Intraspecific variation in brain size and architecture: population divergence and phenotypic plasticity

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Academic dissertation

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“The chief function of the body is to carry the brain around.”

Thomas A. Edison

Contents

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

I. Gonda, A., Herczeg, G. and Merilä, J. 2009. Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)? *Journal of Evolutionary Biology* 22, 1721–1726.

II. Gonda, A., Herczeg, G. and Merilä, J. 2011. Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation? *BMC Evolutionary Biology* 11, 75.

III. Gonda, A., Trokovic, N., Herczeg, G., Laurila, A. and Merilä, J. 2010. Predation- and competition-mediated brain plasticity in *Rana temporaria* tadpoles. *Journal of Evolutionary Biology* 23, 2300–2308.

IV. Gonda, A., Välimäki, K., Herczeg, G. and Merilä, J. 2011. Brain development and predation: plastic responses depend on evolutionary history. *Biology Letters* doi: 10.1098/rsbl.2011.0837

V. Gonda, A., Herczeg, G. and Merilä, J. 2009. Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B* 276, 2085–2092.

VI. Gonda, A., Herczeg, G. and Merilä, J. Intraspecific brain size variation in the wild: a review. *Manuscript.*

Contributions (Authors are listed in alphabetic order.)

	I	**II**	**III**	**IV**	**V**	**VI**
Original idea, experimental design	AG, GH, JM	AG, GH, JM	AG, AL, JM	AG, GH, JM, KV	AG, GH	AG, GH, JM
Field and laboratory work	AG, GH	AG, GH	AG, GH, AL, NT	AG, KV	AG, GH	-
Data analysis	AG, GH	AG, GH	GH	AG, GH	AG, GH	-
Manuscript preparation	AG, GH, JM	AG, GH, JM	AG, GH, AL, JM, NT	AG, GH, JM, KV	AG, GH, JM	AG, GH, JM

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Abstract

Brain size and architecture exhibit great variation among different species, and also have the ability to express high ontogenetic and environmentally induced adaptive plasticity. Yet, studies on population variation in brain size and architecture, as well as brain plasticity induced by ecologically relevant biotic factors have been largely overlooked to date. In this thesis I aim to address the following main questions: (i) do locally adapted populations differ in brain size and architecture, (ii) can the biotic environment (*viz.* social environment, predation, food availability) induce brain plasticity, and (iii) do locally adapted populations differ in levels of brain plasticity induced by the biotic environment?

In the first two studies I report large variation in both absolute and relative brain size, as well as in the relative sizes of different brain parts, among divergent nine-spined stickleback (*Pungitius pungitius*) populations. Some traits show habitat-dependent divergence, implying natural selection being responsible for the observed patterns. Namely, marine sticklebacks have relatively larger *bulbi olfactorii* (chemosensory centre) and *telencephala* (involved in learning) than pond sticklebacks. Further, I demonstrate the importance of common garden studies in drawing firm evolutionary conclusions that are not hampered by ontogenetic and environmental effects.

In the following three studies I show how the social environment and perceived predation risk shapes brain development. In the tadpoles of common frog (*Rana temporaria*), I demonstrate that under the highest *per capita* predation risk situation (predator present – low density), tadpoles develop relatively smaller brains than in less risky situations, while high tadpole density results in enlarged *tectum opticum* (visual brain centre). Visual contact with conspecifics induces enlarged *tecta optica* in nine-spined sticklebacks, whereas when only olfactory cues from conspecifics are available, *bulbus olfactorius* becomes enlarged. Further, perceived predatory risk results in decreased *hypothalamus* (complex function including the regulation of foraging behaviour) development in sticklebacks. In two cases, I also demonstrate how stickleback populations adapted to different environments diverge in brain plasticity. Group-living has a negative effect on relative brain size in the aggressive, competition-adapted pond sticklebacks, but not in the non-aggressive, predation-adapted marine sticklebacks. Perceived predation risk induces enlargement of *bulbus olfactorius* in pond sticklebacks, but not in marine sticklebacks who have larger *bulbi olfactorii* than pond fish regardless of predation.

My results show that brain size and architecture, as well as the capacity for brain plasticity, can evolve over a relatively short evolutionary time period (< 8000 years). These studies also identify putative environmental factors / selective forces likely to be responsible for the observed patterns. Further, I show how common ecological interactions can shape brain development. In all, these studies demonstrate how microevolution can help to explain the enormous variation observed in the brains of wild animals – a point-of-view which I high-light in the closing review chapter of my thesis.

1. Introduction

A fundamental goal of evolutionary biology is to understand the ultimate and proximate causes behind the phenotypic variation observed among higher taxa, species, populations and individuals in the wild. Environmental variation resulting in spatially variable selective pressures can induce phenotypic and genotypic divergence within species (Mayr 1963; Endler 1977). Systematic, environment-dependent phenotypic variation can be a result of two main processes: (i) phenotypic plasticity, wherein different phenotypes can develop from the same genotype (e.g. West-Eberhard 2003), and (ii) local adaptation, wherein selective forces acting on heritable phenotypic variation result in genetic modifications (e.g. Kawecki and Ebert 2004). The vertebrate brain has been a focal trait for biologists from various fields, ranging from neurobiology through developmental biology to evolutionary ecology. However, our current knowledge about the fine-scale, interpopulation variation in brain size and brain architecture is still very limited. Below, I will review what is known about the evolution and plasticity of the brain, and point out some of the gaps in our knowledge which need to be filled to gain a more complete picture of how and why brain size and architecture varies in the wild.

Brain evolution

Large variation in brain size, and in the size of different brain parts, has been reported in several taxa in varying anatomical depth (e.g. Harvey *et al.* 1980; Kotrschal *et al.* 1998; Day *et al.* 2005). Although it is not straightforward to say what exactly brain size tells us, it is believed to be a good proxy for cognitive ability and intelligence in general (Gibson 2002; Striedter 2005). Consequently, size is widely used in evolutionary studies of the brain. Changes in brain size are usually the result of changes in neuron number rather than in neuron size or connectivity (Striedter 2005). Energetic constraints stemming from the extreme cost of developing and maintaining brain tissue (Aiello and Wheeler 1995) should impose strong selective forces against non adaptive increases. Hence, the size of a given brain architecture is expected to be a good estimate of its importance, and might reflect the way a given species or population has adapted to its environment and prevailing selective regime (Winter and Oxnard 2001; Gonzalez-Voyer and Kolm 2010).

Evolutionary studies of the brain have applied various methods and proxies to estimate brain size (Box 1). Most of these studies have been conducted at the macroevolutionary level, comparing the nervous system of different species or even higher taxa. These correlative interspecific studies have demonstrated positive correlations between brain size and life history traits (e.g. maternal investment: Pagel and Harvey 1988b; Barton and Capellini 2011; Isler 2011; Weisbecker

Box 1. Brain metrics

When comparing overall brain size, at least three different metrics can be used: absolute and relative brain sizes, as well as encephalization quotient.

Absolute brain size varies across five orders of magnitude in vertebrates (e.g. Striedter 2005; Deaner *et al.* 2007). It has increased (sometimes decreased) repeatedly in the course of evolution (Striedter 2005). In general, whenever absolute brain size increases, it does so by increasing the number of neurons. Comparing absolute brain size among distant taxa can be meaningless because body size is also highly variable, and the internal architecture of the brain can be very different (Kotrschal *et al.* 1998). However, the bigger the brain is in absolute terms, the more elements it generally contains. Further, since the power of the brain mainly depends on the number of its elements (Byrne and Bates 2007), absolute brain size can be a good measure of cognitive ability. Thus, when comparing closely related species – or individuals of the same species – in the absence of large structural difference in the brain and body size, absolute brain size can be a good proxy of intelligence and cognitive ability (Gibson 2002; Striedter 2005).

Relative brain size refers to a metric in which body size is taken into account. As brain size does not increase linearly with body size, simply dividing brain size with body size (proportional brain size) can be misleading. As in many other organs (Scmidt-Nielsen 1984), the brain size scales allometrically with body size (e.g. Lande 1979). If brain size is plotted against body size on a double logarithmic scale, the best fitting slope will be less than one (e.g. Lande 1979; Harvey and Bennett 1983; Martin and Harvey 1985; Pagel and Harvey 1988a; Striedter 2005). Hence, the relationship between brain and body size is usually hypoallometric. Relative brain sizes can be compared by using statistical models including body size as a covariate. Large variation has been reported in relative brain size (Bauchot *et al.* 1977; Kotrschal *et al.* 1998) and in general, relative brain size tends to increase in independent lineages during the course of evolution (Striedter 2005). As relative brain size takes both body size and allometry into account, this metric can be used for comparing brain size of diverse taxa. In fact, relative brain size is the most widely used metric in evolutionary studies of brain size.

Encephalization quotient has also been used to control for body size in comparisons of brain size among different taxa (e.g. Jerison 1973; Marino 1997; Lordkipanidze *et al.* 2007; Silox *et al.* 2009; Vasallo and Echeverria 2009). There are several proposed methods for estimating encephalization quotient, but the first one described by Jerison (1973) is the most widely used. It is calculated by dividing measured brain volume by the brain volume expected based on body size, predicted from the allometric relationship of brain and body size from available data over a wide range of taxa (involving as many species/taxa as possible).

and Goswami 2010a,b; parental care type: Gonzalez-Voyer 2009), invasion success (birds: Sol *et al.* 2005; mammals: Sol *et al*. 2008), environmental complexity (fish: Pollen *et al*. 2007; Lisney *et al.* 2008; Shumway 2008, 2010; Gonzalez-Voyer and Kolm 2010; bats: Safi and Dechmann 2005) and behaviour (e.g. food hoarding: Garamszegi and Eens 2004a). Conversely, negative correlations suggest possible trade-offs between brain size and the size of other organs (gut size: Aiello and Wheeler 1995; testis size: Pitnick *et al.* 2006). Further, the strong neural demand stemming from living in complex social groups (the so called "social brain hypothesis") is the most accepted theory behind the unexpectedly large brains of primates, particularly in humans (Dunbar 1998; Dunbar and Shultz 2007a,b; Perez-Barberia *et al.* 2007).

While interspecific studies of brain size variation undoubtedly form the basis of our knowledge regarding the possible evolutionary factors shaping the brain, intraspecific studies could provide an additional understanding of fine scale processes. First, as populations of the same species are likely to have diverged relatively recently, they are likely to be found within the same/similar environmental context as at their time of divergence. Hence, the selective forces behind observed population differentiation may be easier to identify than in the case of divergence at higher taxonomic levels. Further, in an interpopulation framework the heritability of brain size or architecture variation, as well as the selective forces acting on such variation, can be quantified (Lynch and Walsh 1998).

In accordance with interspecific studies, recent intraspecific comparisons have shown that brain size correlates with different environmental factors. For example, in food caching birds, good memory for finding the hidden food (and hence those brain parts that play role in memory storage) might have a direct fitness consequence, especially under harsh or changing environmental conditions (Krebs et al. 1989). Indeed, environmental severity has been found to correlate positively with hippocampus (plays role in spatial memory) size and neuron number in the black-capped chickadee (*Poecile atricapillus*; Pravosudov and Clayton 2002; Roth and Pravosudov 2009), even when one of the environmental factors of harshness, day length, is controlled for (Roth *et al*. 2011). Brain size correlates positively with water oxygen level in fish (Chapman *et al.* 2008) while the size of song control nuclei correlates positively with song repertoire size in birds (Canady *et al.* 1984; Garamszegi and Eens 2004b). Some life history traits like migratory behavior have also been shown to correlate positively with brain size in brown trout (*Salmo trutta*; Kolm *et al.* 2009), or with hippocampus size in a comparative study on subspecies of the white-crowned sparrow (*Zonotrichia leucophrys*; Pravosudov *et al.* 2006).

Although the number of intraspecific studies relying on

population comparisons has started to increase recently, the number of interpopulation studies is as yet extremely low compared to the number of interspecific studies (Fig. 1). Thus, there is an urgent need to establish model systems where the fine details of the microevolution of brain size and architecture can be studied.

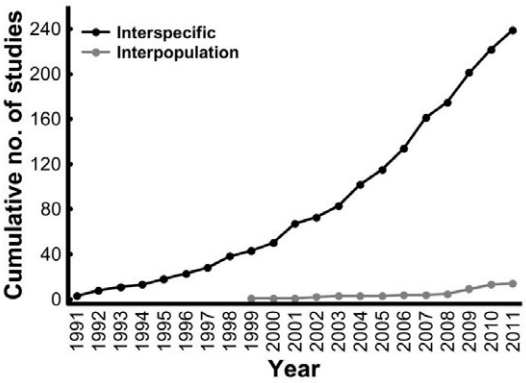

Figure 1. Cumulative number of evolutionary studies focussing on variation in brain size and architecture by comparing species or higher taxa ('Interspecific') *vs.* comparing populations of a single species ('Interpopulation'). Data are based on a literature search in ISI Web of Science, using the search terms: "brain size" and "evolution". Note that studies for 2011 depict the situation as of July.

Brain plasticity

While phenotypic plasticity in general can have unwanted effects, it is often adaptive and increases individual fitness (e.g. Ghalambor *et al.* 2007). The size and architecture of the brain is variable not only on an evolutionary scale, but also on an ontogenetic scale. Besides ontogenetic changes related simply to aging and growth allometry, environmentally induced, plastic responses in brain development are of common occurrence during the lifespan of individual organisms (Wagner 2003; Lisney *et al.* 2007). Phenotypic plasticity has been reported several times in both brain size (Diamond *et al.* 1966; Rosenzweig and Bennett 1969), and in the fine neuroanatomical measures of the brain (Kempermann *et al.* 1997; Nilsson *et al.* 1999). For instance, size of the brain and certain brain parts can change in response to natural environmental variation (e.g. seasonally: Tramontin and Brenowitz 2000), and can be experimentally induced in the lab (Table 1; for reviews see: van Praag *et al.* 2000; Mohammed *et al.* 2002). The song control nuclei of birds are larger during the breeding season (Nottebohm 1981), while a shift in habitat, diet or behaviour can also alter the relative size of the main sensory brain areas in fishes (Wagner 2003; Lisney *et al.* 2007). Experimentally increased abiotic environmental complexity can result in increased brain size (Diamond *et al.* 1966; Rosenzweig and Bennett 1969), in elevated number of hippocampal neurons (Kempermann *et al.* 1997), or in elevated neurogenesis in rodents (Kempermann *et al.* 1997; Nilsson *et al.* 1999; Table 1). Further, captive rearing can also result in decreased brain size, as has been shown in fishes (Kihslinger *et al.* 2006; Burns and Rodd 2008; Burns *et al.* 2008; Table 1).

Table 1. Brain plasticity studies investigating the effect of different abiotic and biotic environmental factors on brain development.

Environmental factor	Affected brain region	Taxon	Reference
enriched environment	total brain	Norway rat, *Rattus norvegicus*	Diamond *et al.* 1966
	hippocampal neurons	House mouse, *Mus muscuslus*	Kempermann *et al.* 1997
	neurogenesis	Norway rat, *R. norvegicus*	Nilsson *et al.* 1999
	neuron proliferation	Coho salmon *Oncorhyncus kisutch*	Lema *et al.* 2005
	cerebellum	Steelhead trout, *O. mykiss*	Kihslinger and Nevitt 2006
	hippocampus	Human, *Homo sapiens*	Maguire *et al.* 2000
	several brain areas and activities	Human, *H. sapiens*	Draganski and May 2008
captive rearing	total brain	Guppy *Poecilia reticulata*	Burns *et al.* 2008
	olfactory bulb and telencephalon	Chinook salmon, *O. tshawytscha*	Kihslinger *et al.* 2006
	optic tectum and telencephalon	Guppy *P. reticulata*	Burns and Rodd 2008
	total brain, several brain parts	Nine-spined stickleback, *Pungitius pungitius*	Gonda *et al.* 2011 (**II**)
social environmnent	optic tectum, bulbus olfactorius	Nine-spined stickleback, *P. pungitius*	Gonda *et al.* 2009 (**V**)
	sensory brain areas	Common frog, *Rana temporaria*	Gonda *et al.* 2010 (**III**)
	neuronal recruitment	Zebra finch, *Taeniopygia guttata*	Adar *et al.* 2008
predation pressure	olfactory bulb	Nine-spined stickleback, *P. pungitius*	Gonda *et al.* 2011(**IV**)
	overall brain	Common frog, *R. temporaria*	Gonda *et al.* 2010 (**III**)

However, a common problem in interpretation of spatial variation in

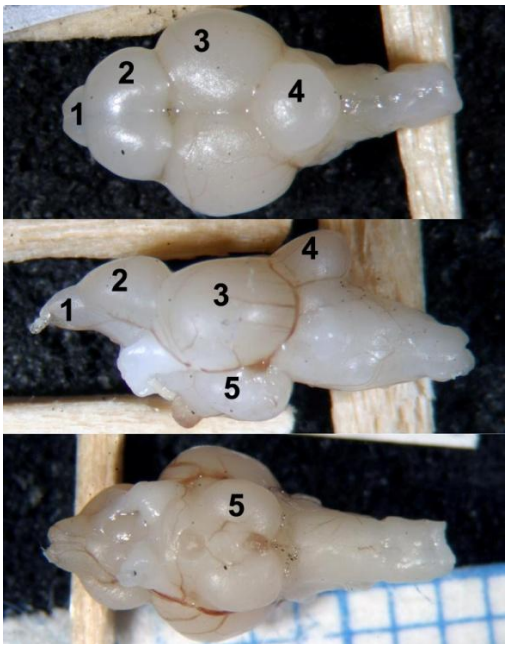

Figure 2. Illustration of the brain parts measured in nine-spined sticklebacks.
1. *Bulbus olfactorius*, 2. *Telencephalon*, 3. *Tectum opticum*, 4. *Cerebellum* 5. *Hypothalamus*

brain architecture in the wild is that local adaptation and phenotypic plasticity cannot be disentangled (e.g. Møller 2010; Wilson and McLaughlin 2010). As a consequence, our knowledge of adaptive phenotypic plasticity in brain size and architecture from the wild is sparse at best. Further, most of the experimental studies inducing brain plasticity have focused on abiotic factors such as environmental complexity (Table 1), and we know almost nothing about the effects of many ecologically relevant biotic environmental factors, such as predation or competition, on brain development. Finally, population divergence in the degree of brain plasticity has been rarely studied (but see Chrispo and Chapman 2010).

2. Aims of this work

The unifying theme of this thesis was to identify the causes and consequences of intraspecific, geographic variation in brain size and architecture. The main aims of my PhD research were roughly threefold: (i) to document habitat-dependent, intraspecific variation in brain size and architecture in the wild; (ii) to determine if brain plasticity could be induced by ecologically relevant, biotic environmental factors; and (iii) to explore habitat-dependent population divergence in brain

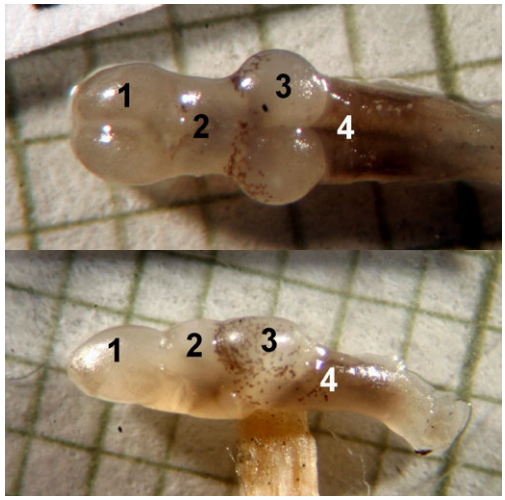

Figure 3. Illustration of the brain parts measured in common frog tadpoles.
1. *Telencephalon*, 2. *Diencephalon*, 3. *Tectum opticum*, 4. *Medulla oblongata*.

plasticity. A more practical long-term goal was to establish a model system wherein microevolution of the brain

Box 2. A synopsis of the known functions of different brain parts in study taxa

Fishes (see also Fig. 2): The *bulbus olfactorius* is the foremost brain part, located in front of the telencephalon. In the nine-spined stickleback, the two are fused. It transmits olfactory cues, allowing fish to detect the presence of conspecifics and competitors of other species, or function as an alarm system, alerting to predators. Olfactory cues also play an important role in different behaviours such us breeding, shoaling and foraging (Östlund-Nilsson *et al*. 2007). The *telencephalon* is a paired structure that receives sensory inputs from all sensory organs, as well as from the hypothalamus (Kotrschal *et al*. 1998). It plays a crucial role in different types of learning processes, such as avoidance learning or spatial learning, and might have a very similar function to the mammalian hippocampus in memory and cognitive mapping (Riedel 1998). Similar to the telencephalon, the *tectum opticum* consists of two hemispheres, and is involved in processing visual cues coming from the retina (Kotrschal *et al*. 1998). Hence, it can be regarded as the main visual brain centre. The *cerebellum* is located posterior and superior to the tectum opticum, and has diverse functions. It has an impact on motor coordination, eye movement and spatial orientation (Kotrschal *et al*. 1998), but is also involved in classical conditioning and spatial cognition (Rodriguez 2005). The *hypothalamus* is the lowermost part of the stickleback brain, and is intimately linked to the hormonal system and behaviour (Kotrschal *et al*. 1998), including reproductive (Sower *et al*. 1993) and feeding behaviours (Kulczykowska and Sánchez Vázquez 2010).

Tadpoles (see also Fig. 3): The *telencephalon* converts sensory inputs to motor outputs (Hoff *et al*. 1999), and might be involved in cutaneous reflex and/or in a defensive behaviour such as freezing (Stehouwer 1987). The *diencephalon* – located posterior to the telencephalon – controls homeostasis, and is involved in sensing hunger and thirst (Hoff *et al*. 1999). It is also known to be involved in the process of metamorphosis (Remy 1962). The *tectum opticum* is connected to the retina and it is the centre of vision. The *medulla oblongata* plays a role in the respiratory system functioning, as well as in auditory and lateral line sensory systems (Torgerson *et al*. 2001; McCormick 1999; Jacoby and Rubunson 1983).

could later be studied with cutting-edge quantitative genetic and functional genomics tools. Finally, I have compiled existing studies on intraspecific variation in brain size and architecture into a review to provide an overview of what is currently known – and not known – about this topic, and to lay out future avenues of research.

In **Chapter I**, I used a common garden experiment to test if nine-spined sticklebacks (*Pungitius pungitius* Linneaus 1758) from coastal marine *vs.* pond populations exhibit genetically-based differences in brain size, and in the size of different brain parts (Fig. 2; for functions, see Box 2). **Chapter II** is an extension of the first chapter, in which I studied if patterns revealed in the lab can also be found in nature, and directly tested for the differences between wild-caught and lab-reared sticklebacks from the same populations. In **Chapter III**, I

investigated the effect of perceived predation risk and intraspecific competition on brain development (Fig. 3; for functions, see Box 2) in tadpoles of common frog (*Rana temporaria* Linneaus 1758).

Box 3. The main questions and answers of the thesis	
Are there genetically based differences in brain size and architecture among different nine-spined stickleback populations?	Yes, there is large variation in brain size and architecture among different nine-spined stickleback populations reared in common garden.
If so, do these differences correspond to differences in habitats occupied by different populations?	In the case of two brain parts, there is a habitat specific pattern: marine fish have significantly larger *bulbus olfactorius* and *telencephalon* than pond fish.
Is there variation in brain size and architecture among different nine-spined stickleback populations in the wild?	Yes, there is large variation in brain size and architecture among different wild nine-spined stickleback populations.
Does divergence in brain size and architecture in the wild match the patterns observed in the lab?	Patterns form the wild and from the lab show incongruence.
Can environmentally induced plasticity obscure genetically based patterns?	Yes. Common garden studies are needed to draw firm evolutionary conclusions about brain size evolution within a species.
Do common ecological factors such as predation or competition influence brain development in common frog tadpoles?	Yes. Under the highest *per capita* perceived predation risk, tadpoles developed smaller brains, while competition affected mainly the sensory centres of the brain.
Do perceived predation risk and food supply influence brain development in nine-spined sticklebacks?	Predation risk influenced brain development while food supply did not.
If yes, does the plastic response depend on population origin?	Predation induced plasticity in the *bulbus olfactorius* was habitat dependent, only seen in pond sticklebacks; in the hypothalamus, the effect was population independent.
Does social environment (living in group *vs.* living alone) affect brain development in nine-spined sticklebacks?	Yes, fish develop larger *bulbus olfactorius* when reared alone and larger *tectum opticum* when they develop in group.
Is there any habitat specificity in brain plasticity induced by social environment?	Pond fish suffer from reduced brain development in group rearing, while marine fish show the opposite trend.

In **Chapter IV**, I studied brain plasticity induced by perceived predation risk and food availability in nine-spined sticklebacks from coastal marine and pond populations. In **Chapter V**, I investigated the effect of sociality on brain development in nine-spined sticklebacks from coastal marine and pond populations. A list of the main study questions is shown in Box 3.

Finally, in **Chapter VI,** I provide a review of intraspecific brain variation, with a special focus on the most promising future avenues of studying brain evolution.

3. **Materials and methods**

Study system

The primary model species used in this thesis was the nine-spined stickleback. It is a small teleost fish with a wide geographic distribution (e.g. Bănărescu and Paepke 2001). This species is an excellent model for investigating adaptive divergence as it occupies diverse habitat types from marine environments through large lakes to isolated ponds, where it can persist as the only fish species. Coastal marine sticklebacks belong to a diverse fish fauna, including several predatory fish species, and live in a structurally complex environment, while pond sticklebacks may live in the absence of piscine predators in a very simple, relatively homogenous abiotic environment. Work with this system has demonstrated habitat-dependent population divergence between marine and pond sticklebacks in body size (Herczeg *et al.* 2009a), growth strategy (Herczeg *et al.* 2011), reproductive output (Herczeg *et al.* 2010a), behaviour (Herczeg *et al.* 2009b; Herczeg and Välimäki 2011), body armour and body shape (Herczeg *et al.* 2010b), and cost of group living (Herczeg *et al.* 2009c).

Taken together, these results suggest that marine sticklebacks are primarily predation-adapted whereas

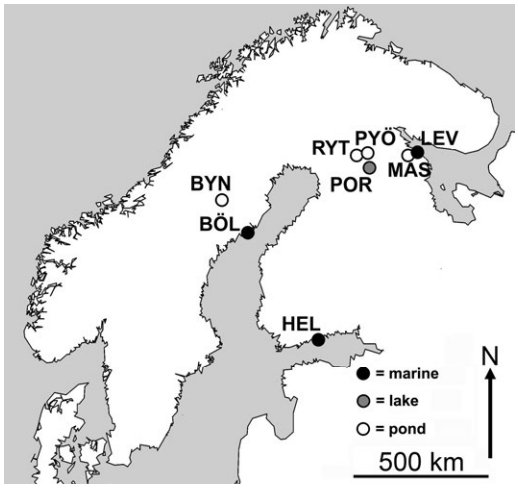

Figure 4. Map of the sampling localities. BÖL = Bölesviken, Baltic Sea, Sweden; HEL = Helsinki, Baltic Sea, Finland; LEV = Levin Navolok Bay, White Sea, Russia; POR = Porontima, Finland; BYN = Bynästjärnen, Sweden; PYÖ = Pyöreälampi, Finland; RYT = Rytilampi, Finland; MAS = Mashinnoje, Russia.

in pond populations, intraspecific competition is the dominant biotic interaction affecting fitness, with predation and interspecific competition being largely negligible (Box 4). Naturally, marine and pond habitats also differ in factors other than predation and competition, salinity being one obvious difference. However, besides the fact that the

coastal marine areas (especially in the northern Baltic Sea) are characterised by very low salinity, nine-spined sticklebacks from large lakes are closer to marine than to pond sticklebacks in term of size and longevity (Herczeg *et al*. 2009a), as well as morphologically (Herczeg *et al.* 2010b). Hence, salinity appears to be of limited importance in explaining the divergence between marine and pond populations from the Fennoscandian region, with within-habitat population replicates being isolated both geographically (Fig. 4) and in most cases also genetically (Shikano *et al.* 2010).

My second model species (used in **III**) was the common frog. This anuran can be found throughout the northern hemisphere, including Fennoscandia (e.g. Gasc *et al.* 1997). Tadpoles have been used extensively as models in studies of phenotypic plasticity, and in particular in studies focussing on predation and intraspecific competition induced phenotypic plasticity in growth (Relyea and Hoverman 2003), time of metamorphosis (Relyea and Hoverman 2003), body shape (e.g., Skelly and Werner 1990; McCollum and Van Buskirk 1996; Van Buskirk and Relyea 1998) and behaviour (e.g. Relyea 2002). My work represents the first study in which predation and competition induced brain plasticity has been tested.

Box 4. Nine-spined stickleback 'ecomorphs'

Predation-adapted
Nine-spined sticklebacks in coastal marine environments (the lower fish in the picture) are small (Herczeg *et al.* 2009a), grow quickly (Herczeg *et al.* 2011), and produce small clutches (Herczeg *et al.* 2010a). They develop full body armour (Herczeg *et al.* 2010b), and do not face growth costs associated with group living (Herczeg *et al.* 2009c). Behaviourally, they are inactive feeders, non-aggressive, risk averse, and not explorative (Herczeg *et al.* 2009b; Herczeg and Välimäki 2011).

Competition-adapted
Nine-spined sticklebacks in isolated ponds (the upper fish in the picture) can become giants (Herczeg *et al.* 2009a), grow slowly (Herczeg *et al.* 2011), and females may produce clutches ca. three times larger than marine conspecifics (Herczeg *et al.* 2010a). Pond fish have reduced, or absent, body armour (Herczeg *et al.* 2010b) and group living inflicts large costs to growth (Herczeg *et al.* 2009c). Finally, they are active feeders, take high risks, behave aggressively, and are active explorers (Herczeg *et al.* 2009b; Herczeg and Välimäki 2011).

Brain measurements

All experimental animals (fish and tadpoles) were euthanized with an overdose of MS 222 (tricaine methanesulphonate), and weighted immediately *post mortem*. I used only adult fish, and tadpoles at developmental stage 25 (Gosner 1960). Body length was measured either directly from the animals (**I**, **II**, **V**), or from digital photos (**III**, **IV**). Brains were dissected from crania and digitally photographed from the dorsal, lateral and ventral views. In fish, brain size and the sizes of five brain parts (*viz. bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus*; Fig. 2, Box 2) were calculated from their length, width and height (the largest distance enclosed by the given brain part) using the ellipsoid model (Pollen *et al.* 2007; Gonzalez-Voyer and Kolm 2010).

In tadpoles, only two dimensions of the different brain parts could be measured (*viz.* length and width of *telencephalon*, *diencephalon* and *tectum opticum*, and depth and width of *medulla oblongata*; Fig. 3, Box 2) due to the lack of clear borders between the brain parts in some dimensions. Here, the size of the brain and different brain parts were estimated from principal component analyses of the measures.

4. Results and discussion

Population variation in brain size and size of the main brain parts

In the first two chapters, I investigated variation in brain size and the size of different brain parts of sticklebacks (Fig. 2, Box 2) from coastal marine vs. pond populations (Box 4), both in a controlled lab environment (**I**) and in

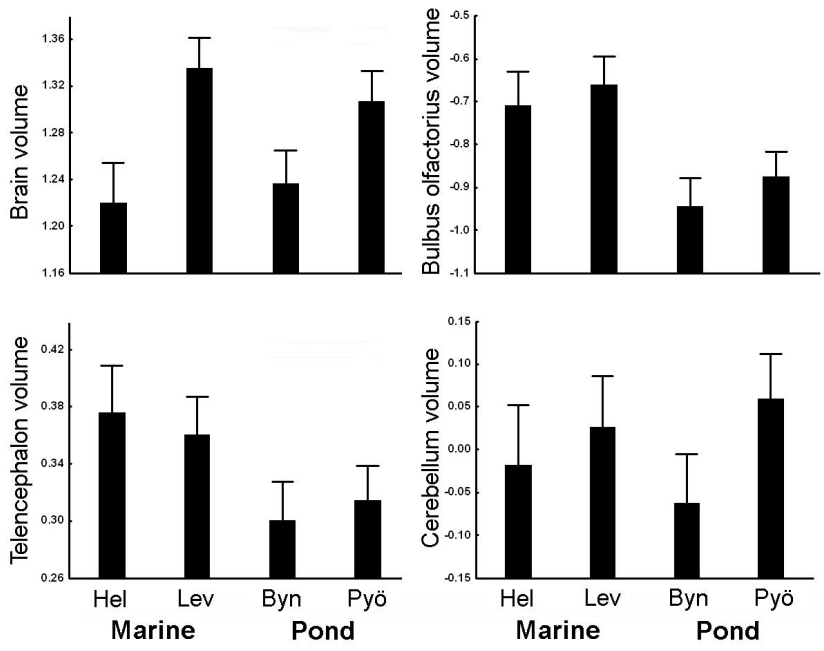

Figure 5. Population differences in brain volume (corrected for body length and weight), and in the volumes of different brain parts (corrected for body length, weight and brain volume) in common garden nine-spined sticklebacks (*Pungitius pungitius*). Means + 95% Confidence Intervals are shown. 'Hel' denotes Baltic Sea at Helsinki, 'Lev' White Sea at Levin Navolok Bay, 'Pyö' Pyöreälampi pond and 'Byn' Bynästjärnen pond.

the wild (**II**). In the common garden experiment (**I**), I found significant divergence in relative brain size, and relative sizes of *bulbus olfactorius*, *telencephalon* and *cerebellum* (Fig. 5) between two marine (Baltic and White Seas) and two isolated pond populations (separated by more than 500 km, Fig. 4). These findings strongly suggest that both brain size and architecture are capable of evolving over relatively short time scales, given that the post-glaciation invasion of the species in Fennoscandia happened less than 8000 years ago (Eronen 2001). Further, I found systematic, habitat-dependent population divergence in the sizes of the *bulbus olfactorius* and *telencephalon*, both being larger in marine than in pond fish. This genetically based and habitat-dependent pattern of population divergence suggests that natural selection is the likely cause behind it (e.g. Clarke 1975; Endler 1986; Schluter and Nagel 1995; Foster 1999; McGuigan *et al.* 2005). This study was the first to provide evidence for interpopulation brain divergence likely to be caused by natural selection. Interestingly, I also found that relative brain sizes in the White Sea and Pyöreälampi pond fish were larger than those from the Baltic Sea and Bynästjärnen pond fish (Figs. 4). Since the original study, more molecular data has become available supporting the view that these two clusters of populations differing in relative brain size belong to different genetic lineages/clusters (*viz.* White Sea drainage *vs.* Baltic Sea drainage; Merilä and Shikano unpublished). In the light of this new data, I suggest that the relative brain size divergence in my system might also have had a historical component.

The results in **I** did not include any information about the brain size and architecture patterns in the wild. Therefore, in **II**, I compared the brains of wild-caught sticklebacks from three marine and four pond populations, including also one large lake population. Because the populations used in **I** were also represented in this sample, I could also directly compare wild and common garden samples from the same populations. I found significant population differentiation in total brain, *telencephalon*, *tectum opticum* and *cerebellum* sizes. However, only the *telencephalon* divergence observed in **I** was also found in the wild data. Further, I found that pond fish had larger brains than marine (and the lake) fish, a systematic pattern which was absent in the common garden material (**I**). The direct comparison of wild-caught and lab-reared fish revealed that brain size decreased in pond fish reared in a common environment, whereas brain size in marine fish was unchanged (Fig. 6). Furthermore, all studied brain parts (Fig. 2, Box 2) developed relatively smaller when fish were reared in the lab. Taken together, this suggests that much of the variation observed in the wild can be related to environmentally induced plasticity, concealing the 'true' genetic patterns of differentiation. These results further

highlight the fact that there is a great deal of intraspecific variation in brain size and architecture. However, the results also emphasize the fact that when a plastic trait such as brain size is considered – especially in intraspecific comparisons, or at lower taxonomic levels– controlled, common garden experiments are required for evolutionary inference. Although this has been reiterated in other contexts (e.g. Merilä 2010; Alho *et al*. 2010), results in **II** provide the first demonstration of this point in the context of brain studies.

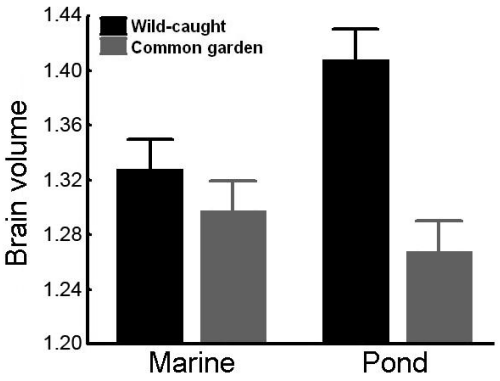

Figure 6. Habitat-specific differences in brain size (corrected for body length and weight) between wild-caught and common garden nine-spined sticklebacks (*Pungitius pungitius*). Means ± SE are shown.

Taken together, results in **I** and **II** support the notion that large within species variation in brain size and architecture exists between locally adapted populations. In some cases this differentiation appears to have a genetic basis, and is most likely the result of directional natural selection. Hence, it provides a solid basis for future studies designed to test the evolutionary significance of differentiation in brain architecture. For example, selection experiments could be used to investigate directly putative selective forces leading to between-habitat brain size differentiation, while use of quantitative genetics methods could allow differentiating between different microevolutionary processes, such as natural selection and genetic drift, as causes of observed patterns (e.g. Merilä and Crnokrak 2001; Ovaskainen *et al*. 2011).

Brain size plasticity induced by biotic environmental factors

In **III**, I investigated the effects of perceived predation risk (presence / absence of chemical and visual cues of dragonfly larvae preying on tadpoles) and competition (high / low tadpole densities with similar food resources) on brain development in common frog tadpoles (see Fig. 3, for functions, see Box 2). I found that both of these common ecological factors (i.e. predation and competition) affected tadpoles' brain development. The most salient finding of this study was that tadpoles under the highest *per capita* perceived predation risk (i.e. in the low density / predator present treatment combination) developed relatively smaller brains than tadpoles grown in the other treatment combinations. This pattern can be explained by variation in energy availability, and its effect on brain development. Brain is the most expensive tissue to develop and maintain (Aiello and Wheeler 1995). I suggest that high perceived predation risk suppressed activity (e.g. Laurila

2000; Van Buskirk and Arioli 2005; Teplitsky and Laurila 2007), which in turn resulted in reduced food intake (Werner and Anholt 1993), and consequently, in an energy deficit having a negative impact on brain development. However, energy loss due to physiological stress (Stoks *et al.* 2005; Steiner 2007; Slos and Stoks 2008) is also a plausible explanation. I also found that tadpoles developed larger *tecta optica* at high densities, while the size of the *medulla oblongata* was larger in the low density treatment. Hence, tadpoles might relay on visual cues in large densities, and use auditory or lateral line sensory systems when only few conspecifics are present. A simple developmental trade-off between the two brain parts might have been present too: such trade-offs have been reported at the evolutionary level (Barton *et al.* 1995; Barton and Harvey 2000). Interestingly, we found later that the larval treatments also had carryover effects: the effect of larval density on the *tectum opticum* was still present after metamorphosis, showing that brain plasticity can last over different life stages (Trokovic *et al.* 2011). Whether this was adaptive or a result of developmental constraints remains to be investigated.

In **IV** and **V**, I investigated the effects of perceived predation risk (chemical cues of Eurasian perch, *Perca fluviatilis*; **IV**), food supply (high *vs.* low; **IV**) and social environment (rearing in groups or alone; **V**) on the brain development of nine-spined sticklebacks. Fish developed smaller *hypothalami* under perceived predation risk than in the predator-free environment. The *hypothalamus* is known to have a very complex regulatory role. For example, it regulates reproductive behaviour (White and Fernald 1993), and controls foraging behaviour (Kulczykowska and Sánchez Vázquez 2010). As predation often restricts activity level, and thus access to food (Sih 1982; Lima and Dill 1990), the decreased activity under high perceived predation risk is a possible explanation for variation in *hypothalamus*. However, how it was translated to size decrease in such a functionally complex brain part is hard to interpret. Finally, food supply did not affect brain development. This is surprising given the high energy needs for brain development (Aiello and Wheeler 1995). The observation that relative brain size was not affected by food manipulation, despite its strong effect on growth in general (Välimäki and Herczeg 2011), suggests brain development is a high priority. Nine-spined sticklebacks developed larger *tecta optica* and smaller *bulbi olfatorii* when reared in groups, compared to fish reared alone (chemical cues were still available in the latter treatment). This is easily explainable by the difference of the relative roles of different sensory systems in the different treatments. However, a developmental trade-off between the two sensory centres is also conceivable (Barton *et al.* 1995; Barton and Harvey 2000).

In summary, I found that the most common biotic environmental factors could induce phenotypic plasticity in both brain size and the size of relevant brain parts. Considering how costly it is to grow and maintain brain tissue (Aiello and Wheeler 1995), and that the size of a given brain part often reflects its importance (Striedter 2005; Kihslinger and Nevitt 2006; Kihslinger *et al.* 2006; Lisney *et al.* 2007), it is highly likely that the observed changes were adaptive. To date, experimentally induced brain plasticity has mainly been studied in response to variation in abiotic environment (Table 1; mammals: Diamond *et al.* 1966; Rosenzweig and Bennett 1969; fish: Kihslinger and Nevitt 2006), or as a response to complex learning tasks in humans (Table 1; Maguire *et al.* 2000). My work has shown that there is an equally great potential for biotic factors to also induce brain plasticity, at least in lower vertebrates.

Population variation in brain plasticity

In **IV** and **V**, I specifically addressed the question of whether there was population variation in phenotypic plasticity of brain size, and whether this variation was habitat-specific by applying predation, food and social treatments (as described in the previous section) to both predation-adapted marine and competition-adapted pond sticklebacks in common garden experiments. Among the three treatments (note that the results came from two separate studies), manipulation of perceived predation risk and social environment resulted in habitat-dependent phenotypic plasticity in brain development, while manipulating available food did not induce any plasticity. In **IV**, I found that while marine sticklebacks had generally larger *bulbi olfactorii* than ponds fish (in accordance with results from **I**), only pond fish reacted to perceived predation risk by increasing their relative *bulbus olfactorius* size.

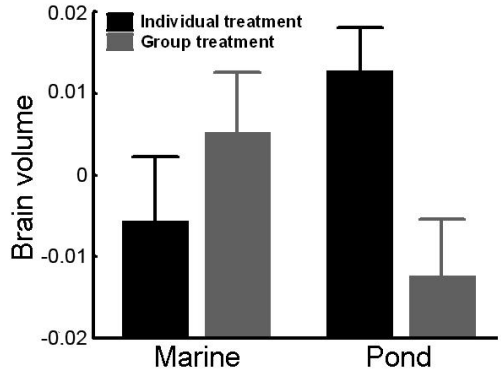

Figure 7. Social environmental effect on brain size (corrected for body length and weight) in nine-spined stickleback (*Pungitius pungitius*). Means ± SE are shown. A significant habitat-dependent treatment effect was found.

This result suggests that olfactory sense may be sufficiently important in the marine environment such that a large *bulbus olfactorius* has become canalized in this environment (e.g. Pfennig *et al*. 2010). Conversely, olfaction may be less important in the piscine predator free ponds, hence *bulbus olfactorius* is small in pond sticklebacks. However, pond populations appear to have evolved the capacity for phenotypic plasticity of this trait. This is based on the interesting – and yet unexplainable –

fact that predation-adapted nine-spined sticklebacks with canalized *bulbus olfactorius* size represent the ancestral form. Thus, the descendant pond sticklebacks have seemingly developed the ability for *bulbus olfactorius* plasticity parallel to decreasing the overall size of this brain part.

The social environment treatments resulted in a strong pattern (**V**): pond sticklebacks developed relatively smaller brains in groups than when reared alone, while there was no such effect in marine fish (or rather an opposite trend was found; Fig. 7). Interestingly, pond fish in the group treatment developed relatively smaller brains in addition to considerably smaller bodies, compared to individually reared pond fish (Herczeg *et al.* 2009c). These patterns suggest that intraspecific competition adapted pond fish, noted for high aggression (Herczeg *et al.* 2009b; Herczeg and Välimäki 2011), face a large cost of group living, even in the absence of ecological or reproductive constraints (all fish were fed *ad libitum*, and were kept out of reproductive condition). It is, thus, plausible to suggest that marine fish are adapted for shoaling / schooling as a means of antipredatory behaviour in contrast to the highly competitive pond sticklebacks which appear to gain no benefit from groupings, and as such, most likely remain solitary.

Taken together, **IV** and **V** provide evidence for habitat-dependent expression of brain plasticity. In both cases, I found plastic response only in the pond habitat, with fish from the marine environment being unaffected by treatments. As genetically-based, habitat-dependent patterns are likely the result of natural selection (Clarke 1975; Endler 1986; McGuigan *et al.* 2005), I suggest that the presence / absence of the ability to express plasticity in neural development may be a trait under selection. However, the link between phenotypic plasticity and local adaptation in brain development surely warrants further investigations.

5. Overview and future directions

In **VI**, I reviewed the available literature on intraspecific brain size variation, and outlined some directions for future research that would advance our understanding of brain microevolution. Based on my own studies (**I-V**; Trokovic *et al.* 2011) and the compiled literature, I found that (i) many studies have demonstrated that brain size is highly variable at the intersepcific level, while studies at the intraspecific level have only recently begun to accumulate (Fig. 1). These intraspecific evolutionary studies may provide new and probably closer insights into the factors driving brain evolution in animals. I further found that (ii) brain plasticity has also been shown to occur in nature, and is also inducible by experimental manipulation in the lab. However, while the effects of the abiotic environment have been studied very extensively, only a handful of studies have investigated the effects of the biotic environment (except my own

studies: Fowler *et al.* 2002; Lipkind *et al.* 2002; Adar *et al.* 2008). Finally, although it is expected that brain plasticity itself can differ among populations, and studying such differences could help us identifying important factors contributing to brain size and architecture variation, I am only aware of one other study (Crispo *et al.* 2010) – apart from my own (**IV, V**) – that has investigated population divergence in brain size plasticity. Although within species comparisons have recently begun to receive some attention (Fig. 1), many opportunities for intraspecific studies into brain evolution remain as yet underutilized. Here, I refer mainly to two main directions which would be very important and fruitful to pursue: quantitative genetics and histological-cytological approaches.

First of all, comparisons of brain size and architecture differences among populations inhabiting different selective environments could provide an explicit means to differentiate in between the causative effects of different microevolutionary processes, such as natural selection *vs.* genetic drift (e.g. Merilä and Crnokrak 2001). By comparing levels of population differentiation in quantitative traits (Q_{ST}) with the degree of differentiation in neutral genetic markers (F_{ST}), one can shed light on the causes of population differentiation (e.g. Leinonen *et al.* 2008). With properly designed common garden experiments (see e.g. Falconer and Mackay 1995; Lynch and Walsh 1998), the quantitative genetic components of phenotypic variation (heritability, dominance, maternal effects, etc.) in brain size and architecture could be estimated, and compared among spatially and/or temporarily different samples, or between treatments. Further, the genetic variance-covariance matrix (**G**; Lande 1979) between the different brain parts could be established on the same material, providing direct tests of the hypotheses of constrained *vs.* independent brain evolution (Finlay and Darlington 1995; Barton and Harvey 2000). Moreover, this could open the possibility to estimate the probable future directions of evolution, particularly if coupled with additional experiments, designed to estimate the strength of selection acting on different phenotypes (e.g. predation experiments). Finally, by applying functional genomics (genome scans: Schlötterer 2003; Storz 2005; Vasemägi and Primmer 2005; quantitative trait loci [QTL] mapping: Weller 2001; Erickson *et al.* 2004; Slate 2005), the genomic regions, and ultimately the genes coding for brain variation could be identified.

Second, integrating methods of neurobiology (e.g. basic histological staining methods e.g. Zhang *et al.* 2011 or advanced molecular methods, such as anti-body labelling, enzyme histochemistry or immunofluorescens methods; e.g. Sallinen *et al.* 2009) with the tools of evolutionary biology (detailed above) could provide a novel opportunity for finding direct causative links between different selective forces and brain functions. One of the most difficult and largely unaddressed

questions in studies of brain evolution (both on intra- and interspecific scales) is establishing the link between variation in brain morphology and brain function. As neurobiologists have already described the function of several structures (different nuclei, cell types) in the brain, establishing the causes of evolutionary divergence in such well understood brain architectures would give us an ultimate picture of brain evolution.

6. Acknowledgements

First, I would like to thank to my primary supervisor Juha Merilä for mentioning that "studying the brains of these environmentally divergent fish would be interesting" when I was an assistant in his group. As I was always interested in the nervous system, and delighted by the evolutionary ecology of nine-spined sticklebacks, this half sentence made me sleepless for weeks and got me started on my scientific life. I would also like to thank you, Juha, for offering me the possibility of conducting my PhD studies in your group, providing me with an overwhelming amount of information / literature, and your trust in my project and in me, even when I was not always able to explain exactly what I would have liked. Special thanks for showing me that a yacht trip in the Baltic can easily challenge tropical waters in a nice summer day (the number of such days per year is another question).

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Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)?

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Adaptive brain size divergence in nine-spined sticklebacks (*Pungitius pungitius*)?

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Abstract

Most studies seeking to provide evolutionary explanations for brain size variability have relied on interspecific comparisons, while intraspecific studies utilizing ecologically divergent populations to this effect are rare. We investigated the brain size and structure of first-generation laboratory-bred nine-spined sticklebacks (*Pungitius pungitius*) from four geographically and genetically isolated populations originating from markedly different habitats. We found that the relative size of bulbus olfactorius and telencephalon was significantly larger in marine than in pond populations. Significant, but habitat-independent population differences were also found in relative brain and cerebellum sizes. The consistent, habitat-specific differences in the relative size of bulbus olfactorius and telencephalon suggest their adaptive reduction in response to reduced (biotic and abiotic) habitat complexity in pond environments. In general, the results suggest that genetically based brain size and structure differences can evolve relatively rapidly and in repeatable fashion with respect to habitat structure.

Introduction

Large variation in brain size or structure has been reported in a number of taxa (e.g. Harvey *et al.*, 1980; Kotrschal *et al.*, 1998; Day *et al.*, 2005). Most of these studies have looked for interspecific correlations between brain architecture and various ecological factors and/or behavioural and life-history traits correlated with fitness (e.g. Garamszegi & Eens, 2004a; Sol *et al.*, 2008). For instance, brain size correlates positively with habitat complexity (Pollen *et al.*, 2007) and with social complexity and diet in cichlids (Gonzalez-Voyer *et al.*, 2009) and with bower complexity in bowerbirds (Madden, 2001). Furthermore, negative correlations among brain size and size of other organs (Aiello & Wheele, 1995; Kaufman, 2003; Pitnick *et al.*, 2006) have also been reported. However, contradictory results across higher taxa are common. For instance, forebrain size correlates positively with habitat complexity both in fishes and in primates (Clutton-Brock & Harvey, 1980; Huber *et al.*, 1997), whereas establishment success in novel environments correlates positively with brain size in birds (Sol *et al.*, 2005) but not in fishes (Drake, 2007).

Despite some criticism (Healy & Rowe, 2007), interspecific correlative studies form the cornerstone of our understanding of brain size evolution. However, intraspecific studies could provide additional and more fine-tuned means to test ideas and observations born out of interspecific studies. First, comparisons of brain size/structure differences among populations of the same species inhabiting different selective environments could provide explicit means to differentiate between different microevolutionary processes, such as natural selection and genetic drift (e.g. Merilä & Crnokrak, 2001), as causes of observed differentiation. Second, population level differences can be very informative: compared to species, populations are more likely to be found in the selective habitats where the observed differences have evolved.

Besides the well-described plasticity in brain size and structure in response to environmental differences (e.g. Maguire *et al.*, 2000; Tramontin & Brenowitz, 2000; Kihslinger & Nevitt, 2006), most intraspecific population

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comparisons made so far have been based on wild caught individuals (Garamszegi & Eens, 2004b; Karlen & Krubitzer, 2006). However, one study, based on laboratory lines of the medaka (*Oryzias latipes*) has reported genetically based intraspecific variation in brain size and architecture (Ishikawa *et al.*, 1999), whereas another study found genetically based brain structure differences in certain brain parts between laboratory-reared populations of guppies (*Poecilia reticulata*) originating from two rivers (Burns & Rodd, 2008). Hence, although intraspecific variation in brain size has often been found, very little is still known about genetic and adaptive basis of population differences in relative brain size and size of different parts.

Nine-spined sticklebacks (*Pungitius pungitius*) provide an excellent model for intraspecific comparisons, as they occupy markedly different habitats ranging from marine environments through large lakes to small, isolated ponds, where they are often the only fish species present (e.g. Bănărescu & Paepke, 2001). Hence, large differences can be found both in biotic (e.g. diversity of prey, competitors and predators) and abiotic (e.g. habitat structure) habitat components.

The aim of this study was to test whether differences in ecological conditions, expected to select for differences in brain morphology, have resulted in divergence of the relative brain size and structure in nine-spined sticklebacks. We predicted that fish from ponds, in the absence of predatory fish, under no or weak interspecific competition, and living in habitats with negligible structural heterogeneity have evolved relatively smaller brains or brain parts related to memory and learning compared with their conspecifics in the sea. For these purposes, we reared laboratory-born fish from two marine and two pond populations in common garden settings until they reached adult size. As far as we are aware, this is the first attempt to test for genetically based habitat-specific population differentiation in brain size and brain architecture from the wild.

Materials and methods

Sampling, breeding and rearing

We collected adult nine-spined sticklebacks from four populations (Fig. 1) during May and June 2007. The two isolated ponds (surface area < 5 ha) were Pyöreälampi (Finland) and Bynästjärnen (Sweden) where the only representatives of other fish species were a few, recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi (information from the Oulanka Research Station). Our sample areas became free from ice around 8000 years ago after the last glaciation (e.g. Eronen *et al.*, 2001) so the colonization of these ponds must have happened after that. The marine samples came from the Baltic Sea (Helsinki, Finland) and White Sea (Levin Navolok Bay, Russia; Fig. 1). Marine nine-spined sticklebacks belong to a diverse fish community consisting of a large number of potential predators and competitors. In turn, in the ponds there are no predatory fish present, and the interspecific competition posed by other fish species is absent or negligible. Predation by aquatic insects and cannibalism at very early stages might be relevant in both habitats. The ponds exhibit very simple physical structure when compared to marine environments.

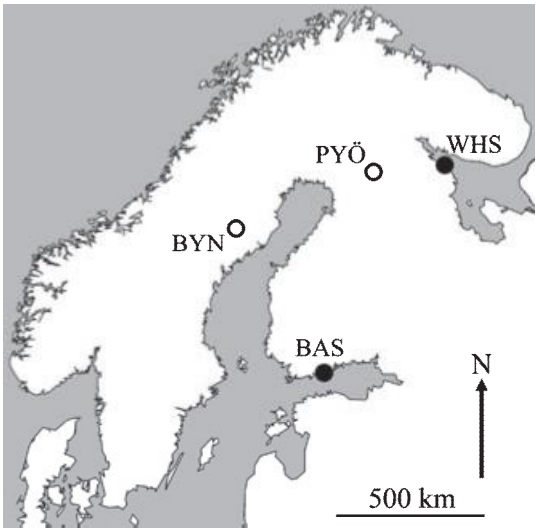

Fig. 1 Map showing the location of the study populations. Full circles denote marine populations: BAS, Baltic Sea population near Helsinki; WHS, White Sea population at Levin Navolok Bay; open circles denote ponds: PYÖ, Pyöreälampi (small isolated pond); BYN, Bynästjärnen (small isolated pond).

After collection, fish were moved to the aquaculture facilities of the University of Helsinki. Artificial crosses were made at the end of June. Five full-sib families were produced from every population *in vitro*. After hatching, each family was divided into two replicates and a maximum of 40 fish per replicate were placed in 10 L aerated plastic tanks. After 2–3 weeks, the fish were moved to similar tanks with mosquito nets at their sides that were placed into 140 L big plastic tanks (eight 10 L tanks in each) with an open, one-way water-flow. Family replicates were placed in different large tanks. After another 3–4 weeks, population pools were formed with families and replicates being equally represented. We aimed to get 100 offspring (= 5 families per population and 20 individuals per family) from each population [from the Baltic Sea, we could only get 93 (26, 24, 21, 16 and 6 per family) individuals]. Plastic tanks (140 L) were divided into halves by mosquito net and set with an open, one-way waterflow. Each population pool was divided into two replicates, and replicates were placed randomly into halves of the 140 L plastic tanks. Water temperature was set to 17 °C. Fish were fed live brine shrimp (*Artemia* sp.) nauplii first, and then frozen

crustaceans (*Cyclops* sp.) and bloodworms (Chironomidae sp.) *ad libitum*. We started with a 24 h light photoperiod, and changed it during 12 days gradually to a 12 h light/12 h dark photoperiod 12 weeks after hatching to avoid fish turning into reproductive condition. Better mimicking of the natural photoperiod change would have been very challenging to achieve due to the latitudinal differences in the source populations. We note that the number of replicate populations per habitat ($N = 2$) and independent families per population ($N = 5$) are limited, but considering that our populations are geographically (Fig. 1) and genetically (pairwise F_{st}-s between 0.1 and 0.8; T. Shikano, G. Herczeg & J. Merilä, unpublished data) isolated, the data should be adequate for initial tests of the questions posed.

Brain measurements

Fish were overanaesthetized (with tricaine methanesulphonate) at the age of 5 months. A randomly chosen 15 individuals (seven and eight from the two replicates respectively) per population were measured. After measuring body weight to the nearest 0.01 g with a digital balance and standard length to the nearest 0.01 mm with digital callipers, brains of the fish were dissected and put into 4% formalin – 0.1 M phosphate-buffered saline solution. After 48 h fixation, photographs were made of the brains from dorsal, right lateral and ventral aspects with a digital camera (Canon EOS 10D; Canon Inc., Tokyo, Japan) connected to a dissecting microscope (Wild M5A; Wild, Heerbrugg, Switzerland). For bilateral structures, only the right parts were measured.

Width, height and length of the brain and five different brain parts – bulbus olfactorius, telencephalon, optic tectum, cerebellum and hypothalamus – were measured from the digital photographs using tpsDig 1.37 (Rohlf, 2002) software. They were defined as the greatest distance enclosed by the given structure. For a detailed description of the measurements see Pollen *et al.* (2007), whose measurement procedures we followed. We calculated the volume of the different brain parts according to the ellipsoid model (e.g. Huber *et al.*, 1997; Pollen *et al.*, 2007):

$$V = (L \times W \times H)\pi/6 \qquad (1)$$

where V denotes the volume estimate, and L, W and H are the length, width and height of the given structure respectively. Although this model might not account for fine-scale variations in brain shape, it is a suitable measure for our purposes as we compared populations of the same species where drastic shape variations are not expected. The validity of the gross brain measures employed was tested by Pollen *et al.* (2007) and proved to provide consistent estimates of brain region volumes. For paired structures we used a doubled volume estimate of right side measurements. The volume of the total brain was estimated both with the equation suggested by Pollen *et al.* (2007):

$$V = (L \times W \times H)\pi/(6 \times 1.23) \qquad (2)$$

and by summing the volumes of the different parts. The type of estimation did not alter the results qualitatively. Hence, only the results from the ellipsoid model are reported. Repeatability (R) of the volume estimates based on three independent measurements of 20 brains was high ($R > 0.86$, $P < 0.001$).

Analyses

To remove the allometric brain-body size effect (Northcutt *et al.*, 1978) all measures were $\log_{10}$ transformed. As the log standard length–log body weight relationship differed between the populations [General Linear Model (GLM), population × log standard length interaction: $F_{3,52} = 3.34$, $P = 0.03$], we corrected for both log standard length and weight in our subsequent analysis. First, a univariate GLM was used to test the difference in relative brain size between the populations. Log brain volume was defined as the dependent variable, population as the fixed factor and log body weight and log standard length as covariates. Pairwise *post-hoc* tests (LSD tests) were used in determining the significant population differences. Second, to test directly for habitat dependence, we used a General Linear Mixed Model (GLMM). Here, we entered log brain volume as dependent variable, habitat type (marine vs. pond) as fixed factor, population nested within habitat type as a random factor, and log body weight and log standard length as covariates.

We conducted a multivariate GLM to test for differences in brain structure with (log) brain parts as dependent variables, population as a fixed factor, and log body weight, log standard length and log brain volume as covariates. Upon significant multivariate effects, we ran univariate tests followed by LSD tests. To test directly for habitat specificity, we applied separate GLMMs on the variables found significant in the univariate tests (viz. telencephalon, bulbus olfactorius, cerebellum). Here, the given brain part was the dependent variable, habitat type the fixed factor, population nested within habitat type the random factor, whereas log body weight, log standard length and log brain volume were the covariates. All analyses were carried out with the SPSS 16.0 for Windows software package (SPSS Inc., Chicago, IL, USA).

Results

Independently of the body size effects (log body weight: $F_{1,54} = 38.47$, $P < 0.0001$; log standard length: $F_{1,54} = 22.11$, $P < 0.0001$) there were significant differences in relative brain size between the populations ($F_{3,54} = 12.62$, $P < 0.0001$; Fig. 2a). *Post-hoc* comparisons revealed

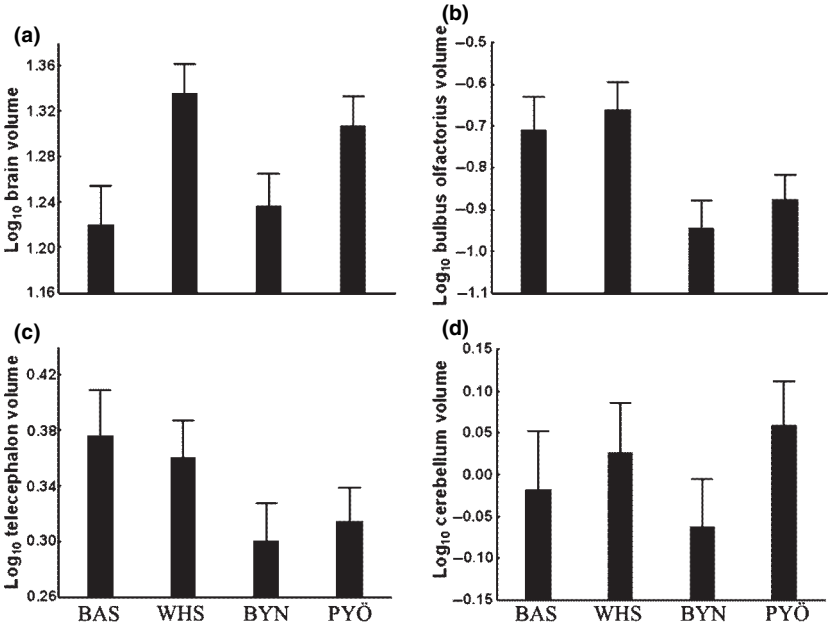

Fig. 2 Population differences in brain volume (corrected for body length and weight), and in the volume of different brain parts (corrected for body length, weight and brain volume). Least squares means + 95% confidence intervals are shown. BAS, Baltic Sea at Helsinki; WHS, White Sea at Levin Navolok Bay; PYÖ, Pyöreälampi (small isolated pond); BYN, Bynästjärnen (small isolated pond).

a habitat-independent pattern (Fig. 2a; White Sea > Baltic Sea; Pyöreälampi > Bynästjärnen; Pyöreälampi > Baltic Sea; White Sea > Bynästjärnen; all $P < 0.002$; all other pairs did not differ). Result of the GLMM analyses supported the results of GLM: after correcting for body size effects (log body weight: $F_{1,54.512} = 37.64$, $P < 0.0001$; log standard length: $F_{1,55.586} = 20.68$, $P < 0.0001$) there were no significant differences among the habitat types ($F_{1,2.085} = 0.01$, $P = 0.92$). Population within habitat type was also nonsignificant ($Z = 0.94$, $P = 0.35$).

After correcting for log weight (Wilks' $\lambda_{5,49} = 0.86$, $P = 0.18$), log standard length (Wilks' $\lambda_{5,49} = 0.91$, $P = 0.42$) and log brain volume (Wilks' $\lambda_{5,49} = 0.12$, $P < 0.0001$), our multivariate GLM revealed significant differences in sizes of different brain parts between the populations (Wilks' $\lambda_{15,135.669} = 0.39$, $P < 0.0001$). In the subsequent univariate analyses, we found significant population differences in three brain parts: bulbus olfactorius ($F_{3,53} = 12.05$, $P < 0.0001$; Fig. 2b), telencephalon ($F_{3,53} = 4.56$, $P = 0.006$; Fig. 2c) and cerebellum ($F_{3,53} = 4.00$, $P = 0.012$; Fig. 2d). Size of the optic tectum ($F_{3,53} = 1.76$, $P = 0.17$) and hypothalamus ($F_{3,53} = 1.10$, $P = 0.36$) did not differ significantly between populations. Pairwise *post-hoc* comparisons revealed a systematic difference between fish from marine and pond environments; marine fish having larger bulbus olfactorius (between habitat types: all $P < 0.008$; within habitat types: all $P > 0.1$) and telencephalon (between habitat types: all $P < 0.017$; within habitat types: all $P > 0.42$). However, the pattern in cerebellum differences was less clear, only the two small pond populations being significantly different from each other ($P = 0.002$; all other $P > 0.08$). Results of GLMM analyses supported the results of the GLM analyses. Habitat-specific differences were found in case of telencephalon ($F_{1,5.565} = 10.18$, $P = 0.021$) after correcting for size effects (log body weight: $F_{1,25.517} = 0.04$, $P = 0.85$; log standard length: $F_{1,11.507} = 0.13$, $P = 0.72$; log brain volume: $F_{1,25.892} = 115.18$, $P < 0.0001$), whereas populations did not differ within habitat types ($Z = 0.33$, $P = 0.74$). Also, we found habitat-specific differences in the case of bulbus olfactorius ($F_{1,3.776} = 28.69$, $P = 0.007$) after correcting for size effects (log body weight: $F_{1,18.135} = 0.001$, $P = 0.97$; log standard length: $F_{1,4.412} = 3.44$, $P = 0.10$; log brain volume: $F_{1,19.554} = 3.16$, $P = 0.09$), whereas populations did not differ within habitat types ($Z = 0.21$, $P = 0.83$). In the case of the cerebellum, we did not find habitat-specific differences ($F_{1,2.362} = 0.005$, $P = 0.95$), after correcting for size effects (log body weight: $F_{1,49.137} = 3.95$, $P = 0.05$; log standard length: $F_{1,35.594} = 2.85$, $P = 0.10$; log brain volume: $F_{1,44.853} = 22.40$, $P < 0.0001$), whereas populations did not differ within habitat types ($Z = 0.79$, $P = 0.43$).

Discussion

Our results indicate that different populations of the same species can differ genetically in their relative brain size, and in the relative sizes of different brain parts. In particular, the observed divergence in the size of bulbus olfactorius and telencephalon was explained by habitat type (marine vs. pond) rather than by population origin. This is the first data to suggest habitat-dependent genetically based intraspecific divergence in brain architecture among natural populations. This habitat-specific

nature of the differences suggests that natural selection is the agent behind the observed divergence. As our study area was covered with ice sheets until about ca. 8000 years ago (Eronen *et al.*, 2001), the results also indicate that observed differences have evolved relatively rapidly.

Independent evolution of the same phenotype in natural populations occupying similar habitats strongly implies natural selection as the causal agent (e.g. Clarke, 1975; Endler, 1986; Schluter & Nagel, 1995; Foster, 1999; McGuigan *et al.*, 2005). The repeated, habitat-specific differences in the relative size of two main brain regions, bulbus olfactorius and telencephalon, strongly suggest adaptive basis of observed differentiation. As the brain morphology differed between the habitat types systematically with marine populations having larger bulbus olfactorius and telencephalon than fish from ponds, we hypothesize that the (i) lack of interactions with other fish species (competitors and/or predators) and/or (ii) reduced environmental complexity in pond environments have relaxed selection on neural processes supported by bulbus olfactorius and telencephalon and resulted in their reduction – a pattern easily understandable in the light of the extremely high development and maintenance costs of brain tissue (Aiello & Wheele, 1995). Similar adaptive reductions in brain parts have been demonstrated in interspecific comparisons (Niven, 2005).

Previous interspecific studies on fish have found positive correlation between telencephalon size and habitat complexity, as well as between bulbus olfactorius size and water turbidity (Huber *et al.*, 1997). Telencephalon has also been found to be larger in polygamous than in monogamous species (Pollen *et al.*, 2007). Telencephalon-ablated fish exhibit significantly diminished rates of learning and habituation (e.g. Laming & McKinley, 1990) and display a deficit in avoidance learning (Portavella *et al.*, 2003). In-depth anatomical analyses indicate that the telencephalon is involved in spatial and emotional learning (for a review, see e.g. Broglio *et al.*, 2003). Even considering that correlations do not allow us to draw firm conclusions about causation, our results together with inference from previous studies suggest that the complexity of biotic environment (e.g. number of fish species), habitat complexity and the importance of learning abilities may be key factors in explaining telencephalon size differences between our nine-spined stickleback populations. The bulbus olfactorius differences may relate to lower visibility in the marine environment, and/or reduced utility of chemosensory functions in the isolated ponds lacking predatory fish.

Significant cerebellum size differences between the pond populations are also intriguing. The cerebellum appears to play a role in spatial orientation, motor coordination and eye movement (Kotrschal *et al.*, 1998), and correlates positively with species diversity and habitat complexity in cichlid fishes (Pollen *et al.*, 2007). In our case, the observed pattern cannot be explained with habitat differences, and thus, requires further investigations.

We also found that mean relative brain volume differed between the study populations, but not in a habitat-specific fashion. In theory, the population pairs with larger (Pyöreälampi and White Sea) and smaller (Bynästjärnen and Baltic Sea) relative brain volumes might share common ancestors (they indeed belong to different drainages) that were genetically differentiated in brain size already before the current populations were formed. Yet, it is also true that we have not measured or identified all possible environmental and ecological differences that might exist between different populations. The fact that fish from different populations, born and reared under standardized laboratory conditions in the absence of any energetic constraints, develop brains of different size suggest that there are some yet unidentified selection pressures which underlie these differences. Identification of these differences remains a challenge for future studies.

In summary, we found genetically based differences in relative brain size and brain architecture among wild nine-spined stickleback populations occupying contrasting habitats. The systematic differences in the relative volume of bulbus olfactorius and telencephalon between habitat types suggest that the divergence is caused by habitat-specific natural selection. This inference is supported by the fact that the direction of differences accords with expectations based on what is known about the function of these brain structures. However, further studies with more extensive replication, as well as confirmatory studies with other species, are needed to establish the generality of the findings.

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Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation?

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Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*) - local adaptation or environmentally induced variation?

Abigél Gonda*, Gábor Herczeg and Juha Merilä

Abstract

Background: Most evolutionary studies on the size of brains and different parts of the brain have relied on interspecific comparisons, and have uncovered correlations between brain architecture and various ecological, behavioural and life-history traits. Yet, similar intraspecific studies are rare, despite the fact that they could better determine how selection and phenotypic plasticity influence brain architecture. We investigated the variation in brain size and structure in wild-caught nine-spined sticklebacks (*Pungitius pungitius*) from eight populations, representing marine, lake, and pond habitats, and compared them to data from a previous common garden study from a smaller number of populations.

Results: Brain size scaled hypo-allometrically with body size, irrespective of population origin, with a common slope of 0.5. Both absolute and relative brain size, as well as relative telencephalon, optic tectum and cerebellum size, differed significantly among the populations. Further, absolute and relative brain sizes were larger in pond than in marine populations, while the telencephalon tended to be larger in marine than in pond populations. These findings are partly incongruent with previous common garden results. A direct comparison between wild and common garden fish from the same populations revealed a habitat-specific effect: pond fish had relatively smaller brains in a controlled environment than in the wild, while marine fish were similar. All brain parts were smaller in the laboratory than in the wild, irrespective of population origin.

Conclusion: Our results indicate that variation among populations is large, both in terms of brain size and in the size of separate brain parts in wild nine-spined sticklebacks. However, the incongruence between the wild and common garden patterns suggests that much of the population variation found in the wild may be attributable to environmentally induced phenotypic plasticity. Given that the brain is among the most plastic organs in general, the results emphasize the view that common garden data are required to draw firm evolutionary conclusions from patterns of brain size variability in the wild.

Background

During the past few decades, studies on diverse taxa have demonstrated that both absolute and relative brain size, as well as absolute and relative sizes of different brain parts, are highly variable and correlate with several environmental factors [mammals e.g. [1-3], birds e.g. [4,5] and fishes e.g. [6,7]]. Most of these studies, which form the basis of our current knowledge about brain size evolution, have used correlative approaches at the interspecific level. However, several recent studies have investigated differences in brain architecture among populations of the same species [8-17]. By using inter-population comparisons, microevolutionary processes can be investigated explicitly because most populations are likely to be found in the environment that actually shaped their brains. This is an unlikely situation in the case of comparisons based on species, which might have gone through adaptive divergence after splitting from common ancestor. An additional benefit from interpopulation comparisons as compared to interspecific comparisons is that the former allow adopting approaches to separate the relative roles of genetic drift and natural selection on observed differentiation [e.g. [18,19]].

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Further, even fewer studies have compared brains of individuals from different populations reared under standardized settings to exclude the possible effects of phenotypic plasticity [but see: [10,12]]. This is surprising considering the fact that phenotypic plasticity in overall brain size, in addition to the size of different brain regions, has often been demonstrated [e.g. seasonality: [20,21]; spatial learning: [22,23], environmental heterogeneity: [24,25]]. Moreover, how this plasticity might influence population and species comparisons in terms of neural architecture has yet to be explored. Therefore, direct comparisons of patterns based on data collected from wild populations with those based on data from standardized common garden settings are needed to establish if any evolutionary inferences can be made from wild collected data in such a highly plastic organ as the brain.

The nine-spined stickleback (*Pungitius pungitius*) is an excellent model species for intraspecific comparative studies and exploring adaptive divergence. It occupies markedly different habitats, ranging from marine environments through large lakes to isolated ponds wherein they are often the only fish species present [e.g. [26]]. Hence, large differences can be found both in biotic (e.g. diversity of prey, competitors and predators) and abiotic (e.g. habitat structure) habitat components. These differences are expected to impose different selection pressures on complex behaviours and memory, and thus, also on the neural architecture. This is especially true in light of the high energetic costs of developing and maintaining large brains [27]. Our recent studies, utilizing common garden reared nine-spined sticklebacks, have demonstrated genetically-based and habitat-related divergence in (i) size of different brain parts [12] and (ii) brain plasticity in response to the social environment [13]. However, patterns found in the wild have not been reported, and the fit between patterns of variation in common garden and wild collected data has never been tested.

Brain size scales allometrically with body size, both on ontogenetic and on evolutionary scales [e.g. [28-32]]. The slope of the allometric relationship between brain size to body size (both variables plotted on a logarithmic scale) is higher in prenatal than adult stages in mammals [28]. Furthermore, the slope of this relationship tends to be steeper at higher taxonomic ranks [ca. 0.75 across mammalian orders; e.g. [29,30]] compared to closely related species, or in intraspecific comparisons (ca. 0.2-0.5; [31,32]). Although some intraspecific studies in brain-body size allometry exist [e.g. [33]], only very few investigations have been conducted within a single vertebrate species, perhaps due to a lack of sufficient within-species size variation among adults. Since nine-spined sticklebacks living in ponds have repeatedly evolved into giants [34,35], the species (representing tenfold body weight differences between adult individuals) also provides an excellent model to study intraspecific brain-body size allometry.

Our aim was to explore population divergence in brain size and in the size of different parts of the brain (*viz.* telencephalon, optic tectum, cerebellum, hypothalamus) in wild-caught nine-spined sticklebacks from different habitats, and to compare the observed patterns with previously reported common garden results [12]. We sampled fish from eight Fennoscandian populations (Figure 1) originating from three habitat types (*viz.* marine, lake and pond environments) to test (i) for differences in the size of the brain and different brain parts among wild populations, and (ii) whether observed differences were habitat specific. Furthermore, to establish whether data collected from the wild can be used for evolutionary inference, (iii) we tested whether data collected from the wild and common garden experiments for fish originating from the same populations are concurrent, and if not, (iv) whether observed differences are population- or habitat-specific. We expected that the higher biotic and abiotic variability of marine and lake environments as compared to pond environments have selected for relatively large brains. We also expected to find habitat-dependent differences in brain parts important in perception, learning and spatial memory, and that the stimulus-poor laboratory environment would reduce the brains of common garden fish compared to

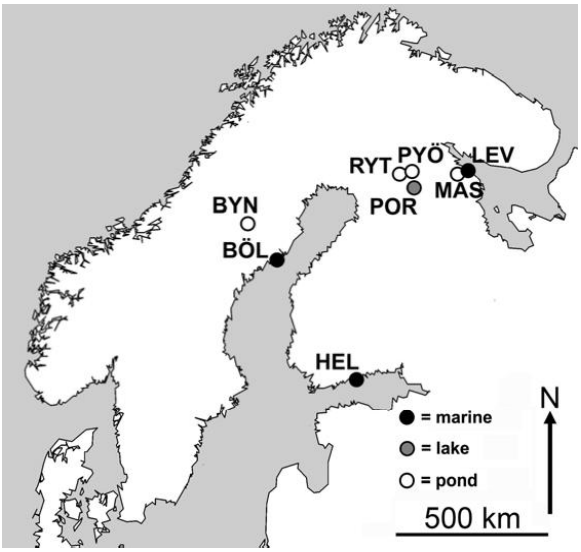

Figure 1 Map of the sampling localities. BÖL = Bölesviken, Baltic Sea, Sweden; HEL = Helsinki, Baltic Sea, Finland; LEV = Levin Navolok Bay, White Sea, Russia; POR = Porontima, Finland; BYN = Bynästjärnen, Sweden; PYÖ = Pyöreälampi, Finland; RYT = Rytilampi, Finland; MAS = Mashinnoje, Russia.

their wild conspecifics. We also explored the brain-body size allometric relationship in fish from different populations, expecting hypoallometry with a relatively shallow slope (<0.5).

Results

Variation in absolute brain size

Dissected brains were fixed and photographed under standardized conditions. Absolute brain size was estimated from measurements taken from digital photographs (dorsal, lateral and ventral views) by using the ellipsoid model [12,36]. General Linear Model (GLM) results revealed a significant population effect in absolute brain size ($F_{7, 112}$ = 153.68, P < 0.001). Average brain sizes of marine and lake fish were similar, but smaller than those of pond fish, the latter also being highly variable (Figure 2). General Linear Mixed Model (GLMM) analyses revealed a significant habitat ($F_{1, 5}$ = 11.84, P = 0.018) and non-significant population within habitat effect (Z = 1.55, P = 0.12). Pond fish had brains almost twice as large as marine fish (Least Squares [LS] mean ± Standard Error [SE]: marine = 18.59 ± 3.43 mm^3; pond = 34.22 ± 2.97 mm^3).

Brain size co-varied with body weight, but independently of population origin and standard length (GLM; population: $F_{7, 96}$ = 1.50, P = 0.18; log body weight: $F_{1, 96}$ = 7.63, P = 0.007; log standard length: $F_{7, 96}$ = 0.52, P = 0.47; population × log body weight: $F_{7, 96}$ = 0.79, P = 0.60; population × log standard length: $F_{7, 96}$ = 1.48, P = 0.18). The log brain size - log body weight regression revealed hypoallometry (i.e. both β = 0 and β = 1 were rejected: R^2 = 0.88, β = 0.50, SE[β] = 0.02, P < 0.001; Figure 3), indicating that brain size increased at half the rate of body size.

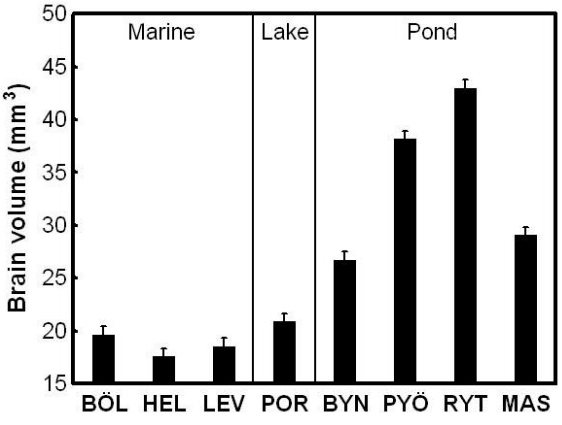

Figure 2 Population variation in absolute brain size in nine-spined sticklebacks. Means ± SE are shown. For population abbreviations, see Fig. 1.

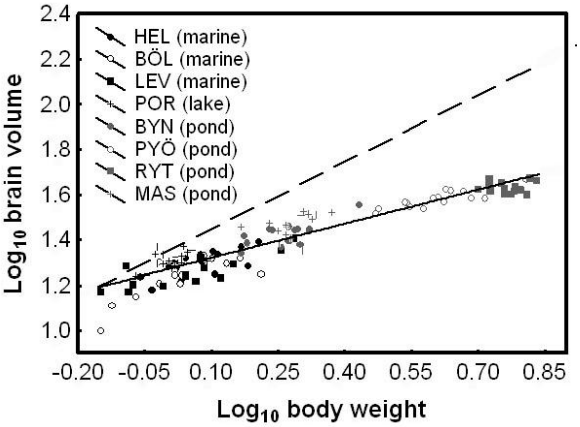

Figure 3 Allometric relationship between brain size and body size in nine-spined sticklebacks. Since we detected no significant population-specific relationship, only the common slope (solid line; β = 0.50) is shown. The dashed line denotes isometry. For population abbreviations, see Fig. 1.

Variation in relative brain size and brain part size

To study variation in relative brain size, we corrected the absolute brain size estimates to body length and body weight. We detected significant differences in relative brain size both at the habitat (GLMM; habitat: $F_{1, 6.89}$ = 10.38, P = 0.015; log standard length: $F_{1, 57.02}$ = 0.27, P = 0.54; log body weight: $F_{1, 93.48}$ = 28.82, P < 0.001; population [habitat]: Z = 0.95, P = 0.34), and population level (GLM; population: $F_{7, 110}$ = 10.79, P < 0.001; log standard length: $F_{1, 110}$ = 0.02, P = 0.89; log body weight: $F_{1, 110}$ = 30.14, P < 0.001). Pond (and the single lake) populations had larger relative brain sizes than marine populations (Figure 4a).

The sizes of different brain parts (telencephalon, optic tectum, cerebellum, hypothalamus) were also estimated from the digital photographs using the ellipsoid model [12,36]. We did not consider absolute size, but corrected our estimates with body length, body weight and absolute brain size. The multivariate GLM revealed a significant population effect on the relative sizes of different brain parts (population: Wilks' $\lambda_{28, 380}$ = 0.53, P < 0.001; log body weight: Wilks' $\lambda_{4, 105}$ = 0.91, P = 0.043; log standard length: Wilks' $\lambda_{4, 105}$ = 0.95, P = 0.024; log brain volume: Wilks' $\lambda_{4, 105}$ = 0.15, P < 0.001). Subsequent univariate tests indicated significant population differences in relative telencephalon ($F_{7, 108}$ = 3.53, P = 0.002), optic tectum ($F_{7, 108}$ = 2.81, P = 0.010), and cerebellum ($F_{7, 108}$ = 2.59, P = 0.016) sizes, but not in hypothalamus ($F_{7, 108}$ = 1.47, P = 0.18) size (Figure 4b-d). Our GLMMs revealed that neither optic tectum (habitat: $F_{1, 10.17}$ = 0.44, P = 0.52; log standard length: $F_{1, 59.62}$ = 0.26, P = 0.61; log body weight: $F_{1, 88.93}$ = 0.50, P = 0.48; log brain volume: $F_{1, 99.99}$ = 262.72, P <

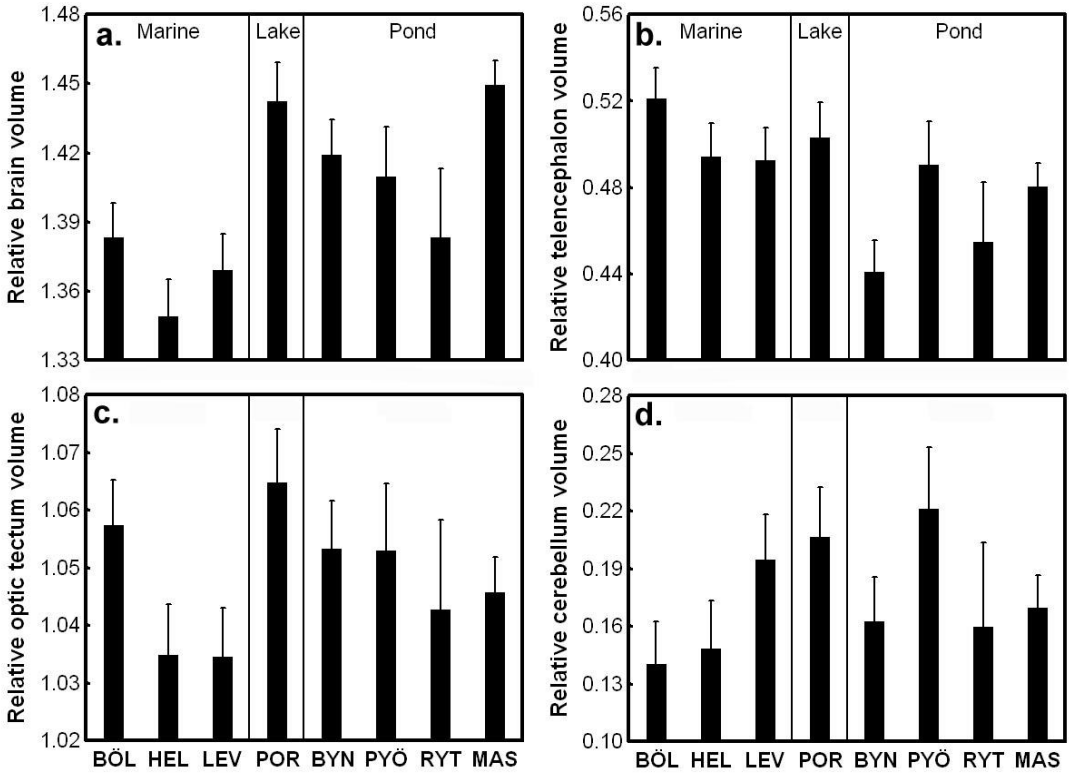

Figure 4 Population variation in relative brain size and relative size of different brain parts. Least Squares means ± SE are shown. Relative brain size is corrected for standard length and body weight while relative size of different brain parts are corrected for standard length, body weight and brain volume. For population abbreviations, see Fig.1.

0.001; population[habitat]: $Z = 1.01$, $P = 0.31$) nor cerebellum (habitat: $F_{1,\ 10.02} = 0.03$, $P = 0.87$; log standard length: $F_{1,\ 64.12} = 0.35$, $P = 0.056$; log body weight: $F_{1,\ 93.92} = 7.16$, $P = 0.009$; log brain volume: $F_{1,\ 99.91} = 32.39$, $P < 0.001$; population[habitat]: $Z = 1.12$, $P = 0.26$) showed significant habitat-dependency in their relative size. However, in the case of telencephalon, the habitat effect approached significance (habitat: $F_{1,\ 9.08} = 3.95$, $P = 0.078$; log standard length: $F_{1,\ 61.39} = 5.11$, $P = 0.027$; log body weight: $F_{1,\ 93.10} = 1.37$, $P = 0.24$; log brain volume: $F_{1,\ 99.91} = 122.53$, $P < 0.001$; population [habitat]: $Z = 1.05$, $P = 0.29$). Marine fish tended to have larger telencephala than pond fish, while no systematic trend could be observed in the other brain parts (Figure 4b-d).

Comparison of wild and common garden brains

We compared relative brain size and relative brain part size (see above) of fish from two marine (Helsinki, Baltic Sea and Levin Navolok Bay, White Sea; Figure 1) and two pond (Bynästjärnen, Sweden and Pyöreälampi, Finland; Figure 1) populations to data from the same populations reared in a common garden experiment [12]. Fish origin (i.e. wild-caught *vs*. common garden) had a habitat specific effect on relative brain size (GLMM; origin: $F_{1,\ 113.11} = 70.99$, $P < 0.001$; habitat: $F_{1,\ 2.49} = 0.65$, $P = 0.49$; origin × habitat: $F_{1,\ 113.83} = 38.36$, $P < 0.001$; log standard length: $F_{1,\ 105.99} = 5.75$, $P = 0.018$; log body weight: $F_{1,\ 113.95} = 42.12$, $P < 0.001$; population[habitat]: $Z = 0.84$, $P = 0.40$). The population-level GLM supported this result. It revealed a population-specific origin effect (origin: $F_{1,\ 110} = 70.41$, $P < 0.001$; population: $F_{3,\ 110} = 5.55$, $P = 0.001$; origin × population: $F_{3,\ 110} = 14.51$, $P < 0.001$; log standard length: $F_{1,\ 110} = 9.56$, $P = 0.003$; log body weight: $F_{1,\ 110} = 46.65$, $P < 0.001$). Relative brain size was similar for wild-caught and common garden marine fish, whereas pond fish had relatively larger brains in the wild than in the laboratory (Figure 5).

A multivariate GLM revealed significant, simple effects of population and origin on the relative size of brain parts, but no interaction between variables (origin: Wilks' $\lambda_{4,\ 106} = 0.74$, $P < 0.001$; population: Wilks' $\lambda_{12,\ 280.7} = 0.68$, $P < 0.001$; origin × population: Wilks' $\lambda_{12,\ 280.7} = 0.91$, $P = 0.57$; log standard length: Wilks' $\lambda_{4,\ 106} = 0.93$, $P = 0.12$; log body weight: Wilks' $\lambda_{4,\ 106} = 0.89$, $P = 0.012$; log brain volume: Wilks' $\lambda_{4,\ 106} = 0.107$, $P < 0.001$). All brain parts were affected by origin, as revealed by the subsequent univariate tests ($5.91 < F_{1,\ 109}$

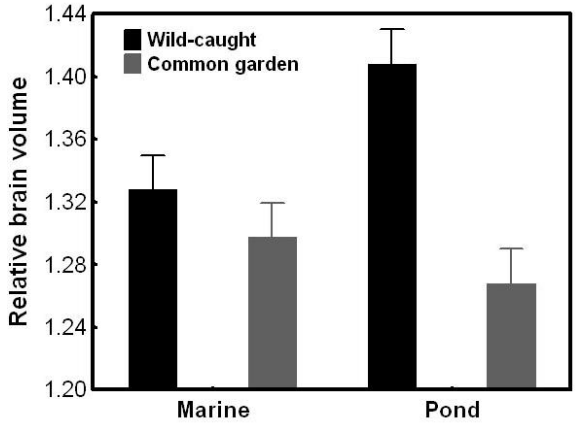

Figure 5 Habitat specific differences in relative brain size between wild-caught and common garden nine-spined sticklebacks. Least Squares means ± SE are shown. Relative brain size is corrected for standard length and body weight.

< 10.27, 0.002 <*P* < 0.017). Wild-caught fish had relatively larger telencephalon, optic tectum, cerebellum and hypothalamus volumes than their common garden reared conspecifics (Figure 6a-d).

Discussion

We showed that there is large variation in absolute brain volume, relative brain volume and relative volume of the telencephalon, optic tectum and cerebellum across wild nine-spined stickleback populations. Brain size patterns in the wild show habitat specificity both in absolute and relative scales: pond fish have larger brains than marine fish. Further, we found a marginally significant trend in the relative telencephalon size: marine fish tend to have larger telencephala than pond fish. The hypoallometric relationship between brain size and body size (slope = 0.5) is in accordance with a previous study on tropical fish [37]. We also found that wild-caught pond fish have larger brains than laboratory-reared pond fish, whereas no differences were observed between wild-caught and laboratory-reared marine conspecifics. The relative sizes of all brain parts were smaller in common garden than in the wild in all populations. These findings indicate that even though large brain size and brain part size variation exist in the wild, both in absolute and relative terms, patterns in nature may differ from those gathered in a standardized common garden and in some cases even in a habitat-dependent way. This strongly suggests that environmental effects on brain development can obscure and confound evolutionary inference based on purely phenotypic data collected from the wild. Hence, our results under-line the importance of not basing evolutionary inference on phenotypic patterns of brain size variation unless the environmental sources of variation have been controlled for - a point reinforced by other studies

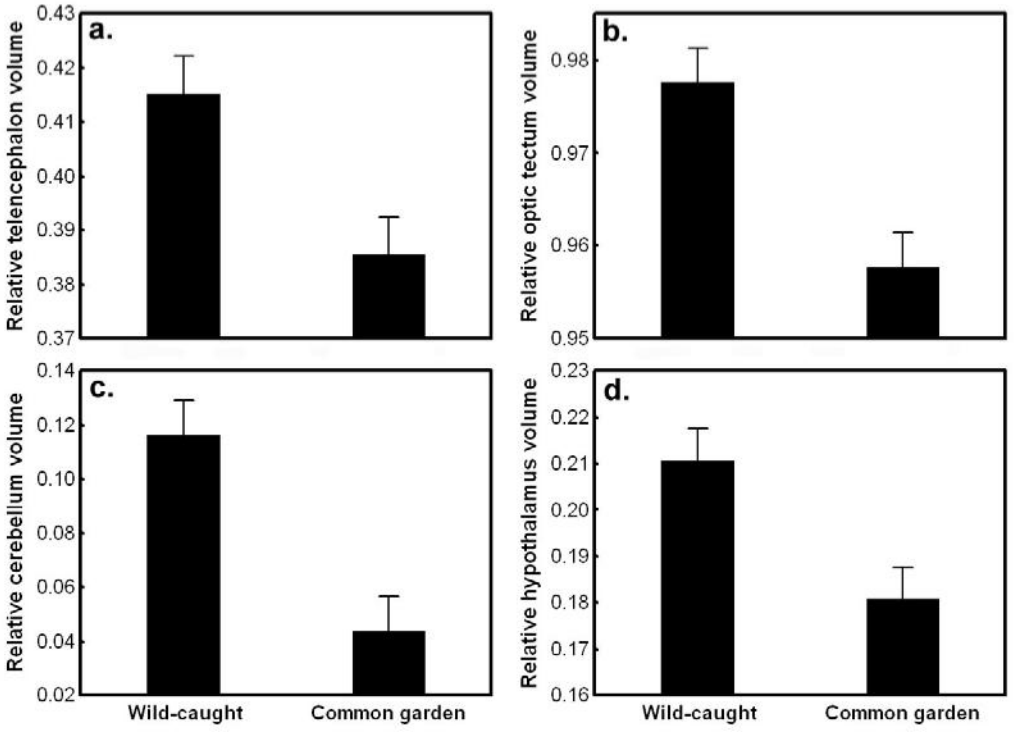

Figure 6 The relative size of different brain parts in wild-caught and common garden nine-spined sticklebacks. Least Squares means ± SE are shown. Relative size of different brain parts are corrected for standard length, body weight and brain volume.

focussing on differentiation in morphological and life history traits [38-40].

We found large habitat-specific population variation in absolute brain size: all marine populations (and a single lake population) had similarly sized brains that were nearly twofold smaller than those of pond fish. Within the pond habitat, there was large variation in average brain size. Although most studies have investigated only relative brain size variation (correcting brain size for variation in body size), absolute brain size can also account for differences in behaviour and/or habitat use. This is evident in comparisons of closely related species [see e.g. [32]], as the brains of these species tend to be more similar than those of distant taxa. Indeed, absolute brain size variation is routinely utilized in studies of primate and human evolution [e.g. [41]]. In general, increased brain size is attributable to an increase in neuron number, and not in neuron size [32]. Further, increases in absolute brain size result in decreased proportional connectivity [32]. Obviously, larger bodies need larger brains to be controlled at a similar level [32]. Hence, it is not surprising that pond populations that have evolved to giants [34,35] have also much larger brains than smaller sized marine or lake populations.

Previous findings have demonstrated a shallower hypoallometric slope at the intraspecific level and among closely related species than across broader taxonomic groups (mammals, intraspecific: 0.2-0.4, broad interspecific: 0.66, [28]; fishes, intraspecific: 0.44, intrafamiliar: 0.5, broad interspecific: 0.66, [37]). In accordance with these results we found a hypoallometric relationship between brain and body size with a slope = 0.5. In mammals, it has been demonstrated that on a broad evolutionary time scale, there has been greater net directional selection on brain size than on body size, while the short-term differentiation in brain *vs*. body size in closely related mammalian taxa has resulted from directional selection acting mostly on body size with changes in brain sizes being largely correlated responses [32]. Further, Gonzalez-Voyer et al. [42] demonstrated in Tanganyikan cichlids that even strongly correlated traits, such as brain and body size, can evolve independently from each other, and that body size may be under stronger selection than brain size during adaptive radiation. In the case of the nine-spined stickleback system, habitat-dependent body size diversification has been demonstrated [34,35], and body weight differences among these recently differentiated populations can be tenfold. Hence, it seems feasible to suggest that the observed brain size divergence might have been a correlated response to body-size divergence.

To assess body-size-independent brain size trends, we also investigated brain size differences relative to body size. Similarly to results for absolute brain size, and in contrast to our expectations, pond sticklebacks had relatively larger brains than marine sticklebacks. Intraspecific variation in relative brain size and brain architecture also appears to be strongly correlated with different ecological factors and/or life history traits. For example, environmental harshness has been shown to correlate positively with the size and neuron number of the brain region linked with memory storage (hippocampus) in the black-capped chickadee, *Poecile atricapillus*, a food caching species for which good memory can be essential for survival [14]. Garamszegi & Eens [8] found a positive correlation between song length and repertoire size and both relative and absolute volumes of different song nuclei. By comparing two subspecies of the white-crowned sparrow, the migratory *Zonotrichia leucophrys gambelii* and non-migratory *Z. l. nuttalli*, Pravosudov et al. [43] found that migratory subspecies had larger hippocampus and more hippocampal neurons. Habitat-independent, genetically based intraspecific variation in brain architecture has also been found both in wild guppy (*Poecilia reticulata*) populations reared in common environments [11], and in laboratory lines of the medaka (*Oryzias latipes;* [10]).

Marine nine-spined sticklebacks are members of a diverse fish fauna, and as such, are faced with numerous predators and interspecific competitors. In contrast, pond fish communities are much simpler. Structural heterogeneity in the pond environment is also much lower than that found in marine environments. These environmental factors are all known to be important in shaping brain evolution. For instance, predation pressure has been shown to affect brain size evolution in Mallorcan bovids [44], diet affected brain size evolution of bats [45], both environmental complexity and social features sculpt the brain architecture of cichlid fish [36], while living in large and socially complex groups is the most accepted hypothesis for the evolution of the extremely large brain of humans [46]. Hence, we expected selective pressures stemming from predation, interspecific competition, and habitat complexity to result in relatively larger brains in marine populations. Moreover, assuming that body size divergence (pond fish > marine/lake fish [34,35]) preceded correlated brain size divergence, we also expected pond fish to have similar or smaller brains, in relative terms, than marine or lake fish. Our previous common garden experiment based on a subset of the populations used here revealed no habitat-dependence in relative brain size [12]. Therefore, the pattern found in the current study (pond fish > marine fish) is highly unlikely to be a result of selection on brain size itself. Further, while we found no habitat-dependence in the common garden setting, strong population differentiation in relative brain size in a habitat-independent

way was detected (selective force unknown; [12]). Therefore, the plasticity resulting in the habitat-dependent wild *vs*. common garden difference cannot be habitat-specific itself. In a controlled laboratory experiment we found that group rearing had a negative effect on brain development in pond but not in marine fish [13]. Hence, the hypothesis that the aggressive, bold and anti-social pond fish [47,48] have larger relative brain sizes due to ontogenetic phenotypic plasticity as a response to fierce intraspecific competition must be rejected. Another possible explanation for larger relative brains in pond than in marine populations can be found from differences in ontogenetic allometry: pond fish living under negligible predation can become twice as old as marine fish [34], and an ontogenetic change in body *vs*. brain growth might explain this pattern. However, this issue requires further investigation.

Not only absolute and relative brain size, but also the relative size of different brain parts of nine-spined sticklebacks varied in the wild. Significant population differences were found in the relative sizes of the telencephalon, optic tectum and cerebellum. Further, we found marginally significant ($P < 0.08$) habitat-specificity in the relative size of the telencephalon, with marine fish tending to develop larger telencephala than pond fish. This is in accordance with results from our previous common garden study [12]. The telencephalon is larger in monogamous species, and shows a trend towards a positive correlation with rock size in the habitats in Tanganyikan lake cichlids [36], suggesting that both social and environmental heterogeneity may select for larger telencephalon. However, quite surprisingly, generalist limnetic populations of three-spined sticklebacks (*Gasterosteus aculeatus*) that use plankton as a main food source have larger telencephala than benthic-foraging populations of the same species as measured in samples from the wild [16]. The optic tectum is relatively larger in fish that prey on fish or other fast-moving prey, and clear water fishes develop larger optic tecta than species inhabiting turbid waters [6]. Cerebellum size correlates positively with the number of sympatric species in a fish community, and hypothalamus size is larger in monogamous than polygamous cichlids [36]. However, we did not find habitat specificity in the relative size of the optic tectum or the cerebellum, neither in the present, nor in the previous common garden study [12].

There are some incongruence between the present study and our previous work [12]. Here we found habitat-specific brain size divergence and population divergence in relative optic tectum size that was not seen in the common garden study. Only the habitat-dependence of relative telencephalon size found in the common garden study could be detected in the data from wild fish. A direct comparison between common garden and wild brains from the same populations revealed a habitat-dependent effect: pond (but not marine) fish had relatively larger brains in the wild than in the common garden. Further, the relative size of all brain parts was smaller in the laboratory than in the wild, perhaps due to a stimulus-poor environment during brain development. The most plausible explanation for the differences among common garden and wild data resides on phenotypic/ontogenetic plasticity in brain architecture. The potential for plastic responses to environmental heterogeneity is very high in fish [49-51]. Neurogenesis persists long into adulthood in fish [51-53] and contributes to lifelong growth of the brain. Hence, the fact that pond fish can live nearly twice as long as marine or lake fish may result in bias originating from plain ontogenetic plasticity or allometry. Furthermore, local random environmental variation may induce plasticity that could conceal genetic trends. Therefore, common garden studies seem to be of particular importance in studies of brain evolution. For instance, in this study system erroneous evolutionary conclusions could be drawn from the habitat-specificity (implying local adaptation) of relative brain size in the data from the wild given that observed differences cannot be reproduced under common garden conditions (showing that the differences are environmentally induced).

Finally, we showed that relative brain size and brain architecture are different between wild-caught and common garden sticklebacks from the same populations. The negative effect of domestication on brain size is well known both as a result of genetic adaptation and phenotypic plasticity [54,55]. In a recent paper, Burns et al. [56] demonstrated that laboratory rearing caused a significant decrease in the relative brain size of guppies (*P. reticulata*). Interestingly, we found that laboratory rearing had a negative effect on brain size in pond but not in marine nine-spined sticklebacks. The reason for this difference is unknown and warrants further investigations. We also found that all brain parts (corrected for both body and brain size) were smaller in common garden than in nature, a pattern congruent with general expectations. The reason for this can be a phenotypically plastic response to the comparatively stimulus poor laboratory environment.

Conclusion

In summary, we found large variation both in absolute and relative brain size, and brain architecture, among nine-spined sticklebacks in the wild. However, the patterns differed markedly from those found previously under standardized common garden settings, being most probably a result of environmental or age effects prevailing in the wild. Further, we found that the

difference between wild or common garden samples can be habitat specific. Considering the extreme plasticity of the fish brain, drawing evolutionary inference from wild-collected material alone can be challenging, and easily misleading. To understand brain size/structure variation in the wild, more intraspecific, common garden based studies, especially those that attempt to separate genetic and environmental contributions to brain development are needed.

Methods

Sampling and husbandry

Adult fish were collected from eight populations during May and June of 2007. Three habitat types were covered: marine samples came from Helsinki (Baltic Sea, Finland), Bölesviken (Baltic Sea, Sweden) and Levin Navolok (White Sea, Russia); Rytilampi (Finland), Pyöreälampi (Finland) and Bynästjärnen (Sweden) are isolated ponds, and Iso-Porontima (Finland) is a lake (Figure 1). This region started to deglaciate around 8000 years ago [e.g. [57]], thus, the populations are younger than this. Fish were collected with seine nets and minnow traps. After collection, they were moved to the aquaculture facilities of the University of Helsinki. Prior to taking brain measurements, fish were kept in standardized environment for approximately three months: temperature (14°C) and photoperiod (12 h light, 12 h dark) were held constant, and feeding (*ad libitum*) with bloodworms (Chironomidae sp.) was similar for all population groups. We note that the ca. three months common garden keeping (to standardize body condition, which is highly variable during spring in nature) might have caused some plastic responses induced by the artificial environment. However, this effect is highly unlikely to be profound. The experiment was conducted under license of the Animal Experiment Board in Finland, reference number: STH379A.

Sampled habitat types differed markedly, both in terms of biotic and abiotic aspects. In marine and lake habitats, nine-spined sticklebacks belong to a diverse fish community consisting of a large number of potential fish predators and interspecific competitors. Conversely, ponds lack predatory fish, and interspecific competition is absent (Rytilampi and Bynästjärnen), or negligible (Pyöreälampi; where a few, small-sized whitefish [*Coregonus lavaretus*] were recently introduced). While predation by aquatic insects and cannibalism at very early stages might occur in both habitats, there are two facts indicating large differences in predation caused mortality at later-than-fry stages: pond fish (i) show marked reduction in their defensive body armour (pelvic apparatus; [58]) and (ii) have a much longer lifespan than marine fish (6-7 years *vs*. 3-4 years; [34]). The structural complexity of the marine and large lake environment exceeded that of the study ponds which exhibit very simple structure (*viz*. negligible vegetation, and only a few rocks or fallen logs at the bottom of the pond). Although we did not quantify the abundance of nine-spined sticklebacks, it was evident from catch numbers and relative effort that population densities in ponds exceed those in the marine environment.

Brain measurements

The entire procedure, from dissection through photography and measurement of whole brains and brain regions, followed exactly those outlined in Gonda et al. [12,13]. Fish (N = 15 per population) were euthanized with an overdose of MS 222 (tricaine methanesulfonate). Body weights were measured to the nearest 0.01 g with a digital balance and standard length (from the tip of the mouth to the end of the caudal peduncle) to the nearest 0.01 mm with digital callipers (for population variation in body weight and standard length see Additional file 1). Freshly dissected brains were fixed in 4% buffered formalin (0.1 M phosphate buffered saline) solution for 48 h. After fixation, digital photos were taken.

Width, height and length of the brain and four different parts of the brain - telencephalon, optic tectum, cerebellum and hypothalamus - were measured from the digital photographs using tpsDig 1.37 [59] software. They were defined as the greatest distance enclosed by the given structure. As the brains could not be cut off from the spinal cord at comparable positions in every individual, the end of the brain was defined as the perpendicular projection of the cerebellum on the medulla. We calculated the volume of the different brain parts according to the ellipsoid model [e.g. [60,36]]. Total brain volume was estimated with two different methods: first, with the equation of the ellipsoid model suggested by Pollen et al. [36]; second, we calculated brain volume by summing the volumes of the different parts. Both methods gave qualitatively similar results, thus, only the results from the ellipsoid model are reported. Repeatability (R) of the volume estimates was calculated from three repeated independent measurements of three independent photographs of a subsample of brains (N = 20). All volume variables were highly repeatable ($R > 0.86$, $P < 0.001$).

Analyses

Absolute brain size was compared among populations using a General Linear Model (GLM) with brain volume as dependent variable and population as fixed factor. To test the habitat effect directly, we also ran a General Linear Mixed Model (GLMM) with brain volume as a dependent variable, habitat type (marine vs. pond) as a fixed effect, and population, nested within habitat type,

as a random factor. Note that in this and the subsequent (see below) tests of habitat effects we excluded the single lake population and only compared three marine with four pond populations. To account for an allometric brain size - body size relationship, all metric variables were $\log_{10}$ (hereafter log) transformed. Because a GLM with log body weight as a dependent variable revealed population dependent patterns in the log standard length - log body weight relationship (population: $F_{7,\ 104} = 13.02$, $P < 0.001$; standard length: $F_{1,\ 104} = 152.73$, $P < 0.001$; population × standard length: $F_{7,\ 104} = 14.56$, $P < 0.001$), subsequent analyses of total brain size or brain part size included both log standard length and log body weight for size correction.

To study body - brain size allometry, we performed a GLM with log brain volume as the dependent variable, population as a fixed factor, and log standard length and log body weight as covariates, including factor × covariate interactions. We also ran a simplified GLM with only log body weight as a covariate, but the results remained qualitatively the same (data not shown). As only log body weight was significant in the model (see Results) the slope of the log brain size - log body weight correlation was determined by linear regression.

To assess relative brain size trends, we applied two approaches. First we ran a GLMM using log brain volume as the dependent variable, with habitat fixed and population nested within the habitat (random) factor, and log body weight and log standard length as covariates. Second, to compare populations directly, we ran a GLM with log brain volume as a dependent variable, population as a fixed factor and log body weight and log standard length as covariates. To compare the relative size of different brain parts, we ran a multivariate GLM with the brain parts as dependent variables, population as a fixed factor, and log body weight, log standard length and log brain volume as covariates. We note that random factors could not be properly computed in the multivariate context, and therefore, habitat effects could not be tested directly. In cases of a significant multivariate effect, related univariate tests were also performed. Upon significant univariate effect, we ran GLMMs testing for habitat effects with the given brain part as dependent variables, habitat as a fixed effect and population, nested within habitat, as a random factor, with log body weight, log standard length and log brain volume as covariates.

Finally, using data (N = 15 per population) from our previous common garden experiment [12] we compared a restricted set of populations for which we had data from both the wild and common garden. These populations were Helsinki (Baltic Sea, Finland), Levin Navolok Bay (White Sea, Russia), Bynästjärnen (pond, Sweden) and Pyöreälampi (pond, Finland). For these data we did not address absolute size. We compared relative brain volume by first running a GLMM with log brain volume as the dependent variable, habitat (marine *vs*. pond), origin (wild *vs*. common garden) and their interaction as fixed factors; population nested within habitat as a random factor, and log standard length and log body weight as covariates. Second, to compare populations, we ran a GLM with log brain volume as the dependent variable, population, origin and their interaction as fixed factors, and log standard length and log body weight as covariates. To compare the relative size of different brain parts, we ran a multivariate GLM with the log brain parts as dependent variables, population, origin and their interaction as fixed factors, and log standard length, log body weight and log brain volume as covariates. Upon significant multivariate effects, the related univariate tests were considered. Because we did not find significant population × origin interaction in the multivariate GLM (see Results), we did not address habitat × origin effects with GLMMs.

As in all GLMs and GLMMs testing for population or habitat divergence in relative brain volume or relative brain part volume the covariates were only used for correction, we did not include factor-covariate interactions. All statistical analyses were performed with the SPSS 18.0 for Windows package (SPSS Inc., Chicago, Illinois, USA).

Additional material

Additional file 1: Body size of the nine-spined sticklebacks (*Pungitius pungitius*) used in this study. Standard length and body weight of the nine-spined stickleback (*Pungitius pungitius*) individuals used in the present study in the different populations. Mean ± SD and the minimum - maximum range are presented.

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Authors' contributions

AG collected data for the study, dissected the brains, took photos of them, measured the sizes of the brains and the sizes of the different brain parts and participated in the design of the study and writing of the manuscript. GH performed the statistical analyses, participated in the design of the study and helped in the writing of the manuscript. JM participated in the design and coordination of the study and helped in the writing of the manuscript. All authors read and approved the final manuscript.

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III

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Predation- and competition-mediated brain plasticity in *Rana temporaria* tadpoles

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Abstract

An increasing number of studies have demonstrated phenotypic plasticity in brain size and architecture in response to environmental variation. However, our knowledge on how brain architecture is affected by commonplace ecological interactions is rudimentary. For example, while intraspecific competition and risk of predation are known to induce adaptive plastic modifications in morphology and behaviour in a wide variety of organisms, their effects on brain development have not been studied. We studied experimentally the influence of density and predation risk on brain development in common frog (*Rana temporaria*) tadpoles. Tadpoles grown at low density and under predation risk developed smaller brains than tadpoles at the other treatment combinations. Further, at high densities, tadpoles developed larger optic tecta and smaller medulla oblongata than those grown at low densities. These results demonstrate that ecological interactions – like intraspecific competition and predation risk – can have strong effects on brain development in lower vertebrates.

Introduction

Phenotypic plasticity is an important and taxonomically widespread phenomenon providing means to cope with environmental heterogeneity in an adaptive fashion (e.g. Schlichting & Pigliucci, 1998; Miner *et al.*, 2005; Callahan *et al.*, 2008). The majority of plasticity studies have focused on behavioural, morphological and life history traits; however, the plasticity of internal organs has only recently begun to receive increasing attention (e.g. Piersma & Drent, 2003).

The brain is the centre of the nervous system and, as such, an extremely important organ in vertebrates. Laboratory studies have demonstrated brain plasticity at different neuroanatomical levels and life stages in several taxa, including mammals, fishes and reptiles (e.g. Kempermann *et al.*, 1997; Font *et al.*, 2001; Zupanc, 2001). For instance, there is strong evidence for seasonal plasticity in the size of certain neural structures (Nottebohm, 1981; Tramontin & Brenowitz, 2000) and that mental and physical training (e.g. Gould *et al.*, 1999; Rhode *et al.*, 2003) can influence neural architecture. The effects of environmental complexity (reviewed in Van Praag *et al.*, 2000) and social environment (Fowler *et al.*, 2002; Sorensen *et al.*, 2007; Adar *et al.*, 2008; Gonda *et al.*, 2009) on brain development have also been demonstrated. Two lines of evidence suggest that these plastic modifications can be of adaptive value. First, previous studies in brain development have demonstrated that those parts of the brain that are likely to be important in a particular context develop more than those of less importance (Kihslinger & Nevitt, 2006; Kihslinger *et al.*, 2006; Lisney *et al.*, 2007). Second, as the brain is the most expensive tissue to develop and maintain (Aiello & Wheeler, 1995) energetic constraints should impose strong selection against nonadaptive modifications of brain. However, while environmentally induced plasticity in brain development appears to be common, studies of the ecological and evolutionary relevance of this plasticity are slow to accumulate. The understanding of how ecological interactions may modify brain architecture is almost nonexistent, for example, we are not aware of any studies investigating the effect of

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predation risk or competition on brain architecture. Further, factorial experiments investigating independent and joint effects of different treatments are lacking. Studies incorporating these major ecological factors would be valuable in understanding the importance of plasticity in brain architecture for natural populations.

Predation risk and intraspecific competition often induce plastic modifications in morphology and behaviour in a variety of organisms (Miner *et al.*, 2005; Callahan *et al.*, 2008). Amphibian larvae show strong plastic responses to intraspecific competition and exhibit a wide range of phenotypic plasticity in response to predation risk (Miner *et al.*, 2005). While the tadpoles' responses can vary among specific predator types (e.g. Van Buskirk, 2001; Laurila *et al.*, 2006), predation risk posed by larval dragonflies tends to induce smaller body size, deeper tail and tail muscle, and lowered activity (e.g. Skelly & Werner, 1990; McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998). These modifications are adaptive, as they increase survival under predation risk (McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998). Intense intraspecific competition, on the contrary, induces larger bodies, shallower tails and higher activity (Relyea, 2002). Modifications of other structures and internal organs, including mouthparts and the gut, have also been reported (Relyea & Auld, 2004, 2005). While these competitor-induced modifications increase the competitive ability of tadpoles, they also increase vulnerability to predation (Relyea, 2002). Although the brain is important for processing sensory stimuli key to the detection and behavioural avoidance of predators, as well as in feeding (e.g. Köhler & Moyà-Solà, 2004), whether predation risk or competitors induce plastic changes in the brain architecture remains unexplored in tadpoles or in other taxa.

The aim of this study was to explore the potential effects of predation risk and intraspecific competition on brain development of common frog (*Rana temporaria*) tadpoles. Previous studies have shown that *R. temporaria* tadpoles express behavioural and morphological plasticity in response to both predators and competitors (e.g. Laurila *et al.*, 1998, 2004; Van Buskirk, 2001; Teplitsky & Laurila, 2007). We raised tadpoles in a two-factor experiment, manipulating levels of intraspecific competition and predation risk, and asked the following questions: Does intraspecific competition and/or predation risk influence brain size of tadpoles? Which brain regions are affected by these treatments? Do predation risk and competition have synergistic effects on brain size? Are brain phenotypes correlated with the treatment-induced morphological phenotypes? We considered two possible levels of effects on brain development. First, considering energetic constraints (the brain is the most expensive tissue to develop and maintain, e.g. Aiello & Wheeler, 1995), we predicted that (i) high intraspecific density that was likely to be resulted in high competition for low per capita food resources would imply an energetic constraint on overall brain development and (ii) the presence of a predator would also constrain brain development, especially at low density where foraging activity is reduced because of the perceived high per capita predation risk. Second, considering that those parts of the brain that are likely to be important in a particular context develop more than those of less importance (e.g. Lisney *et al.*, 2007), we predicted that the size of brain structures related to perception or learning (e.g. telencephalon, optic tectum) will increase with increased social complexity and higher competition, as well as in the presence of predators.

Materials and methods

Experimental animals

We collected *R. temporaria* eggs from a population in central Sweden (Stora Almby, Uppsala municipality, Sweden, 59°51′N, 17°28′E, and altitude 40 m) on 9 April 2008. Approximately 500 freshly laid eggs were collected from each of 12 families and immediately transported to the laboratory in Uppsala. The eggs were reared in family-specific 3 L vials containing reconstituted soft water (APHA1985; changed every 3 days) at a constant temperature of 18 °C. Hatched tadpoles were maintained in these vials until they reached developmental stage 25 (complete absorption of external gills; Gosner, 1960). Late-instar dragonfly larvae (*Aeshna* sp.), which are voracious predators of the tadpoles, were collected from ponds near Uppsala and used as predators in the experiment.

Experimental design

The experiments were conducted in plastic tanks (36 × 40 × 90 cm) placed in a fenced field in Uppsala. The tanks were established 2 weeks before the beginning of the experiments, to allow algal growth. The tanks were filled with 90 L of water, 10 g of dried leaves (*Betula* sp., *Populus tremula*) and 4 g of rabbit pellets, inoculated with 1 L of pond water, and covered with mosquito net to prevent colonization by insects. On April 21, we pooled 100 seemingly healthy tadpoles from each of the 12 clutches into a bucket and then allocated the appropriate number of tadpoles to each tank.

We manipulated total density of tadpoles (high density = 50 tadpoles/tank; low density = 10 tadpoles/tank; these densities are within the natural range of tadpole density in *R. temporaria* (Laurila, 1998; A. Laurila, personal observation) and predator presence. In the predator treatment, one dragonfly larva was placed in a cylindrical cage (diameter 8 cm; height 21 cm) made of transparent plastic film with a double net bottom (mesh size 1.5 mm) and hung 6 cm over the tank bottom. This allowed the tadpoles to receive both visual and chemical cues from the predator, whereas the predator was unable

to catch the tadpoles. In the no-predator treatment, the cage was left empty. During the experiment, the tadpoles relied on the resources provided in the beginning (leaves, rabbit pellets) and on the algae growing in the tanks. Predators were fed with *R. temporaria* tadpoles (ca. 300 mg) every other day. Each treatment combination was replicated eight times, resulting in a total of 32 experimental units. Treatments were assigned randomly among the tanks.

Body and brain measurements

On day 24 of the experiment, 176 randomly chosen individuals (five from each low density tank and six from each high density tank) were killed with an overdose of MS 222 (tricaine methanesulphonate). Immediately following death, tadpoles were weighed to the nearest 0.01 g with a digital balance, and photographed from dorsal and lateral views, using a digital camera (Nikon D80; Nikon Corp., Tokyo, Japan) equipped with a macro lens (Sigma AF 105 mm f/2.8 EX DG; Sigma Corp, Kanagawa, Japan) in a standardized setup. A millimetre scale was placed in each photograph for scaling. The following measures were later obtained from the digital photographs using tpsDig 1.37 (http://life.bio.sunysb.edu/morph/) software: body length (from the tip of mouth to cloaca), maximum body width, maximum body depth, maximum tail muscle depth, maximum tail depth and tail length (from cloaca to the end of tail). The tadpoles were fixed in 4% formalin – 0.1 M phosphate-buffered saline solution for later dissection of the brains.

Tadpole brains were dissected and put into 4% formalin buffered with 0.1 M phosphate-saline solution. We excluded 12 individuals because of dissection failure, resulting in a total of 164 individuals for brain measurements. After 48 h fixation, dorsal and right lateral views of brains were photographed with a digital camera (Canon EOS 10D; Canon Inc., Tokyo, Japan) connected to a dissecting microscope (Wild M5A; Wild, Heerbrugg, Switzerland). For bilateral structures, only the right-hand side was measured. We could only measure two dimensions for each brain part (length and width of telencephalon, diencephalon and optic tectum, and depth and width of medulla oblongata) because some of the borders of the brain parts could not be identified with accuracy; hence, three dimensional estimations were impossible. Measures were taken from the digital photographs using tpsDig 1.37 software and were defined as the greatest distance enclosed by the given structure. All brains were photographed and measured three times. Repeatability of different brain measurements was high [$R = 0.60–0.95$ (mean = 0.77); $F > 5.60$, $P < 0.001$].

Statistical analyses

To test for the treatment effects on growth in general, we ran General Linear Mixed Models (GLMMs) with total length or body weight as dependent variables, the treatments (predation, density) and their interaction as fixed factors, and replicate (= tank) nested within predation × density as a random factor.

To correct the original body shape variables (body length, body width, body depth, tail length, tail depth, tail muscle depth) for body size, we calculated residuals from regressions between the shape variables and total length. We tested the homogeneity of the slopes of our body shape variables and total length among the different treatment combinations with General Linear Models (GLM ANCOVAs) and found no significant predation × density × total length interaction in any of the cases (all $P > 0.173$), suggesting that the residuals were comparable (see McCoy *et al.*, 2006). To describe body shape with the minimal possible number of independent variables, we ran a Principal Component Analysis (PCA) on the size-corrected variables resulting in two informative PCs (see Results). To test for the treatment effects on body shape, we ran GLMMs with the PC scores as dependent variables, the treatments (predation, density) and their interactions as fixed factors, and replicate nested under predation × density as random factor.

To describe 'brain size' with one variable, we ran a PCA on all variables (length and width of telencephalon, diencephalon and optic tectum, and width and depth of medulla oblongata). Only the first PC was informative, describing brain size (see Results). We followed the same strategy for the separate brain parts, i.e. we ran separate PCAs for the brain parts (telencephalon, diencephalon, optic tectum, medulla oblongata). The first PCs were always informative and described the size of the given structure (see Results). We used these PCs in the subsequent analyses.

To test our hypotheses (treatments effects, relationship with the other treatment-induced morphological changes) in a straightforward manner and to correct for all possible confounding variables, we built complex GLMMs to investigate the patterns in brain development. First, we ran a GLMM with the PC describing brain size as dependent variable, the treatments (predation, density) and their interaction as fixed factors, replicate nested within predation × density as random factor, and total length, body weight and the two shape PCs as covariates. Next, we ran separate GLMMs for the different brain parts, with the PC describing the given brain part as dependent variable, the treatments (predation, density) and their interaction as fixed factors, replicate nested in predation × density as random factor, and total length, body weight, the PC describing brain size and the two shape PCs as covariates. In the case of optic tectum and medulla oblongata, our GLMM indicated the presence of a possible trade-off (see Results); hence, we run an extra GLMM to test this possible trade-off directly. Here, we built a GLMM with the PC describing optic tectum size as dependent variable, the treatments (predation, density) and their interaction as

fixed factors, replicate nested within predation × density as random factor, and the PCs describing brain and medulla oblongata size as covariates. We conducted backward stepwise model selection based on the $P < 0.05$ criteria, but as the model selection did not produce qualitative changes in any of the cases (data not shown), we report results from the original models. SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) for Windows software package was used for all analyses.

Results

General morphology

We found significant effects of predation risk ($F_{1,\ 27.44} = 8.83$, $P = 0.006$), density ($F_{1,\ 27.44} = 6.45$, $P = 0.017$), and also a predation × density interaction ($F_{1,\ 27.44} = 4.94$, $P = 0.035$; Fig. 1a) on total length. The GLMM on body weight revealed a similar trend, but it was not significant (predation: $F_{1,\ 27.38} = 3.97$, $P = 0.056$; density: $F_{1,\ 27.38} = 1.31$, $P = 0.26$; predation × density: $F_{1,\ 27.38} = 3.87$, $P = 0.059$; Fig. 1b). The replicate effect was nonsignificant in both cases ($Z < 1.75$, $P > 0.08$). Tadpoles were significantly longer and tended to be heavier in the absence of predation risk at low density than in any other treatment combination.

The PCA on the size-corrected shape variables revealed two PCs with eigenvectors > 1, which together accounted for 83.27% of the total variance. Both PCs were biologically meaningful, PC1 (60.68% of total variance) described a gradient from relatively small-bodied and long-tailed tadpoles towards relatively large-bodied and short-tailed tadpoles, whereas PC2 (22.59% of total variance) described a gradient from tadpoles with low tails and tail muscles towards tadpoles with high tails and tail muscles (Appendix S1).

The GLMM on the first shape PC revealed a strong density effect ($F_{1,\ 28.61} = 47.62$, $P < 0.001$; Fig. 1c) without any effect of predation risk (predation: $F_{1,\ 28.61} = 0.12$, $P = 0.91$; predation × density: $F_{1,\ 28.61} = 0.81$, $P = 0.38$; Fig. 1c). The replicate effect was nonsignificant ($Z = 0.86$, $P = 0.39$). Tadpoles at low density had relatively longer tails and smaller bodies than tadpoles at high density. The GLMM on the second shape PC revealed significant density and predation effects with a marginally significant interaction term (predation: $F_{1,\ 22.62} = 12.26$, $P = 0.002$; density: $F_{1,\ 22.62} = 24.93$, $P < 0.001$; predation × density: $F_{1,\ 22.62} = 3.87$, $P = 0.086$; Fig. 1d). The replicate effect was nonsignificant ($Z = 0.92$, $P = 0.35$). Tadpoles at low density or under predation risk had deeper tails and deeper tail muscles than at high density or in the absence of predation risk. The marginally significant interaction term suggests that predation risk had a stronger effect at low tadpole density than at high tadpole density (Fig. 1d).

Brain morphology

The PCA on all brain variables retrieved only one PC with eigenvector > 1 accounting for 70.26% of the total variance. This PC was strongly and positively related to all original variables (factor loadings from 0.71 to 0.93); hence, we treated this PC as describing overall brain size.

The GLMM on the overall brain size revealed a density-dependent effect of predation risk and a positive correlation with shape PC2 (predation: $F_{1,\ 34.53} = 1.41$, $P = 0.24$; density: $F_{1,\ 60.24} = 5.51$, $P = 0.022$; preda-

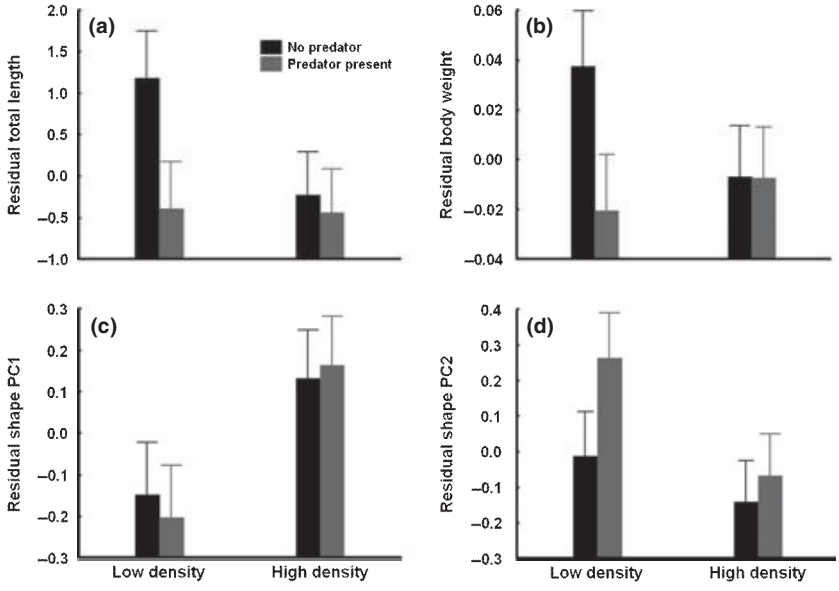

Fig. 1 The effects of density and perceived predation risk on growth and general morphology in *Rana temporaria* tadpoles. (a) length, (b) weight, (c) principal component describing a shape gradient from small-bodied and long-tailed tadpoles towards large-bodied but short-tailed tadpoles, (d) principal component describing a shape gradient from low-tailed and -tail muscled tadpoles towards tadpoles with high tails and tail muscles. Means ± SE are shown. Residuals are calculated from General Linear Mixed Models without the factors density and predation.

tion × density: $F_{1,\ 29.88} = 5.85$, $P = 0.022$; total length: $F_{1,\ 149.99} = 14.93$, $P < 0.001$; body weight: $F_{1,\ 149.72} = 2.42$, $P = 0.12$; shape PC1: $F_{1,\ 153.47} = 0.07$, $P = 0.80$; shape PC2: $F_{1,\ 153.17} = 7.878$, $P = 0.006$). The replicate effect was nonsignificant ($Z = 1.08$, $P = 0.28$). Tadpoles at low density under predation risk developed relatively smaller brains than tadpoles under other treatment combinations (Fig. 2a). Tadpoles having deeper tails and deeper tail muscles had also relatively larger brains (Appendix S2a).

The four PCAs on the different brain parts revealed similar patterns: the first PCs were always strongly and positively related to the original variables (two per brain part; factor loadings from 0.82 to 0.97); hence, we treated them as good size proxies for the given brain part.

We found a significant density effect and a marginally significant predation × density interaction on relative optic tectum size (Table 1). Tadpoles at higher density had relatively larger optic tecta. This effect appeared to be a result of the strong effect of density in the absence of predation risk (Fig. 2b). We also found a significant density effect on the medulla oblongata (Table 1): tadpoles at higher density had relatively smaller medulla oblongata (Fig. 2c). There were no treatment effects on the telencephalon or diencephalon (Table 1). The GLMMs also showed a (i) significant positive correlation with shape PC1 in the medulla oblongata, (ii) significant positive correlation with shape PC2 in the optic tectum and (iii) a marginally significant negative correlation between diencephalon size and shape PC2 (Table 1, Appendix S2b–d). The GLMM testing for direct correlation between the size of optic tectum and medulla oblongata revealed a significant negative relationship

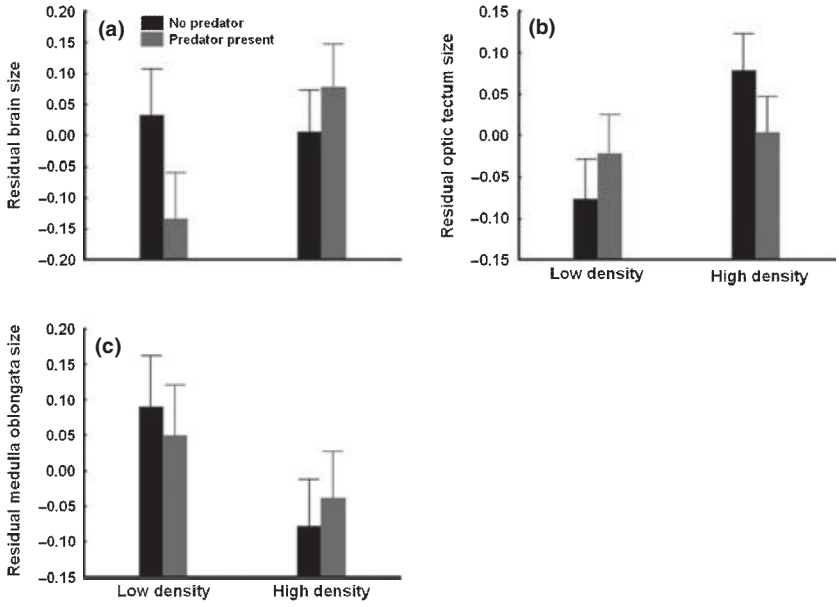

Fig. 2 The effects of density and perceived predation risk on brain development in *Rana temporaria* tadpoles. (a) brain size, (b) optic tectum size, (c) medulla oblongata size. Means ± SE are shown. Residuals are calculated from General Linear Mixed Models without the factors density and predation.

Table 1 Results of the General Linear Mixed Models on the different brain parts.

	Telencephalon		Diencephalon		Optic tectum		Medulla oblongata	
Effect	d.f.	*F*	d.f.	*F*	d.f.	*F*	d.f.	*F*
Predation	1, 32.89	0.23	1, 31.47	1.21	1, 34.63	0.35	1, 36.02	0.71
Density	1, 59.81	0.26	1, 58.01	1.17	1, 61.94	11.05***	1, 63.92	8.75***
Predation × density	1, 29.57	2.72	1, 28.04	0.10	1, 31.29	3.02*	1, 32.55	0.21
Brain size	1, 152.74	263.29****	1, 150.63	130.42****	1, 152.98	292.78****	1, 152.95	158.06****
Total length	1, 149.61	< 0.01	1, 151.51	0.28	1, 148.98	0.93	1, 149.39	< 0.01
Body weight	1, 150.10	1.32	1, 151.85	0.02	1, 149.49	0.04	1, 149.86	0.97
Shape PC1	1, 152.88	1.21	1, 152.73	0.11	1, 152.59	0.25	1, 152.70	4.45**
Shape PC2	1, 150.83	0.39	1, 147.69	3.81*	1, 151.70	5.11**	1, 151.60	0.83

The replicate effects were always nonsignificant ($Z < 1.00$; $P > 0.32$). Shape PC1 describes a gradient from small-bodied and long-tailed tadpoles towards large-bodied but short-tailed tadpoles, whereas shape PC2 describes a shape gradient from low-tailed and -tail muscled tadpoles towards tadpoles with high tails and tail muscles.
*$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, ****$P < 0.001$.

between these traits ($F_{1,\ 155.87} = 30.47$, $P < 0.001$; data not shown).

Discussion

The most salient finding of this study is the influence of ecological interactions on the relative size of the brain and certain brain parts in larval *R. temporaria*, with both density and predation being important factors that shape brain development. In addition, we found that (i) both the presence of predators and high density had a negative effect on growth, (ii) tadpoles raised at high density had relatively larger bodies and shorter tails when compared to those raised at low density and (iii) tadpoles had deeper tails and tail muscles under the predation risk at low density than in the other treatment combinations. Because these results on induced changes in morphology are in accordance with previous studies on tadpoles (McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998; Relyea, 2002; Laurila *et al.*, 2004; Teplitsky & Laurila, 2007), we believe that our results on brain plasticity might also be applied to amphibian larvae in general. Previous studies have found increased survival of induced tadpoles in the presence of lethal predators, which has been linked with the induced beneficial morphology and behaviour (e.g. McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998; Laurila *et al.*, 2006). Similarly, the plastic changes induced by competitors are considered adaptive (Relyea, 2002; Relyea & Auld, 2004, 2005). Our results suggest that the benefits of competitor and predator-induced morphological plasticity are linked with altered neural capacity. Later, we will discuss the implications of this finding while keeping in mind that the demonstration of the potential adaptive value of tadpole brain plasticity has to await for further studies.

We found that predation risk and high intraspecific density that was likely to result in high competition both induced phenotypic plasticity in relative brain size of larval *R. temporaria*. Tadpoles developed relatively smaller brains when they perceived visual and chemical stimuli from a predator but only at low density. Because the brain is energetically the most expensive organ (Aiello & Wheeler, 1995), our results could be explained in terms of energy availability and its impact on brain development (e.g. Taylor & van Schaik, 2007). We suggest that predation risk might be more readily perceived as high at low tadpole density but lower (because of dilution effect) at high density; hence, the presence of a predator might increase risk aversion (manifesting as lowered activity and energy intake) only at low tadpole densities. Perceived predation risk can result in energy deficit not only by lowered activity, as physiological stress responses may also lead to less energy available for development (Stoks *et al.*, 2005; Steiner, 2007; Slos & Stoks, 2008). Another effect, namely that increased competition at high density might make tadpoles more risk-taking, is also conceivable. It has been previously shown that a predation threat affects the activity of tadpoles negatively (Skelly & Werner, 1990; Laurila *et al.*, 1998; Teplitsky & Laurila, 2007), which has been suggested to result in reduced food intake (Werner & Anholt, 1993), and therefore might impose an energetic constraint on brain development.

Seemingly, density alone (i.e. different levels of intraspecific competition) did not pose an energetic challenge that could constrain brain development. As competition may reduce individual food intake at high densities (Anholt & Werner, 1996; Teplitsky & Laurila, 2007), we expected to find negative effects of density on relative brain size. However, this effect was not observed, suggesting that tadpoles at high density did not trade-off relative brain size for increased relative investments into other structures or activities, despite the general growth deficit in this treatment. It has been shown that physical activity directly influences brain size by increasing neurogenesis and decreasing neuronal degradation (Cotman & Berchtold, 2002; Catlow *et al.*, 2009); hence, the level of physical activity per se could be reflected on brain size. Accordingly, more active tadpoles living in the absence of predation and/or under high intraspecific competition (Skelly & Werner, 1990; Anholt & Werner, 1996; Laurila *et al.*, 1998; Teplitsky & Laurila, 2007) might develop relatively bigger brains compared to less active tadpoles living at low density under high individual predation risk. This expectation is also supported by our data.

We found that relative optic tectum (the main centre for vision) size was significantly larger at high than at low tadpole densities. It seems feasible to suggest that increased competition for food at high density imposes higher demands on optic tectum, inducing its growth. Furthermore, vision is involved in communication and perception of social environment (Hoff *et al.*, 1999), and these needs can also be expected to be more pronounced at high densities. A nonsignificant trend ($P < 0.1$) for an interaction between predation and density was found, presuming that predation might have a positive effect on optic tectum size at low density, whereas the opposite trend was observed at high density. Although olfactory cues are especially important in predator detection in tadpoles (Kats & Dill, 1998; Schoeppner & Relyea, 2005), vision also plays some role in both predator detection and localization (e.g. Semlitsch & Reyer, 1992). Hence, the enlargement of the optic tectum under predation risk at low density (with high individual risk) can be expected. The decreased optic tectum development under predation risk at high density is less straightforward to explain. However, trade-offs among different brain parts can occur (Barton *et al.*, 1995; Barton & Harvey, 2000), and size of optic tectum in the predatory treatment at high density could be traded-off with some other – yet unidentified – brain structure required for anti-predator behaviour. An alternative explanation could be that at

high density (where large optic tectum is favoured), predation imposed an energetic constraint, so opposite to the situation at low density, predation constrained maximal optic tectum development.

In contrast to the optic tectum, we found that the size of the medulla oblongata was significantly larger at low density when compared to high density treatment. The medulla oblongata is involved in regulation of respiratory, auditory and lateral line system functions in tadpoles (Torgerson *et al.*, 2001; McCormick, 1999; Jacoby & Rubunson, 1983). Previous studies in brain development have demonstrated that those parts of the brain likely to be important in a particular context develop more than those of less importance (e.g. Kihslinger & Nevitt, 2006; Lisney *et al.*, 2007). It has also been shown from an evolutionary perspective that changes in demand alter the number and size of component elements, making the relative size of different brain parts a reliable predictor of their importance for the organism in question (Kotrschal *et al.*, 1998). We assume that under low intraspecific competition environments where demands for good vision are lower than in high competition environments, other sensory systems such as lateral line and vestibular become more important. As a consequence, tadpoles reared at low densities develop smaller optic tectum and larger medulla oblongata compared to tadpoles reared at high densities. Trade-offs among different brain parts have been shown to occur in different taxa at both evolutionary and ontogenetic levels (e.g. Barton *et al.*, 1995; Barton & Harvey, 2000; Gonda *et al.*, 2009). Hence, an alternative explanation could be that the medulla oblongata is in a trade-off relationship with the optic tectum, so when the relative size of optic tectum became enhanced for higher competitive ability, the medulla oblongata became smaller because of energetic or developmental constraints.

We also assessed the possible relationships between treatment-induced morphological (body shape) and brain differences to evaluate if an increased investment into morphology (e.g. into tail muscles for better locomotive performance) was related to the enhancement of certain brain structures. Interestingly, we found that optic tectum increased with increasing tail and tail muscle depth, and medulla oblongata increased with increasing body size and decreasing tail length. Although these trends might seem to contradict what was found in the analyses of treatment effects on body shape and brain morphology, this is not the case. The correlations between body shape and brain structures discussed here are corrected for the treatment effects and describe treatment-independent relationships. The finding that tadpoles with deeper tail and tail muscle had larger optic tectum suggests that stronger predator-induced morphology is connected to enhanced visual abilities. Tadpoles with relatively larger bodies and shorter tails had relatively larger medulla oblongata; this aligns with the contention that the competition-induced phenotype requires more developed lateral line and vestibular sensory systems.

In summary, our results demonstrate that predation risk and high density that was likely to result in high intraspecific competition – two commonplace ecological interactions – influence brain development of larval common frogs. First, we found that tadpoles developed relatively small brains when reared in a combined treatment of predator risk and low tadpole density, probably as a result of the constrained energy intake because of a risk-averse behavioural strategy (low activity, limited foraging) adopted under high per capita predation risk. Second, we found opposite patterns in relative optic tectum and medulla oblongata size: tadpoles had relatively larger optic tectum and smaller medulla oblongata at high tadpole density. This might be a result of either opposing demands of these brain parts under different situations, or a trade-off between the two structures. It is noteworthy that the density effect on optic tectum was mainly driven by the differences observed in the absence of predation; predation might have opposing effects on the optic tectum at different densities. Our results also raise an interesting question: does larval experience during the aquatic phase affect the brain structure and neural abilities of metamorphosed, terrestrial frogs? Future research is needed to study the adaptive value of brain plasticity, energetic constraints and trade-offs involved in brain development, as well as potential carry-over effects in brain architecture to later life stages.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Factor loadings from the principal component analysis on the body shape variables.

Appendix S2 Correlations between certain brain structures and the level of investments into treatment-induced body shape change.

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Brain development and predation: plastic responses depend on evolutionary history

IV

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Brain development and predation: plastic responses depend on evolutionary history

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Although the brain is known to be a very plastic organ, the effects of common ecological interactions like predation or competition on brain development have remained largely unexplored. We reared nine-spined sticklebacks (*Pungitius pungitius*) from two coastal marine (predation-adapted) and two isolated pond (competition-adapted) populations in a factorial experiment, manipulating perceived predatory risk and food supply to see (i) if the treatments affected brain development and (ii) if there was population differentiation in the response to treatments. We detected differences in plasticity of the bulbus olfactorius (chemosensory centre) between habitats: marine fish were not plastic, whereas pond fish had larger bulbi olfactorii in the presence of perceived predation. Marine fish had larger bulbus olfactorius overall. Irrespective of population origin, the hypothalamus was smaller in the presence of perceived predatory risk. Our results demonstrate that perceived predation risk can influence brain development, and that the effect of an environmental factor on brain development may depend on the evolutionary history of a given population in respect to this environmental factor.

Keywords: competition; predation; brain plasticity; brain size; *Pungitius*; stickleback

1. INTRODUCTION

The vertebrate brain is an organ with great capacity for plastic neuro-anatomical changes (e.g. [1–3]). Brain parts that are likely to be important in a particular context develop more than those of less importance [4]. Further, as the brain is the most expensive tissue to develop and maintain [5], energetic constraints should impose strong selection against non-adaptive modifications. Surprisingly, studies on the effect of biotic environment on brain development are scarce [6–8]. In particular, studies in which the effects of common ecological interactions have been tested—such as predation and competition—are notably absent from the literature [9,10].

The evolution of the brain has attracted more scientific interest than brain plasticity induced by ecologically relevant environmental factors. However, most studies of brain evolution have relied on interspecific comparisons, looking for correlations between brain architecture and fitness-related traits [11–13]. Studies of interpopulation differences in brain architecture from an evolutionary perspective have started to appear only recently [14–16]. Still, research integrating plasticity into the evolutionary perspective, by studying population variation in environmentally induced brain plasticity, is scarce [8,17].

In this study, we aimed to investigate the effects of two important ecological factors—predation pressure and food limitation—on the brain development of different nine-spined stickleback (*Pungitius pungitius*) populations. Nine-spine sticklebacks living in isolated ponds (zero piscine predation) are often more aggressive, bolder, long-living giants with reduced body armour and increased costs of group living when compared with marine sticklebacks facing high piscine predation [8,18–21]. These patterns suggest that marine nine-spined sticklebacks are mainly adapted to avoid predation, whereas pond fish are adapted to intraspecific competition. Here, we compared the brain development of sticklebacks from two pond and two coastal marine populations in the presence or absence of predator chemical cues, and subjected to two different levels of food supply.

2. MATERIAL AND METHODS

Adult nine-spined sticklebacks were collected in 2009 from two isolated ponds (Abbortjärn, Sweden, 64°29′ N, 19°26′ E; Pyöreälampi, Finland, 66°15′ N, 29°26′ E) and two low-salinity, coastal marine habitats from the Baltic Sea (Nyköping, Sweden, 58°39′ N; 17°06′ E; Helsinki, Finland, 60°13′ N, 25°11′ E). Pond habitats were small (less than 5 ha) and contained no sympatric predatory fish, whereas marine environments are characterized by more diverse ecological communities, replete with several piscine predators. Sample sites were separated by more than 500 km. Crosses (six to eight per population) were done *in vitro* during July and August. Fish were reared individually in 1.4 l plastic tanks within four Allentown Zebrafish Rack Systems (Aquaneering Inc., San Diego, CA, USA, hereafter 'rack'), each equipped with physical, chemical, biological and UV filters and a closed water circulation system. All rearing took place in freshwater. The photoperiod was set to 14 L : 10 D cycle (light : dark), and the water temperature was held constant at 12°C.

Fish were distributed into four treatment combinations of two perceived predation risk and two food levels in a full-factorial, randomized block design. The water reservoir of each rack was connected to a separate 150 l tank. In the predation treatment, two 10–15 cm long perch (*Perca fluviatilis*) were placed in two randomly chosen tanks while the other two tanks contained only water. Hence, in the predation treatment olfactory cues from a fish predator abundant in the Baltic Sea and Fennoscandian freshwater habitats were either present or absent. In the food treatment, predation treatments were randomly divided into high (two ad libitum feedings per day) and low (one ad libitum feeding per two days) food groups. Feeding was started with live brine shrimp nauplii (*Artemia* sp.), and was gradually changed to frozen bloodworms after 80 days. Each full-sib family was represented in each treatment combination.

At the age of 34 weeks—when fish approached adult size—individuals were euthanized by MS-222, photographed under standardized conditions, and weighed to the nearest 0.01 g. Standard length was measured from pictures using tpsDig v. 2.15 [22]. Brains of the fish were dissected, fixed in 4 per cent formalin—0.1 M phosphate-buffered saline solution, and photographed with a digital camera connected to a dissecting microscope from dorsal, lateral and ventral viewpoints. Size of the brain and five brain parts (*viz.* bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus) were measured from the digital photographs with tpsDig v. 2.15 [22] (see also the electronic supplementary material). The volume of the total brain and the different brain parts was estimated according to the ellipsoid model, which estimates volume based on the length, width and height of the object using correction factors [8,14,15].

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Table 1. Results of the GLMMs. *F*-statistics and degrees of freedom are shown. Note that sex and its interactions were only included in the model to control for sex-related variation, and they are not discussed further.

effect	total brain	bulbus olfactorius	telencephalon	tectum opticum	cerebellum	hypothalamus
habitat (H)	3.89 (1,2.23)	29.24* (1,3.15)	0.50 (1,2.37)	<0.01 (1,2.39)	2.64 (1,2.68)	0.96 (1,3.08)
predation (P)	<0.01 (1,170)	1.97 (1,170)	1.41 (1,169)	0.02 (1,169)	0.06 (1,170)	3.89[a] (1,171)
food (F)	0.34 (1,170)	0.65 (1,170)	0.79 (1,169)	0.59 (1,169)	0.38 (1,170)	0.01 (1,170)
sex (S)	124.54*** (1,171)	6.09* (1,171)	25.96*** (1,170)	3.27 (1,170)	2.44 (1,170)	8.56** (1,171)
H × P	0.02 (1,170)	4.14* (1,171)	0.11 (1,170)	0.10 (1,170)	0.03 (1,170)	0.40 (1,171)
H × F	2.62 (1,170)	0.05 (1,169)	0.02 (1,169)	0.27 (1,169)	0.02 (1,169)	0.04 (1,169)
P × F	2.09 (1,170)	0.28 (1,171)	0.23 (1,170)	1.86 (1,170)	0.32 (1,170)	0.10 (1,171)
S × H	0.54 (1,170)	0.30 (1,170)	0.14 (1,169)	0.26 (1,169)	0.49 (1,169)	0.32 (1,170)
S × P	0.03 (1,170)	0.56 (1,170)	1.02 (1,169)	0.13 (1,169)	0.07 (1,170)	0.01 (1,170)
S × F	0.23 (1,170)	7.52** (1,169)	0.02 (1,169)	0.11 (1,169)	0.07 (1,169)	0.15 (1,169)
length	0.97 (1,172)	3.87 (1,155)	0.29 (1,170)	1.76 (1,171)	0.60 (1,170)	1.15 (1,150)
weight	86.74*** (1,169)	1.91 (1,65)	0.44 (1,155)	1.29 (1,158)	5.24* (1,144)	1.32 (1,54.4)
total brain	—	45.80*** (1,158)	151.07*** (1,171)	303.12*** (1,171)	208.45*** (1,170)	74.75*** (1,153)

*$p > 0.05$; **$p > 0.01$; ***$p > 0.001$; [a]$p = 0.0502$.

In total, 187 brains were analysed. Some individuals could not be included owing to random mortality (mainly at early life stages), fish escaping the system and problematic dissections. Hence, family effects were not analysed, but rather it was assumed that sampled fish represented an unbiased sample of each source population's genetic pool. All metric variables were $\log_{10}$ transformed. We used general linear mixed models (GLMMs) to analyse variation in brain size and the size of different brain parts. The models were built with habitat (marine versus pond), sex, predation (presence versus absence of perceived predation risk) and food treatments (high versus low) as fixed effects, with population nested in habitat as random factor. Standard length and body weight were both added as covariates because the study populations differ in relative weight [8,14,15]. In the analyses of brain parts, we also added total brain size as a covariate. In all models, we included simple effects, and all two-way interactions between fixed factors. We note that sex was included in the models only to control for sex-related variation. As we focused on the habitat and treatment effects, sex effects will be reported, but not discussed here.

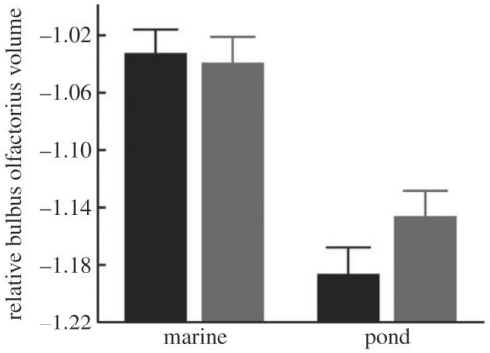

Figure 1. Effects of habitat of origin and perceived predation risk on bulbus olfactorius development. Least-squares means (from the GLMM) ± s.e. are shown. Black bars denote predator absent and grey bars denote predator present.

3. RESULTS

Brain plasticity was habitat-dependent (table 1): predation risk induced development of larger bulbi olfactorii in pond fish, but not in marine fish (figure 1). In general, marine fish had relatively larger bulbi olfactorii than pond fish (figure 1). Predation also had an effect on the development of the hypothalamus (table 1): independent of population, habitat or sex, fish developed smaller hypothalami in the presence of predator (figure 2). The food treatment did not affect brain development. The population effect was always non-significant ($p > 0.18$).

4. DISCUSSION

To date, the effect of predation on brain development has only been assessed in a single population of anurans [9,10]. Here, we found that perceived predation risk in the absence of actual contact with the predator has a significant—and sometimes habitat-dependent—effect on brain development in nine-spined sticklebacks. Surprisingly, while marine sticklebacks had relatively larger bulbi olfactorii than pond fish, perceived predation risk induced plastic modification in the bulbus olfactorius only in the latter. Our results suggest that in marine environments under constant predation risk, a large relative size of bulbus olfactorius might have become fixed, while in ponds, plasticity of the relatively small bulbi olfactorii occurred. Taken together, predation induced bulbus olfactorius enlargement both on the evolutionary and ontogenetic scales. As the brain is an extremely expensive tissue both to develop and maintain [5], the observed patterns support the conjecture that the bulbus olfactorius is an important sensory centre in predation avoidance. Given that predation-adapted sticklebacks are likely to represent the ancestral form, the fact that the plastic response appeared parallel to a decrease in bulbus olfactorius size in the piscine-predator free ponds warrants further investigations. We note that the predation treatment also reduced the aggression and risk-taking of our fish, demonstrating that sticklebacks identified olfactory cues from perch as predation risk [23].

Independent of population, habitat and sex, fish developed smaller hypothalami in the presence of predatory cues than in their absence. The hypothalamus has a wide range of functions [24]. For instance, it regulates reproductive behaviour [25], and it is also the centre of regulating feeding behaviour in fish [26]. Hence, based on our data, it is impossible to determine why perceived predation risk resulted in decreased hypothalamus size. However, considering

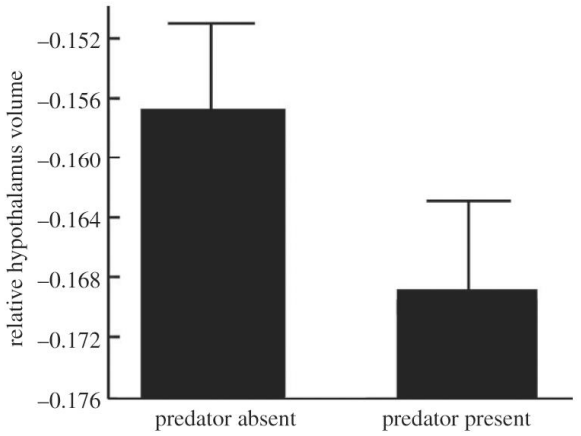

Figure 2. The effect of perceived predation risk on hypothalamus development. Least-squares means (from the GLMM) ± s.e. are shown.

that feeding behaviour can be regulated by different stress factors such as predation [26], and that nine-spined sticklebacks from the present experiment decreased their aggression and risk-taking levels in the presence of perceived predation risk [23], it seems possible that decreased hypothalamus size is somehow linked to the altered behavioural activity.

In summary, we found that perceived predation risk affected brain development, and that the effect can depend on a population's evolutionary history with predation. Available energy did not affect brain development. Interestingly, stickleback populations evolving under negligible predation had the ability to react to chemical predatory cues, while predation-adapted populations have evolved towards fixation of larger sized neural chemosensory centre (bulbus olfactorius). In the case of the hypothalamus, the negative effect of perceived predation risk affected all fish similarly, suggesting that the predation-induced reduction of functions regulated by the hypothalamus was general.

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Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain

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Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain

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The influence of environmental complexity on brain development has been demonstrated in a number of taxa, but the potential influence of social environment on neural architecture remains largely unexplored. We investigated experimentally the influence of social environment on the development of different brain parts in geographically and genetically isolated and ecologically divergent populations of nine-spined sticklebacks (*Pungitius pungitius*). Fish from two marine and two pond populations were reared in the laboratory from eggs to adulthood either individually or in groups. Group-reared pond fish developed relatively smaller brains than those reared individually, but no such difference was found in marine fish. Group-reared fish from both pond and marine populations developed larger tecta optica and smaller bulbi olfactorii than individually reared fish. The fact that the social environment effect on brain size differed between marine and pond origin fish is in agreement with the previous research, showing that pond fish pay a high developmental cost from grouping while marine fish do not. Our results demonstrate that social environment has strong effects on the development of the stickleback brain, and on the brain's sensory neural centres in particular. The potential adaptive significance of the observed brain-size plasticity is discussed.

Keywords: brain size; brain architecture; group living; nine-spined stickleback; phenotypic plasticity; *Pungitius*

1. INTRODUCTION

Several forms of plasticity in brain architecture have been demonstrated at different neuroanatomical levels and life stages in numerous taxa, including mammals, birds and fishes (e.g. Diamond *et al.* 1966; Rosenzweig & Bennett 1969; Kempermann *et al.* 1997; Tramontin & Brenowitz 2000; Zupanc 2001; Draganski & May 2008). During the past few decades, experimental studies have shed light on the effects of abiotic and biotic environmental complexities on the development of neural architecture (reviewed in Van Praag *et al.* 2000; Mohammed *et al.* 2002). For instance, rodents kept in stimulus-rich environments increased their brain size (Diamond *et al.* 1966; Rosenzweig & Bennett 1969), had more hippocampal neurons (Kempermann *et al.* 1997) and showed an elevated level of neurogenesis (Kempermann *et al.* 1997; Nilsson *et al.* 1999) as compared with those kept in a stimulus-poor environment. Chinook salmon (*Oncorhynchus tshawytscha*) reared in overly simplified hatchery conditions developed smaller bulbi olfactorii and telencephala as compared with wild conspecifics (Kihslinger *et al.* 2006). Kihslinger & Nevitt (2006) demonstrated that simply adding a few rocks in the rearing tanks resulted in increased cerebellum size in salmon (*Oncorhynchus mykiss*) alevins, while structural complexity of the abiotic environment affected the rate of cell proliferation in the telencephalon of juvenile coho salmon (*Oncorhynchus kisutch*; Lema *et al.* 2005). Structurally enriched environment has also been shown to increase foraging skills and learning ability in Atlantic salmon (*Salmo salar*; Brown *et al.* 2003). Obviously, brain architecture and behaviour are expected to be correlated both within and between species. In line with that expectation, Burns & Rodd (2008) have demonstrated a negative correlation between 'hastiness' and telencephalon size in guppies (*Poecilia reticulata*). On a larger scale, several comparative studies have revealed that behavioural and neural complexity appears to evolve in concert (e.g. Lefebvre *et al.* 1997; Reader & Laland 2002; Gonzalez-Voyer *et al.* 2009).

Social environment is also implicated as an important factor in shaping the ontogeny and evolution of brain architecture. For instance, the large brains of primates are thought to be a consequence of living in complex societies (Dunbar & Shultz 2007). Coevolution of sociality and brain size has also been demonstrated in other mammals (Perez-Barberia *et al.* 2007), while recent studies have started to uncover the importance of parental care-type and pair-bonding in the brain-size evolution of fishes (Pollen *et al.* 2007; Gonzalez-Voyer *et al.* 2009). However, apart from these interspecific comparative studies, only very few experimental studies have investigated the effects of sociality on brain architecture. Social isolation was found to decrease the number of new neurons in the dentate gyrus of prairie voles (*Microtus ochrogaster*; Fowler *et al.* 2002), while greater social complexity increased neuronal recruitment in birds (Lipkind *et al.* 2002; Adar *et al.* 2008).

Adult neurogenesis is limited to a few areas of the brain in mammals (Gould *et al.* 1999; Hastings *et al.* 2000, 2001), yet several studies have demonstrated its more

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widespread occurrence in birds (e.g. Reh & Fischer 2001). In comparison with mammals and birds, neurogenesis persists longer into adulthood in reptiles (Font *et al.* 2001) and fishes (Zupanc & Horschke 1995; Zupanc 2001, 2006), contributing to lifelong growth of brain size and thereby the potential for plastic responses to environmental heterogeneity (Birse *et al.* 1980; Raymond & Easter 1983; Zupanc & Horschke 1995). Hence, fishes provide an excellent model for neural plasticity studies. The effect of abiotic environment on the development of brain architecture in salmonid fishes has been demonstrated (e.g. Kihslinger & Nevitt 2006; Kihslinger *et al.* 2006). However, despite the widespread occurrence of group living in numerous fish taxa (e.g. Pitcher & Parrish 1993; Krause & Ruxton 2002), no studies of the potential effects of social environment on brain architecture in fishes have been conducted. Likewise, studies investigating the possibility that genetically based population-level differences in brain development are due to sociality are as yet to be conducted. Such differences could be expected to occur if the costs and benefits of grouping differ among populations residing in different selective environments.

The aim of this study was to investigate the long-term effects of social environment on brain development of nine-spined sticklebacks (*Pungitius pungitius*), and to compare the effects between populations originating from contrasting environments. This was done by comparing the relative size of brains and five different brain regions of adult fish subjected to different social environment treatments in the laboratory from hatching until adulthood. We were interested in addressing the following questions. (i) Is there any difference in relative brain size of nine-spined sticklebacks reared either individually or in groups? (ii) Which parts of the brain are the most affected by these conditions? (iii) Are there any population or habitat-specific differences in the detected patterns? The latter could be expected because the study populations originated from two contrasting environments (*viz.* marine and pond environments) where the costs and benefits of grouping are different. Pond fish grow faster (G. Herczeg, A. Gonda & J. Merilä, unpublished data), are more aggressive, are bolder, have higher drive to feed (Herczeg *et al.* 2009*a*), and probably, as a consequence, display a higher cost of grouping (Herczeg *et al.* 2009*b*) than their marine conspecifics. Hence, we could formulate two main hypotheses. First, we hypothesize that there should be a habitat-specific treatment effect on relative brain size due to the habitat-specific differences in the costs of grouping (i.e. pond fish should have smaller brains when reared in groups than when reared individually). Second, we hypothesize about habitat-independent treatment effects on brain parts involved in communication or, more generally, in perception of the social environment. Here, we expected that the tectum opticum (the visual centre) will be enlarged in group-reared fish as compared with individual-reared conspecifics.

2. MATERIAL AND METHODS

(a) *Field sampling and study populations*

Adult nine-spined sticklebacks were collected from late May to early June of 2007, immediately before the peak of the breeding season, with the aid of minnow traps and seine nets from four populations representing two contrasting habitat types. Although it is known that sampling can introduce a bias towards bolder-than-average fish (Biro & Dingemanse 2009), this effect is hard to avoid, and we believe that the role of this possible bias is negligible in our case. These were two marine populations from the Baltic Sea near Helsinki (Finland) and the White Sea in Levin Navolok Bay (Russia), and two pond populations from Bynästjärnen (Sweden) and Pyöreälampi (Finland; figure 1). The marine sampling sites were shallow coastal bays close to creek inlets (Baltic Sea being a brackish water environment), representing low-salinity sea habitats. Even though we could sample only two replicate populations per habitat type, the large geographical (above 500 km) and genetic (based on highly polymorphic microsatellite markers; T. Shikano, G. Herczeg & J. Merilä, unpublished data) distance made them truly independent. The surface area of ponds was less than 5 ha and their maximum depth around 10 m. The habitats differ in several respects: nine-spined stickleback is the only fish species in the ponds apart from a small number of recently introduced small-bodied whitefish (*Coregonus lavaretus*) in Pyöreälampi. Based on diet analyses (e.g. Kahilainen *et al.* 2004), these whitefish are a potential competitor but not a predator of the nine-spined sticklebacks. It is noteworthy that we never caught a single whitefish among the thousands of sticklebacks during our extensive sampling in Pyöreälampi. Thus, pond sticklebacks experience no fish predation and no (or negligible) interspecific competition. By contrast, marine sticklebacks face several types of predatory fishes and interspecific competitors. These differences have resulted in entirely different evolutionary constraints of group living in the populations used here: pond fish suffer from reduced growth when kept in groups while marine fish do not (Herczeg *et al.* 2009*b*).

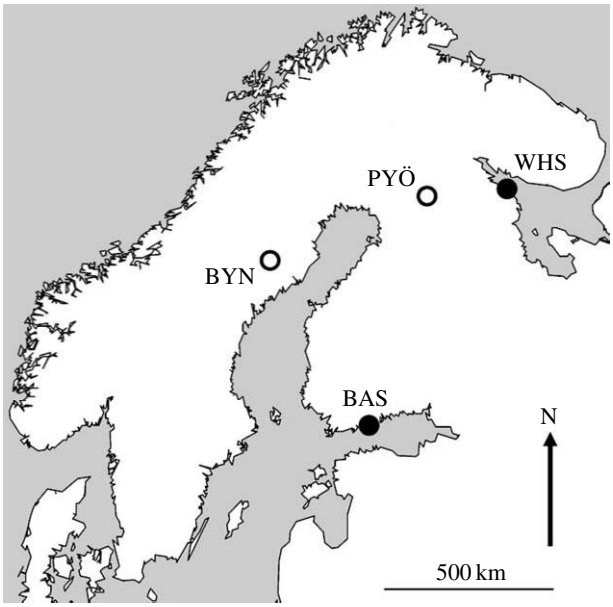

Figure 1. Map showing the location of the study populations. BAS, Baltic Sea, Finland; WHS, White Sea, Russia; PYÖ, Pyöreälampi, Finland; BYN, Bynästjärnen, Sweden. Open circles, small isolated ponds; filled circles, marine populations.

(b) *Breeding conditions and experimental design*

After collection, adult fish were transported to the aquaculture facilities of the University of Helsinki and kept at 17°C under permanent light and fed with frozen bloodworms

(Chironomidae sp.) until a sufficient number of fish had attained reproductive condition. Both wild-caught adults and all their offspring (see below) were kept and raised in freshwater. Five artificial crosses per population were made in the last week of June. The clutches were placed into 1.4 l tanks of two Allentown Zebrafish Rack Systems (hereafter 'rack'; Aquaneering Inc., San Diego, CA, USA). Racks had closed water circulating systems with multi-level filtering (physical, chemical, biological and UV filters) and inbuilt thermostats. Unfertilized eggs were removed daily. After hatching, 10 fish per family (i.e. 50 fish per population) were placed individually and randomly into the 1.4 l tanks of the two racks (hereafter individual treatment). The transparent plastic tanks were separated from each other with white panels to block visual contact between neighbours. Chemical contact could not be blocked due to the closed water system.

Of the remaining fish, in the second treatment (hereafter group treatment), a maximum of 80 individuals (depending on the size of the family) per family were divided into two replicates and placed in well-aerated 10 l plastic tanks. After three to four weeks, fish were transported to similar 10 l tanks with mosquito nets at the sides, and these tanks were placed in larger plastic tanks ($76\times54\times40$ cm, length, width and height, respectively; eight 10 l tanks in each) set with an open, one-way water flow. The 10 l tanks were placed randomly into the large tanks, and replicates within family were placed into different large tanks. After another three to four weeks (depending on the day of fertilization) 20 fish per family were chosen (replicates equally represented) and pooled within populations, resulting in pools of 100 fish per population. From the Baltic population, equal family representation and reaching $n=100$ were impossible because of the low number of individuals in the original families and the subsequent mortality. Here, 93 fish were pooled (26, 24, 21, 16 and 6 per family, respectively). Each new population pool was divided into two replicates. The replicates were placed randomly into halves of the larger ($76\times54\times40$ cm) plastic tanks halved by mosquito net and set with an open, one-way water flow. The replicates within populations were placed into different tanks. The water volume was set to 140 l in the larger tanks; hence, the *per capita* water volume (1.4 l), or in other words the fish density, was similar between treatments from this point onwards. In short, only chemosensory clues of conspecifics were present in the individual treatment, while visual, chemosensory and tactile cues were all present in the group treatment.

In both treatments, the temperature was set to 17°C throughout the experiment. We changed from a 24-hour light (natural at high latitudes in summer) cycle to a 12 L : 12 D periodism gradually during the course of one week after week 12. Owing to the latitudinal differences between the populations (figure 1), we did not attempt to mimic the natural light regimes any more closely. Fish were *ad libitum* fed two times per day. Feeding was started with live brine shrimp (*Artemia salina*); as the fish grew, we switched to frozen copepeods (*Cyclops* sp.) and then to frozen bloodworms. No gravel or other physical structures were presented in the rearing environments.

(c) *Brain measurements*

At the age of five months, when fish reached adult size (standard length from the tip of the nose to the tail base=4–7 cm; e.g. Bănărescu & Paepke 2001), 15 individuals from every population and every treatment were killed by an overdose of MS 222 (tricaine methanesulphonate). Individuals from the individual treatments represented families equally, and individuals from the group treatment were selected randomly from the mixed population pools (replicates represented evenly). After over-anaesthetizing the fish, their body weight was measured to the nearest 0.01 g with a digital balance and their standard length to the nearest 0.01 mm with a digital calliper. Then the brains of the fish were dissected and put into a 4 per cent formalin–0.1 M phosphate-buffered saline solution for 48 hours of fixation. After that, digital photographs were taken of the brains from three viewpoints (dorsal, right lateral and ventral) with a digital camera (Canon EOS 10D, Canon Inc., Tokyo, Japan) through a connected dissecting microscope (Wild M5A, Heerbrugg, Switzerland). A scale was positioned in each photograph for later measurements. Brains were positioned symmetrically and in a horizontal position by eye. We estimated the repeatability of our measurements based on three repeated independent measures of a subsample of brains ($n=20$) and found that all measurements (see below) were highly repeatable (all $r>0.8$).

The size of the brain and five different brain parts—bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus—were measured from the digital photographs with TPSDIG v. 1.37 software (Rohlf 2002). The width, height and length of each structure were taken and defined as the greatest distance enclosed by the given structure. The measures were perpendicular to the midline in the case of width, parallel to the projection of the brain in the case of length and perpendicular to the projection of the brain in the case of height. A detailed description of the measurements is given by Pollen *et al.* (2007), whose measurement procedures we followed. The volume of the total brain and the different brain parts was estimated according to the ellipsoid model (e.g. Huber *et al.* 1997; Pollen *et al.* 2007). This model might not account for fine-scale changes in brain shape, but it should be suitable for the purpose of our study as we compared populations of the same species where large shape changes are not expected. These estimates were validated by Pollen *et al.* (2007), who found that they provided consistent volume estimates of different brain regions. The volume (V) of the different brain parts was calculated as

$$V=(L\times W\times H)\pi/6, \tag{2.1}$$

where L, W and H denote the length, width and height of the given structure, respectively. For paired structures we used a doubled volume estimate of right side measurements. The total volume of the brain was estimated in two different ways. First, we used the equation

$$V=(L\times W\times H)\pi/(6\times1.23) \tag{2.2}$$

(Pollen *et al.* 2007); and, second, we simply summed the volumes of the different parts. The method of calculation did not influence the results qualitatively. Hence, only the results from the ellipsoid model are reported. We note that this method did not allow us to analyse fine-scale structural differences within brain parts, but significant differences at measures used would indeed indicate large treatment and/or population effects.

(d) *Data analyses*

All morphological variables were log transformed to correct for the allometric relationship between brain size and body size (Northcutt *et al.* 1978) and to achieve a linear

relationship between them. The transformed values were used in all analyses. A general linear mixed model (GLMM) was used to test for the habitat and treatment effects on brain size. Because we found a marginally significant difference in the body weight–standard length relationship between the populations (GLM ANCOVA: $F_{3,112}=2.38$, $p=0.073$), we corrected for both body weight and standard length in our analyses. In the GLMM, brain volume was the dependent variable, treatment and habitat fixed factors, body weight and standard length covariates, and population nested in habitat type a random factor.

Since the different parts of the brain were not independent, a multivariate GLM was conducted to test for the treatment effects at the population level. In this analysis (MANCOVA), the size of bulbus olfactorius, telencephalon, tectum opticum, cerebellum and hypothalamus were defined as dependent variables, treatment and population as fixed factors and body weight, standard length and brain volume as covariates.

In all models, we included the interaction between the fixed factors. Analyses were carried out with the SPSS v. 16.0 for Windows (SPSS Inc., Chicago, IL) software package.

3. RESULTS

After correcting for size effects (body weight: $F_{1,112.55}=49.991$, $p<0.0001$; standard length: $F_{1,113.09}=34.866$, $p<0.0001$), a habitat-specific treatment effect on brain size was found (habitat×treatment interaction: $F_{1,112.16}=7.816$, $p=0.006$; figure 2). The main effects of treatment ($F_{1,112.34}=2.035$, $p=0.157$) and habitat ($F_{1,2.07}=0.146$, $p=0.738$) were insignificant, as was the effect of population within habitat type ($Z=0.978$, $p=0.328$). Pond fish grew smaller brains in the group treatment than in the individual treatment, while no such effect was observed in marine fish (figure 2).

After correcting for size effects (body weight: Wilks's $\lambda_{5,105}=0.922$, $p=0.124$; standard length: Wilks's $\lambda_{5,105}=0.921$, $p=0.117$; brain volume Wilks's $\lambda_{5,105}=0.145$, $p<0.0001$), multivariate GLM revealed a significant treatment (Wilks's $\lambda_{5,105}=0.828$, $p=0.001$) and population effects (Wilks's $\lambda_{15,290}=0.39$, $p<0.0001$) on different brain parts. The treatment×population interaction was insignificant (Wilks's $\lambda_{15,290}=0.92$, $p=0.86$). Univariate analyses of the data revealed significant treatment effect on two brain parts, the bulbus olfactorius ($F_{1,109}=11.22$, $p=0.001$; figure 3) and the tectum opticum ($F_{1,109}=7.72$, $p=0.006$; figure 3). The bulbus olfactorius was significantly larger, while the tectum opticum was smaller in fish from the individual treatment than in fish from the group treatment. The treatments did not affect the size of telencephalon ($F_{1,109}=0.16$, $p=0.69$), cerebellum ($F_{1,109}=2.52$, $p=0.115$) or hypothalamus ($F_{1,109}=0.288$, $p=0.593$). The treatment-independent population differences are not in the focus of the present paper and will be discussed elsewhere.

4. DISCUSSION

Our results demonstrate that social environment can have marked effects on the development of the nine-spined stickleback's brain. Fish originating from pond populations developed smaller brains when reared in groups than when reared alone, while fish originating from marine

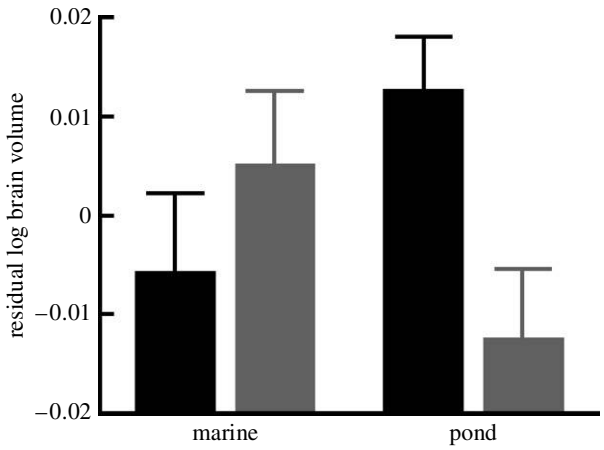

Figure 2. Social environmental effect on brain volume (mean+s.e.). Significant habitat-dependent treatment effect was found. Black bars, individual treatment; grey bars, group treatment.

populations showed a (insignificant) trend towards the opposite. According to our knowledge, this is the first time an interpopulation difference in brain-size plasticity during ontogenesis has been demonstrated. The fact that the difference in the level of plasticity was habitat- and not population-specific suggests that habitat-specific natural selection is the likely cause of the observed difference (cf. Clarke 1975; Endler 1986; McGuigan *et al.* 2005). We further discovered that social environment affected the development of different brain regions (*viz.* bulbus olfactorius and tectum opticum) in a similar manner in all populations. Individually reared fish receiving information from their conspecifics only via chemical cues developed significantly larger bulbi olfactorii than fish grown in groups. By contrast, group-reared fish subject to visual, chemical and tactile sensory inputs from conspecifics grew significantly larger tecta optica than individually reared fish. Size of telencephalon, cerebellum and hypothalamus appeared to be unaffected by social environment.

Population differences in learning and memorizing abilities (e.g. Mackney & Hughes 1995; Nelson *et al.* 1995; Girvan & Braithwaite 1998; Brown & Braithwaite 2005) and in brain architecture have been demonstrated in some taxa (Garamszegi & Eens 2004; Pravosudov *et al.* 2006; Brown *et al.* 2007; Burns & Rodd 2008; A. Gonda, G. Herczeg & J. Merilä, unpublished data). However, we are not aware of any study that would have investigated interpopulation variation in neural plasticity. In the present study, population differences in plasticity in response to social environment occurred between populations from two markedly different habitats in which the cost of sociality is expected and known to differ (Herczeg *et al.* 2009*a*,*b*).

Because marine nine-spined sticklebacks are under heavy fish predation throughout their lifespan, grouping can be beneficial in reducing predator-caused mortality and, assuming that food is patchier in marine than in pond environments, in increasing foraging efficiency too (Pitcher & Parrish 1993; Krause & Ruxton 2002). By contrast, intraspecific competition is expected to be one of the main biotic selective forces in pond sticklebacks. In fact, fish from ponds are more aggressive, bolder and have higher drive to feed (Herczeg *et al.* 2009*a*) than their

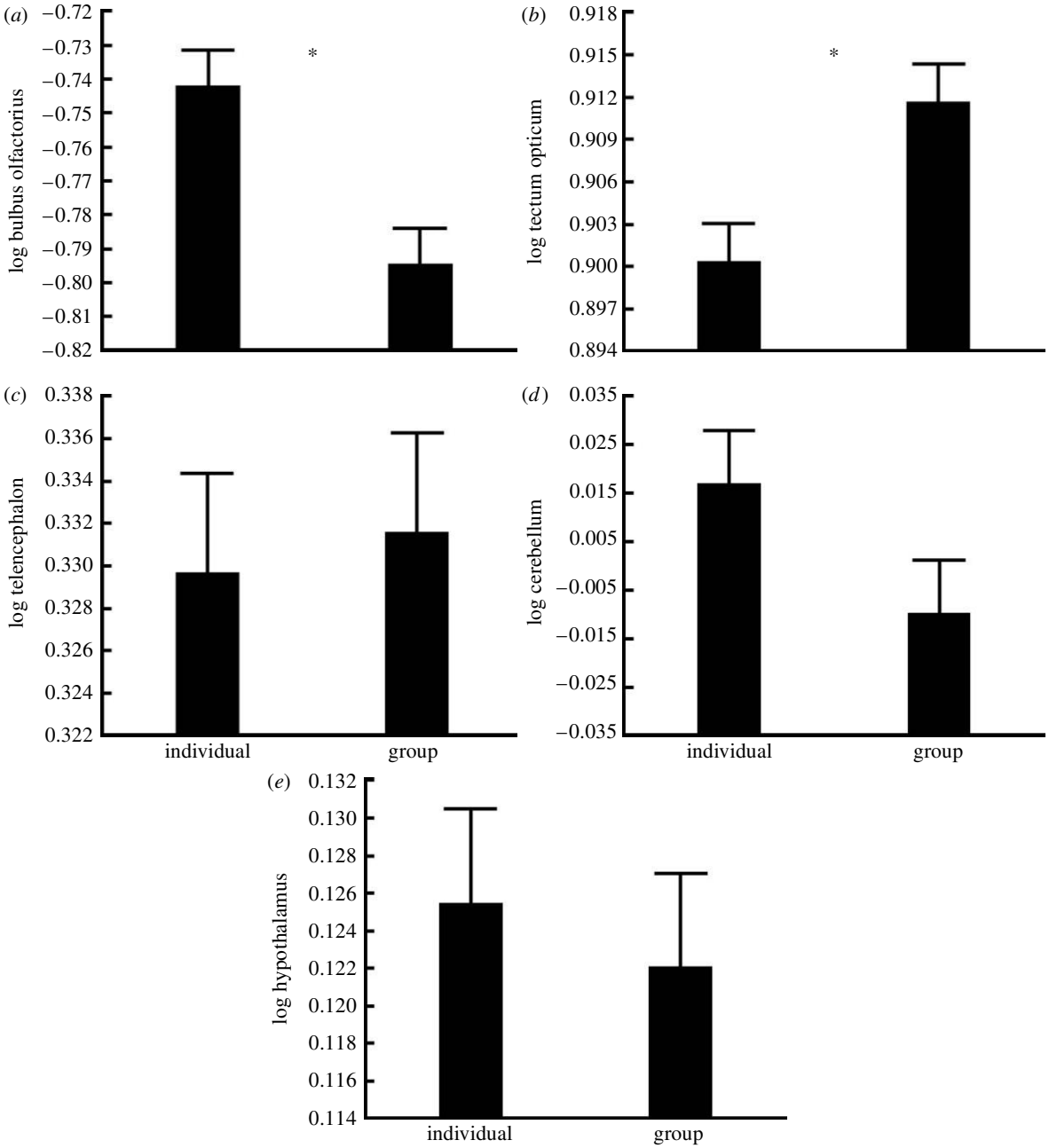

Figure 3. (*a–e*) Least-squares mean (+s.e.) size of brain parts in different social environmental treatments in different populations. Significant treatment effects are marked with asterisks.

marine conspecifics. Furthermore, and probably as a consequence, pond fish face high costs of grouping in terms of growth even when constraints originating from food limitation, predation, parasitism or reproduction are ruled out (Herczeg *et al.* 2009*b*). This happens irrespective of the fact that pond fish occur in high densities (G. Herczeg & A. Gonda, personal observation) and that, under stress, both pond and marine fish tend to group (Herczeg *et al.* 2009*b*). Therefore, group living or permanent contact with conspecifics can be considered to better reflect the natural situation for both marine and pond fish than living in isolation (which is hard to imagine in the studied habitat types), but also more stressful for pond than for marine fish. Considering that the brain is the most expensive tissue to develop and maintain (e.g. Aiello & Wheele 1995), the results showing that pond fish had smaller relative brain size when kept in groups than when kept alone, while marine fish showed some tendency towards opposite patterns, are not unexpected. However, it is interesting that the cost of grouping could manifest as a reduction in brain size in a situation where—due to the increased need during social interactions—one could actually expect larger brains to be developed. The fact that we used laboratory-reared fish suggests that the among-population patterns are likely to have a genetic basis, even though the possibility of maternal effects cannot be ruled out in our design. Furthermore, we found repeated, habitat-specific, population-independent differences that strongly support the role of natural selection in shaping the pattern (e.g. Clarke 1975; Endler 1986; Schluter & Nagel 1995; Foster 1999; McGuigan *et al.* 2005). We suggest that selection did not act directly on brain plasticity, but rather on the causes behind the differences in grouping costs between habitat types (e.g. behaviour), manifested as energetic constraints on brain development.

Previous studies in brain development have demonstrated that those parts of the brain that are likely to be important in a particular context develop more than those of less importance (Kihslinger & Nevitt 2006; Kihslinger *et al*. 2006; Lisney *et al*. 2007). It has also been shown that changes in demand alter the number and size of component elements, making the relative size of different brain parts a reliable predictor of their importance for the organism in question (Kotrschal *et al*. 1998). In our experiment, individually reared fish could only get information from their conspecifics by chemical cues, while visual, chemical and tactile cues were all available for group-reared fish. Our treatments were extremely simple in terms of abiotic complexity (we applied empty plastic tanks). Hence, one could expect that olfactory centres will be enlarged in the individual treatment, while visual centres will be enlarged in the group treatment. Our results are in line with these expectations: individually reared fish had larger bulbi olfactorii coupled with smaller tecta optica than their group-reared conspecifics, irrespectively of population origin. Evolutionary trade-offs between olfactory and visual centres of the primate brain have been shown at the interspecific level (Barton *et al*. 1995; Barton & Harvey 2000), but not in fish (Van Staaden *et al*. 1995; Huber *et al*. 1997). Our results support the existence of such a trade-off at the ontogenetic level: fish in a certain treatment not only enhanced the growth of the more-used structure, but also reduced the less-used one. These responses make sense considering the extremely high cost of developing and maintaining brain tissue (Aiello & Wheele 1995).

In summary, the results demonstrate that social environment—i.e. solitude versus membership of a group of conspecifics—has a marked effect on the development of the nine-spined stickleback brain. Individually reared pond fish developed relatively larger brains than their group-reared conspecifics from the same populations, while no such effect (or rather a tendency towards the opposite) was detected in marine fish. This pattern might arise from the higher costs of sociality in pond fish than in marine fish, originating in the lower benefits of grouping and higher drive for intraspecific competition in pond than in marine nine-spined sticklebacks (Herczeg *et al*. 2009*a*,*b*). Furthermore, we found that individually kept fish developed larger bulbi olfactorii but smaller tecta optica than fish kept in groups, irrespective of population origin. This finding supports the contention that the relative size of certain brain parts is related to their relative importance. Our study provides the first evidence for habitat-specific difference in brain-size plasticity, and emphasizes the importance of social environment in shaping brain architecture, with special emphasis on the main neural sensory centres.

The experiments were done under the licence of the Helsinki University Animal Experimentation Committee.

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Intraspecific brain size variation in the wild: a review

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VI

Manuscript

Intraspecific brain size variation in the wild: a review

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Abstract. The brain is a trait of central importance for organismal performance and fitness, and displays a great deal of evolutionary and ontogenetic variability both within and among different taxa. This variability can be ascribed to (i) genetically based differences caused by selective (cf. adaptive) or neutral processes (e.g genetic drift), (ii) environmentally induced phenotypic plasticity, or (iii) some combination of the two. However, still little is known about the ultimate and proximate causes of brain size variability in the wild. To date, evolutionary studies of brain size variation have mainly utilized comparative methods applied to analyses at the level of species or higher taxa. Even though these studies have uncovered several interesting correlations and identified possible drivers of brain size evolution, they suffer from the difficulty of separating causality from correlation. In the other extreme, studies of brain plasticity have focussed mainly – and understandably - on within population patterns. Between these extremes lie interpopulational studies of brain size variation. These studies, focussing on brain size variation among populations of the same species that occupy different habitats or selective regimes, form a rapidly growing field of investigations which is reviewed here. These studies can help us to understand brain evolution by providing a test-bed for ideas born out of interspecific studies, as well as aid in uncovering the relative importance of genetic and environmental factors shaping variation in brain size and architecture. Aside from providing the first in depth review of published interpopulational studies of brain size variation, we discuss the problem and prospects embedded with interpopulational studies of brain size variation. In particular, the following topics were identified as deserving further attention: First, studies focusing on disentangling the contributions of genes, environment and their interactions on brain variation within and among populations will be helpful in differentiating between selective and neutral explanations for observed variability in brain size. Second, studies applying quantitative genetic tools to evaluate the relative importance of genetic and environmental factors on brain features at different ontogenetic stages can be rewarding given the expected links between trait heritability and fitness and that between fitness and brain variability. Third, apart from utilizing simple gross estimates of brain size, future studies could benefit from use of neuroanatomical, neurohistological and/or molecular methods in characterizing variation in brain size and architecture.

Keywords: brain size, natural selection, neural architecture, population differentiation

Introduction

The brain has always been of interest to almost every field of biology dealing with animals due to its role in shaping the outcome of almost any contact between an individual organism and its environment. One of the simplest, yet often used, proxies for the brain's evolutionary state of development is its size (Box 1). Even though the significance of the overall brain size – or even the size of the main brain parts – and what exactly they tell us about the individual or species intelligence and cleverness is debated (Healy and Rowe 2007), overall brain size is in general believed to be a good proxy of intelligence and cognitive ability (Gibson 2002; Striedter 2005). Energetic constraints, stemming from the fact that the brain tissue is extremely expensive to maintain (Aiello and Wheeler 1995), should impose strong selective pressure against non-adaptive variability and changes. Hence, an increase in brain size can happen only when the benefits of a larger brain outweigh the cost of production and maintenance. For example, selection for increased cognitive ability should favour increased brain size, but only when enough resources can be secured to cover the increased energetic needs without loss in other aspects of fitness. For the same energetic reason as above, the size of a given brain part might be a good indicator of its importance, and reflect the way the given species or population has adapted to its environment and prevailing selective regime (Winter and Oxnard 2001; Gonzalez-Voyer and Kolm 2010).

However, choosing the right method and variable for comparisons is very important (e.g. Deaner *et al.* 2007; Healy and Rowe 2007). For brain size comparisons, at least three different variables can be used: absolute brain size, relative brain size, and the encephalization quotient (Box 1). For quantifying brain size, several well established methods are available, from simply weighing brains to volume calculations based on serial sectioning (Box 2).

Enormous variation in brain size – both in absolute and relative terms – has been reported in a number of taxa (e.g. mammals: Harvey *et al.* 1980; fish: Kotrschal *et al.* 1998; birds: Day *et al.* 2005). Our current knowledge about variation in brain size and architecture in the wild is based on two main lines of research. First, on interspecific comparative studies focussing on relationships between brain size and environmental parameters as well as between brain size and behaviour and/or life history trait variation (e.g. environmental complexity: Pollen *et al.* 2007; food hoarding: Garamszegi and Eens, 2004a; social complexity: Dunbar and Shultz 2007a,b; parental care-type and pair-bonding: Gonzalez-Voyer *et al.* 2009a). Second, on studies of adaptive phenotypic plasticity in brain size (reviewed in: van Praag *et al.* 2000; Mohammed *et al.* 2002). These two areas form the fundament of our current knowledge of brain size variation. In the following, we will (i)

Box 1. Defining and measuring brain size

Absolute brain size varies across five orders of magnitude in vertebrates (e.g. Striedter 2005; Deaner *et al.* 2007). It has increased (in some cases decreased) several times independently during the course of evolution (Striedter 2005). In general, whenever absolute brain size increases, it increases through changes in the number rather than size of neurons. Comparison of absolute brain size among distant taxa can therefore be meaningless because of brain-body size allometry and large variation in body size. Further, the internal structure/organization of the brain can be markedly different among distantly relative species (e.g. among fish: Kotrschal *et al.* 1998). However, when comparing closely related species, populations, or individuals of the same species, absolute brain size can be a good proxy of intelligence and cognitive ability (Gibson 2002; Striedter 2005). Further, larger brains (in absolute terms) contain more elements, and since the cognitive capacity of the brain mainly depends on the number of its elements (Byrne and Bates 2007), this also makes absolute brain size a good measure of cognitive ability.

Relative brain size refers to brain size corrected for variation in body size or its correlates. As brain size does not increase linearly with body size, simple division of brain size by body size (proportional brain size) can be misleading. Instead, as with many other organs (Schmidt-Nielsen 1984), brain size scales allometrically with body size (e.g. Lande 1979). If brain size is plotted against body size on a double logarithmic scale, the best fitting line will have a slope that is less than one (e.g. Lande 1979; Harvey and Bennett 1983; Martin and Harvey 1985; Pagel and Harvey 1988a; Striedter 2005). Hence, the relationship between brain and body size is hypoallometric. Relative brain sizes can be compared by including body size as a covariate in the statistical model. Large variation in relative brain size has been reported (Bauchot *et al.* 1977; Kotrschal *et al.* 1998) and in general, tends to increase in independent lineages in the course of evolution (Striedter 2005). As relative brain size takes body size into account, and also accounts for the above mentioned allometric relationship, this metric can be used for comparing brain size of taxa that differ in body size. In fact, relative brain size is the most widely used metric in evolutionary studies of brain size.

Encephalization quotient has also been used to control for body size in comparisons of brain size among different taxa (e.g. Jerison 1973; Marino 1997; Lordkipanidze *et al.* 2007; Silox *et al.* 2009; Vasallo and Echeverria 2009). There are several proposed methods for estimating encephalization quotient, but the first one described by Jerison (1973) is the most widely used. It is calculated by dividing the actually measured brain volume with the brain volume expected based on body size, estimated from the allometric relationship of the brain and body size from available data on a wide range of taxa (involving as many species/taxa as possible).

review the current state of knowledge about the factors shaping brain variation in the wild, (ii) introduce the emerging field of intraspecific brain evolution focusing on interpopulation variation in brain size and size of brain parts as well as on the interpopulation variation of the plasticity of these traits. Finally, (iii) we outline further avenues for studies aimed to increase our understanding of brain evolution and factors driving it.

Box 2. Measuring brains – methods

Volume of the brain and different brain parts. The undoubtedly easiest and in some cases the most ethical (cf. no need to sacrifice the animals) way to gather data on brain size variation is to use measures of skull (from collections) or head (on live specimens) volume. An obvious advantage of this method is that it allows brain size estimation when the brain itself is not available (e.g. Köhler and Moya-Sola 2004; Ashwell 2008). It has been shown that brain size estimates by this method are reliable in birds (Iwaniuk and Nelson 2002; Moller 2010). However, this method cannot be used in every taxa. For instance, in some fish the brain fills only a part of the neurocranium (Kotrschal *et al.* 1998). Brain volume can also be simply measured by the fluid displacement method (Karlen and Krubitzer 2006), by filling up the skulls with lead shot (Marino *et al*. 2006; Iwaniuk and Nelson 2002) or by use of the ellipsoid model (e.g. Pollen *et al.* 2007; Gonda *et al.* 2009a,b; 2011a). As to the latter, photographs of the brain are taken from three views (*viz.* dorsal, lateral and ventral) and the length, width and height of the brain (or different brain parts) are measured from the photographs. The volume is calculated by an equation assuming ellipsoid form. One can also estimate brain volume using computed tomography (CT) and X-ray, often used in studies of museum material (e.g. Macrini *et al.* 2007; Madden 2001). However, the restricted availability and high costs of these methods may explain why they are less common. Size estimates can also be inferred by applying landmark-based geometric morphometrics using photographs (for details, see: Park and Bell 2010). Finally, the most precise but also the most time consuming method for estimating the volume of the overall brain (or any brain part) is based on serial histological sectioning (e.g. Airey and DeVoogd 2000; Wilson and McLaughlin 2010). Even though this method yields the most accurate measures of the size of the brain and brain regions, the much quicker method based on the ellipsoid model may also give very good estimates of brain size (Pollen *et al.* 2007), and allow much higher sample sizes to be obtained.

Brain weight. Another simple but informative brain measurement is the weight of the brain. It is a widely used variable in evolutionary studies (e.g. Sol *et al.* 2002; Safi *et al.* 2005) with the obvious limitation that the weight of different parts cannot be usually obtained.

First pillar: Macroevolution and comparative studies

An enormous amount of macroevolutionary research has been conducted on different taxa in attempts to understand the major evolutionary forces behind brain size evolution (e.g. Clutton-Brock and Harvey 1980; Kotrschal *et al.* 1998; Striedter 2005; Shumway 2010). Giving a full overview on this topic is outside of the scope of this treatment (see: Healy and Rowe 2007 for a good summary of research in this area so far). However, we will briefly review the main findings and the proposed selective forces that shape the evolution of brain size and architecture, as they provide templates for further interpopulation comparisons and form a basis for comparing macroevolutionary and microevolutionary patterns. Correlations have been revealed between brain size or size of different brain structures and different environmental factors (e.g. Pollen *et al.* 2007), life history (e.g. Gonzalez-Voyer *et al.* 2009a; Isler 2011; Barton and Capellini 2011), behavioural (Ratcliffe *et al.* 2006; Aviles and Gramszegi 2007) and morphological traits (body size: Gonzalez-Voyer *et al.* 2009b; gut size: Aiello and Wheeler 1995; testis size: Pitnick *et al.* 2006) on interspecific (or higher) level after controlling for phylogenetic non-independence. However, most of these studies are done on primates and birds. Specifically, the evolution of the exceptionally large relative brain size of primates (and especially humans) has mainly been studied in light of sociality (e.g. Dunbar and Shultz 2007a,b). Social complexity, requiring life in large and complex groups or in pair bonds is accepted as the main driver of primate, especially human, brain size evolution (also known as "social brain hypothesis" e.g. Dunbar 1998; Dunbar and Shultz 2007a,b; Perez-Barberia *et al.* 2007). Apart from the increase in overall brain size, the size of the neocortex and hippocampus has received special attention. This is because the neocortex in primates (and especially in humans) has increased disproportionally during its evolution, and the hippocampus plays an important role in memory and learning, which have always been of human interest (Striedter 2005). In the case of birds, most of the focus has been on brain size or size of the forebrain, especially the telencephalon and the hippocampus for the same reason as in primates. The main correlates and suggested drivers behind the evolution of these neural structures are suggested to be selection forces stemming from migration and foraging innovation (e.g. Lefebvre *et al.* 1997; Sol *et al.* 2005a,b).

Even though the above mentioned comparative studies form the cornerstone of our current knowledge about brain size evolution, they are by nature correlative and therefore causations are hard to prove with the approaches used.

Second pillar: Adaptive phenotypic plasticity in brain size

Animals can adapt to their environment through genetic changes, but also ontogenetic phenotypic

plasticity allows adaptive adjustment or acclimation to prevailing environmental conditions (e.g. Ghalambor *et al.* 2007). Studies on brain development have demonstrated that those parts of the brain that are likely to be important in a particular context develop more than those of less importance in that context (Kihslinger and Nevitt 2006; Kihslinger *et al.*, 2006; Lisney *et al.*, 2007). Again, as the brain is the most expensive tissue to develop and maintain (e.g. Aiello and Wheeler 1995), energetic constraints should impose strong selection against nonadaptive modifications of brain. Hence, phenotypic plasticity in the brain can be expected to have an adaptive value.

Plastic changes in the brain size occur in nature. For instance, there is strong evidence for seasonal plasticity in the size of certain neural structures (e.g. in the song control centre of songbirds, Nottebohm 1981; Tramontin and Brenowitz 2000), in the anatomy of the human hypothalamus and hippocampus (Hofman and Swaab 2002), in the volume of hypothalamic nuclei in humans (Hofman and Swaab 1992) and in the hippocampal morphology of the white footed mouse *Peromyscus leucopus* (Pyter *et al.* 2005). Mental and physical training also appear to influence neural architecture (e.g. Patel *et al.* 1997; Gould *et al.* 1999a,b; van Praag *et al.* 2000; Brown *et al.* 2003; Rhode *et al.* 2003; Draganski and May 2008). For instance, the size of the posterior hippocampus of London cab drivers increases with time spent as a cab driver (Maguire *et al.* 2000). Additionally, hippocampus-dependent learning has been shown to increase the number of newly generated cells of the hippocampus in rats (Gould *et al.* 1999a,b), spatial learning induced neurogenesis in the hippocampus of birds (Patel *et al.* 1997), and voluntary running resulted in enhanced neurogenesis in the hippocampus of adult mice (van Praag *et al.* 1999; Brown *et al.* 2003; Rhode *et al.* 2003). Change in social status altered the size of song control centres of songbirds (Voigt *et al.* 2007) and the size of somatostatin-containing neurons in fish (Hofmann and Fernald 2000), while social rank has been found to correlate with forebrain cell proliferation rate in fish (Sorensen *et al.* 2007). Further, the size of brain parts that are of importance in certain life stages can also change reversibly. For example, shifts in habitat, diet or behaviour can alter the relative size of the main sensory brain areas in fish (Wagner 2003; Lisney *et al.* 2007), while changes in the size of different brain parts during pregnancy in women is likely to reflect the different need for the function that given brain part is responsible for (Oatridge *et al.* 2002).

Besides naturally occurring plastic changes, brain plasticity can be induced experimentally as well. Such experimental studies have shed light on the effects of abiotic and biotic environmental complexity on brain development (reviewed in: van Praag *et al.* 2000; Mohammed *et al.* 2002). Some of the main studies are compiled in Table 1. For example, rodents exposed to enriched (stimulus rich) abiotic environments resulted in

Table 1. Experimental studies on brain plasticity investigating the effects of different abiotic and biotic environmental factors. Studies on the effect of abiotic environmental factors are only a representative subset of studies, while all studies (to our knowledge) on the effects of biotic environment are listed.

Environment	Factor	Affected brain region	Species	Ref.
abiotic	enriched environment	brain size	Norway rat, *Rattus norvegicus*	1, 2
		hippocampal neurons	House mouse, *Mus musculus*	3
		neurogenezis	House mouse, *M. musculus*; Norway rat, *R. norvegicus*	3, 4
		cell proliferation in the telencephalon	Coho salmon, *Oncorhyncus kisutch*	5
		size of the cerebellum	Steelhead trout, *O. mykiss*	6
	captive rearing	brain size, size of the optic tectum and telencephalon	Guppy, *Poecilia reticulata*	7, 8
		size of the olfactory bulb and telencephalon	Chinook salmon, *O. tshawytscha*	9
		size of several brain parts, the size of the overall brain	Nine-spined stickleback *Pungitius pungitius*	10
	training	hippocampus	Human, *Homo sapiens*	11
		several brain areas and activities	Human, *H. sapiens*	12

Table 1. continued.

Environment	Factor	Affected brain region	Species	Ref.
biotic	social environment	optic tectum, bulbus olfactorius	Nine-spined stickleback, *P. pungitius*	13
		sensory brain areas	Common frog, *Rana temporaria*	14, 15
		number of new neurons in the dentate gyrus	Prairie vole, *Microtus ochrogaster*	16
		neuronal recruitment	Zebra finch, *Taeniopygia guttata*	17, 18
		size of the brain and the proportion of different brain areas	Desert locust, *Schistocerca gregaria*	19
	predation pressure	olfactory bulb	Nine-spined stickleback, *P. pungitius*	20
		overall brain size	Common frog, *R. temporaria*	21

References: (1) Diamond *et al*. 1966; (2).Rosenzweig and Bennett 1969; (3) Kempermann *et al.* 1997, (4) Nilsson *et al.* 1999; (5) Lema *et al*. 2005; (6) Kihslinger and Nevitt 2006; (7) Burns and Rodd 2008; (8) Burns *et al.* 2008; (9) Kihslinger *et al*. 2006; (10) Gonda *et al.* 2011a; (11) Maguire *et al.* 2000, (12) Draganski and May 2008; (13) Gonda *et al.* 2009a; (14) Gonda *et al.* 2010; (15) Trokovic *et al.* 2010; (16) Fowler *et al.* 2002; (17) Lipkind *et al.* 2002, (18) Adar *et al.* 2008; (19) Ott and Rogers 2010; (20) Gonda *et al.* 2011a; (21) Gonda *et al.* 2010

increased brain size (Diamond *et al.* 1966; Rosenzweig and Bennett 1969), more hippocampal neurons (Kempermann *et al.* 1997) and elevated level of neurogenesis (Kempermann *et al.* 1997; Nilsson *et al.* 1999) compared to those living in stimulus poor environments (Table 1).

Captive rearing has been shown to reduce brain size in guppies (Burns and Rodd 2008; Burns *et al.* 2008), size of the olfactory bulb and telencephalon in the Chinook salmon (Kihslinger *et al.* 2006) and guppies (Burns and Rodd 2008), and the relative size of every main brain part as well as the size of the whole brain in particular habitats in nine-spined sticklebacks (Gonda *et al.* 2011a; Table 1). Kihslinger and Nevitt (2006) showed that adding only a single rock in the rearing tank can increase the size of the cerebellum of salmons at very early life stages, while changes in cell proliferation in the telencephalon (although without changes in the size of the given brain part) can be induced by environmental complexity in juvenile Coho salmon (Lema *et al.* 2005). These later studies are of a special importance, as they may have important implications to fish aquaculture and reintroduction programs (Box 3).

Different biotic environmental factors have also been shown to influence brain development, but the number of studies on this effect is still far lower than those of the abiotic environment – all studies on the effects of biotic environment are listed in Table 1. Furthermore, many commonplace and ecologically important biotic interactions such as social environment, predation risk or competition have rarely been investigated (but see e.g.: Gonda *et al.* 2009a, 2010, 2011b; Trokovic *et al.* 2011). It has been shown that social environments can alter brain development, especially the sensory brain areas, both in the nine-spined stickleback (Gonda *et al.* 2009a) and the common frog (*Rana temporaria;* Gonda *et al.* 2010; Trokovic *et al.* 2011). Individually reared fish developed smaller optic tectum and larger bulbus olfactorius than group reared fish, and in some highly aggressive populations group-rearing resulted in decreased overall brain size (Gonda *et al.* 2009a). The development of the main sensory brain areas were also affected by density in both tadpoles and metamorphosed froglets (Gonda *et al.* 2010; Trokovic *et al.* 2011). Social isolation decreased the number of new neurons in the dentate gyrus of prairie voles (Fowler *et al.* 2002) while social complexity increased neuronal recruitment in birds (Lipkind *et al.* 2002; Adar *et al.* 2008). The change in density between life phases of desert locusts alters the size of the brain and the proportion of different brain areas; solitarious locusts have smaller brains as compared to and gregarious locusts (Ott and Rogers 2010). Perceived predation risk resulted in decreased size of the olfactory bulb in some populations of nine-spined sticklebacks (Gonda *et al.* 2011b) while common frog tadpoles developed smaller brains under predation risk in low density (= high per capita predation risk) than in high

Box 3. Effects of hatchery rearing on brain development, behaviour and fitness in fish

Domestication and captive breeding have been shown to influence the brain size or size of different brain parts in several domesticated taxa (mammals: Kruska 2005; Yamaguchi *et al.* 2009; birds: Guay and Iwaniuk 2008; Rehkämper *et al.* 2008; fish: Kihslinger and Nevitt 2006). One of the best-studied taxa in this respect is hatchery reared fishes, because of the potential economical and conservational importance that hatchery induced changes can have. Hatchery/captive rearing has been shown to result in reduced overall brain size (Kihslinger and Nevitt 2006; Burns *et al.* 2008) as well as reduced size of different brain parts in fish (e.g. Kihslinger *et al.* 2006; Gonda *et al.* 2011a). Different parts of the brain play a role in different stages of predator avoidance; the telencephalon is involved in learning, while sensory brain parts are involved in detecting the predators (e.g. *bulbus olfactorius*: chemical cues; *optic tectum*: visual cues). Hatchery rearing has also been shown to induce non-adaptive behavioural modifications such as decreased predator avoidance (Balaa and Blouin-Demers 2011) and feeding success (Wintzer and Motta 2005). These behavioural changes have been shown to have direct fitness consequences: once released into the wild, hatchery reared fish survive far worse than their native conspecifics (Thorstad *et al.* 2011). This might be because anti-predator behaviours have both innate and learned parts, and both chemical and visual cues are needed for their normal development (Kelley and Magurran 2003).
Fitness of hatchery reared fish can be increased via increasing their behavioural flexibility or foraging skills by environmental enrichment (Strand *et al.* 2010; Moberg *et al.* 2011), while anti-predator avoidance can be improved by lowering the rearing densities (Brockman *et al.* 2010) or enriching the rearing environment by simulated predator attack (Roberts *et al.* 2011). Likewise, brain size / size of different brain parts can be increased by increased environmental complexity (Kihslinger and Nevitt 2006). However, no study to date has established a firm link between predator avoidance behaviours and brain development – albeit the evidence above suggests that such are likely to exist. By first manipulating brain size (and behaviour) and then exposing fish to free ranging predators (e.g. Leinonen *et al.* 2011), it would be possible to test whether plastic responses in brain size inflicted by captive rearing are causally related to reduced fitness in the wild.

density or in the absence of predator (Gonda *et al.* 2010).

Beyond comparative studies and phenotypic plasticity

The above detailed interspecific correlative studies form the cornerstone of our present knowledge about how brain size/architecture evolved, and studies on phenotypic plasticity have highlighted the importance of ontogenetic variation in brain development. However, these pillars together are still far from providing a complete picture about the

processes resulting in the observed brain variation in the wild. The proposed factors that might shape the brain both on evolutionary and ontogenetic scales are well established in most cases (e.g. Dunbar 1998; Shumway 2010), but several critical questions remain unanswered. Are the present environmental factors imposing selective pressures on the brain the same as the ones that originally lead to the present forms? What is the heritability of brain size and how is it influenced by environmental variability? Likewise what is the relative importance of phenotypic plasticity *vs.* local adaptation in explaining variation in brain size and architecture in the wild? In other words, to what extent is the variation we see among wild populations in brain architecture caused by differences in the genetic constitution of the population, rather than environmentally induced plasticity? Can brain plasticity itself be under selection and expressed differently in different populations? Within the genetically based patterns, what is the relative importance of natural selection *vs.* drift in explaining the observed differentiation? Are brain size and architecture differences coded by a small number of genes with major effects, or rather by a large number of genes with small effects? Are there strong genetic correlations between the sizes of different brain parts, i.e. strong constraints on evolution of brain architecture? What are the fitness consequences of individual variation in brain size?

The list could be continued, and it is clear that a number of fundamental evolutionary questions about brain variation simply cannot be answered by interspecific evolutionary or intrapopulation plasticity studies. To fill the gap between the two, and to answer most of the questions listed above, population comparisons within a single species – coupled with studies of within population variation – are needed. In other words, evolutionary studies should be scaled down to the inter- or even intrapopulation level, while plasticity studies need to be scaled up to the interpopulation level to provide answers to questions posed.

First missing pillar: microevolutionary studies based on population comparisons

Macroevolutionary brain studies rely on the assumption that variation between species is much higher than variation within species. Even though extensive within species brain size variation has been reported by several studies (e.g. Kolm *et al.* 2009; Moller 2010) variation between species is indeed likely to be larger than that within species in most cases (Garamszegi and Eens 2004a; Garamszegi *et al.* 2005). However, the intraspecific variation in brain size and architecture is still very informative and important for our understanding of evolutionary processes. Contrary to studies on the species level, evolutionary studies on brain size at intraspecific level have only recently started to receive the attention of evolutionary biologists (Fig. 1; Table 2; e.g. Roth and Pravosudov 2009; Kolm *et al.* 2009; Gonda *et al.* 2009b, 2011a; Crispo and Chapman 2010). As

with all new research areas, the first studies are explorative and are paving the road for more in depth studies to come. In the case of evolutionary studies of brain size at the intraspecific level, early studies have used rather rough brain size measurements (e.g. Burns and Rodd 2008; Møller 2010). Although these proxies of brain size are believed to be good estimates of intelligence and cognitive ability (see above), more refined techniques (see 'Future') can improve the resolution and provide more fine-tuned analyses of specific hypothesises to be tested. Perhaps more importantly, as compared to interspecific studies, intraspecific studies provide numerous conceptual advantages in testing hypotheses about the evolution of brain size and architecture.

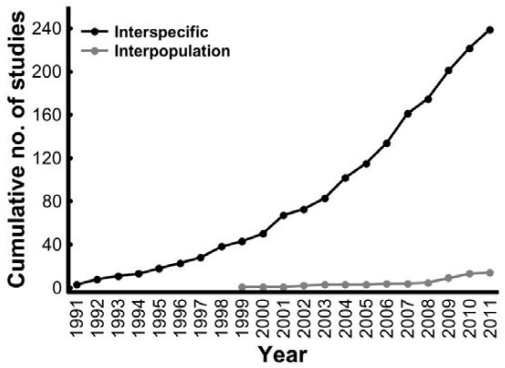

Figure 1. Cumulative number of evolutionary studies focussing on variation in brain size and architecture by comparing species or higher taxa ('Interspecific') *vs.* comparing populations of a single species ('Interpopulation'). Data are based on a literature search in ISI Web of Science, using the search terms: "brain size" and "evolution". Note that studies for 2011 depict the situation as of July.

Firstly, comparisons of brain size and architecture differences among populations of the same species inhabiting different selective environments could provide explicit means to differentiate between various microevolutionary processes, such as natural selection and genetic drift (e.g. Merilä and Crnokrak, 2001), as causes of observed differentiation. By comparing the levels of population differentiation in quantitative phenotypic traits (Q_{ST}) with the degree of differentiation in neutral genetic markers (F_{ST}), one can probe the causes of differentiation (e.g. Leinonen *et al.* 2008). If $Q_{ST} > F_{ST}$, the patterns/differences in the given phenotypic trait among population inhabiting different habitats are likely to reflect local adaptation (i.e. evolutionary divergence). If $Q_{ST} = F_{ST}$, this indicates that the observed differences do not exceed what would be expected due to genetic drift alone. On the other hand, if the $Q_{ST} < F_{ST}$, the examined populations have diverged less than expected by drift alone, and the populations are likely to be under similar selective pressures (Merilä and Crnokrak 2001). Thus far, this approach has not been applied in any study of brain evolution, and hence, formal tests of adaptive differentiation are as yet lacking.

Apart from the Q_{ST}-F_{ST} comparisons, there is another way to test for links between the phenotypic expression of a trait and selective forces shaping the phenotypic appearance of that trait: selection experiments. Selection experiments have been frequently employed to study the functional significance of phenotypic variation of different traits (e.g. Reznick and Ghalambor 2005; Leinonen *et al.* 2011). However, no

Table 2. Synopsis of evolutionary studies of brain variability based on interpopulation comparisons. "Proposed correlates" identifies the factor that might have contributed to the observed divergence in the brain, "Sampling" tells whether the studies were done on wild-caught animals (W) or on animals reared in controlled laboratory environment (common garden, CG).

Taxon	Trait	Proposed correlates	Method	Sample	Ref.
Human, *Homo sapiens*	brain size	intelligence quotient	magnetic resonance imaging	W	e.g. 1
Marsh wrens *Cistothorus palustris*	song control nuclei	song learning, repertoire size	histology	W	2
White-crowned sparrow, *Zonotrichia leucophrys*	hippocampus size and neuron numbe	migratory behaviour	histology	W	3
Black-capped chickadee, *Poecile atricapillus*	hippocampus size and neuron number	latitude, temperature, snow cover, day length	histology	W	4-6
Dwarf Victoria mouthbreeder, *Pseudocrenilabrus multicolor victoriae*	brain mass, plasticity	oxygen level of water, dispersal potential	weighing	CG	7, 8
Brown trout, *Salmo trutta*	brain size and architecture	mating strategy, sex	volume calculation on photos	W	9
Three-spined stickleback, *Gasterosteus aculeatus*	brain size and architecture	foraging strategy (limnethic, benthic), sex	shape analysis on photos	W	10
Nine-spined stickleback, *Pungitius pungitius*	brain size and architecture	predation, environmental complexity	volume calculation on photos	W&CG	11, 12
Honey bee, *Apis mellifera*	total brain and mushroom body size	learning performance	histology	W	13
Small white, *Pieris rapae*	total brain and mushroom body size	learning	histology	CG	14

References: (1) Ruhston and Ankey 1996; (2) Canady *et al.* 1984; (3) Pravosudov *et al.* 2006; (4) Pravosudov and Clayton 2002; (5) Roth and Pravosudov 2009; (6) Roth *et al.* 2011; (7) Crispo and Chapman 2010; (8) Chapman *et al.* 2008; (9) Kolm *et al.* 2009; (10) Park and Bell 2010; (11) Gonda *et al.* 2011a; (12) Gonda *et al.* 2009b; (13) Gronenberg and Couvillon 2010); (14) Snell-Rodd *et al.* 2009

study has as yet used this kind of experimental approach to verify the actual impact of a particular brain phenotype on individual performance or fitness. For example, one obvious context where such experiments could be revealing would be to study the possible negative influence of hatchery induced changes in brain size on the ability of fish to avoid predation by free ranging predators (Box 3). There is another reason why intraspecific comparative studies can be more informative and provide us with more detailed answers about the evolutionary forces behind brain size evolution than the otherwise undeniably important interspecific comparative studies. This resides in the fact that most populations are likely to be found in the selective environment that actually shaped their brains, while this is less likely to be the case in species comparisons: adaptive divergence after splitting from a common ancestor might have broken the link between a given brain phenotype and the selection pressure under which it evolved. Hence, population comparisons can help us to identify the most important environmental factors selecting for size and structural changes in the brain, and by studying recently established populations / recent radiations, natural selection acting on the brain can be 'caught in action'.

Based on interpopulation comparisons, environmental variables that might have contributed to the reported brain size/architecture divergence, as well as to correlated life history and/or behavioural traits, have been identified (Table 2). For example, in food hoarding animals, good memory (and hence the associated neural basis) is essential for survival, especially under harsh environmental conditions. Indeed, environmental harshness correlates with the size and neuron number of hippocampus in the black-capped chickadee (*Poecile atricapillus*; Pravosudov and Clayton 2002; Roth and Pravosudov 2009), even when one of the environmental factors of harshness (the day length) was controlled for (Roth *et al.* 2011). In two other studies, a difference in the predatory regime was the main proposed factor behind brain architecture divergence in nine-spined sticklebacks (Gonda *et al.* 2009b, 2011a). Brain comparisons between populations and the main findings of those studies are summarized in Table 2.

Brain evolutionary studies that were based on comparisons of individuals of the same population, or several populations but neglect population origin, might be of less direct importance in the context of local adaptation. However, such studies (e.g. MacDoughall-Shackleton *et al.* 1998; Møller 2010; Wilson and MacLaughin 2010) have identified interesting behavioural and life history traits which might be worth investigating on the interpopulation level. For example, the correlation between size of song control centres in the brain and song repertoire in songbirds has received much attention (e.g. Ward *et al.* 1998; Airey and DeVoogd 2000; Garamszegi and Eens 2004b), and sometimes yielded conflicting results (for review see Garamszegi and Eens 2004b). However, Canady *et al.* (1984),

studying marsh wrens (*Cistotohorus palustris*) both in nature and in the lab, were among the first to show among population variation in song brain centres. Also fish with different foraging behaviours differ in their brain architecture: actively foraging brook chars (*Salvelinus fontinalis*) have larger telencephalons than their less active conspecifics (Wilson and McLaughlin 2010). Different proxies of brain size (brain mass and head size) in the barn swallow (*Hirundo rustica*) were also shown to be in positive correlation with several factors, including migratory behaviour, offspring defence, recapture probability (i.e. learning), sex and social environment (Møller 2010).

Some quantitative genetic work has already been done to study the heritability of brain size and architecture mainly in humans and primates. Differences in gross brain morphology were found to be heritable ($h^2 \approx 0.66 – 0.97$) on the basis of analyses utilizing known pedigrees or exploiting the possibilities in human twins (e.g. Peper *et al.* 2007; Hulshoff Pol *et al.* 2006). Likewise, heritabilities of brain size, cerebral volume and grey matter volume in baboons, *Papio hamadryas*, were found to be high ($h^2 \approx 0.67 – 0.86$; Rogers *et al.* 2007). Similar results have been found in zebra finches (*Taeniopygia guttata*), where brain weight and telencephalon volume were also highly heritable ($h^2 \approx 0.49 – 0.63$), and size of some song control nuclei had lower but still significant heritabilities ($h^2 \approx 0.03 – 0.16$) based on the application of 'animal model' analyses on full-sib families (Airey *et al.* 2000). These studies are promising, as they indicate high evolvability of different brain traits in distant taxa. At the same time, they raise interesting questions from the evolutionary point of view: if the variation in the brain size and size of different brain parts has important consequences on fitness, how are we to explain these high heritabilities? Namely, traits with close association to fitness are expected to have low heritabilites (Mousseau and Roff 1987; Merilä and Sheldon 1999). Given the functional importance and the energetic constraints of maintaining brain tissue, it is intriguing that the heritabilites of brain size traits appear be this high.

We see many possibilities in quantitative genetic studies of brain size variation, especially in species where large-scale breeding experiments are possible. As compared to studies of primates and humans, in which experimental work is difficult and logistically constrained, organisms with shorter generation times – such as small-sized fish and possibly some amphibians – might provide promising models for quantitative genetic work. However, whichever species one chooses to utilize, one of the limiting factors in studies of brain variability resides in obtaining high resolution data on brain size variation. Hence, as Houle *et al.* (2010) recently pointed out, high-throughput phenotyping methods need to be developed to meet the demand of measuring hundreds (preferably thousands) of brains.

Taken together, intraspecific studies on brain variation have started to accumulate (Fig. 1). These studies suggest that there is a great deal of

variation in brain phenotypes both among and within populations, as well as covariation between brain phenotypes and environmental (and behavioural or life history traits) variables within a single species. Furthermore, the quantitative genetic studies thus far indicate high heritability of brain size and the size of different brain parts, which together with the functional – and therefore also evolutionary – significance of brain variation suggest ample opportunity for local adaptation in brain traits. However, the evidence for local adaptation in brain size and architecture from the wild is still scant. While some of the studies have utilized common garden approaches, most of the studies have relied on wild-caught animals and the genetic – and hence – adaptive basis of the observed differentiation remains questionable (e.g. Gonda *et al.* 2011a).

Second missing pillar: brain plasticity from an evolutionary perspective

As highlighted above, phenotypic plasticity in brain size has been demonstrated several times. However, it is still debated if phenotypic plasticity itself is an evolvable trait or just the first step toward adaptation (West-Eberhard 2003; DeWitt and Scheiner 2004; de Jong 2005; Pigluicci *et al.* 2006; Pfennig *et al.* 2010). Work done on brain plasticity so far is not placed to challenge any of these views. Contrary to the large amount of brain plasticity studies done at the within population level, we are aware of only three studies investigating the evolution of brain plasticity. Nine-spined sticklebacks showed habitat-dependent population divergence in brain plasticity induced by sociality (Gonda *et al.* 2009a): pond sticklebacks (which are the only fish species in the ecosystem) developed relatively smaller brains in groups than in isolation, while marine sticklebacks (which are members of a diverse fish fauna with numerous predators) showed an opposite trend. It was suggested that under heavy piscine predation, marine sticklebacks developed some mechanisms that eliminate the social stress stemming from aggressive encounters. Further, another study showed that nine-spined sticklebacks from pond environment increased the size of their bulbi olfactorii in the presence of predation pressure while this brain part remained the same in marine fish, however marine fish in general developed larger brain than pond fish (Gonda *et al.* 2011b). The results suggest that predation pressure increase the size of the olfactory brain centre both on evolutionary and ontogenetic scale. A third study showed that African cichlids (*Pseudocrenilabrus multicolor victoriae*) with higher dispersal potential have more plastic (and also smaller) brains than their conspecifics without high dispersal potential (Crispo and Chapman 2010). Finally, though not directly addressing the question of population variation in brain plasticity, it has been found that the effect of captive-rearing can be habitat-specific in nine-spined sticklebacks, where pond fish developed smaller brains in captivity than in the wild, while marine fish

developed similar brains both in the wild and in the lab (Gonda *et al.* 2011a).

Based on the above studies, we can expect that environmentally induced phenotypic plasticity in the brain can show habitat-dependent population variation under common garden settings. Patterns emerging from common garden experiments are likely to have a genetic basis, while the habitat-dependence suggests that natural selection is the driving force. However, more studies addressing geographic variation in brain plasticity, and possible population differences in the degree of plasticity, are needed to form a better view of evolutionary potential of brain plasticity itself.

Future

We have provided an overview of the published studies on intraspecific variation in brain size and architecture, and shown that there is a considerable evolutionary potential for brain divergence within species. This within species variation provides possibilities to address evolutionary questions about brain size divergence that could not be tested with interspecific evolutionary comparative studies, or with intrapopulational plasticity studies. Unfortunately, the relatively low number of intraspecific evolutionary studies suffers from similar problems as the interspecific ones: most of them are correlative and the results are sometimes conflicting. However, considering that studying intraspecific brain size variation is an emerging field (Fig. 1), one should perhaps focus on the future possibilities rather than on the shortcomings of present and past work. By focusing on brain evolution within species, it is possible to improve our understanding of the mechanisms behind brain evolution, as both the key ingredients of the evolutionary process – inheritance and selection – can be quantified and studied in detail. In fact, the array of possibilities is bewildering, but here we aim to point out a two main lines of research that could lead to significant immediate progress.

The first major advance would come from applications of quantitative genetic tools on brain size variation. It is now already clear that for drawing solid evolutionary inference, data should be collected from common garden material to avoid the confusion between genetically based differences and phenotypic plasticity (Gonda *et al.* 2011a). Most of the brain evolutionary studies, both on inter- and intraspecific levels, have been based on wild caught animals of perhaps different age and/or life stages, with an implicit assumption that brain size is constant during the life of an individual. However brain size and architecture can change seasonally, during the life of an individual or can be altered by changing environmental conditions (Pyter *et al.* 2005; Macrini *et al.* 2007). Environmentally induced phenotypic plasticity can often obscure the genetically based differences of a trait and might lead to false conclusions of studies based on purely wild caught samples (e.g. Merilä 2010; Alho *et al.* 2010) – an effect already demonstrated in brain variation (Gonda *et al.* 2011a).

Furthermore, ontogenetic changes (e.g. Wagner 2003; Lisney *et al.* 2007; Macrini *et al.* 2007) as well as seasonal plasticity of the brain (Nottebohm 1981; Tramontin and Brenowitz 2000; Hofman and Swaab 1992, 2002; Pyter *et al.* 2005) can also be controlled in common garden conditions. Common garden studies, however, also offer other advantages than just ruling plasticity out. With adequate breeding designs (see e.g. Falconer and Mackay 1995; Lynch and Walsh 1998) the different quantitative genetic components (additive genetic, maternal, environmental effects, dominance, etc.) of phenotypic variation could be disentangled both within and among populations. Further, by measuring different brain traits on the same individuals, the genetic correlations between traits could be estimated, and the competing constraint *vs.* independent brain evolution hypotheses (Finlay and Darlington; 1995; Barton and Harvey 2000) could be directly tested. Construction of the genetic variance-covariance matrix (**G** matrix: Lande 1979) would allow estimation of the lines of least resistance (cf. Schluter 1996) and thus aid in our understanding of the constraints of brain evolution. Combining estimates of heritabilities, genetic correlations and the **G** matrix with estimates of natural or sexual selection on different brain phenotypes would make a detailed reconstruction of the evolutionary process possible. Further, proper common garden material from several populations would allow us to estimate the actual quantitative genetic variation within and among populations, which, together with similar estimates of the neutral genetic variation would provide a direct test of the roles of natural selection *vs.* genetic drift behind genetically based population divergence (Merilä and Crnokrak, 2001; Leinonen *et al.* 2008). Finally, and ultimately, with the current genomics tools, approaches such as genome scans (Schlötterer 2003; Storz 2005; Vasemägi and Primmer 2005) or quantitative trait locus (QTL) mapping (Weller 2001; Erickson *et al.* 2004; Slate 2005), can be used to identify the genomic regions containing the genes coding for brain variation.

The second line of quick advances might result from applying the well-established, simple and sophisticated methodology from neurobiology to the above described evolutionary framework. As the brain is the most expensive tissue from the energetic point-of-view (Aiello and Wheeler 1995), any increase in its size should be more beneficial than the cost of developing and maintaining it (e.g. Safi and Dechmann 2005). However, given the many functions brain serve, linking variation in brain size to variation in any other (e.g. behavioural) traits can be difficult (Healey and Rowe 2007). Further, even though the different brain parts might evolve in concert and not be entirely independent (Finlay and Darlington 1995), not all changes in all brain parts might be detectable by measuring overall brain size. Studying the size of different brain parts might bring us closer to identifying functional relationships between the given neural structures and the factors

that are important in their evolution. However, the functions of the main brain parts are very diverse (e.g. Kotrschal *et al.* 1998; Striedter 2005). Hence, using the volume of a part of the brain and correlating it with some e.g. behavioural trait, such as the hippocampus with food hoarding, can still be just a "proxy for more relevant and subtle changes in the structure of the brain underlying changes in behaviour" (Roth *et al.* 2010). Methods from neurobiology are available from basic histological methods to cutting-edge molecular tools. Basic methods include different staining methods (e.g. Nissl staining; Nissl 1898) that allow one to calculate the volume of more specific brain regions within brain parts with functions defined, or calculate neuronal densities. Further a by the help of a newly developed method one can count neurons and other cell types in the brain (Herculano-Houzel and Lent 2005) that provide us with a powerful tool as the number of neurons might reflect the importance of a given brain structure more than it's pure size. The more advanced methods consist of, for example, parallel application of different neuro-histochemical methods to visualise specific cells or components of the neurons in the brain such as anti-body labelling, enzyme histochemistry or immunofluorescence methods (Sallinen *et al.* 2009). These later methods/techniques have already resulted in valuable application of easily available model systems (e.g. zebrafish, *Danio rerio*) to study very complex and important problems such as neurodegenartive human diseases (Panula *et al.* 2010; Xi *et al.* 2011). Such truly interdisciplinary approaches (note that the tools and knowledge are readily available for both quantitative genetics and neurobiology) would bring the understanding of both the processes and detailed function of brain evolution into reach.

Conclusion

The enormous brain size and architecture variation observed in nature has attracted a lot of attention in different fields of biology, including evolutionary biology. Thus far, the two main pillars of our understanding on brain variation have been macroevolutionary comparative studies of species or higher taxa and plasticity studies within populations. Interpopulation comparisons of brain size and architecture, as well as brain plasticity represent a more recent and still developing line of research in evolutionary neurobiology. This new line of research brings studies on brain size and architecture closer to mainstream evolutionary biology research where the study of spatial or geographic variation has been one of the fundaments of evolutionary investigations. By tapping into the approaches and methods from this well established field of research, we envision that intraspecific studies in evolution can soon flourish and help us towards better understanding of the evolution and functional significance of variation in brain size and architecture.

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