ISSN 1403-5227

LICENTIATAVHANDLING

ZOOLOGISKA INSTITUTIONEN

Stockholms Universitet S-106 91 Stockholm

Stockholms universitet

LEARNING, BEHAVIOURAL FLEXIBILITY AND COGNITIVE AGEING IN FEMALE GUPPIES *(POECILIA RETICULATA*) ARTIFICIALLY SELECTED FOR RELATIVE BRAIN SIZE

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Ämne: Etologi

Löpnummer: 2021:1

Learning, behavioural flexibility and cognitive ageing in female guppies (*Poecilia reticulata*) artificially selected for relative brain size

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Datum för seminarium: 9.4.2021

Learning, behavioural flexibility and cognitive ageing in female guppies (*Poecilia reticulata*) artificially selected for relative brain size

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The thesis is based on the following articles, which are referred to in the test by their Roman numerals:

I Boussard, A., Buechel, S.D., Amcoff, M., Kotrschal, A. and Kolm, N. (2020). Brain size does not predict learning strategies in a serial reversal learning test. *J Exp Biol.* **223**, jeb224741. doi: 10.1242/jeb.224741.

II Boussard, A., Amcoff, M., Buechel, S.D., Kotrschal, A. and Kolm, N. (2021). The link between relative brain size and cognitive ageing in female guppies (*Poecilia reticulata*) artificially selected for variation n brain size. *Exp Gerontol.* **146**, 111218. doi: 10.1016/j.exger.2020.111218.

Candidate contribution to thesis articles*

	I	**II**
Conceived the study	**Significant**	**Significant**
Designed the study	**Substantial**	**Substantial**
Collected the data	**Substantial**	**Substantial**
Analysed the data	**Substantial**	**Substantial**
Manuscripts preparation	**Substantial**	**Substantial**

*** Contribution Explanation**

Minor: contributed in some way, but contribution was limited.

Significant: provided a significant contribution to the work.

Substantial: took the lead role and performed the majority of the work.

Abstract

There are many factors that cause variation in cognitive abilities and how such abilities are preserved through increasing age. One proximate mechanism that has been linked to this variation, especially at the interspecific level, is relative brain size. In this thesis I investigated the role of relative brain size in two aspects of cognition, advanced learning abilities and cognitive ageing. More specifically, **Paper I** tested the ability to learn from earlier experience and adopt an efficient learning strategy, while **Paper II** assessed if there are cognitive consequences with age that vary with quantitative differences in brain size. For this, I used female guppies (*Poecilia reticulata*) artificially selected for relative brain size in a reversal learning paradigm. In **Paper I**, I found no relationship between the ability to adopt an efficient learning strategy and relative brain size. However, large-brained females performed at a higher level and had a better explicit long-term memory. From **Paper I**, I conclude that when cognitive divergence is driven by relative brain size at the intraspecific level, it is mostly quantitative in nature, while more qualitative divergence might be found mainly at the interspecific level. **Paper I** indicates that although a larger brain generates enhanced cognitive abilities and thus most likely influence brain size evolution, not all patterns found at a higher phylogenetic scale can be found also at the intraspecific level. The results from **Paper II** indicate that there are no overall cognitive consequences that vary with increasing age. Interestingly, cognitive levels were predicted by relative brain size in young females, but this effect disappeared with increasing age. From this, I speculate that the slight decrease in performance in large-brained females could be caused by differences in life history strategy. Large-brained females appear to invest more into cognition and development of brain tissue early in life, while small-brained females allocate more resources to somatic maintenance. There might thus be a three-way link between cognitive ageing and relative brain size on one hand and life history on the other. The two studies in this thesis highlight factors that drive and constrain the evolution of brain size at the intraspecific level.

Introduction

The vertebrate brain varies greatly in size (Butler and Hodos, 1996; Jerison, 1973; Tsuboi et al., 2018). Developing and maintaining brain tissue is costly and will thus act as a constraint in brain size evolution (Striedter, 2005). Increased brain size has for instance a negative impact on gut size (Aiello and Wheeler, 1995; Navarrete et al., 2011; Isler and van Schaik, 2009; Liao et al., 2016; Kotrschal et al., 2013; Tsuboi et al., 2015), reproductive rate (Gonzalez-Voyer et al., 2016; Isler and van Schaik, 2009, Kotrschal et al., 2013), longevity (Kotrschal et al., 2019), and innate immune responses (Kotrschal et al., 2016). On the other hand, a driving force in brain size evolution is the cognitive advantages that are potentially balanced by the aforementioned costs (Striedter, 2005). A larger brain may increase cognitive ability and increase survival rate (Benson-Amram et al., 2016, Kotrschal et al., 2015; Lefebvre et al., 2002; Reader and Laland, 2002; Sol et al., 2007; Wagnon and Brown, 2020), improve predator avoidance (Møller and Erritzøe, 2014; Shultz and Finlayson, 2010; van der Bijl et al., 2015), and facilitate mate assessment (Bloch et al., 2018; Corral-Lopez et al., 2017). These contradictory selective pressures are generally thought to have given rise to the enormous existing variation in brain size within and among (Striedter, 2005). This thesis focuses on one of the strongest candidate factors to promote evolutionary increases in brain size, namely cognition.

How variation in brain size is related to variation in cognitive abilities has fascinated scientists over generations. In its broadest sense, cognition is the process when animals acquire and process information about the external environment and act on it (Shettleworth, 2010). Just like brain size, cognitive abilities vary greatly at all taxonomic levels. More simple cognitive abilities are shared by all vertebrates, while more complex forms are found in a minority of recently evolved species such as primates and corvids (Emery and Clayton, 2004; Seed et al., 2009; Shettleworth, 2010). When comparing cognitive abilities and brain size between species, a pattern of enhanced cognitive abilities being linked to increased brain size emerge. For instance, bower complexity in male bower birds (Madden et al., 2001), inhibitory control in birds and mammals (Kabadayi et al., 2016; MacLean et al., 2014), problem solving ability in carnivores (Benson-Amram, 2016), and tool use in birds and primates (Lefebvre et al., 2002; Reader and Laland, 2002) have all been related to increased brain size. One possible explanation to why a larger brain is sometimes advantageous in various cognitive contexts, is that a larger brain is often richer in neurons (e.g. Marhounová et al., 2019; Olkowitcz et al.,

2016). In fact, neuron number have proven to explain much variation in cognitive abilities (Dicke and Roth, 2016; Herculano-Houzel, 2017). When trying to understand what causes variation in cognitive abilities it might therefore be useful to assess both brain size and neuron number.

In **Paper I**, I tested the hypothesis that an advanced form of learning, behavioural flexibility, can be predicted by brain size also at the intraspecific level. Behavioural flexibility, defined as the ability to repeatedly change a behavioural pattern (Bond et al., 2007; Mackintosh, 1974), is an interesting aspect of cognition. Behavioural flexibility enables animals to adjust to an unpredictable and fluctuating environment (Day et al., 1999; Jones, 2005; Shettleworth, 2010). As the level of unpredictability and fluctuation differs between species and even populations, variation in behavioural flexibility is expected. Ultimately, variation in behavioural flexibility across species has been explained by feeding ecology (Day et al., 1999), spatially heterogenous environment (Jones, 2005), and social complexity (Ashton et al., 2018; Bond et al., 2007). At the proximate level, differences in brain size have been associated with variation in behavioural flexibility (Bitterman, 1965; Buechel et al., 2018; Elias, 1970). What is especially interesting with behavioural flexibility is that regardless of brain size, animals from insects to humans can adjust to changes by a fixed stimulus-response action pattern (Bitterman, 1965; Gonzalez et al., 1967; Hunter and Kamil, 1971; Sherry and Strang, 2015). However, some animals can learn from previous experience and generalize this information across situations in order to faster adjust to abrupt environmental changes (Bond et al., 2007; Strang and Sherry, 2014; van Horik and Emery, 2018). This process can be referred to as developing an efficient learning strategy (Mackintosh, 1974). Developing an efficient learning strategy is more cognitively demanding than using a fixed stimulus-response action pattern to adjust to unpredictability and fluctuation (Shettleworth, 2010). The former is mainly found in large brained species, whereas the latter (with some exceptions) is more likely to be found in small-brained species (Bitterman, 1965; Bond et al., 2007; Gonzalez et al., 1967; Hunter and Kamil, 1971; Sherry and Strang, 2015; Strang and Sherry, 2014; van Horik and Emery, 2018). This pattern provides an interesting opportunity to test the effect of brain size on two aspects of behavioural flexibility. First, the level of behavioural flexibility can be tested to investigate quantitative differences between species and/or populations. Second, the ability to develop an efficient learning strategy can be tested to investigate qualitative differences and cognitive

divergence between species and/or populations. Variation between species in the ability to adopt an efficient learning strategy is often associated with differences in brain size (Bitterman, 1965; van Horik and Emery, 2018). However, experimental tests at the intraspecific level of the proximate mechanisms causing this variation is relatively scarce. Given the possible effect on fitness provided by greater behavioural flexibility (Lefebvre and Bolhuis, 2003; Lefebvre et al., 2004; Timmermans et al., 2000), the link to brain size might reveal important insights into the driving forces behind brain size evolution.

In **Paper II**, I investigated another interesting aspect of cognition, namely how cognitive abilities are preserved into higher age. As a consequence of degradation of brain structures, certain cognitive abilities gradually decline with increasing age (Hedden and Gabrieli, 2004; Kandel et al., 2013; Marner et al., 2003; Phillips et al., 2019; Ritchie et al., 2015; Scahill et al., 2003). This process is referred to as cognitive ageing. Interestingly, cognitive ageing occurs in numerous invertebrate and vertebrate species, despite the great variation in brain organization and cognitive abilities across lineages (Behrends et al., 2007; Hirsch and Peretz, 1984; Ritchie et al., 2015; Tapp et al., 2003; Voytko et al., 1999; Yu et al., 2006; Zwoinska et al., 2013; Zyzak et al., 1995). Studies in birds and humans suggest that a larger brain suffers less from degradation (Katzman, 1993; Satz, 1993; but see Stern, 2009; Vágási et al., 2016). Accordingly, a larger brain might slow down the rate and onset of cognitive ageing. This means that increased brain size might decouple physical from chronological age. Since cognitive abilities are important for fitness and reproductive success (Dukas, 2005; Dukas and Bernays, 2000; Dukas and Duan, 2000; Egas and Sabelis, 2001; Grieco et al., 2002; Sherman and Visscher, 2002; Steidle, 1998) and most species reproduce throughout their life-span, cognitive ageing has the potential to be important in brain size evolution. Also, large-brained species generally live longer (Allman et al., 1993; Barrickman et al., 2008; González-Lagos et al., 2010, 2016; Isler and van Schaik, 2009; Jiménez-Ortega et al., 2020; Sol et al., 2007; 2016; Yu et al., 2018). This suggest a three-way link between brain size, life-span and cognitive ageing, at least on a larger phylogenetic scale. At the same time, recent results suggest that this pattern is reversed between close related species and at the intraspecific level (Eckerstöm-Liedholm, 2019; Kotrschal et al., 2019). Life-history theory predicts faster senescence in short lived individuals (Kirkwood and Holliday, 1979; Williams, 1957). Hence, we have two different potential predictions at different taxonomic scales. First, if a larger brain

causes a shorter life-span, cognitive ageing rate would be faster. This might be a constraint in brain evolution. Second, a slower cognitive ageing rate due to a larger brain and a longer life-span might facilitate brain size evolution.

Aim

Although there is a growing body of experimental evidence supporting the link between enhanced cognitive abilities and increased brain size, much of the existing evidence is still based on correlations between species. However, natural selection acts on individual phenotypes (Thornton and Lukas, 2012). What has largely been missing fully to understand the proximate mechanisms causing cognitive divergence and also the driving forces and constrains in brain size evolution, is experimental studies at the intraspecific level. Therefore, the aim with **Paper I** was to experientially test the effect of brain size on divergence in the ability to adopt efficient learning strategies. Although many aspects of behavioural flexibility have been investigated across multiple species (Ashton et al., 2018; Bitterman, 1965; Bond et al., 2007; Day et al., 1999; Sherry and Strang, 2015; van Horik and Emery, 2018), no studies have looked at the effect of brain size within a laboratory bred population with controlled differences in brain size and other relevant aspects. How cognitive abilities are preserved with increasing age have the potential to be important in brain size evolution. Hence, in **Paper II**, the aim was to investigate how two aspects of cognition, associative learning and behavioural flexibility, are preserved with increased age using the above-mentioned populations. More specifically, we examined the ´brain reserve´ hypothesis, that predicts that a larger brain slows down the onset and rate of cognitive ageing (Katzman, 1993; Satz, 1993; but see Stern, 2009). The effect of brain size on cognitive ageing is well studied in humans, but it is less well understood if evolutionary increases in brain size also affect the rate and onset of cognitive ageing in other species.

Study system

The guppy (*Poecilia reticulata*) is a small tropical freshwater fish native to northeast South America. Guppies have an interesting biology and are highly adaptive to many different environments and have therefore become a very popular aquarium species for both aquarium enthusiasts and for researchers. The ease at which guppies can be kept and bred under

laboratory conditions together with the ecology and a rich behaviour repertoire (such as a highly promiscuous mating system, variable antipredator behaviour and shoaling dynamics), make guppies an excellent study system for studies in ecology and ethology. In order to address the above stated questions, I used the 7th generation female guppies artificially selected for small and large relative brain size (Kotrschal et al., 2013), with matching number of neuron numbers (Marhounová et al., 2019). In short, descendants to wild-caught guppies from high-predation areas in the Quare River, Republic of Trinidad and Tobago, were kept in large (ca 200 individuals) populations in mixed sex tanks prior to the selection procedure. Three independent replicate populations with 75 breeding pairs each was set up by using fish from the mixed sex tanks. Following offspring production, the breeding pairs were euthanized and their brains dissected out. Offspring to breeding pairs with the largest and smallest brains (brain weight relative to body length) were used to create the “up” and “down” selected lines per replicate population. After five generations of selection, a >15% difference in brain size and >11% difference in neuron number were established between the treatment selection lines (Marhounová et al., 2019).

Methods

Paper I

In **Paper I**, I tested if the ability to adopt an efficient learning strategy vary with relative brain size at the intraspecific level. I assessed learning ability by means of a serial reversal learning paradigm. I used 96 female guppies (small-brained n = 48, large-brained n = 48). The subjects were trained to discriminate between two colour stimuli. After a fixed amount of trials (i.e. 30 trials) and a two-day pause, the reward contingency was reversed. The reward contingency was reversed ten times and each reversal constituted of 30 trials. The ease at which an animal can switch between reward contingencies is a common method to test the ability to learn from experience and adopt an efficient learning strategy (Bitterman, 1965; Bond et al., 2007; Shettleworth, 2010). I scored if the subjects did a correct or non-correct choice each trial and analysed the individual binary outcome variables. Since previous studies suggest that brain size predicts advanced learning abilities in general (Benson-Amram et al., 2016; Kabadayi et al., 2016; MacLean et al., 2014; Reader and Laland, 2002) and also during reversal learning (Bitterman, 1965; Buechel et al., 2018; Elias, 1970; van Horik and Emery, 2018), I hypothesized that there would be a difference in learning ability between the small- and large-brained female guppies. I predicted that large-brained females would be faster to switch between reward contingencies over serial reversals than their small-brained conspecifics.

Paper II

In **Paper II**, I investigated if there are cognitive consequences that vary with brain size and neuron numbers in a non-human animal. The ´brain reserve´ hypothesis predicts that quantitative differences in brain morphology slow down the onset and rate of cognitive ageing (Ritchie et al., 2015; Stern, 2009; Wolf et al., 2004). Therefore, I used 288 female guppies with >15% difference in relative brain size and >11% difference in neuron number (Marhounová et al., 2019), from three different ecological relevant age groups, 4-6 months old (n = 96), 12 months old (n = 96) and 24 months old (n = 96) in a reversal learning paradigm. Each age group was equally divided between small- and large-brained females. This set up enabled me to test for potential decline in two aspects of cognition, initial colour discrimination ability and behavioural flexibility. As in **Paper I**, the subjects were first trained to discriminate between two colour stimuli to test initial colour discrimination ability. After the subjects had

successfully learnt to discriminate between the two colours, the reward contingency was reversed. How fast an animal recover from the reversal and learn the new contingency is a common measure of behavioural flexibility (Bitterman, 1965; Bond et al., 2007; Shettleworth, 2010). The scoring and analyses were identical as in **Paper I**. I hypothesised that there would be a difference in cognitive performance between the brain size selected lines. In accordance with the ´brain reserve´ hypothesis, I predicted that small-brained female guppies would show a steeper decline in initial colour discrimination ability and behavioural flexibility with increasing age.

Results

Paper I

Initial colour discrimination

Initial colour discrimination was not predicted by relative brain size. Neither learning rate nor performance differed significantly between the brain size selected lines. This was expected since a previous experiment failed to detect any difference in a similar binary colour discrimination task (Buechel et al., 2018). Importantly, the females learnt to discriminate between the two colours (trial: $\chi^2(1) = 63.22$, $p < 0.001$). During the last six trials in this part of the experiment small- and large-brained females reached similar performance levels (raw data mean±s.e. small-brained females; 93±3.7%, large-brained females; 98±2.0%).

Serial reversal learning

During serial reversals, I found no evidence for an ability in female guppies to generalize information across situations by a learnt rule. This means that both small- and large-brained female guppies failed to develop an efficient learning strategy (brain size × reversal: $\chi^2(1) = 0.18$, $p = 0.68$: Figure 1). Instead, learning rate and performance was negatively affected and the error rate increased over serial reversals (reversal: $\chi^2(1) = 17.91$, $p < 0.001$). Although both brain size selected lines failed to progressively improve over serial reversals, large-brained females performed at a higher level than small-brained females (brain size × training session: $\chi^2(1) = 6.44$, $p = 0.01$).

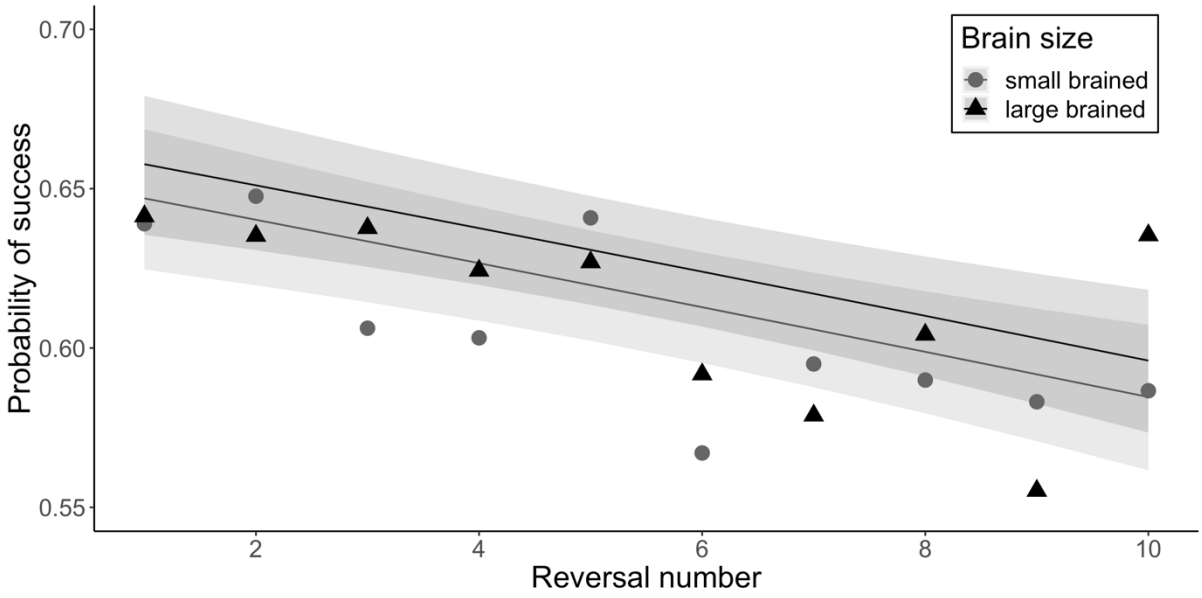

Figure 1. Performance in serial reversal learning. Performance and learning rate were measured as the proportion of correct responses in each trial across ten serial reversals. We found no evidence for the hypothesis that relative brain size predicts the ability to progressively improve performance over serial reversals and thereby develop an efficient learning strategy (brain size * reversal; $\chi^2(1) = 0.18$, $p = 0.68$). Raw mean data (based on 26 763 observations in total) for 48 small-brained and 48 large-brained female guppies across ten serial reversals. The logistic regression slope estimates for small-brained (grey line) and large-brained (black line) females and 95% confidence interval (shading) are predictions obtained from a GLMM with binominal error distribution.

Explicit long-term memory

The first trial of each reversal was preceded by a two-day pause. This enabled me to test for explicit long-term memory differences in associative learning between small- and large-brained female guppies (Bailey et al., 1996). Large-brained females performed at lower level the first trial in nine out of the ten reversals (brain size: $\chi^2(1) = 10.99$, $p < 0.001$: Figure 2). Importantly, the performance in the last six trials of each reversal did not differ significantly between the brain size selected lines (brain size: $\chi^2(1) = 0.14$, $p = 0.71$). This suggests that large-brained females had a stronger memory of what was learnt during the previous reversal and hence performed at a lower level when the reward contingency was reversed. The increase in performance during the first trial of each reversal with increasing reversals is an indication of an increased error rate and thus extinction.

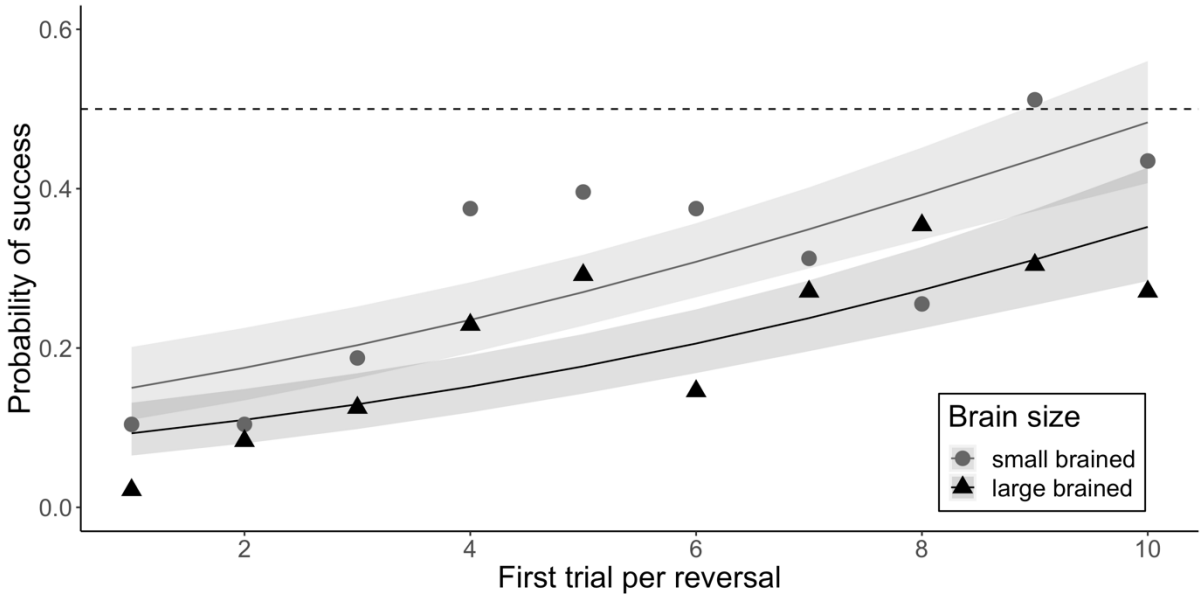

Figure 2. Explicit long-term memory. Memory was measured as the proportion correct responses on the first trial of each reversal (preceded by a two-day pause). We found that relative brain size predicted long-term memory, as large-brained females made more errors on the first trial of each reversal (brain size; $\chi^2(1) = 10.99$, $p < 0.001$). Raw mean data (based on 948 observations in total) and the logistic regression slope estimates for 48 small-brained and 48 large-brained female guppies in a serial reversal learning assay. The dotted horizontal line represents the 50% performance level. The logistic regression slope estimates for small-brained (grey line) and large-brained (black line) females and 95% confidence interval (shading) are predictions obtained from a GLMM with binominal error distribution.

Paper II

Initial colour discrimination

The brain size × trial interaction and the main effect of brain size were non-significant in the youngest, the middle aged and the oldest age groups (Figure 3). This means that there was no difference in initial colour discrimination learning rate between small- and large-brained females in any of the three age groups. Trial was highly significant in all three age groups ($p < 0.001$), which indicate that all females learnt to discriminate between the two colours. No obvious decline in learning rate between the three age groups could be detected. The last six trials during this part of the experiment all three groups reached similar performance levels (raw data mean±s.e. 93.5±1.0%$_{youngest}$, 90.0±1.3%$_{middle}$, 94.1±1.0%$_{oldest}$).

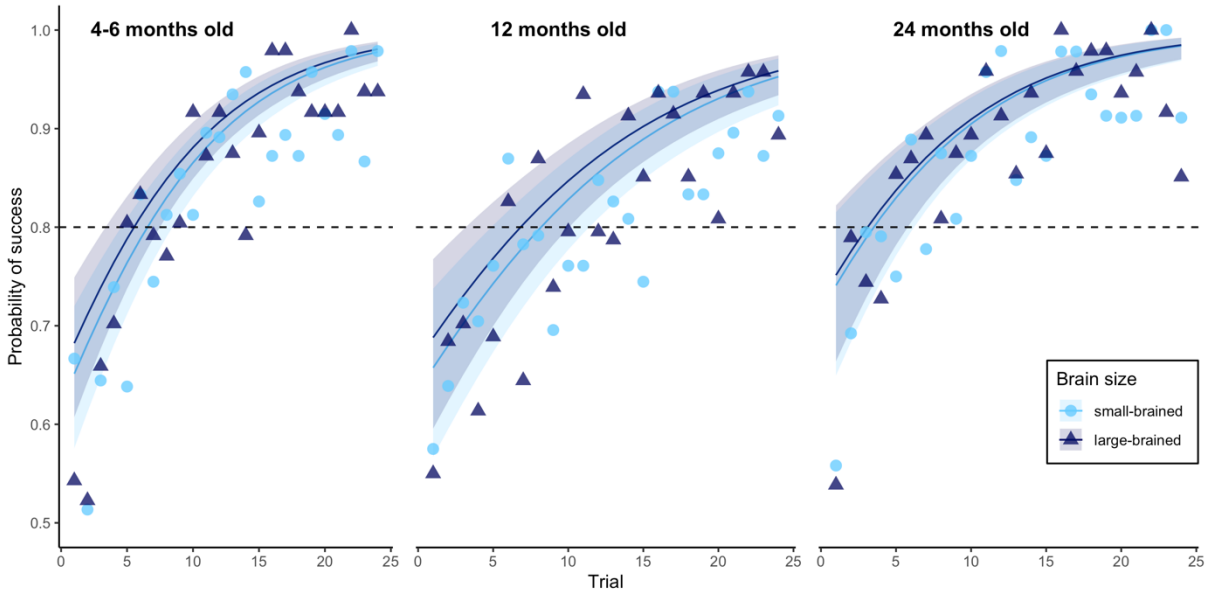

Figure 3. Initial colour discrimination. Proportion of correct responses each trial in a binary colour discrimination task in 288 female guppies of three different age groups. Circles, clear blue line and 95% CI (shading) signify raw mean data and GLMM predictions for small-brained females; diamonds, dark blue line and 95% CI (shading) signify raw mean data and GLMM predictions for large-brained females. Means and model predictions were established from 6912 observations in total across 24 trials. The dotted horizontal line represents the 80% correct responses level. We found no difference in colour discrimination learning rate between small- and large-brained females, or any change with increasing age.

Reversal learning

When the reward contingency was reversed, large-brained females learnt the new contingency faster and did more correct responses than small-brained females in the youngest age group (brain size × trial; $\chi^2(1) = 5.17$, $p = 0.02$: brain size; $\chi^2(1) = 4.23$, $p = 0.04$: Figure 4). This effect disappeared in the middle aged and the oldest groups, which means that small- and large-brained females learnt at the same rate in these two groups. Behavioural flexibility can thus be predicted by brain size early in life, but not at later life stages in female guppies. As in the initial colour discrimination part of the experiment, the three different age groups reached similar performance levels on average in the last six trials in the reversal learning part (raw data mean±s.e. $87.0\pm1.4\%_{youngest}$, $88.0\pm1.4\%_{middle}$, $97.0\pm1.4\%_{oldest}$). This suggests that there is no overall decline in behavioural flexibility within the ecological relevant life-span in female guppies.

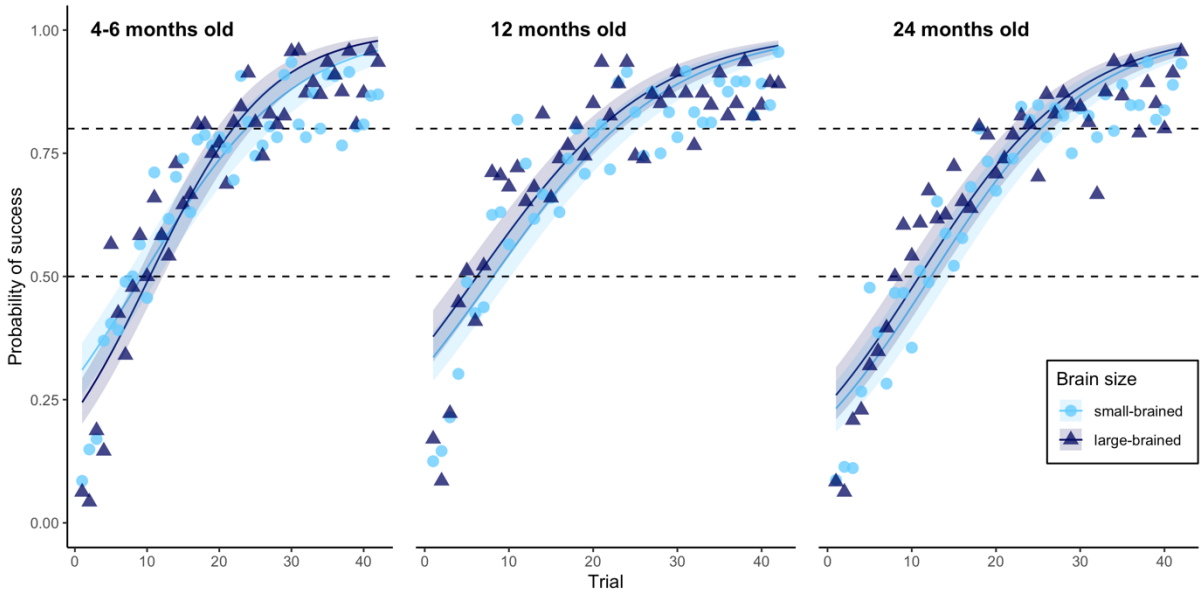

Figure 4. Reversal learning. Proportion of correct responses in each trial in a colour reversal learning task in small- and large-brained female guppies of three different age groups. Circles, clear blue line and 95% CI (shading) signify raw mean data and GLMM predictions for small-brained females; diamonds, dark blue line and 95% CI (shading) signify raw mean data and GLMM predictions for large-brained females. Means and model predictions were established from 12 054 observations in total across 42 trials. The dotted horizontal lines represent the 50% and the 80% correct response levels. We found that learning rate and correct responses were predicted by relative brain size in the youngest group (brain size × trial; $\chi^2(1) = 5.17$, $p = 0.02$: brain size; $\chi^2(1) = 4.23$, $p = 0.04$). In the middle age and the oldest group neither learning rate nor proportion correct responses were predicted by relative brain size.

Discussion

The main theme in this thesis has been to investigate how fast evolution of relative brain size affects cognitive abilities and how these are preserved with increasing age. Both **Paper I** and **Paper II** use the reversal learning paradigm to investigate binary discrimination ability and behavioural flexibility. Unique guppy populations artificially selected for differences in relative brain size was used to experimentally test a potential proximate mechanism causing variation in the above-mentioned aspects of learning. Since this field is dominated by comparative studies and other correlational studies these results are an important addition.

Paper I investigates if relative brain size predicts the ability to adopt an efficient learning strategy during serial reversal learning. Contradictory to my predictions, relative brain size did not predict the ability to adopt an efficient learning strategy. That is, none of the brain size

selected lines were able to generalize information across situations by a learnt rule and thereby failed to progressively improve over serial reversals. This suggests that female guppies are restricted to fixed stimulus-response action patterns to adjust to unpredictable changes in the environment. According to theory, there is a trade-off between behavioural flexibility and memory (Tellos-Ramos et al., 2019). Under natural conditions, female guppies have to assess and remember potential mates, where to find food, and shelter from predators (Houde, 1997). The ecological challenges of female guppies might therefore facilitate memory rather than advanced learning abilities. Another possible explanation for the inability to adopt an efficient learning strategy might be the small magnitude in brain size difference at the intraspecific level. The differences in brain size, and/or brain region size that is involved in behavioural flexibility, is not large enough within a species to create the patterns found at a larger phylogenetic scale (Bitterman, 1965). However, large-brained females performed at a slightly higher level during serial reversals and had a better explicit long-term memory than small-brained females. This suggests that while cognitive divergence can be of a qualitative nature at larger phylogenetic scale, it is mainly quantitative at the within species level.

Paper II investigates if cognitive ageing rate can be predicted by differences in brain size, i.e. relative brain size and neuron number in this study, as proposed by the ´brain reserve´ hypothesis (Stern, 2009). During reversal learning, large-brained females learnt the new reward contingency at a faster rate than small-brained females in the youngest age group. This positive effect of brain size on learning rate disappeared in the middle aged and oldest group. Behavioural flexibility levels might thus align with increasing age in these selection lines. Cognitive ageing rate might therefore be slightly faster in large-brained females than in small-brained females. This contrasts with the predictions from the ´brain reserve´ hypothesis (Stern, 2009). The shorter life-span in large-brained compared to small-brained guppies (Kotrschal et al., 2019) might explain this pattern. Large-brained female guppies might invest more resources into important fitness-related traits early in life, while small-brained guppies invest relatively more into somatic maintenance. Despite the slight decrease in learning rate in large-brained females, no obvious overall negative effect of age was evident neither during initial colour discrimination nor during reversal learning. That means that the performance levels were overall quite similar between the age groups and no decline in the cognitive abilities tested here could be detected. The cognitive abilities affected by ageing vary greatly

between species (Bartus et al., 1979; Lai et al., 1995; Valenzano et al., 2007; Voytko, 1999). One possible explanation would be variation in degradation in brain sub-structures depending on the biological significance of a particular cognitive trait (Bond et al., 2007; Day et al., 1999; Sherry and Strang, 2015). Another possible explanation is that variation in cognitive ageing is caused by differences in social complexity. Long-lived group living species with a heterogeneous age structure might allow for a decline in certain cognitive abilities in older individuals, as this can be compensated for by younger individual. In solitary species or species with a homogeneous age structure, such as guppies (Croft et al., 2003), selection might be stronger to preserve cognitive abilities into higher age.

To conclude, the results from **Paper I** indicate that a relatively larger brain can indeed generate enhanced cognitive abilities in demanding contexts. However, at the intraspecific level a larger brain does not generate any additional learning ability in terms of more efficient learning strategies, unlike the commonly observed pattern between species with differences in relative brain size. In **Paper I**, I therefore propose that an evolutionary increase in relative brain size cause quantitative cognitive divergence within species and more qualitative cognitive divergence at a higher taxonomic level. **Paper II** indicates that variation in cognitive ageing cannot be explained by quantitative differences in brain size, as proposed by the ´brain reserve´ hypothesis. Instead, other intrinsic factors might affect the onset and rate of cognitive ageing. Since large-brained individuals tend to have shorter life-span, at least at the intraspecific level, I propose that the slightly faster cognitive ageing rate in these individuals may act as a constraint in brain size evolution.

Acknowledgement

I would like to thank my supervisor Niclas Kolm for all support during these projects and I am grateful that the supervision is always matched to each unique individual. I also want to thank my co-supervisor John Fitzpatrick, my follow-up group of David Wheatcroft and Rhonda Snook, and all current and previous member of the Kolm lab for interesting discussions and insightful comments on my projects. A big thanks to Mirjam Amcoff who is always very helpful with everything from lab practicalities to discussing projects ideas and much more. Least, but

not last, I would like to thank Loke von Schmalensee for everything you teach me about statistics.

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RESEARCH ARTICLE

Brain size does not predict learning strategies in a serial reversal learning test

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ABSTRACT

Reversal learning assays are commonly used across a wide range of taxa to investigate associative learning and behavioural flexibility. In serial reversal learning, the reward contingency in a binary discrimination is reversed multiple times. Performance during serial reversal learning varies greatly at the interspecific level, as some animals adopt a rule-based strategy that enables them to switch quickly between reward contingencies. A larger relative brain size, generating enhanced learning ability and increased behavioural flexibility, has been proposed to be an important factor underlying this variation. Here, we experimentally tested this hypothesis at the intraspecific level. We used guppies (*Poecilia reticulata*) artificially selected for small and large relative brain size, with matching differences in neuron number, in a serial reversal learning assay. We tested 96 individuals over 10 serial reversals and found that learning performance and memory were predicted by brain size, whereas differences in efficient learning strategies were not. We conclude that variation in brain size and neuron number is important for variation in learning performance and memory, but these differences are not great enough to cause the larger differences in efficient learning strategies observed at higher taxonomic levels.

KEY WORDS: Cognitive ability, Behavioural flexibility, Memory

INTRODUCTION

Cognitive ability varies greatly at all taxonomic levels (Shettleworth, 2010; Thornton and Lukas, 2012). More advanced cognitive abilities may enable an animal to use previous experience to develop efficient learning strategies (Hunter and Kamil, 1971; Mackintosh, 1974, 1988; Rumbaugh et al., 1996; Wilson et al., 1985). Here, we define an efficient learning strategy as the ability to generalize obtained information from earlier successful responses across situations by a learnt rule. By developing and adopting efficient learning strategies, an individual is able to switch faster between contingencies and solve novel problems than if restricted to, for instance, a fixed stimulus–response action pattern (Bitterman, 1965; Gonzalez et al., 1967). Differences in this aspect of cognition are well studied across a wide array of species. For example, macaws (*Diopsittaca nobilis*) outperform caiques (*Pionites melanocephala*) in both colour association and reversal learning tasks (van Horik and Emery, 2018), pinyon jays (*Gymnorhinus cyanocephalus*) solve spatial and visual serial discrimination problems faster than nutcrackers (*Nucifraga columbiana*) and scrub jays (*Aphelocoma californica*) (Bond et al., 2007), and bumblebees (*Bombus* spp.) outperform honeybees (*Apis* spp.) in odour discrimination problems (Sherry and Strang, 2015). The capacity to generalize information across situations and adopt an efficient learning strategy clearly differs between species. However, the proximate mechanisms causing this divergence remain unclear. Natural selection acts on the individual and the cause of individual variation and its potential consequences in cognitive evolution have been largely overlooked (Thornton and Lukas, 2012). To understand what causes the above-stated divergence in efficient learning strategies, it is important to also examine the proximate predictors of learning performance at the intraspecific level.

Reversal learning assays have been used in taxa ranging from insects to humans (Ashton et al., 2018; Bitterman, 1965; Bond et al., 2007; Buechel et al., 2018; Day et al., 1999; Izquierdo et al., 2016; Liu et al., 2016; Sherry and Strang, 2015). There are several strengths of reversal learning assays. First, reversal learning assays test several aspects of learning ability. Second, the neurological mechanisms underlying performance are comparable across multiple species. Third, the experimental protocol is largely standardized across different species. Initially, animals are trained in a binary discrimination task (e.g. visual, olfactory or spatial cues). After either a fixed number of trials or a pre-decided learning criterion, the reward contingency is reversed. In order to be rewarded, the animal thus has to inhibit the response towards the originally rewarded stimulus (A+) and switch to the previously unrewarded stimulus (B−). In this case A+ B− becomes A− B+. The switch in reward contingency is thought to be more cognitively demanding and involves different cognitive processes and brain regions from those for the initial discrimination task (Frank et al., 1972; Izquierdo et al., 2016; López et al., 2000; Watanabe, 2012; Buechel et al., 2018). The initial discrimination tests for associative learning ability, while the ability to reverse and learn the new reward contingency tests behavioural flexibility (Bitterman, 1965; Bond et al., 2007; Izquierdo et al., 2016; Shettleworth, 2010). In serial reversal learning, the reward contingency is reversed multiple times, which is considered to be even more demanding and specifically tests for differences in the formation of efficient learning strategies (Bitterman, 1965; Bond et al., 2007; Shettleworth, 2010). During serial reversals, some animals progressively improve their performance and make fewer errors as they continuously relearn the new reward contingency (Bitterman, 1965; Bond et al., 2007; Sherry and Strang, 2015). The ability to improve requires that the individual generalizes information based on earlier experience and adopts an efficient learning strategy to maximize the rewarded responses (Bitterman, 1965; Bond et al., 2007; Shettleworth, 2010).

In reversal learning assays, a striking variation in performance, post-reversal recovery rate and degree of efficient learning strategies

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Received 6 March 2020; Accepted 10 June 2020

Journal of Experimental Biology (2020) 223, jeb224741. doi:10.1242/jeb.224741

is found, both across and within species (Ashton et al., 2018; Bitterman, 1965; Bond et al., 2007; Buechel et al., 2018; Day et al., 1999; Elias, 1970; Lucon-Xiccato and Bisazza, 2014; Sherry and Strang, 2015; van Horik and Emery, 2018). In serial reversal assays, some species perform very well in serial reversal learning, whereas others fail to both relearn and progressively improve over serial reversals (Bitterman, 1965; Lucon-Xiccato and Bisazza, 2014; Patrick et al., 1967; Warren, 1960). One key component that has been put forward to predict performance in serial reversal learning is relative brain size (Bitterman, 1965; Buechel et al., 2018; van Horik and Emery, 2018; Elias, 1970). This idea is partially supported at the intraspecific level, where artificially selected large-brained guppies and mice outperformed their small-brained conspecifics (Buechel et al., 2018; Elias, 1970). However, serial reversal learning may be considered cognitively more demanding than reversal learning as, in order to progressively improve over serial reversals, at least one additional cognitive process is required, i.e. the ability to generalize information by a learnt rule. To date, it is still unknown whether intraspecific variation in relative brain size also causes variation in performance and efficient learning strategies during serial reversal learning.

To test the hypothesis that relative brain size predicts the ability to adopt an efficient learning strategy, we used female guppies, *Poecilia reticulata* W. Peters 1859, artificially selected for small and large relative brain size and with known differences in neuron numbers (Marhounová et al., 2019), in a serial reversal learning assay. Previous experiments with brain size-selected lines have shown that large-brained guppies outperform small-brained guppies in a number of cognitively demanding tasks (Herczeg et al., 2019). These include associative numerical learning (Kotrschal et al., 2013a), spatial cognition (Kotrschal et al., 2015a) and reversal learning (Buechel et al., 2018). The apparent enhanced cognitive abilities of large-brained guppies have also been shown to be advantageous in ecologically relevant situations such as mate choice assessment (Bloch et al., 2018; Corral-Lopez et al., 2017), predator inspection behaviour (van der Bijl et al., 2015) and survival under predator threat (Kotrschal et al., 2015b). We quantified individual performance in binary colour discrimination over 10 serial reversals. As the ability to generalize previously gained information and adopt an efficient learning strategy is cognitively demanding, we hypothesized that a larger relative brain size will generate cognitive advantages facilitating performance in this test. We thus expected large-brained females to make fewer errors and adopt an efficient learning strategy that would result in a faster reversal learning rate over serial reversals.

MATERIALS AND METHODS

Brain size-selected guppies

The experiment was performed in accordance with ethical applications approved by the Stockholm Animal Research Ethical Permit Board (Dnr: N173/13 and 223/15). We used n=96, 7–8 month old female offspring from 7th generation guppies artificially selected for relative brain size, i.e. brain mass relative to body length (Kotrschal et al., 2013a), with associated differences in neuron number (Marhounová et al., 2019). In the 5th generation, the artificial selection had resulted in 15.4% difference in relative brain size and 11.9% difference in neuron number (Marhounová et al., 2019). Briefly, descendants of wild-caught guppies from high-predation areas in the Quare River, Republic of Trinidad and Tobago, were used to set up three independent breeding stocks (replicates). From each of these, one up- and one down-selected line were created, resulting in six brain size selection lines; 75 breeding pairs per replicate formed the parental strains. For details on the artificial selection, see Kotrschal and colleagues (2013a). Only females were used in this experiment as males have been difficult to motivate with a food reward (Fuss and Witte, 2019; Kotrschal et al., 2013b). Fish were kept with constant aeration, in 25±1°C water temperature, on a 12 h:12 h dark:light cycle. Fish were fed 6 days per week with flake food and live *Artemia nauplii*.

Experimental apparatus

We used the experimental set-up described by Buechel and colleagues (2018) and first used by Lucon-Xiccato and Bisazza (2014). The 7 l experimental tanks were divided into a home compartment and a conditioning chamber (Fig. 1). To avoid unnecessary handling stress, individuals were held in the

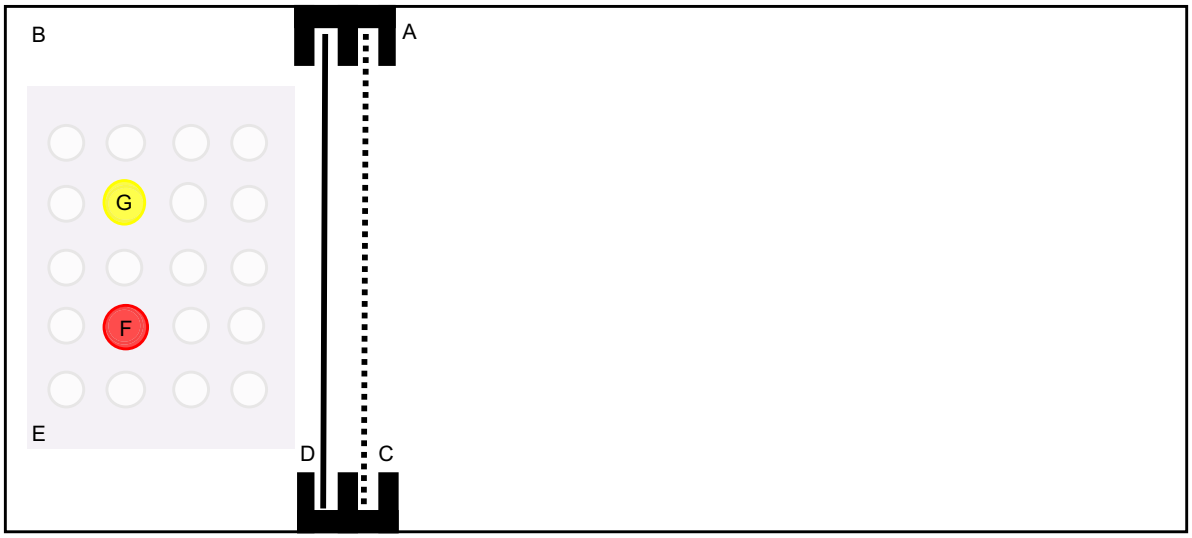

Fig. 1. Schematic diagram of the serial reversal learning set-up. The tank consisted of a home compartment (A) and a conditioning chamber (B). These were separated by a transparent sliding door (C) and an opaque sliding door (D). All training took place in the conditioning chamber. A white plate (E) with 20 holes (10 mm in diameter, 5 mm deep) was placed at the bottom in the conditioning chamber. Animals were trained to discriminate between a red plastic disc (F) and a yellow plastic disc (G) and find an *Artemia* underneath the rewarded stimulus.

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experimental tanks for the duration of the experiment. The fish were physically isolated but visual contact was allowed between adjacent home compartments to avoid negative isolation effects on learning (Bouton, 2007; Petrazzini et al., 2012). However, visual contact between adjacent conditioning chambers was prevented to avoid social learning effects (Brown and Laland, 2002; Laland and Williams, 1997; Reader et al., 2003). All training took place in the conditioning chamber. The home compartment and the conditioning chamber were divided by an opaque and a transparent sliding door. The opaque doors prevented the females from perceiving any visual cues from the arrangement by the experimenter. The transparent doors allowed the females to habituate to and assess the arrangement before entering the conditioning chamber. The trial started with the opening of the opaque door; 10 s later, we opened the transparent door. To prevent observer bias, the experiment was conducted blind and experimental tanks were only identified by running numbers.

Pre-training

First, the females were trained to dislodge a black plastic disc (14 mm in diameter) placed on a white plate with 20 equispaced holes (10 mm in diameter, 5 mm deep) to access one frozen adult *Artemia* underneath. During the first trials, the disc only partially covered the well. Over successive trials, we increased well coverage until it was completely covered. All females could easily dislodge the disc after 30 trials. This set-up is ecologically relevant as it makes use of guppies' natural behaviour to forage underneath leaves and other plant parts (Houde, 1997).

Colour discrimination learning

We trained 96 females (48 each of small and large brained) to discriminate between one red and one yellow disc and to associate one of the stimuli with the food reward. Half of all small- and large-brained females were trained to associate the red stimulus with a food reward, whereas the other half of all small- and large-brained females were trained to associate the yellow stimulus with the food reward. We chose stimuli colour in consideration of mate and food choice preferences of female guppies. Female guppies are known to prefer orange colours as they signal high quality in both mates and food (Houde, 1997; Rodd et al., 2002). The disc with the rewarded conditioned stimulus (CS+) was moveable, whereas the unrewarded conditioned stimulus (CS−) was unmovable because of a glued-on knob fixed in the well. In order to control for olfactory cues, food was placed underneath both the rewarded and the unrewarded disc. As mentioned above, the CS+ colour was counterbalanced and randomly distributed across the brain size-selected lines and replicates to control for any innate preference and colour bias. The females were given six trials per day over five consecutive days (i.e. 30 trials), with a 2 day pause prior to each reversal. To prevent side bias, the position (left- or right-hand side) of the CS+ was randomly chosen for each trial, with no more than two consecutive trials in the same position. For each trial, we scored the first push on either of the discs as either correct or incorrect. If a female did not push any of the discs within 120 s, that trial was counted as a no-choice trial. The time limit was chosen based on our experience in training guppies in this set-up (Buechel et al., 2018) and for logistic reasons such that relevant information was collected while permitting a relatively large sample size. For incorrect and no-choice trials, we gave each female 15 min to make a correct choice before we moved the rewarded disc 5 mm to the side to allow easy access to the food. This ensured that all females received the same number of positively reinforced trials throughout the experiment. During training, all females were tested in a randomized order, with trials typically running between 08:15 h and 17:00 h.

Serial reversal learning assay

Following the initial colour discrimination training, the reward contingency was reversed, i.e. CS+ became CS− and vice versa, for 10 serial reversals. The trial procedure and duration were identical to the procedure described in the colour discrimination learning. We established a fixed number of trials (30) per reversal with reference to the previously established fast colour discrimination ability in female guppies (Buechel et al., 2018; Lucon-Xiccato and Bisazza, 2014).

Data analysis

We performed all statistical analyses in R statistical software (version 3.5.1, http://www.R-project.org/), with the *glmer* function in *lme4* packages, version 1.1-18-1 used for mixed modelling (http://lme4.r-forge.r-project.org). In order to determine how relative brain size might explain variation in learning ability, we used generalized linear mixed models (GLMMs) with binomial error distributions and logit link functions (0=incorrect response, 1=correct response). We analysed the initial colour discrimination task and the serial reversal tasks separately, as the initial colour discrimination task tests associative learning ability, whereas serial reversal learning also tests behavioural flexibility and efficient learning strategies. Continuous variables were always centred at midpoint prior to analyses. Brain size was structured as a two-level categorical factor (small and large). Initially, all models included brain size nested in replicate as a random effect, but replicate returned a zero variance that caused singular fit. To control for a potential effect of replicate we thus included it as a fixed effect in all models, but dropped it from the models as it was not significant ($P>0.3$) and inclusion of replicate did not improve model fit (decreased Akaike information criterion, AIC). Non-significant interactions ($P>0.1$) were excluded until the lowest AIC was met. Statistical significance was obtained by using the ANOVA function, specifying Type III Wald χ^2 tests, in the *car* package (Fox and Weisberg, 2019).

The full model testing initial colour discrimination included the fixed effects rewarded colour, the interaction between brain size and trial, as well as random slope for fish ID [glmer syntax final model: success∼brain size+trial+rewarded colour+(trial|fish ID)].

The full model testing the hypothesis that reversal learning rate is predicted by relative brain size initially included trial number as a fixed effect. Trial caused scaling problems and was therefore dropped from the model and replaced by session number (one to five). Each session included six trials for each reversal. The full model included the fixed effects rewarded colour, and the interactions between brain size and session, and brain size and reversal as well as random slope for fish ID [*glmer* syntax final model: success∼brain size×session+reversal+rewarded colour+(reversal|fish ID)].

The last trial of each reversal was followed by a 2 day pause prior to the next reversal, as described above, which constitutes an opportunity to measure explicit long-term memory (Bailey et al., 1996). By comparing performance on the first trial of each reversal, we could thus test for differences in long-term memory. The full model testing the performance in the first trial per reversal included the fixed effects rewarded colour, the interaction between brain size and reversal, as well as random intercept for fish ID [*glmer* syntax final model: success∼brain size+reversal+rewarded colour+(1|fish ID)]. To test whether differences in performance in the first trial of each reversal were caused by differences in memory retrieval, we fitted an additional GLMM that compared the performance on the fifth day of each reversal between small- and large-brained females. The full model included the fixed effects rewarded colour,

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brain size and reversal, as well as random intercept for fish ID [*glmer* syntax full model: success~brain size+reversal+rewarded colour+(1|fish ID)].

RESULTS

Colour discrimination learning

In the colour discrimination part of the experiment, animals learnt to associate a colour stimulus with a food reward (trial: χ^2_1=63.22, P<0.001). However, matching previous results, neither learning rate (slope of the learning curve) nor performance was predicted by brain size. During the last day of colour discrimination training, small- and large-brained females reached similar performance levels, with a raw data mean±s.e. of 93±3.7% correct responses for small-brained females versus 98±2.0% correct responses for large-brained females. We found no main effect of brain size (χ^2_1=0.20, P=0.65). Females trained on the red stimulus learnt to associate the stimulus colour with the reward at a faster rate than females trained on the yellow stimulus (χ^2_1=19.35, P<0.001); note, that rewarded stimulus colour was counterbalanced between brain size and replicates (see Materials and Methods).

Reversal learning rate and performance across serial reversals

The brain size×reversal interaction was not significant (χ^2_1=0.18, P=0.68; Fig. 2), and was therefore excluded (as mentioned in Materials and Methods). This means that there was no evidence for differences in the ability to progressively improve and thereby adopt an efficient learning strategy between small- and large-brained females. For both brain size-selected lines, learning rate and performance during each reversal was negatively correlated with increasing number of reversals; error rate increased over serial reversals (χ^2_1=17.91, P<0.001). Performance on the last day of the final reversal was slightly above chance with a raw data mean±s.e. of 62.9±0.08% for small-brained versus 68.3±0.07% for large-brained females. Across reversals, the interaction between brain size and session number was significant (χ^2_1=6.44, P=0.01), suggesting a steeper average learning curve in large-brained compared with small-brained females within a week-long reversal. It was easier to switch from the rewarded red to the rewarded yellow stimulus than vice versa across reversals (χ^2_1=301.96, P<0.001). The model also revealed a significant main effect for session, but not for brain size (session: χ^2_1=791.99, P<0.001; brain size: χ^2_1=0.83, P=0.36).

Explicit long-term memory

On the first trial in nine out of the 10 reversals, large-brained females made significantly more errors and therefore performed at a lower level than small-brained females (χ^2_1=10.99, P<0.001; Fig. 3), suggesting an enhanced explicit long-term memory in large-brained females. Importantly, the performance on the last day of training for each reversal did not differ between the brain size-selected lines (χ^2_1=0.14, P=0.71), which indicates that the memory of what was learnt in the previous reversal was better stored in large-brained compared with small-brained females. The model for the last day of each reversal also revealed significant main effects for reversal and colour (reversal: χ^2_1=57.64, P<0.001; colour: χ^2_1=68.40, P<0001). For both brain size-selected lines, performance on the first trial of each reversal increased with increasing reversals (χ^2_1=42.66, P<0.001). Note that an increase in performance in the first trial of each reversal was an effect of increasingly lower performance levels at the end of the previous reversal. Memory retrieval was affected by rewarded stimulus colour (χ^2_1=14.73, P<0.001), suggesting that across reversals, the yellow stimulus was better stored in the long-term memory.

DISCUSSION

We investigated the performance of small- and large-brained female guppies in a serial reversal learning assay. We found that large-brained females learnt the new reward contingency at a faster rate and also stored and retrieved information more efficiently in their explicit long-term memory as compared with their small-brained conspecifics. However, none of the brain size-selected lines progressively improved their performance over serial reversals. Rewarded colour was a strong predictor of performance and learning rate for both brain size-selected lines.

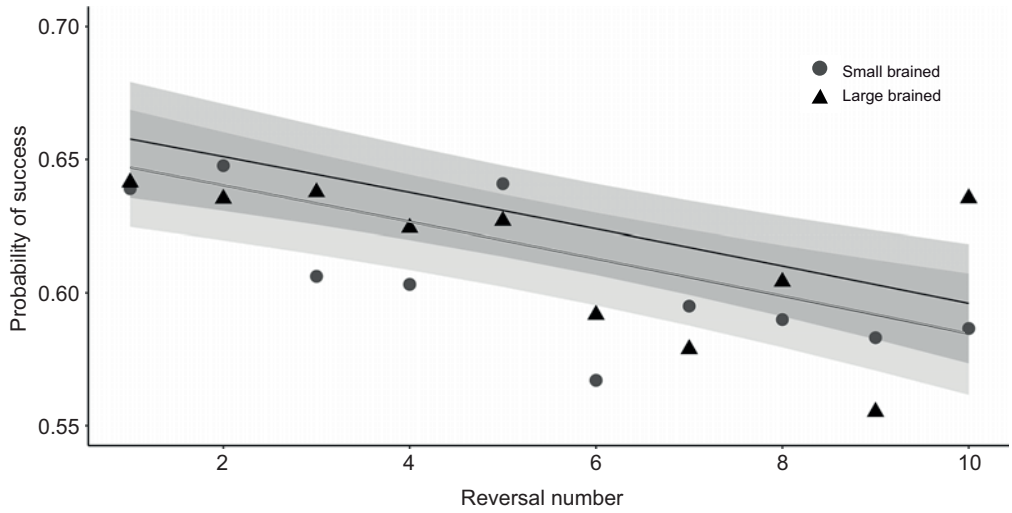

Fig. 2. Performance in serial reversal learning. Performance and learning rate were measured as the proportion of correct responses in each trial across 10 serial reversals (see Materials and Methods). We found no evidence for the hypothesis that relative brain size predicts the ability to progressively improve performance over serial reversals and thereby develop an efficient learning strategy (brain size×reversal; χ^2_1=0.18, P=0.68). Raw mean data (based on 26,763 observations in total) for 48 small-brained and 48 large-brained female guppies across 10 serial reversals. The logistic regression slope estimates for small-brained (grey line) and large-brained (black line) females and 95% confidence interval (shading) are predictions obtained from a GLMM with binominal error distribution.

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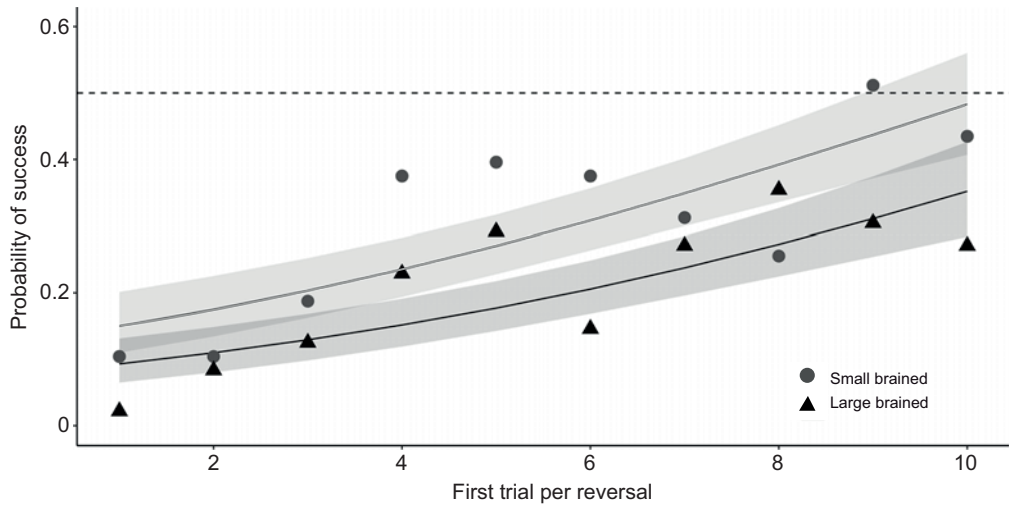

Fig. 3. Explicit long-term memory. Memory was measured as the proportion of correct responses on the first trial of each reversal (preceded by a 2 day pause). We found that relative brain size predicted long-term memory, as large-brained females made more errors on the first trial of each reversal (brain size; χ^2_1=10.99, P<0.001). Raw mean data (based on 948 observations in total) and the logistic regression slope estimates for 48 small-brained and 48 large-brained female guppies in a serial reversal learning assay. The dotted horizontal line represents the 50% performance level. The logistic regression slope estimates for small-brained (grey line) and large-brained (black line) females and 95% confidence interval (shading) are predictions obtained from a GLMM with binominal error distribution.

Differences in relative brain size, with associated differences in neuron number, affected variation in two distinct aspects of cognition in our experiment, as relative brain size predicted associative learning rate over serial reversals and predicted explicit long-term memory storage. However, learning rate during the initial discrimination did not differ significantly between the brain size-selected lines, something that has been observed in earlier assays on these selection lines (Buechel et al., 2018). Performance on the last day of training across serial reversals did not differ between the brain size-selected lines, whereas performance on the first trial of each reversal did. Large-brained females made more errors on the first trial across serial reversals, yet they reached the same performance level as small-brained females on the last day of each reversal. From this, we conclude that associative learning rate was faster in large-brained than in small-brained females in the more cognitively demanding reversal learning context; the significant interaction between brain size and session number corroborates this argument. Previous experimental studies have also found positive relationships between learning rate in demanding contexts and relative brain size, both in the brain size-selected guppies (Buechel et al., 2018) and in mice artificially selected for increased brain mass (Elias, 1970). The lower performance in the first trial of each reversal is most likely explained by differences in memory capacity. What is learnt towards the end of each reversal seems to be better stored in the explicit long-term memory of large-brained females. Phylogenetic comparative studies have revealed a correlation between whole-brain size and different aspects of memory storage, for instance between bird species (Garamszegi and Eens, 2004). Taken together, our results are mainly consistent with previous findings at the interspecific level in that relative brain size is advantageous in particularly demanding cognitive contexts (Benson-Amram et al., 2016; MacLean et al., 2014; Boire et al., 2002; Reader and Laland, 2002), whereas more fundamental aspects of cognition are probably less related to relative brain size (Buechel et al., 2018). However, we found no support for the proposal that relative brain size predicts the ability to learn from previous experience at the within-species level, as we found no difference in the ability to adopt an efficient learning strategy. The learning rate in both small-brained and large-brained females was instead strongly impaired over an increasing number of reversals, which almost led to extinction in the last reversals. Female guppies of the brain size-selected lines showed neither signs of stability in what was learnt during early reversals nor any flexibility to make new associations when contingencies were reversed repeatedly. We conclude that female guppies only rely on associative learning processes and apparently lack the ability to take advantage of previously successful discrimination in order to adopt an efficient learning strategy.

There are at least two possible explanations for why learning rate was impaired by serial reversals in both of the brain size-selected lines. First, ecological requirements create species-specific challenges that generate divergence in cognitive abilities (Shettleworth, 2010). Continuous changes in feeding opportunities or social complexity are ultimately thought to generate a flexible behaviour repertoire (Bond et al., 2007; Day et al., 1999; Sherry and Strang, 2015). In accordance with the existence of trade-offs between cognitive abilities, as proposed by Tellos-Ramos and colleagues (2019), the ecology of the guppy might favour spatial memory rather than the advanced learning abilities investigated here. Second, cognitive processes involved in the ability to adopt an efficient learning strategy are controlled by a specific region in the telencephalon (Frank et al., 1972; Izquierdo et al., 2016; López et al., 2000; Watanabe, 2012). Primates have among the largest relative telencephalon sizes in the animal kingdom (Finlay and Darlington, 1995), and telencephalon neuron number and density in parrots and many songbirds are equivalent to those of primates (Olkowitcz et al., 2016). Interestingly, species from these taxa are also known to typically perform well in serial reversal assays (Cauchoix et al., 2017; Boogert et al., 2011; Gosette, 1968; Gosette et al., 1966; van Horik and Emery, 2018). Brain region size typically varies between but not within species (Finlay and Darlington, 1995; Gould et al., 2013; Healy and Krebs, 1992; Lucas et al., 2004). Neither neuron density nor relative (to the rest of the brain) telencephalon volume differ between the brain size-selected lines

(Kotrschal et al., 2017; Marhounová et al., 2019), which might explain why we found quantitative (i.e. a slightly higher learning rate) but not qualitative (i.e. negative effect of increasing reversals in both brain size-selected lines) differences in learning ability between the brain size-selected lines. We speculate that more advanced learning abilities are explained by the evolution of increased telencephalon size and/or neuron number, rather than by relative brain size and total number of neurons. Alternatively, individual variation in relative brain size and neuron number is too small for the detection of differences in advanced learning abilities in these selection lines.

The positive effect on discrimination learning rate with increasing number of reversals is generally substantially smaller in fish (Engelhardt et al., 1973; Fuss and Witte, 2019; Levin and Vergara, 1987; Lucon-Xiccato and Bisazza, 2014; Mackintosh and Cauty, 1971; Squier, 1969; Wodinsky and Bitterman, 1957) than in other vertebrates (Bitterman, 1965; Bond et al., 2007; Mackintosh and Cauty, 1971). Although there are examples of a positive effect on discrimination learning rate across reversals (see for example Engelhardt et al., 1973; Mackintosh and Cauty, 1971; Wodinsky and Bitterman, 1957), the majority of studies in fish have failed to show any progressive improvement over serial reversals (Behrend et al., 1965; Behrend and Bitterman, 1967; Bitterman et al., 1958; Bitterman, 1965; Engelhardt et al., 1973; Fuss and Witte, 2019; Gonzalez et al., 1967; Levin et al., 1984; Lucon-Xiccato and Bisazza, 2014; Patrick et al., 1967; Portavella and Vargas, 2005; Warren, 1960). Our results are thus consistent with many previous serial reversal learning assays in other species of fish. Another aspect of the results found in fish in this context is that, even when they show improvement over increasing reversals, they rarely improve their performance over serial reversals beyond the level of performance in the initial discrimination. It can thus be questioned whether the fish tested really understand 'the principle of reversal' (*sensu* Shettleworth, 2010).

A surprising finding during this experiment was the strong effect of the rewarded stimulus. During the initial colour discrimination part, female guppies from both brain size-selected lines made more errors with the yellow compared with the red stimulus. Red is well known to be a signal of strong biological significance for female guppies as, under natural conditions, it predicts feeding opportunities and male quality (Houde, 1997). It is thus not surprising that we found a bias for red, something that has been shown before in a learning context (Buechel et al., 2018). Interestingly, across serial reversals, more errors were made with the red compared with the yellow stimulus. This suggests a shift in this pre-existing bias, induced by an increased number of reversals. One possible explanation for this shift might be that as red is an important stimulus for female guppies, not being rewarded for pushing the red disc might be perceived as a strong negative experience. Over time, this might decrease the willingness to push the red disc. A shift in the pre-existing bias for important stimuli has been shown to change after both positive and negative experience in butterflies (Westerman et al., 2012) and spiders (Hebets, 2003; Hebets and Vink, 2007; Svensson et al., 2010) in mating contexts. However, whether the shift from red to yellow bias in this experiment is an experimental artefact or whether it has an adaptive value should be investigated further.

Although the relative brain size was not measured directly in the fish used in this experiment, all of the previous assays of relative brain size in both earlier and the current generation of selection have shown substantial differences (typically 12–15% differences in later generations) in relative brain size (Kotrschal et al., 2013a,b, 2017), brain volume (Kotrschal et al., 2017; Marhounová et al., 2019) and neuron numbers (Marhounová et al., 2019) between these selection lines. We therefore assume that these differences remain in the fish used in the current study.

To conclude, it is increasingly clear that in demanding contexts, a relatively larger brain size provides individuals with an enhanced cognitive ability. However, differences in learning ability and behavioural flexibility between the brain size-selected lines were not large enough to enable detection of the differences found at higher taxonomic levels (Bitterman, 1965; Bond et al., 2007). We speculate that the mechanisms causing variation in the ability to adopt an efficient learning strategy require larger variation in brain size, neuron number or region structure size than the differences in relative brain size observed in these selection lines. Additionally, based on both the results of the present study and those of previous experimental studies (Buechel et al., 2018; Elias, 1970), we hypothesize that while a relatively larger brain increases performance in species-specific tasks, it does not provide any additional learning ability. We thus propose that brain size-driven cognitive divergence within species is mostly quantitative in nature.

Acknowledgements
We thank Anna Rennie for fish housekeeping, John Fitzpatrick, Wouter van der Bijl, Joacim Näslund and Loke von Schmalensee for statistical advice, and Lotta Wallander for help during the experiment.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.B., N.K.; Methodology: A.B., A.K., N.K.; Formal analysis: A.B.; Investigation: A.B.; Resources: N.K.; Writing - original draft: A.B.; Writing - review & editing: A.B., S.B., M.A., A.K., N.K.; Visualization: A.B.; Supervision: N.K.; Project administration: S.B., M.A.; Funding acquisition: N.K.

Funding
This project was funded by grants to N.K. from the Swedish Research Council (Vetenskapsrådet; grant 2016-03435), and Knut and Alice Wallenberg Foundation (Knut och Alice Wallenbergs Stiftelse; grant 102 2013.0072). Deposited in PMC for immediate release.

Data availability
All data are available from the Dryad digital repository (Boussard et al., 2020): dryad.5mkkwh72s

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Experimental Gerontology 146 (2021) 111218

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

The link between relative brain size and cognitive ageing in female guppies (*Poecilia reticulata*) artificially selected for variation in brain size

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ARTICLE INFO

Section editor: Thomas Foster

Keywords:
Senescence
Behavioural flexibility
Reversal learning

ABSTRACT

Cognitive ageing is the general process when certain mental skills gradually deteriorate with age. Across species, there is a pattern of a slower brain structure degradation rate in large-brained species. Hence, having a larger brain might buffer the impact of cognitive ageing and positively affect survival at older age. However, few studies have investigated the link between relative brain size and cognitive ageing at the intraspecific level. In particular, experimental data on how brain size affects brain function also into higher age is largely missing. We used 288 female guppies (*Poecilia reticulata*), artificially selected for large and small relative brain size, to investigate variation in colour discrimination and behavioural flexibility, at 4–6, 12 and 24 months of age. These ages are particularly interesting since they cover the life span from sexual maturation until maximal life length under natural conditions. We found no evidence for a slower cognitive ageing rate in large-brained females in neither initial colour discrimination nor reversal learning. Behavioural flexibility was predicted by large relative brain size in the youngest group, but the effect of brain size disappeared with increasing age. This result suggests that cognitive ageing rate is faster in large-brained female guppies, potentially due to the faster ageing and shorter lifespan in the large-brained selection lines. It also means that cognition levels align across different brain sizes with older age. We conclude that there are cognitive consequences of ageing that vary with relative brain size in advanced learning abilities, whereas fundamental aspects of learning can be maintained throughout the ecologically relevant life span.

1. Introduction

Cognitive ageing is the process of age-related decline of certain cognitive abilities, caused by degradation of brain structures (Hedden and Gabrieli, 2004; Kandel et al., 2013; Marner et al., 2003; Phillips et al., 2018; Ritchie et al., 2015; Scahill et al., 2003). Cognitive ageing is widespread throughout the animal kingdom and studied among diverse species (Behrends et al., 2007; Hirsch and Peretz, 1984; Ritchie et al., 2015; Tapp et al., 2003; Yu et al., 2006; Zwoinska et al., 2013; Zyzak et al., 1995). For example, humans process information at a slower rate and show a severe decline in reasoning and working memory with increasing age (Ritchie et al., 2015), dogs progressively decline in associative learning and behavioural flexibility with increasing age (Tapp et al., 2003), and associative learning and memory declines with increasing age in nematodes (Zwoinska et al., 2013). The causes and consequences of many aspects of cognitive ageing are very well studied in humans and are focused on how to prevent and cure age-related cognitive diseases. However, if evolutionary increases in vertebrate brain size also affect the rate and onset of cognitive ageing is less well understood. Here, we focus on the cognitive consequences of ageing in a laboratory bred guppy population with known differences in brain size.

Learning is an important ability that ranges from simple non-associative learning shared by all bilateral species to more advanced cognitive abilities such as casual reasoning and imagination described in humans, great apes and corvids (Emery and Clayton, 2004; Seed et al., 2009; Shettleworth, 2010). There is ample evidence for the positive effects of learning on survival and reproductive success (Dukas, 2005; Dukas and Bernays, 2000; Dukas and Duan, 2000; Egas and Sabelis, 2001; Grieco et al., 2002; Sherman and Visscher, 2002; Steidle, 1998). Considering the important fitness effects of learning, any decline should

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https://doi.org/10.1016/j.exger.2020.111218
Received 7 October 2020; Received in revised form 10 December 2020; Accepted 16 December 2020
Available online 26 December 2020

be disadvantageous. There should thus be strong natural selection for preservation of important aspects of learning ability throughout life time. Within the species tested, the magnitude of cognitive ageing is highly variable, i.e. chronological and physical age can be decoupled. At the proximate level there are both extrinsic and intrinsic sources of variation in cognitive decline. A limited number of studies have investigated why chronological age is not always synchronized with physical age within an ecologically relevant life span in animals. At the intraspecific level, differences in life-history traits, ontogeny and environmental factors have been put forward as important predictors of the onset and rate of cognitive decline. For instance, in honey bees (*A. mellifera*) foragers show a faster decline in olfactory associative learning than nurse bees (Behrends et al., 2007), short-lived female nematodes (*C. remanei*) decline faster than longer-lived males in olfactory associative learning (Zwoinska et al., 2013), and higher water temperature cause faster decline in visual associative learning rate than lower water temperature in the African turquoise killifish (*Nothobranchius furzeri*) (Valenzano et al., 2006). One intrinsic factor that has been suggested to cause variation in cognitive decline is quantitative variation in various aspects of brain size. According to the 'brain reserve' model, higher individual quantitative levels of brain size, neuronal number, number of synapses etc. allow individuals to cope better with brain structure degradation and thereby preserve cognitive abilities into higher age (Katzman, 1993; Satz, 1993; but see Stern, 2009). Hence, a larger 'brain reserve' might delay and slow down cognitive decline compared to a smaller 'brain reserve'. In humans, whole brain volume and/or the volume of certain structures were smaller in individuals with age-related cognitive diseases (Ritchie et al., 2015; Wolf et al., 2004). Theory and empirical findings together imply that quantitative differences in brain morphology might secure brain functionality and thereby preserve cognitive abilities into higher age. However, the link between several aspects of brain morphology and age-related cognitive decline is largely unexplored in non-human animals.

Here, we investigate if ageing has cognitive consequences that vary with quantitative differences in brain size and neuron numbers by means of an experimental approach. We used the reversal learning paradigm to test cognitive performance in three different chronological age groups of female guppies artificially selected for small and large relative brain size, with established substantial differences in brain size (>15%) and neuron number (>11%) (Kotrschal et al., 2013, 2017; Marhounová et al., 2019). Previous experiments on these brain size selected lines have shown that large-brained guppies outperform small-brained guppies in experiments testing various cognitive abilities known to decline with age (Buechel et al., 2018; Kotrschal et al., 2013, 2015a). Since differences in cognitive ability and quantitative measurements of brain morphology is so well established between these up- and down selected lines they emerge as a highly suitable model system to test cognitive performance between different age groups. Based on the 'brain reserve' hypothesis, we expect that with increasing age large-brained females decline in colour association learning ability and behavioural flexibility at a slower rate than small-brained females.

2. Materials and methods

2.1. Experimental design

To examine how relative brain size impacts cognitive ageing, we used individuals with known differences in brain size and neuron number in a cross-sectional design (i.e. individuals from different age groups are compared). We opted to use a cross-sectional design rather than test the same individuals at different ages since the latter might lead to over- or underestimating cognitive ageing if the same test is used multiple times (Harada et al., 2013).

In order to test cognitive ageing, we used the reversal learning paradigm. Reversal learning is a common test of associative learning ability and behavioural flexibility that is frequently used across taxa (Bond et al., 2007; Buechel et al., 2018; Day et al., 1999; Izquierdo et al., 2016; Liu et al., 2016; Sherry and Strang, 2015). During reversal learning, individuals are initially trained to discriminate between two stimuli, rewarded A+ and unrewarded B-. After reaching either a learning criterion or a fixed number of trials, the reward contingency is reversed, i.e. A+ B- becomes A- B+. In order to continue to be rewarded, the animal has to inhibit the previous rewarded response and form a new association. The rate at which individuals learn the new reward contingency has been seen as an indication of behavioural flexibility (Izquierdo et al., 2016). The reversal learning paradigm thus tests for at least two aspects of cognition: the initial part tests for associative learning ability, the reversal part tests for behavioural flexibility.

2.2. Subjects

We used 7th generation female guppies artificially selected for relative brain size (Kotrschal et al., 2013). In short, guppies were up- or down selected for brain weight relative to body length from three independent breeding stocks (replicates), resulting in six lines in total. For more details on the selection regime see Kotrschal et al. (2013). After five generations of selection, a 15.4% difference in relative brain size between up- and down selected lines was established (Marhounová et al., 2019). Recent assays on brain morphology have also shown a difference in brain volume (Kotrschal et al., 2017; Marhounová et al., 2019) and an 11.9% difference in neuron number (Marhounová et al., 2019) between up- and down selected lines. The brains of the fish used in this experiment were not measured directly. Since repeated previous quantification of brain weight, brain volume and neuron number have all shown significant differences in earlier as well as the current generation of selection (we are currently on the 7th generation of selection), we assumed that the previously established differences occur also in the fish used here (Kotrschal et al., 2013, 2017; Marhounová et al., 2019).

We used in total 288 females from three different age groups; 4–6, 12 and 24 months of age, from here on these groups are referred to as the youngest, the middle age and the oldest group. These ages were chosen since they represent the life span from sexual maturation until maximal life length under natural conditions (Reznick et al., 2001). The number of females used were balanced across the three age groups as well as the up- and down selected lines per replicate (i.e. 96 females, 48 small and large-brained, per age group). As all fish from the same generation are bred within a short time range (i.e. three months), the three different age groups were tested at different time points. Only females were used in this experiment since guppy males in general are more difficult to motivate with a food rewards as compared to females due to differences in body size and life history (Houde, 1997; Fuss and Witte, 2019; Kotrschal et al., 2013).

Fish were kept with constant aeration, in 25 ± 1 °C water temperature, on a 12:12 h dark: light cycle, java moss (*Taxiphyllum sp.*) and water snails (*Planorbis sp.*) to facilitate the elimination of organic waste. Fish were fed six days per week with flake food and live *Artemia nauplii*.

2.3. Apparatus

All females were kept individually in 7 L experimental tanks that were divided into a home chamber and a testing chamber (Fig. 1). These were separated by a transparent and a grey sliding door. The grey door prevented the females from perceiving any cues when the experimenter prepared each new trial. The transparent door allowed the females to assess the arrangement in the conditioning chamber prior to each trial. A trial started with opening of the grey door and 10 s later the transparent door was opened. The females had visual contact with each other between the home chambers, to minimize potential negative effects of social isolation (Lombardi-Brandão et al., 2015; Petrazzini et al., 2012), but were prevented from contact between testing chambers to avoid social learning (Brown and Laland, 2002; Reader et al., 2003). A white plate with 20 equispaced wells (10 mm in diameter, 5 mm deep) was

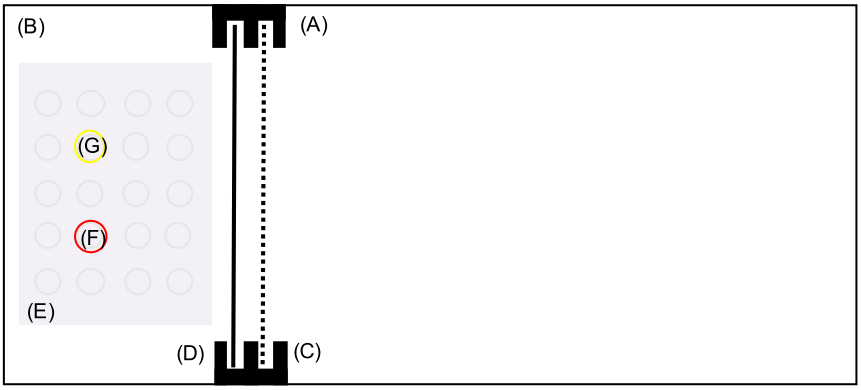

Fig. 1. Schematic diagram of the experimental set-up. All fish were housed individually in experimental tanks throughout the whole experiment. The tanks consisted of a home chamber (A) and a testing chamber (B). These were separated by a transparent (C) and a grey (D) sliding door. A white plate (E) with holes was placed at the bottom in the testing chamber. Fish were trained to discriminate between a red (F) and a yellow plastic disc (G) and to find a frozen and thawed *Artemia* reward underneath the rewarded stimulus colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

placed at the bottom in the testing chamber. Prior to the cognitive assays all females were pre-trained to dislodge a black plastic disc (14 mm in diameter, 2 mm thick) placed adjacent to the mid well on the white plate and find an adult frozen and thawed *Artemia* reward underneath. During the first trials of the pre-training, the disc only partly covered the well. During consecutive trials we successively covered the whole well. All females learnt to dislodge the black disc within 30 trials. Moving an object to find food underneath is part of the behavioural repertoire of guppies since under natural conditions guppies are known to forage underneath plant parts etc. (Houde, 1997). The experimenter was blind to the selection line of all females in order to avoid any potential observer bias.

2.4. Cognitive assay

Initially, we trained the females in a binary colour discrimination task. We used, red and yellow colour stimuli, in consideration of the ecology of female guppies. Orange colours are important cues during mate choice and foraging (Houde, 1997; Rodd et al., 2002). Red and yellow have also successfully been used in similar experiments using female guppies (Buechel et al., 2018; Fong et al., 2019; Lucon-Xiccato and Bisazza, 2014). To control for potential colour preferences, the rewarded stimuli were balanced for each age group across the up- and down selected lines per replicate. That is, half of all small and large-brained females respectively learnt to associate yellow with the *Artemia* reward and the other half learnt to associate red with the *Artemia* reward. To ensure that the females learnt to discriminate between the stimuli, the position of the rewarded stimulus, right vs left, was haphazardly chosen by rolling a dice, but making sure that no more than two consecutive trials were performed in the same position. The rewarded stimulus (S+) was free to slide from the well, whereas the unrewarded stimulus (S-) was unmovable due to a glued-on silicon knob that protruded down into the well. To control for odour effects, an *Artemia* reward was placed under both the rewarded and the unrewarded stimulus. For each trial, we scored the first push a female did on either of the disc as a correct (1) or incorrect (0) response. If the female did not push any of the discs within 120 s, that trial was recorded as a non-choice trial, but we allowed the female to solve the task to provide all females with the same number of positively reinforced trials. The females were given one daily six-trial session during four days, i.e. 24 trials in total.

To test behavioural flexibility, the reward contingencies were reversed following the fixed number of trials (i.e. 24) in the initial colour discrimination, i.e. S+ became S- and vice versa. The training protocol was equivalent to the protocol described in the initial colour discrimination, but the females were given seven days of daily six-trial sessions, i.e. 42 trials in total. Three individuals, one large-brained from the middle age group and two small-brained from the oldest group, were excluded from the reversal learning part due to acclimatization problems to the new reward contingency (after making several errors during the first reversal trials they showed high stress levels and were removed in accordance with our ethical permits).

2.5. Data analysis

Statistical analyses were performed using R statistical software (v 3.5.1, http://R-project.org/). To assess the impact of relative brain size on learning rate and flexibility between three different age groups, we used generalized linear mixed effects models (GLMMs) with binomial error distribution (1 = correct response, 0 = incorrect response) and logit link functions, as implemented with the *glmer* function in the *lme4* package (Bates et al., 2014). We fitted separate models for the initial colour discrimination and the reversal learning since they test different aspects of cognition. More specifically, for each cognitive assay and age group we modelled correct/incorrect response as a function of the fixed effects brain size (small, large), trial number, rewarded colour (red, yellow), brain size × trial and brain size × rewarded colour, were the brain size × trial interaction test for differences in slope (i.e. learning rate) between small and large-brained females and the brain size × rewarded colour interaction test for differences in slopes between small and large-brained females depending on rewarded colour. Since the reversal learning part is strongly affected by the performance in the colour discrimination, and we detected differences in colour discrimination across the three different age groups (see results, Section 3.1.), we fitted separate models for the three different age groups. To account for repeated measurements, the random effect fish ID was included. Since it is biologically reasonable to expect each unique individual to learn differently from other individuals, we fitted individual learning curves for each fish, i.e. random intercept and slope (Schielzeth and Forstmeier, 2009). In all initial full models, we fitted a random intercept for brain size nested in replicate. Replicate returned a zero-variance estimate. This zero-variance caused singular fit to the models. To still control for replicate we thus fitted it as a fixed factor, but dropped it from further analyses since it was non-significant ($p > 0.4$ for all models) and inclusion of replicate did not improve model fit ($\Delta AIC > 2$ for all models). [*glmer syntax* for all full models: correct/incorrect response ~ brain size + trial + rewarded colour + brain size × trial + brain size × rewarded colour + (trial | fish ID)].

The continuous predictor variable 'trial' was standardized to zero mean prior to running models. To ensure model convergence, we used the optimizer *bobyqa* (*Nelder_Mead* was used for the model testing initial colour discrimination in the middle age group) and increased the number of iterations, as implemented with the *glmerControl* function. Model selection was done backwards based on Akaike's information criterion, only non-significant interactions were subject to any exclusion (Bolker, 2008). Statistical significance was obtained by using the ANOVA function, specifying Type III Wald chi-square tests, in the *car* package (Fox and Weisberg, 2019).

3. Results

3.1. Initial colour discrimination

We found that relative brain size did not predict colour discrimination learning rate or the probability of average correct responses in any of the three age groups (Table 1). Small and large-brained females learnt to discriminate between red and yellow at equal rate in the youngest, middle aged and oldest group (Fig. 2). Importantly, trial number predicted correct responses (Table 1), suggesting that all females learnt the initial discrimination task (Fig. 2). Rewarded colour predicted the probability of correct responses (Table 1). Females trained on red responded correctly more often than females trained on yellow in the youngest (raw data mean ± s.e. 94.0 ± 0.07% correct responses for females trained on red versus 74.8 ± 1.3% correct responses for females trained on yellow) and the middle age groups (raw data mean ± s.e. 87.1 ± 1.0% correct responses for females trained on red versus 75.9 ± 1.3% correct responses for females trained on yellow). In the oldest group, females trained on yellow responded correctly more often than females trained on red (raw data mean ± s.e. 91.2 ± 0.8% correct responses for females trained on yellow versus 84.0 ± 1.1% correct responses for females trained on red).

During the last six trials, the youngest and oldest groups reached similar mean performance levels while the middle age group reached a slightly lower level (raw data mean ± s.e. 93.5 ± $1.0\%_{youngest}$, 90 ± $1.3\%_{middle}$ and 94.1 ± $1\%_{oldest}$).

3.2. Behavioural flexibility

We found that relative brain size predicted behavioural flexibility in the youngest group, such that large-brained females learnt the new reward contingency at a faster rate and made more correct choices (Table 2; Fig. 3). Brain size interacted with colour, indicating that large-brained females had a preference for red in the youngest age group (Table 2). Behavioural flexibility was not predicted by relative brain size in the middle age group (Table 2; Fig. 3). The model revealed a significant main effect of trial, suggesting that all females learnt the new reward contingency, and also for rewarded colour, suggesting that red was a more salient stimulus (Table 2). In the oldest group, behavioural flexibility was not predicted by relative brain size. Small and large-brained females learnt the new reward contingency at equal rate and did equal amount of correct responses (Table 2, Fig. 3). The model also revealed significant effects of trial and rewarded colour, suggesting that all females learnt the new reward contingency and that females trained on yellow did more correct responses (Table 2).

Overall, the three different age groups reached similar mean performance levels the last six trials (raw data mean ± s.e. 87 ± $1.4\%_{youngest}$, 88 ± $1.4\%_{middle}$ and 87 ± $1.4\%_{oldest}$).

Table 1
Results from independent GLMMs testing for the effect of relative brain size on initial colour discrimination ability across three different age groups of female guppies. The columns provide chi-squared values (χ^2), degrees of freedom (d.f.) and associated significance values (p) for the fixed effects brain size (small, large), trial number (1–24) and rewarded colour (red, yellow). Significant ($p < 0.05$) effects are highlighted in bold. We also report the logistic regression slope estimates and their standard errors (SE). Brain size small, mid trial (see methods, Section 2.5.) and colour red are set as baseline.

Fixed effects	χ^2	d.f.	p - Value	Estimate ± SE
Youngest group (4–6 months old)				
(Intercept)	245.48	1	<0.001	3.08 (0.20)
Brain size	0.60	1	0.44	0.14 (0.18)
Trial	99.67	1	**<0.001**	0.14 (0.01)
Colour	90.43	1	**<0.001**	−1.87 (0.20)
Middle age (12 months old)				
(Intercept)	114.05	1	<0.001	2.23 (0.21)
Brain size	0.36	1	0.55	0.14 (0.23)
Trial	58.78	1	**<0.001**	0.10 (0.01)
Colour	15.78	1	**<0.001**	−0.91 (0.23)
Oldest group (24 months old)				
(Intercept)	93.91	1	<0.001	2.08 (0.22)
Brain size	0.05	1	0.82	0.05 (0.24)
Trial	52.10	1	**<0.001**	0.13 (0.02)
Colour	12.14	1	**<0.001**	0.86 (0.25)

4. Discussion

The onset and rate of cognitive ageing has been proposed to be affected by individual differences in brain size and neuron number. To test this, we investigated cognitive performance in female guppies from three different age groups artificially selected for small and large relative brain size. We found that a relative larger brain with more neurons did not preserve cognitive abilities into higher age better than a smaller brain as predicted by the 'brain reserve' hypothesis. In contrast, the evident positive effect of relative brain size on behavioural flexibility in the youngest age group disappeared with increasing age. Initial colour discrimination learning rate did not vary with relative brain size across age in these selection lines. Interestingly, we found no obvious effect of age neither during the initial colour discrimination nor during the reversal learning in these female guppies.

In line with a previous study (Buechel et al., 2018), we found a significant interaction between brain size and trial (i.e. differences in slopes and thus learning rate) during reversal learning in the youngest age group, but this effect was not evident in the middle and the oldest age groups. We interpret this as a relative decline in behavioural flexibility in large-brained female guppies. Our results might thus support an evolutionary trade-off between relative brain size and maintenance of advanced cognitive abilities into higher age. One explanation for such a trade-off between relative brain size and cognitive ageing is how resources are allocated differently depending on life-history strategy. Life-history theory predicts that high investment in important fitness-related traits early in life will be traded-off against a faster senescence and a shorter life span. While a longer life span would maintain these traits into higher age (Kirkwood and Holliday, 1979; Williams, 1957). Most notably in this context, a recent comparison in longevity between the small and large-brained guppies revealed a 22% shorter intrinsic life span in the large-brained guppies (Kotrschal et al., 2019). Hence, any cognitive benefits early in life of having a larger, more neuron rich brain, might be lost later in life if general and cognitive ageing occurs faster. The alignment in cognitive levels during reversal learning found here is consistent with other findings in animals with natural or artificial dimorphic life spans. The short-lived sex in nematodes (*C. remanei*) outperformed the long-lived sex in a cognitive assay early in life, but showed a faster cognitive ageing rate (Zwoinska et al., 2013). Artificial selection for improved learning abilities in fruit flies (*Drosophila melanogaster*) reduced life span with 15% in up-selected lines compared to controls (Burger et al., 2008). Similar patterns have been found in long-lived mutants of nematodes (Murakami et al., 2005) and mice (Bartke, 2005). Taken together, this supports a general pattern where cognitive ageing rate appears to be faster in short-lived animals compared to long-lived conspecifics. Furthermore, our results strengthen previous results by Kotrschal et al. (2013, 2019), that evolutionary changes in relative brain size cause changes in life-history strategy also at the intraspecific level. The faster learning rate during reversal learning found in young large-brained females indicate that they allocate more resources into cognition and presumably into the development and maintenance of brain tissue early in life. In contrast, small-brained females appear to allocate more resources into somatic maintenance (Kotrschal et al., 2013, 2016). A higher cognitive ability early in life might increase extrinsic survival and thereby secure future reproductions.

Our results do not support the 'brain reserve' hypothesis. One potential explanation for this could be that the hypothesis predicts patterns in cognitive ageing rate mainly at the interspecific level. Across taxa,

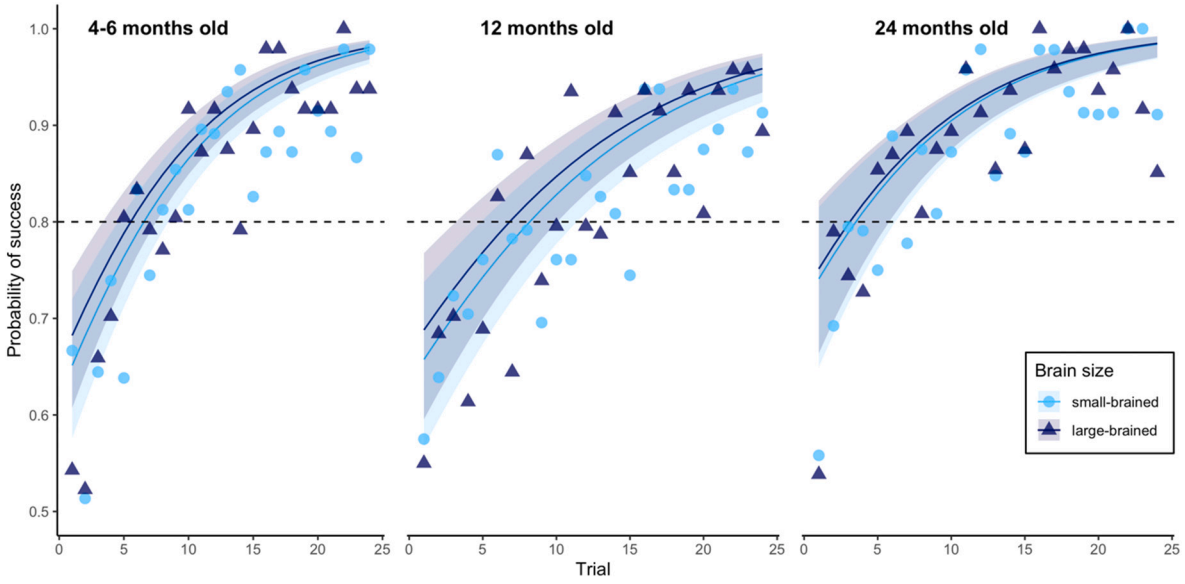

Fig. 2. Initial colour discrimination. Proportion of correct responses each trial in a binary colour discrimination task in 288 female guppies of three different age groups. Circles, clear blue line and 95% CI signify raw data means and GLMM predictions for small-brained females; diamonds, dark blue line and 95% CI signify raw data means and GLMM predictions for large-brained females. Means and model predictions were established from 6912 observations in total across 24 trials. The dotted line represents 80% correct responses level. We found no difference in colour discrimination learning rate between small and large-brained female that changed with increasing age. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Results from independent GLMMs testing for the effect of relative brain size on behavioural flexibility across three different age groups of female guppies. The columns provide chi-squared values (χ^2), degrees of freedom (d.f.) and associated significance values (p) for the fixed effects brain size (small, large), trial number (1–42) and rewarded colour (red, yellow). Significant ($p < 0.05$) effects are highlighted in bold. We also report the logistic regression slope estimates and their standard errors (SE). Brain size small, mid trial (see methods, Section 2.5.) and colour red are set as baseline.

Fixed effects	χ^2	d.f.	p - Value	Estimate ± SE
Youngest group (4–6 months old)				
(Intercept)	37.12	1	<0.001	1.10 (0.18)
Brain size	4.23	1	**0.04**	0.53 (0.26)
Trial	158.83	1	**<0.001**	0.10 (0.01)
Colour	0.04	1	0.83	0.05 (0.22)
Brain size × trial	5.17	1	**0.02**	0.02 (0.01)
Brain size × colour	5.35	1	**0.02**	−0.73 (0.32)
Middle age (12 months old)				
(Intercept)	112.54	1	<0.001	1.51 (0.14)
Brain size	1.61	1	0.20	0.18 (0.14)
Trial	270.76	1	**<0.001**	0.10 (0.01)
Colour	14.80	1	**<0.001**	−0.55 (0.14)
Oldest group (24 months old)				
(Intercept)	21.84	1	<0.001	0.74 (0.16)
Brain size	0.68	1	0.41	0.15 (0.18)
Trial	380.61	1	**<0.001**	0.11 (0.01)
Colour	4.50	1	**0.03**	0.38 (0.18)

absolute and relative brain size have (with a few exceptions) increased considerably during vertebrate evolution (Jerison, 1973; Striedter, 2006; Tsuboi et al., 2018). At the interspecific level, species with a larger brain than expected for their body mass generally have a longer life span (Allman et al., 1993; Barrickman et al., 2008; González-Lagos et al., 2010; Isler and van Schaik, 2009; Jiménez-Ortega et al., 2020; Sol et al., 2007, 2016; Yu et al., 2018). However, this pattern is apparently often reversed at the intraspecific level. For instance, large-brained guppies live significantly shorter than their small-brained conspecifics (Kotrschal et al., 2019). As discussed above, a longer life span often correlates with a slower cognitive ageing rate within several species (Bartke, 2005; Burger et al., 2008; Murakami et al., 2005; Zwoinska et al., 2013). Therefore, we speculate that quantitative differences in brain anatomy, together with general differences in life-history and ecology, explain cognitive ageing rate in accordance with the 'brain reserve' hypothesis at the macroevolutionary level, but not at the intraspecific level. The slower brain degradation in large-brained compared to small-brained species corroborates this speculation (Vágási et al., 2016). Interestingly, the patterns in humans makes for a striking exception to this pattern. There are several possible explanations to this pattern. First, humans exceed their expected brain size for their body weight more than any other vertebrate (Jerison, 1973; Striedter, 2006; Tsuboi et al., 2018). Second, together with the increase in brain size, humans have also extended their life span more than other primates (Allen et al., 2005; Allman et al., 1993). Third, grandmaternal care during post-reproductive life stages may increase inclusive fitness and select for preservation of cognitive abilities into higher age (Allen et al., 2005).

It is clear that also in non-human animals cognitive ageing is a not a general process that causes decline in all aspects of cognition. Rather, cognitive ageing causes decline in certain independent modules of cognitive abilities (Bartus et al., 1979; Hedden and Gabrieli, 2004; Izquierdo et al., 2016; Lai et al., 1995; Voytko et al., 1999). In support of this, we found no obvious decrease in initial colour discrimination learning rate as the mean performance the last six trials was similar across the three different age groups. This suggests that these simpler cognitive abilities are maintained throughout the ecologically relevant life span in guppies that this study encompassed. Also, red and yellow colours are biologically important cues for female guppies (Houde, 1997; Rodd et al., 2002). Selection might therefore be strong to preserve these traits also into higher age. This finding contrasts with what has been found in invertebrates (nematodes: Zwoinska et al., 2013; and honey bees: Behrends et al., 2007) and in the African turquoise killifish (Valenzano et al., 2006), but is supported by findings in rhesus monkeys (Bartus et al., 1979; Lai et al., 1995), all tested within their ecologically relevant life span. Interestingly, behavioural flexibility was also mainly maintained through increased age in our guppy selection lines, as we found no substantial decrease in mean performance the last six trials across the three different age groups. This suggests that, despite the alignment between small and large-brained females with increasing age,

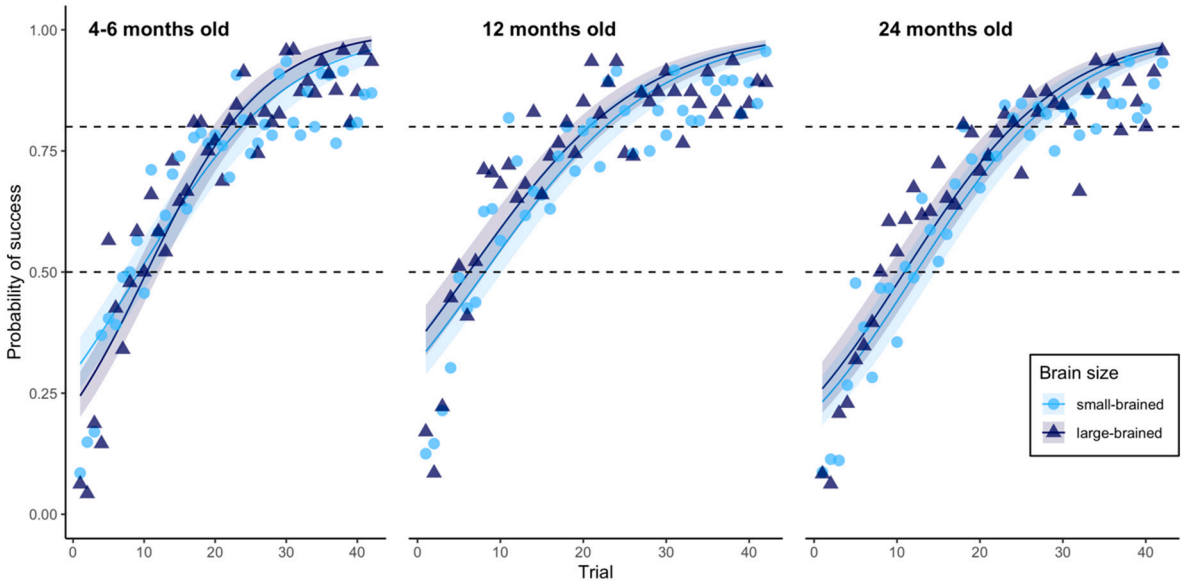

Fig. 3. Reversal learning. Proportion of correct responses each trial in a colour reversal learning task in small and large-brained female guppies of three different age groups. Circles, clear blue line and 95% CI signify raw data means and GLMM predictions for small-brained females; diamonds, dark blue line and 95% CI signify raw data means and GLMM predictions for large-brained females. Means and model predictions were established from 12,054 observations in total across all 42 trials. The dotted line represents 50% and 80% correct responses level. We found that learning rate and correct responses were predicted by relative brain size in the youngest group (brain size × trial; $\chi^2_1 = 5.17$, $p = 0.02$: brain size; $\chi^2_1 = 4.23$, $p = 0.04$). In the middle age and the oldest group neither learning rate nor correct responses were predicted by relative brain size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also more complex cognitive abilities are largely maintained across the ecologically relevant life span in female guppies. Also, this result contrasts with findings in rhesus monkeys, where behavioural flexibility declined with increasing age (Bartus et al., 1979; Lai et al., 1995; Voytko, 1999). Since cognitive decline is caused by degradation in brain structures (Finkel and Holbrook, 2000; Hedden and Gabrieli, 2004; Marner et al., 2003; Phillips et al., 2018), the current and earlier findings imply that there might be interspecific variation in the level of degradation of brain sub-structures. These differences might in turn be caused by interspecific differences of the importance of a particular cognitive trait (Bond et al., 2007; Day et al., 1999; Sherry and Strang, 2015), also in higher age. We speculate that the social complexity of a species might partly explain these differences. For instance, in long-lived group living species with a heterogeneous age structure, the decline in flexibility in older individuals might be compensated by high levels of behavioural flexibility in younger individuals. Hence, selection pressure for preservation of a given cognitive ability might be low. In solitary species or in species with a homogeneous age structures, selection pressures for preservation of various cognitive abilities into higher age might be stronger. Guppies shoal in relatively homogeneous age groups in wild populations (Croft et al., 2003), which might thus explain why we did not find any substantial overall decrease with increasing age in the cognitive abilities tested here. We speculate that social complexity can be a potentially important factor that cause interspecific variation in cognitive ageing rate. However, while this 'sociality allows for cognitive ageing' hypothesis might be relevant for animal species that form groups with heterogeneous age structures remains to be tested.

During the initial colour discrimination, red was a more salient stimulus for the youngest and the middle age groups. Whereas yellow was instead a more salient stimulus for the oldest group. A pre-existing bias for red colours are well known in female guppies, as it is an important signal for male quality and nutrient food resources (Houde, 1997; Rodd et al., 2002). Experience is known to affect pre-existing biases (Hebets, 2003; Svensson et al., 2010; Westerman et al., 2012). However, these guppies are bred under constant laboratory conditions and it is unlikely that this shift is caused by differences in experience between the groups. Alternatively, there might be age-related physiological processes that causes a shift in the salience of a signal. Shift in pre-existing biases and preferences during mate choice have been found to vary with age in both female guppies (Kodric-Brown and Nicoletto, 2001) and female wolf-spiders (FowlerFox et al., 2015; Uetz and Norton, 2007). Therefore, we speculate that this shift in salience is caused by age-related physiological processes rather than confounding environmental conditions. Future tests of visual acuity and colour vision across differently aged guppies are necessary to address this question.

5. Conclusions

Overall, our results show that cognitive ageing is indeed a complex process, generated by a wide array of intrinsic and extrinsic factors that most likely cause extensive variation at all taxonomic levels in the affected neural networks. We conclude that the 'brain reserve' hypothesis does not fully explain the relationship between relative brain size and cognitive ageing at the intraspecific level. Instead, our results indicate that evolving a larger brain might generate important cognitive advantages in certain contexts early in life, but a slightly faster cognitive ageing rate. In addition to the many benefits of evolving a larger brain (Kotrschal et al., 2013, 2015b; MacLean et al., 2014; Sol et al., 2007; van der Bijl et al., 2015), there are also substantial costs associated with increased brain size (Kotrschal et al., 2013, 2016; Raichle and Gusnard, 2002; Tsuboi et al., 2015). It has also previously been shown that there is a negative relationship between relative brain size and life span at the intraspecific level (Kotrschal et al., 2019). We propose that a faster cognitive ageing rate is caused by the shorter life span generated by a larger relative brain size at the intraspecific level and suggest that this can be yet another aspect that constrain the evolution of increased brain size.

Ethics

The experiment was performed in accordance with ethical applications approved by the Stockholm Animal Research Ethical Permit Board (Dnr: N173/13, 223/15, N8/17 and 17362-2019).

CRediT authorship contribution statement

Conceptualization: A.B., N.K.
Methodology: A.B., A.K., N.K.
Formal analysis: A.B.
Investigation: A.B.
Resources: N.K.
Writing original draft: A.B.
Writing review and editing: A.B., S.B., M.A., A.K., N.K.
Visualization: A.B.
Supervision: N.K.
Project administration: S.B., M.A.
Funding acquisition: N.K.

Declaration of competing interest

The authors have no conflicts of interest.

Acknowledgement

We thank Anna Reine, Vivien Holub and Eduardo Nila for fish housekeeping.

Funding

This project was funded by grants to N.K. from the Swedish Research Council (grant 2016-03435), and Knut and Alice Wallenberg Foundation (grant 102 2013.0072).

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