy & Rowe 2007). One reason for this is that different structures in the brain control different things, such as; motor function, sensory processing and cognition. Hence, to correlate cognition with size of the brain without considering the size of the different structures have been suggested to be misleading. However, in the view of the broad definition of cognitive ability one might suggest that cognition in most cases involve most of these structures. Specific cognitive abilities, though, might be controlled or used by a certain structure of the brain to a larger extent than others. In this experiment, however, no such thing is investigated. But, considering the results obtained, even though the size of different structures may play a role, the overall brain size (controlled for body size) obviously does so as well. Some might argue that only testing the fish in one cognition test is not adequate, since there are many ways of testing different types of cognition. However, the chance of testing only the one cognitive ability, in this case quantity discrimination, affected by relative brain size is highly unlikely. In addition, the experimental set-up did not only involve the fish's ability to discriminate between objects, but also the ability to learn and remember, which includes many different aspects of cognition

An associative experiment such as the one performed in the present study, have earlier been suggested to be a relatively simple task for fishes, since they have been able to complete such tasks, also without their forebrain as demonstrated by lesion studies (Braithwaite 2006). However, my experiment is not just a simple association experiment and the ability to discriminate between quantities and also memory has to be used by the fish. To increase the level of cognitive demand, cumulative surface area was also controlled for, since this was earlier shown to be used as a cue by fish (Agrillo *et al.* 2009, Agrillo *et al.* 2010).

Can selection experiments for relative brain size in lower vertebrates lead to such an enhancement in cognitive ability similar to those seen in higher vertebrates, for instance, an adult human's ability to discriminate between quantities? The ability of large-brained guppies to discriminate between 2 and 4 objects is actually better than a human infant's ability to discriminate between quantities (Feigenson *et al.* 2002). In humans two different systems have been suggested to be used for quantity discrimination; one system for small numbers and one system for large numbers. The small number system is an object-tracking system and the large number system is a number estimation system, which is ratio dependent. The size limit for the object tracking system found in six and twelve month old infants have been suggested to be 3. In guppies, however, the size limit has been suggested to be 4 (Agrillo *et al.* 2012, Xu 2003). Thus, guppies only have to use one system when discriminating between 2 and 4, while infants may try to use both the small and large discrimination system and hence end up failing to discriminate such quantities. The methods used for quantity discrimination is not the same in studies of infants and guppies. Nevertheless, this provides interesting information, and points towards the possibility of selecting for advanced cognitive ability also in relatively simple organism such as the guppy.

Furthermore, my results provide interesting insights into sex differences in cognitive ability following selection for relative brain size. Because of sexual dimorphism in guppies these results may be due to, for instance, morphological and behavioural differences. Females are in general larger than males (♀= 18-24 mm and ♂ = 13-19 mm) (Reznick & Miles 1989), and since the brain

increases allometrically with body size (Striedter 2005) adult females used in this experiment will always have a larger absolute brain size than males. If the same cellular scaling rules apply for males and females, the sex with the larger brain would possess higher cognitive abilities, which in this case are the females (Herculano-Houzel *et al.* 2006, 2007, Northcutt 2002). However, to date there has been no investigations if the same cellular scaling rules apply for both sexes within a species.

The behaviour of females and males also differs remarkably. Guppy females have, for example, been found to be more innovative and active in food search than males (Laland & Reader 1999). The reason for this is most likely that females are more dependent on food for their reproductive success, while the limiting factor for males is the accessibility for females (Trivers 1972). Since guppies are viviparous and no post-natal parental care occurs, the differences in investment and also food dependencies between the sexes are very much present in guppies (Reznick & Yang 1993). Because of these differences the experimental set up used in this experiment might have been more suitable for females. Females eat more and hence get more associative training than males. Furthermore, it has been suggested that females eat more due to a higher metabolic need caused by trying to escape males that want to mate (Laland and Reader 1999). However, in this experiment this is unlikely, since the experiment fish were kept separately together with only a non-mature friend fish. On the other hand, when mature females and males are kept together, males spend a lot of time courting females (Farr & Herrnkind 1974). This once again suggests that males do not focus as much on food search as in courtship. Instead, males may only prioritize food search when they are food deprived (Reader & Laland 2000). In the associative training sessions, fishes were allowed to eat during 1.5 hours. This time might have caused saturation of food in males resulting in the associative training being less efficient for them. At the same time, saturation may not have been enabled in females since they are more dependent on food (Laland & Reader 1999, Trivers 1972).

When guppies search for food it has lately been shown that they use vision (Rajaei *et al.* 2012). Since males have highly visual ornamentation and courting displays, vision is also important to females during mate-choice. Male ornamentation varies and usually consists of different colour patterns, such as black, blue and orange spots and lines. The stimuli used in the experiment consisted of black objects, which could have been perceived by females as black dots. Thus females might possess a better visual system to detect these objects than males, which in turn actually may have avoided them if they interpreted them as threatening. The idea that males would feel threatened by the stimuli, and thus not approaching them to the same extent as females, is however unlikely since males often interact considerably in the guppy (Jirotkul 1999, Kodric-Brown 1993). Further investigations to establish definite conclusions about the sex-specific results found in this study will be interesting avenues of future research.

A larger brain and hence higher cognition can be of great beneficial value for an individual in nature, for example in terms of foraging efficiency. The marginal value theorem is often mentioned in terms of foraging theory, and it is used to predict how long an animal should stay and forage in a patch before leaving for the next one (Charnov 1976). If a larger brain is better at estimating the number of food items in the present patch and compare them to number of food items in another patch, individuals with larger brains might be able to maximize foraging efficiency and potentially obtain higher fitness. Further, if females with a relatively larger brain are better at interpreting visual signals, they might also possess a better ability to actively separate males of different quality when choosing a mate.

Of course there are not only benefits rising from a larger brain in forms of higher cognition. Brains are metabolically expensive tissues, and thus a larger brain should be costly to have (Aiello & Wheeler 1995, Nilsson 1996). The costly tissue hypothesis, stated by Aiello and Wheeler (1995), suggests that an increase in relative brain size should be followed by a decrease in size of other

expensive tissues. This is also supported in a species of fish (*Gnathonemus petersii*) (Kaufaman *et al.* 2003). Aiello and Wheeler (1995) further expanded this hypothesis and suggested the energy trade-off hypothesis, which states that any increase in relative brain size should result in a decrease in energy consumption of other physiological aspects such as locomotion and reproduction. An investigation of a possible trade-off between locomotion and relative brain size has been done in birds, where a negative correlation between relative brain size and pectoral muscle (used to power flying in birds) mass was found. It was then argued for that energetic demands of flight may prevent an increase in brain size (Isler & van Schaik 2006) and that migrating birds cannot provide enough energy for a large brain (Sol *et al.* 2005). Furthermore, trade-offs between relative brain size and fecundity have recently been found in the guppy (Kotrschal *et al.* unpublished data). All these costs associated with a larger brain most likely provide the answer to why not all animals have larger brains despite the great beneficial value it might have in some situations.

5. Conclusion

This experiment demonstrates that relative brain size is important for cognitive ability in a vertebrate. It also shows that a difference in cognitive ability can be obtained after only two generations of artificial selection. To my knowledge, no other studies like this have been done earlier. Hence these results provide interesting and useful findings valuable for future studies within the field. The sex-specific differences I found could have been due to overall higher cognitive ability in females in relation to the stimuli, or that the experimental set-up might have been more suitable for females that are more dependent on food than males. Another approach where males are rewarded with swimming together with receptive females instead of getting food might be better to investigate possible cognitive differences between males with different brain size.

Future studies should further investigate possible trade-offs resulting from a relatively larger brain. How and if the neuron numbers differ between sexes and different individuals should also be interesting to look into. It would also be interesting to continue with selection experiments to investigate if the brain size and also cognitive ability somehow reaches a maximum in an organism. The link between cognitive ability, courtship behaviour and mate-choice might also be an interesting avenue of research, since the ability to interpret for example visual signals often is included in these kinds of behaviours.

6. Acknowledgements

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