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THE GENETICS OF BEHAVIOUR AND OTHER ADAPTIVE TRAITS IN NINE-SPINED STICKLEBACKS (*PUNGITIUS PUNGITIUS*)

by

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Alive without breath,
As cold as death;
Never thirsty, ever drinking,
All in mail never clinking.

— *J.R.R. Tolkien, The Hobbit*

ABSTRACT

One of the main goals in current evolutionary biology research is to identify genes behind adaptive phenotypic variations. The advances in genomic technologies have made it possible to identify genetic loci behind these variations, also concerning non-model species. This thesis investigates the genetics of the behaviour and other adaptive traits of the nine-spined stickleback (*Pungitius pungitius*) through the application of different genetic approaches. Fennoscandian nine-spined stickleback populations express large phenotypical differences especially in behaviour, life –history traits and morphology. However the underlying genetic bases for these phenotypical differences have not been studied in detail. The results of the project will lay the foundation for further genetics studies and provide valuable information for our understanding of the genetics of the adaptive divergence of the nine-spined stickleback.

A candidate gene approach was used to develop microsatellite markers situating close to candidate genes for behaviour in the nine-spined stickleback. Altogether 13 markers were developed and these markers were used in the subsequent studies with the anonymous random markers and physiologically important gene markers which are already currently available for nine-spined sticklebacks.

It was shown that heterozygosity correlated with behaviour in one of the marine nine-spined stickleback populations but with contrasting effects: correlations with behaviour were negative when using physiological gene markers and positive with random markers. No correlation was found between behavioural markers and behaviour. From the physiological gene markers, a strong correlation was found between osmoregulation-related gene markers and behaviour. These results indicate that both local (physiological) and general (random) effects are important in the shaping of behaviour and that heterozygosity– behaviour correlations are population dependent.

In this thesis a second linkage map for nine-spined sticklebacks was constructed. Compared to the earlier nine-spined stickleback linkage map, genomic rearrangements were observed between autosomal (LG7) and sex-determing (LG12) linkage groups. This newly constructed map was used in QTL mapping studies in order to locate genomic regions associated with pelvic structures, behaviour and body size/growth. One major QTL was found for pelvic structures and *Pitx1* gene was related to these traits as was predicted from three-spined stickleback studies, but this was in contrast to earlier nine-spined stickleback study. The QTL studies also revealed that behaviour and body size/growth were genetically more complex by having more QTL than pelvic traits. However, in many cases, pelvic structure, body size/growth and behaviour were linked to similar map locations indicating possible pleiotropic effects of genes locating in these QTL regions. Many of the gene related markers resided in the QTL area. In the future, studying these possible candidate genes in depth might reveal the underlying mechanism behind the measured traits.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscripts which are referred to in the text by their Roman numerals:

I Veronika N. Laine, Craig R. Primmer, Gábor Herczeg, Juha Merilä, Takahito Shikano (2012) Isolation and characterization of 13 new nine-spined stickleback, *Pungitius pungitius*, microsatellites located nearby candidate genes for behavioural variation. *Annales Zoologici Fennici* 49:123–128.

II Veronika N. Laine, Gábor Herczeg, Takahito Shikano, Craig R. Primmer (2012) Heterozygosity–behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. *Molecular Ecology* 21:4872–4884.

III Takahito Shikano, Veronika N. Laine, Gábor Herczeg, Johanna Vilkki, Juha Merilä (2013) Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. *Genes, Genomes, Genetics* DOI: 10.1534/g3.113.007237.

IV Veronika N. Laine, Gábor Herczeg, Takahito Shikano, Johanna Vilkki, Juha Merilä: QTL analysis of behavior in nine-spined sticklebacks (*Pungitius pungitius*). *Submitted manuscript.*

V Veronika N. Laine, Takahito Shikano, Gábor Herczeg, Johanna Vilkki, Juha Merilä: Quantitative trait loci for growth and body size in the nine-spined stickleback *Pungitius pungitius* L. *Accepted in Molecular Ecology.*

1. INTRODUCTION

1.1 Adaptive phenotypic variation

Environmental variation causes organisms to adapt differently (Endler 1977). This process leads to discernible phenotypic variation between same species populations and contemporary evolutionary research aims at identifying those differences and understanding its genetic background (Orr 2005; Stapley et al. 2010). Aiding to this, the rapid advances in genomic technologies have made it possible to identify genetic loci facilitating these variations beyond the typical model organisms (Stapley et al. 2010). In addition to genetic factors, biotic factors derived from conspecifics or other species, such as competition and predation, are known to affect population-level variation e.g. in behaviour (Herczeg and Välimäki 2011) and a genetic basis eluding to this phenomenon has been suggested (e.g. Magurran 1990; Shaw et al. 2007; Brown et al. 2007). Furthermore variation in predation pressure can also induce differences in morphological defence systems and life history traits (Roff 1992; Tollrian and Harwell 1999). Hence, it is of utmost importance to investigate many aspects of phenotypically adaptive traits when aiming for the comprehension of the underlying genetic mechanisms.

1.2 Genetics of behaviour

Disentangling the underlying biological mechanisms in different behaviours can help us understand the developmental history, genetic composition, characteristics of the nervous system, physiological state, environmental factors and biochemical reactions an individual has and experiences during its lifetime (Figure 1). (Anholt and Mackay 2009; Krebs and Davies 1997).

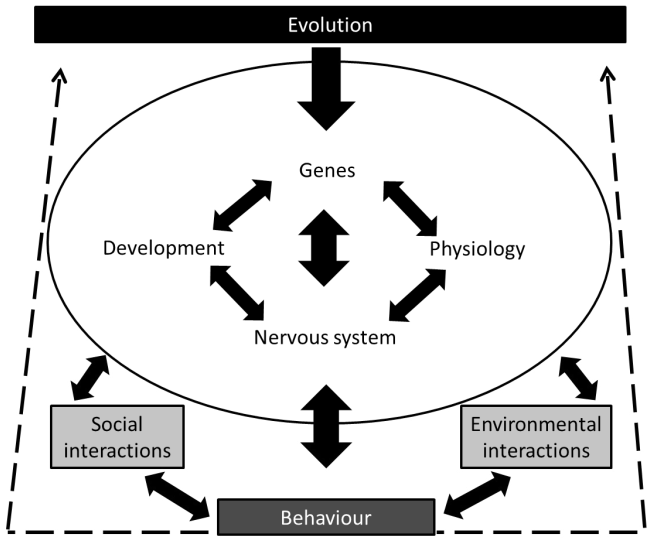


Figure 1. A representation of how behaviour is connected to different aspects of biology (from Anholt & Mackay 2009)

Experimental behavioural studies were first conducted by experimental psychologists Pavlov, Thorndike and Skinner and also by the early behavioural ethologists Lorenz, von Frisch and Tinbergen, all together building the foundations of behavioural research (Anholt and Mackay 2009). The advances in technology and methodological approach have in modern times led to a more comprehensive understanding of behaviour; incorporating insights from various fields of research such as neurobiology, physiology and genetics. The underlying genetic basis provides the foundation for the nervous system to express behaviours between organisms and their social and physical environments. The rapid speed at which genetics developed over the last decades has in particular helped to gain a more profound knowledge of the underlying mechanisms by means of utilizing quantitative genetics.

Genes that affect behaviour can have two kinds of effects. First, some genes are influencing the manifestation of the behaviour i.e. the gene is affecting a trait in which there is little or no variation in the population and the trait is not environmentally determined. This can be studied by mutagenesis approaches as it has been done for example with mice (Belknap et al. 2001). Second, a particular set of genes are contributing to the behavioural variation. With quantitative trait loci (QTL) mapping approach it is possible to identify and estimate the contributions of these genes to the observed phenotypic variance (Boake et al. 2002). The effects can be monitored by creating mutations and this is possible especially in the model organisms. However, it is often only possible to rely on the mutations that have occurred naturally and are manifesting in populations where variations of behaviours are occurring.

One gene can be responsible for many distinct and unrelated phenotypic effects in the phenomenon called pleiotropy (Stearns 2010). Generally there are no genes solely for behaviour and genes termed as "behavioural genes" are not directly controlling behaviour but these genes have instead been influencing the development and function of behaviour by affecting the development of the nervous system related to adult behaviour (Sokolowski 2001). In addition, these genes can affect both behaviour and physiology. Pleiotropic effects have for example been studied in the fruitfly (*Drosophila melanogaster*) e.g. examining genes affecting both behaviour and development (reviewed by Sokolowski 2001 and Anholt and Mackay 2004) and in honeybees (*Apis mellifera*) in the context of reproduction and social behaviour (reviewed by Page et al. 2012). Furthermore a rather surprising example of pleiotropic effects between physiology and behaviour comes from the melanocortin system studies. It has been shown that there is a widespread relationship between melanin-based coloration and other phenotypic traits in vertebrates, e.g. darker wild vertebrates are more aggressive, sexually active and resistant to stress than lighter individuals (reviewed by Ducrest et al. 2008). Thus genetic studies should not only concentrate on the most popular genes studied in behavioural genetics but widen the search to other systems as well.

1.3 Behaviour and fitness

Behavioural traits are known to have important fitness consequences. For example, behavioural type can affect survival, breeding success and offspring quality, among other parameters (Réale et al. 2007). As mentioned, genes can affect the trait's manifestation or the variation but, in addition to measuring these effects, the level of genetic variability can also be of importance. In heterozygosity-fitness correlations the thought is that in some circumstances, high heterozygosity is expected to convey a selective advantage to an individual but there are also cases where high heterozygosity resulted in lower fitness (discussed in: Coltman and Slate 2003; Chapman et al. 2009; Szulkin and David 2011), as have cases of balanced polymorphism (Aeschlimann et al. 2003; Reusch et al. 2001). As an example, in salmonids, individual heterozygosity has been shown to be positively correlated with aggression, competitive performance, dominance rank and predator avoidance (Blanchet et al. 2009; Tiira et al. 2006; Tiira et al. 2003; Vilhunen et al. 2008). However, the fitness consequences of behavioural types can be context-dependent. Aggression can be advantageous in competitive situations, when there is a need to assert dominance, challenge a higher-ranking individual or defend a claim for scarce resources, such as territories. In contrast, high aggressiveness and boldness, for example in predator avoidance situations, can be costly (Anholt and Mackay 2012).

1.4 Factors affecting behavioural studies

Recent evolutionary behavioural ecology is going through a paradigm change regarding the relevance of individual differences in behaviour. In short, individual behavioural differences assessed in the same context and situation are no more seen as measurement errors or as random noise, but rather as a highly relevant biological phenomenon (e.g. Dingemanse et al. 2010). Such behavioural consistency can be studied at several levels (Garamszegi and Herczeg 2012; Herczeg and Garamszegi 2012), one being the consistent individual differences in multiple behaviours, often called behavioural syndromes, where e.g. the more aggressive individuals are also often bolder (Garamszegi and Herczeg 2012; Gosling 2001; Sih et al. 2004b; Sih et al. 2004a). Hence, when several functionally independent behaviours are considered (e.g. risk-taking, aggression and activity), the common component (i.e. behavioural type; Bell 2007) of the behaviours should also be considered besides the single behaviours. Behavioural syndrome studies also suggest that there may be important ecological and evolutionary consequences of such behaviour in the form of e.g. trade-offs between activity and risk of getting eaten by the predator (Réale et al. 2007; Sih et al. 2004a).

In addition to genetic factors, environmental effects have a substantial contribution to phenotypic variation. Humidity, temperature, diet, light/dark cycle in cooperation with age and sex of the individual and also its social environment can affect how an individual behaves in these different situations (Krebs and Davies 1997). Also environmental factors such as environmental toxicants can affect the physiology of an individual and thus, indirectly, the expressed behaviour. For example, exposure to synthetic estrogen was shown to reduce aggressive behaviour in male three-spined sticklebacks (*Gasterosteus aculeatus*), a territorial and rather aggressive fish (Bell 2001). Since courtship and aggression are both important determinants of the lifetime reproductive success of male fish, sublethal effects on the behaviour of individual animals may influence the dynamics of fish populations in aquatic environments where steroidogenic compounds occur. However the effect of toxicants on the behaviour of the fish could also vary due to their differences in genotype. E.g. in the zebrafish (*Danio rerio*) study by Colman *et al.* (2009), the authors detected differences in the response to the growing dose of synthetic estrogen. Hence, it is essential to identify, control or exclude different aspects that can affect the measured behaviour to reveal true genetic differences.

Assessing or controlling the genetic background of the tested individuals is also important to eliminate or reduce genetically induced variation in the expression of behaviours (Anholt and Mackay 2009). In laboratory studies it is possible to control the environmental conditions and also the genetic background due to controlled breeding. However, breeding in laboratory is available only to a limited number of species. By using naturally occurring genetically diverged populations, it is possible to identify

DNA changes affecting phenotypic differences also in other species. For example, genetically diverged populations have previously been successfully used in three-spined sticklebacks to identify genes causing morphological changes between divergent populations (Colosimo et al. 2004; Shapiro et al. 2004). Another suitable way to study genetics of behaviour is to compare domesticated and wild animals, since domestication alters both the behaviour and the genetics of the individual. There are good examples especially from the fish studies (e.g. Lucas et al. 2004; Wright et al. 2006a,b).

In addition to behavioural trials done in laboratory, trials can be conducted in the field under ecologically relevant conditions to understand the significance of patterns. Furthermore, some behaviours are not manifesting in laboratory conditions. Thus, behavioural studies combining both laboratory and field studies might help to make the implications broader and more widely applicable (Adriaenssens and Johnsson 2010).

1.5 Model vs. wild species

In this thesis I regard wild species as which the individuals used in research are descended from recently sampled individuals of a non-domesticated species. In contrast a model species has been reared in the laboratory for many generations. Most behavioural genetic studies have been conducted on model organisms such as mice, *Drosophila* and more recently also domesticated animals have been included (Fitzpatrick et al. 2005; Inoue-Murayama 2009). New models have also been emerging especially for behavioural studies, such as the honeybee, providing valuable insights into social behaviour (Fitzpatrick et al. 2005). Despite the potential ecological and evolutionary significance in wild populations and the fact that more variation in behaviour is seen in the natural setting, the underlying genetic basis of behaviours has rarely been studied in free-living populations. However, there are various tools available currently to change the situation. For instance, ‘candidate gene approach’ can easily be used in the wild populations (see Materials and methods) (Fidler et al. 2007; Fitzpatrick et al. 2005). Furthermore, the most recent developments in genomic techniques enable new ‘whole-genome’ approaches to be undertaken in almost any species, thus removing one of the main limitations of conducting such studies in non-model organisms, i.e. the lack of genome sequence information.

2. AIMS OF THE THESIS

The aim of my PhD was to investigate the genetics of nine-spined stickleback (*Pungitius pungitius*) behaviour and other adaptive traits through the application of different genetic approaches (Figure 2). The overarching goal is to find genetic factors that are affecting the behaviour, life –history traits and morphology of the nine-spined stickleback as the underlying genetic bases for these phenotypical differences in Fennoscandian nine-spined stickleback populations have not been studied in detail. This study is divided into two sections. First I focused on the generation of candidate gene markers for nine-spined stickleback behaviour and on utilizing them and other available markers in a heterozygosity-behaviour correlation study (chapters **I- II**). In the second phase I applied linkage and quantitative trait loci mapping to obtain a deeper understanding of the association of these markers with behaviour and morphology (chapters **III-V**). The results of the project will lay the foundation for further behaviour genetics studies and provide valuable information for our understanding of the genetics of nine-spined stickleback adaptive divergence.

2.1 The nine-spined stickleback as a model species

Fish are well suited for complex behavioural studies as they share a diverse range of behavioural types and adaptations. In addition, vertebrates such as fish may prove useful in genotype–behaviour phenotype association studies, as 'cultural' determinants of behaviour are not expected to be as strong as among humans and it is possible to conduct controlled experiments and behavioural trials. The nine-spined sticklebacks live in a wide range of habitats and local differences in aggressiveness and boldness have been observed. As an example, individuals from small ponds (lacking piscine predators) are bolder and more aggressive than individuals originating from marine environments (Herczeg et al. 2009a). More precisely, behavioural plasticity has been tested in these populations by using both chemical cues of predators and food manipulation (Herczeg and Välimäki 2011). The results showed that evolutionary history, ontogenetic experience and also the sex of the individuals affected the behaviour in laboratory reared pond and marine nine-spined sticklebacks. Furthermore, there also exists evidence for habitat-dependent expression of adaptive phenotypic plasticity as pond fish were more risk-taking than marine fish, but only when they had developed under food shortage.

In addition to behavioural differences, there are also genetic and morphological differences between different nine-spined stickleback populations in Fennoscandia (Herczeg et al. 2010; Shikano et al. 2010c). There is a large variance in genetic variability when comparing pond, lake and marine populations. Because of their small population

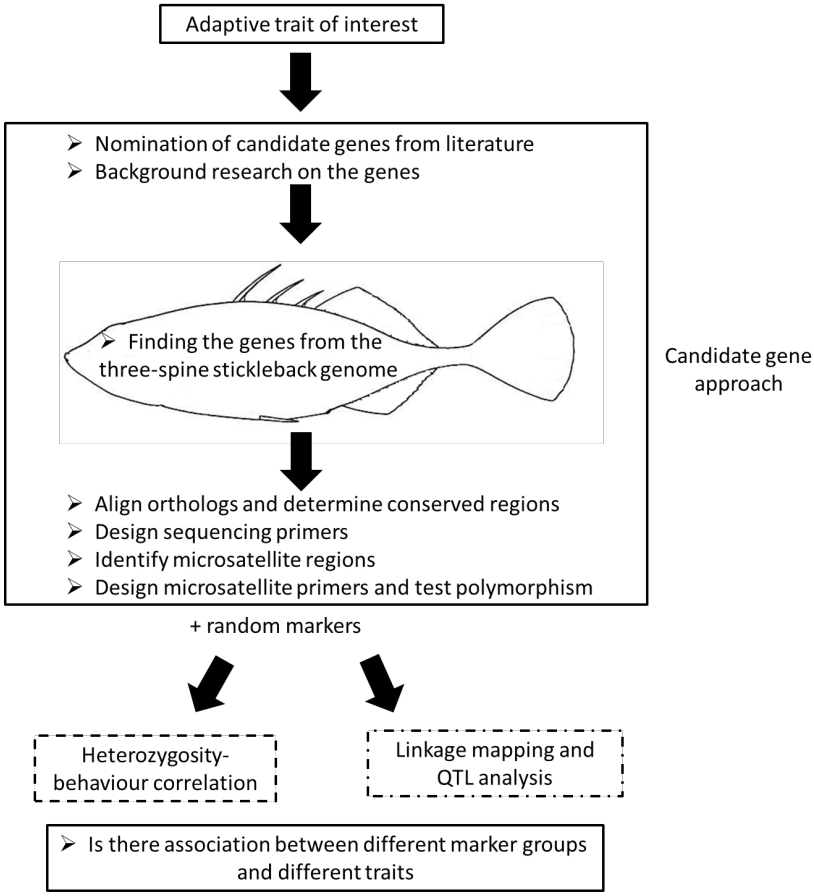


Figure 2. Thesis outline

size and the unpredictable nature of the habitat they live in, the pond populations are more susceptible to inbreeding than marine and lake populations (Shikano et al. 2010c). Furthermore, it has been observed that pond-living fish grow larger and in this case both predation and competition seem to have significant impact on the size differences between different habitat types (Herczeg et al. 2009b; Välimäki and Herczeg 2012). Larger sizes in ponds are the result of divergent growth strategies: while marine fish grow to a small size quickly, pond fish reach giant sizes slowly, even at the cost of delayed maturation (Aikio et al. 2013; Herczeg et al. 2012). To protect themselves from predators, nine-spined sticklebacks have body armour and 8-12 dorsal spines and two pelvic spines (Bănărescu and Paepke 2001). Recent studies have further described differences in armour between pond and marine populations. In ponds, body armour is reduced and the pelvic spine complex can be missing (Herczeg et al. 2010; Mobley et al. 2011) but, surprisingly, there is no evidence of predator-induced expression of plasticity in body armour in these populations (Välimäki et al. 2012). Contrary to marine fish, pond fish are negatively affected by group living. When reared in groups, pond fish

have smaller body size and brains compared to individual rearing, an observation not prevalent in marine fish (Gonda et al. 2009; Herczeg et al. 2009c).

The behavioural differences in nine-spined stickleback populations can be the result of physiological characteristics and the state of the fish in addition to genetic background and adaptation to environmental conditions. For example, weak locomotor ability, sleep deprivation and starvation can all affect the activity and aggression levels of animals (Biro and Stamps 2010; Damsgard and Dill 1998). Because many behaviours such as aggressiveness and exploration are considered to be energetically costly, there should be a correlation between behaviour and metabolic rate and this has been shown in deer mice (*Peromyscus maniculatus*) where a genetic correlation between resting metabolic rate and exploratory behaviour has been found (Careau et al. 2011). In addition to exploratory behaviour, metabolic rate correlates also with an individual's social status (Cutts et al. 2001; Cutts et al. 1999; Cutts et al. 1998; Metcalfe et al. 1995; Yamamoto et al. 1998) and it has been hypothesized that higher metabolic rate ensures rapid growth in salmonids (Metcalfe et al. 1995). In addition, behaviour itself can affect the growth of the individual. Higher growth rate has been found in low-explorative type brown trout (*Salmo trutta*) compared to bolder conspecifics in the wild (Adriaenssens and Johnsson 2010). Intriguingly, this finding is in contrast to the observation that bolder individuals in domestication/laboratory situations grow faster, and more research is warranted to shed light on these contrasting results (Biro and Stamps 2010; Lahti et al. 2002).

When there are olfactory cues present from perch, nine-spined sticklebacks become less aggressive and risk-taking, and their growth is also decreased (Herczeg and Välimäki 2011; Välimäki and Herczeg 2012). Although it seems that predation is not affecting the body armour of nine-spined sticklebacks, in many other studies predation is known to affect the morphological defence (Hill and Hill 2002; Tollrian 1995; Young et al. 2003). In addition, it has been suggested that predation-induced morphological defences, such as body armour and spines, are actually indirect effects of behavioural changes rather than a direct adaptive response (Johansson and Andersson 2009). This seems to indicate that predators often decrease the activity of the prey (Lima and Dill 1990), which is adaptive by decreasing the probability of the prey being killed (Werner and Anholt 1993), but also enables the prey to use the saved energy for example to growth (Donovan and Gleeson 2006; Tolley and Torres 2002) or development of defensive structures like spines. An excellent example of predator induced morphological change is the predator-prey relationship between the northern pike (*Esox lucius*) and the crucian carp (*Carassius carassius*). In the presence of pike, carps develop deeper bodies (Andersson et al. 2006; Vøllestad et al. 2004); an observation leading to two hypotheses: one stating that this is an adaptive response because pikes prefer shallow-bodied carps (Nilsson et al. 1995) or alternatively this is due to an indirect effect where energy saved from reduced activity is

allocated to body growth (Holopainen et al. 1997; Vøllestad et al. 2004; Andersson et al. 2006; Johansson and Andersson 2009; although see Relyea 2002; Laurila et al. 2008). Other fish species have shown similar response to predator cues (Eklöv and Jonsson 2007). However, it should be noted that in addition to saved energy allocated to growth, available food for prey can affect the body shape (Andersson et al. 2006; Hjelm et al. 2001) and also when there are predators around there are less competitors and more food available for prey to grow larger (Holopainen et al. 1997; Johansson and Andersson 2009).

Nine-spined sticklebacks are a suitable model species for evolutionary and ecological genomics and especially for behavioural studies, as there is the possibility to conduct controlled experiments and the fish are easily reared in a laboratory (Merilä 2013). Furthermore, the availability of the genome sequence of a related species, the three-spined stickleback, has enabled us to develop a suite of genomic markers in nine-spined sticklebacks such as of gene-linked microsatellites (Shikano et al. 2010; Laine et al. 2012 (**I**)) and restriction site–associated DNA (RAD) loci (Bruneaux et al. 2013). In addition, both stickleback species are excellent model species for convergent evolution studies where similar phenotypical adaptations have been potentially achieved through different genetic pathways. As outlined above, nine-spined sticklebacks have large morphological and as well as behavioural differences between habitat types. In convergent evolution studies more emphasis has been put on the evolution and genetic basis of morphological differences (Colosimo et al. 2005; Herczeg et al. 2010; Kaeuffer et al. 2012; Peichel et al. 2001; Shapiro et al. 2009; Shapiro et al. 2004) but behavioural aspects, especially risk-taking and exploration related traits, have been getting more and more attention and it is already known that stickleback risk-taking behaviour is influenced by genetic and environmental factors (Bell 2009; Merilä 2013). However, the exact genes affecting the behaviour are not as yet known. Combining genomic tools and data from the behavioural studies can uncover the genetic basis of stickleback behaviour.

3. MATERIALS AND METHODS

3.1 Methodological background

3.1.1 Candidate genes and heterozygosity-fitness/behaviour correlations

In behavioural genetics, a lot of attention has been given to the serotonergic and dopaminergic systems, because these two hormonal systems play many roles in an organism's physiology and behaviour (Berger et al. 2009; Noblett and Coccaro 2005). Recent studies have demonstrated how the knowledge of genes that have been shown to be associated with behavioural traits in "model" organisms (including humans) can be utilized for studying the genetic basis of behavioural variation in wild animal populations through the use of a candidate gene approach (Figure 2) (Fidler et al. 2007; Fitzpatrick et al. 2005). Recently, a zebrafish model was used successfully to identify many candidate genes and pathways to regulating aggression in fish, combining gene expression profiling, behavioural analyses and pharmacological manipulations (Filby et al. 2010).

The candidate gene approach can be used to design molecular markers, for example to use sequence polymorphism information in categorising wild individuals to behavioural groups without measuring the actual behaviour (Fitzpatrick et al. 2005). Earlier studies have also demonstrated that molecular markers located within or nearby target candidate genes can be a useful resource for the identification of genes associated with adaptive phenotypic divergence, especially when there are no other genomic resources available (Shikano et al. 2010a; Tonteri et al. 2010). In addition, markers closely linked to functionally important genes are useful in construction of comparative genetic maps, in which they can be used as comparative anchor-tagged sequence loci (Lyons et al. 1997).

As mentioned in the introduction, genetic diversity can be an important factor affecting behaviour as shown in the studies on salmonids (Blanchet et al. 2009; Tiira et al. 2006; Tiira et al. 2003; Vilhunen et al. 2008). The idea behind heterozygosity-fitness/behaviour correlations studies is the testing of the direct vs. local vs. general effect hypotheses (Hansson and Westerberg 2008; Hansson and Westerberg 2002; Szulkin et al. 2010). The direct effect hypothesis predicts that heterozygote advantage arises because of overdominance at loci which have a direct effect on phenotypes. The predictions of the local effect hypothesis are similar to those of the direct effect hypothesis, except for that the markers are assumed to be closely linked to a fitness-related gene, rather than directly affecting a trait. An example of such a marker may be

an intronic microsatellite. Finally, the general effect hypothesis predicts that individual heterozygosity reflects genome-wide heterozygosity and that this is expected to be correlated with the individual's inbreeding coefficient and, thus, to be associated with fitness.

The heterozygosity-fitness correlation studies have often been using neutral microsatellite markers, but recently, Olano-Marin et al. (2011a; b) added an additional dimension to this research field when they reported contrasting heterozygosity-fitness correlations for loci presumed to be neutral *vs*. functionally important. In their blue tit (*Cyanistes caeruleus*) study population they showed that heterozygosity at 58 gene-linked microsatellites was negatively related to hatching and the local recruitment success of females, while heterozygosity at 21 presumably neutral microsatellite loci was positively associated with adult survival. These authors hypothesized that the negative heterozygosity-fitness correlations observed for functional loci were best explained by the local effect (outbreeding depression) hypothesis. Thus, markers developed from the candidate gene approach can also be used in the heterozygosity-fitness/behaviour correlation studies to shed light on contrasting effects between different marker categories.

3.1.2 Linkage mapping and QTL studies

In QTL mapping studies, the aim is to link the phenotypic variation with the DNA sequence variation. To do this, a cross between phenotypically different strains is created and the segregation of parental polymorphisms is correlated with the phenotypic variation. Statistical methods, like single marker analysis or interval mapping, is used to detect regions in the genome where the segregation of polymorphic markers correlates with the differences in phenotypic values. These QTL regions often contain a large number of genes and it is challenging to identify the genes that are affecting the trait. Increasing the amount of recombinants might improve the resolution where the QTL is and also the testing of whether the potential candidate genes from the QTL region contain polymorphisms that could be associated with the phenotypic variation. (Falconer and Mackay 1996; Lynch and Walsh 1998).

Due to the developments in molecular biology and bioinformatics, it is now easier than before to conduct QTL studies with wild animals (Ellegren and Sheldon 2008; Slate et al. 2009; Slate 2005). Hence, instead of only relying on domesticated animals and model organisms, the study of wild-derived animals provides new opportunities to improve our understanding of the genetic basis of quantitative traits (Kruuk et al. 2008; Slate 2005). Compared to morphological traits, studying the genetic basis of behaviour is challenging, because heritabilities and repeatabilities are relatively low in behavioural traits (Bell et al. 2009; Boake 1989; Mousseau and Roff 1987). However,

QTL mapping has been successfully used in identifying the genomic areas associated with behaviour (Flint and Corley 1996; van Oers and Mueller 2010; Reif and Lesch 2003).

In order to map the phenotypes to genome, a linkage map is needed. Previously, only one mapping study has been conducted with nine-spined sticklebacks, in which several skeletal traits and sex determination was mapped in North-American populations (Shapiro et al. 2009). In multiple three-spined stickleback populations two genes *Pituitary homeobox transcription factor 1* (*Pitx1*) and *Ectodysplasin* (*Eda*) have had a major effect on the evolution of derived pelvic and armour phenotypes, respectively (Cole et al. 2003; Colosimo et al. 2005; Colosimo et al. 2004; Coyle et al. 2007; Cresko et al. 2004; Shapiro et al. 2004). However, in the study of Shapiro et al. (2009) the results revealed that the measured pelvic traits were situated in chromosome regions not previously known to control the corresponding traits in three-spined sticklebacks, suggesting these convergent morphological traits, which are model traits in the evolutionary biology, might have different genetic origins in the two species. Because we see the same phenotypic differences in Fennoscandian nine-spined stickleback populations too, although they represent a different evolutionary lineage from the North-American clade (Aldenhoven et al. 2010; Shikano et al. 2010c), it would be interesting to compare the genetics of these fish with the North-American nine-spined stickleback and also with the three-spined stickleback. Adding microsatellite markers that are linked to candidate genes to the linkage map would help us find potential genes affecting the studied trait (Shikano et al. 2010b). In addition, QTL mapping of both behavioural and morphological traits would reveal possible genomic linkages/ pleiotropism between different traits.

3.2 Nine-spined stickleback samples

The nine-spined sticklebacks used in my studies were from pond and marine populations originating from Fennoscandia (Figure 3). Ponds are free of piscine predators and competitors, while marine populations are sympatric to a number of predatory or competitor fish species. As I was interested in genetically based differences, my studies were based on fish reared in laboratory under common garden settings, in two cross schemes. The samples for studies **I** and **II** were from first generation full-sib families (family setting) and in studies **III-V** second generation hybrids (F_2-cross) were generated.

3.2.1 Studies I-II: family setting

The fish used in studies **I** and **II** were the same as in Herczeg et al. (2009a). Adult nine-spined sticklebacks were collected from two coastal marine (Helsinki, Baltic Sea,

Finland; 60°13′N; 25°11′E and Levin Navolok, White Sea, Russia; 66°18′N; 33°25′E) populations and two isolated pond (Bynästjärnen, Sweden; 64°27′N; 19°26′E and Pyöreälampi, Finland; 66°15′N; 29°26′E) populations. An F_1 laboratory generation was produced and subsequently raised in a common garden environment as outlined in Herczeg et al. (2009a). Five full-sib families from each population were available for the study and no parental individual was used in more than one cross. Ten fish from each family (200 individuals) were housed individually and visual contact between the tanks was blocked by white plastic panels. In this experiment, 79 offspring were used (the extra fish were for other experiments), 17 from Baltic Sea, 22 from the White Sea, 19 from Bynästjärnen and 21 from Pyöreälampi. In study **I** only parental fish were used, while in study **II** I used the offspring in the main analysis.

3.2.2 Studies III-V: F_2-cross

The fish forming the grand-parental generation (F_0) were collected from a marine population from the Baltic Sea (Helsinki, Finland; 60°13'N, 25°11'E) and from a pond population from north-eastern Finland (Rytilampi; 66°23'N, 29°19'E). The breeding conditions are explained in the chapter of this thesis. In short, a female fish from the marine population was mated with a male from the pond population and the resulting F_1-offpring was group-reared. Once the F_1-fish had matured, one randomly selected male and female were selected, and allowed to mate and produce clutches *in vivo*. This pair produced seven successive clutches and the laid clutches were removed from the male's nest and reared in 1.4 L tanks until hatching. About six days after hatching 400 F_2-offspring were placed individually in 1.4 L tanks. Visibility between the tanks was blocked by placing white plastic sheets between the tanks. Hence, the fish were completely predation- and competition-naive and free from parasites potentially altering behaviour during the rearing period. Due to juvenile mortality, 283 fish (reaching adult size) were used in subsequent analyses.

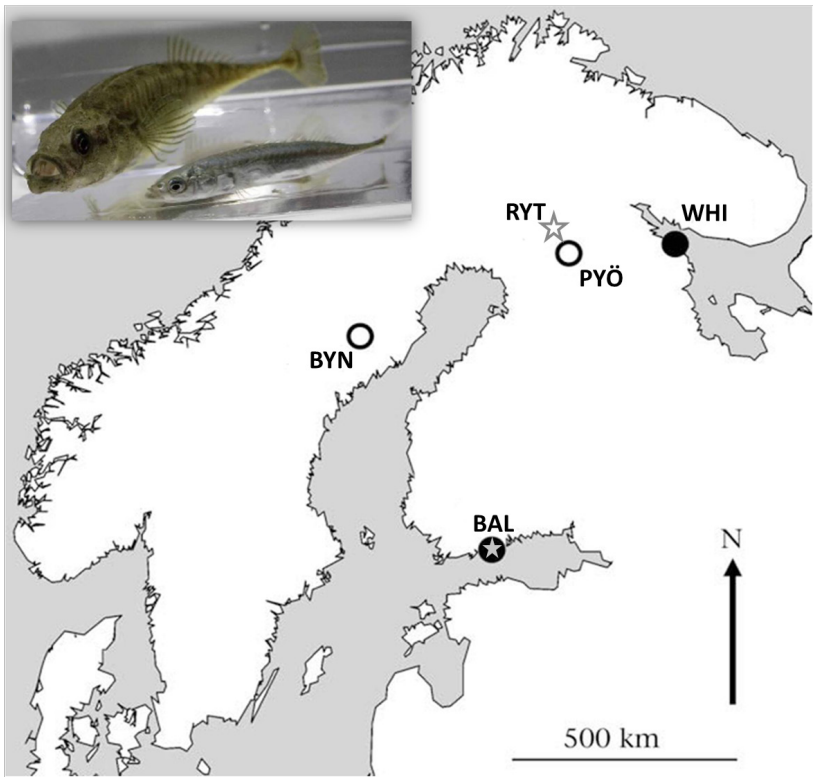


Figure 3. The study populations used in studies **I & II** are marked as circles; open circles denote pond and black circles for marine populations. Stars indicate populations used in the QTL-studies **III-V** (open star for pond and grey for marine). BYN, Bynästjärnen; RYT, Rytilampi; PYÖ, Pyöreälampi; WHI, White Sea; BAL, Baltic Sea. In upper left corner picture there are two fully-grown mature female nine-spined sticklebacks; one from Rytilampi (on the left) and one from the Baltic Sea (on the right).

3.3 Behavioural trials and PCA

3.3.1 Study II: normal feeding, risky feeding, boldness and aggression

The estimates of behaviour used in study **II** were feeding activity, aggression and boldness (see Herczeg et al. 2009a), and these traits were only measured from the offspring. Trials were conducted on 8-month-old fish that had been raised from fertilization individually in 1.4-l tanks of zebrafish racks. Therefore, the tested fish were fully predator- and conspecific naïve. Feeding activity was measured in two contexts: first, as the time until the first bite in a normal feeding event in the morning (hereafter, 'normal feeding'), and second, as the time until the first bite after a simulated attack (hereafter, 'risky feeding'). This second measure can be seen as an alternate measure of boldness in addition to our boldness measurement. Aggression was estimated using two variables, the time spent oriented towards a stimulus fish and the number of attacks the focal fish made towards

the stimulus fish. Finally, boldness was estimated with two variables, the time taken for a fish to appear (i.e. head out) and to completely emerge (i.e. full body out) from a refuge in a novel environment.

3.3.2 Studies III-V: feeding activity, risk-taking and exploration

In the studies **III-V** three behavioural traits were assessed following Herczeg and Välimäki (2011) in the following order: feeding activity (normal feeding), risk-taking (risky feeding) and exploration (boldness). The brackets contain the corresponding trials used in study **II**. Feeding activity is a normal behaviour in a familiar environment, risk-taking is a disturbed activity in a familiar environment and exploration is a disturbed activity in an unfamiliar environment (see details above), hence, all three behaviours described activity / boldness in different contexts. Subsequent behavioural tests of the same individuals were separated by an interval of at least four days. Fish were photographed for the morphological purposes (see below) and behavioural trials were conducted between the last two rounds of photographing separated by 28 days. From 283 offspring tested, 10 individuals were excluded because of unsuccessful behavioural trials.

3.3.3 Principal component analyses

In both datasets all behavioural traits were correlated, thus principal component analyses (PCA) were used to get independent composite variables describing behavioural type. In the study **II** three PC –variables were generated and used solely in the statistical analysis: $PC_{Aggression}$, $PC_{Boldness}$ and $PC_{Complex\ behaviour}$. For $PC_{Aggression}$ higher values indicate more aggressive individuals, and for $PC_{Boldness}$ higher values refer to shyer fish. Because our four behavioural variables, $PC_{Aggression}$, $PC_{Boldness}$, normal feeding and risky feeding (the latter two being based on single variables), were correlated across the pooled sample, another PCA was run ($PC_{Complex\ behaviour}$) on all original variables to combine one or more independent variables describing the behavioural type, which is the configuration of several behaviours at the individual level (Bell 2007). In this, higher values of $PC_{Complex\ behaviour}$ describe more aggressive, bolder and more readily feeding (both normal and risky) fish.

In addition to mapping the original variables in the QTL-studies (**III-V**), two PCs were extracted from combining all the variables and these were also used in the mapping analysis. PC1 was positively correlated with feeding activity, risk-taking and, to a lesser extent, with exploration. Therefore this PC can be viewed as a variable describing the common component of all measured behaviours, i.e. placing the fish along a general shyness-boldness continuum. PC2 was strongly positively correlated with exploration,

but not with feeding activity or risk-taking. Hence, PC2 can be viewed as a variable describing an independent aspect of exploration.

3.4 Morphological measurements for studies III & V

The growth parameters were assessed from standard length data collected from photographs of each individual taken at the following time points: 19, 47, 75, 103, 131 and 159 days (SL1, SL2, SL3, SL4, SL5, SL6) post-hatching, respectively. Three growth parameters were estimated: initial fish length (L_0, mm), asymptotic fish length (L_{max}, mm), and growth constant (k, mm day-1). These parameters were obtained by fitting von Bertalanffy growth curve to the measurements of length (mm) at age t (days), collected from each individual over time points given above (Von Bertalanffy 1938). Initial fish length is a parameter derived from the growth curve, but has no biological relevance in this case. Asymptotic fish length is the estimated maximum size of each individual (the fish will never truly reach it). The growth constant is a relative measure of growth that indicates how quickly the size approaches the asymptote and it is linearly related to maximum growth rate on an absolute scale (mm day-1, Aikio et al. 2013). The size changes between subsequent ages were also analysed (growth increments: GR1, GR2, GR3, GR4, GR5; GR1 = ((SL2-SL1)/SL1)x100, GR2 = ((SL3-SL2)/SL2)x100, etc.). Right after the last photographs were taken and the fish were over-anesthetised, the fresh weight of each fish was recorded and they were photographed laterally with a digital camera. As an additional measure of body size a centroid size (csize) was used, which is the square root of the sum of the squared distances from the centroid of each landmark (Bookstein 1991). The fish were stained and pelvic spine and girdle lengths were measured with digital callipers. Both left and right pelvic girdles and spines were measured twice by the same person, and the averaged values were used for analyses.

3.5 Molecular methods

In study **I,** microsatellite markers were designed by using the candidate gene approach discussed above and shown in Figure 2. Possible candidate genes were identified from the literature and same gene homologues were searched from the three-spined stickleback genome and primers were designed based on the sequence. These primers were tested in nine-spined sticklebacks to locate microsatellite areas. When a possible area was found microsatellite primers were designed and the polymorphism level was determined by using the parental fish from the family dataset.

In the studies **II-V** microsatellite markers were used. The markers were divided into three groups based on their location relative to flanking genes with different functions or being randomly selected: markers that were linked to candidates of behaviourally

important genes (study **I**); markers that were associated with putatively physiologically important genes (Shikano et al. 2010b; Shimada et al. 2011b); and randomly selected markers (Colosimo et al. 2004; Heckel et al. 2002; Largiadèr et al. 1999; Miller et al. 2007; Mäkinen et al. 2008; Peichel et al. 2001; Shapiro et al. 2009; Shikano et al. 2011).

3.6 Data analyses

3.6.1 Study II

Individual genetic diversity based on all the 84 markers was estimated using standardized heterozygosity (SH: Coltman *et al.* 1999). SH is not sensitive to missing alleles (Coltman et al. 1999) and it gives the same weight to all markers, regardless of the number of alleles and the frequency of these alleles (Olano-Marin et al. 2011b). SH was also calculated for different marker function groups separately (12 markers for behaviour gene group, 33 physiologically important gene markers and 39 randomly selected markers). The individual genetic diversities were calculated with GENHET v3 (Coulon 2010) using the parental data for the estimation of baseline allele frequencies for each population separately. To test the effects of the diversity of different marker groups on nine-spined stickleback behaviour, general linear mixed models (GLMMs) were used, as implemented in PROC MIXED (Littel et al. 2006) in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). In addition to multilocus heterozygosity, the associations between single-locus variability and behavioural variables were tested as an alternative means of distinguishing between local and general effects (Hansson and Westerberg 2002). The single-locus heterozygosity (SLH) for every behavioural variable was measured fitting one model per locus. Multiple testing correction was performed via the false discovery rate (FDR) procedure using the program QVALUE (Storey and Tibshirani 2003) with an FDR threshold of 0.05. This program calculates q-values, which are the extensions of FDR describing the proportion of false positives incurred within a set of significant features (Storey and Tibshirani 2003).

3.6.2 Studies III-V

Linkage map was constructed using improved CRI-MAP 2.5 (Green et al. 1990). The logarithm of the odds (LOD) scores for all pairs of markers were obtained using the TWOPOINT option. LOD score threshold of 3.0 was used as a significant criterion for linkage. For each linkage group (LG), the best order of the markers was determined using the BUILD option by beginning with the most informative marker pair. The markers that could not be fitted straight with BUILD and LOD score 4.0 were fitted manually. The FLIPS option (N = 3–5) was used to evaluate the statistical significance of the obtained order. After the best order was determined within each linkage group, double recombination events were detected using the CHROMPIC option. Individuals

with over four recombinations were removed and a second CRIMAP analysis round was conducted. Linkage maps were drawn using MAPCHART 2.2 (Voorrips 1994).

Genomic synteny was investigated by comparing my linkage map with the three-spined stickleback genome (Jones et al. 2012) and the linkage map of North American nine-spined sticklebacks (Shapiro et al. 2009). For the markers developed specifically for nine-spined sticklebacks, microsatellite flanking sequences were subject to BLASTN searches against the three-spined stickleback genome to identify homologous genomic regions. BLAST hits were considered significant at a threshold of $E < 10\text{-}5$. Out of 226 informative markers in my linkage map, 110 were used for the linkage map of North American nine-spined sticklebacks (Shapiro et al. 2009).

A total of 226 markers were used in the QTL analyses. Due to recombination rate differences sex-average linkage map distances were used in QTL mapping. QTL mapping was performed in GridQTL (available at http://www.gridqtl.org.uk/) by using the BCF2 portlet and fitting both additive and dominance effects. Analyses were performed at 1 cM intervals. The percentage of the phenotypic variance explained by the QTL was calculated following Zhou et al. (2006). Sex, clutch information and centroid size were used as covariates when needed. Chromosome and experiment wide significance levels were determined by using permutation tests with 10 000 iterations to evaluate the significance of detected QTL. QTL were considered significant when the F-value was above the 5% experiment-wide threshold and suggestive when it was above the 5% chromosome-wide threshold. Confidence intervals were obtained with bootstrap analysis with 10 000 iterations.

4. MAIN RESULTS AND DISCUSSION

4.1 Candidate genes and heterozygosity-behaviour correlations (I & II)

In the first chapter of my PhD I developed 13 new microsatellite markers which were located close to candidate genes for behaviour in nine-spined stickleback. Their polymorphism levels were tested by using nine-spined stickleback samples from four different populations; two marine (BAL, WHI) and two pond populations (BYN, PYÖ). All loci were polymorphic but lower levels of observed heterozygosities were found in populations from pond habitats when compared to those from marine habitats, which was consistent with earlier studies (Shikano et al. 2010c). These markers add to the anonymous markers and to physiologically important gene markers currently available for nine-spined sticklebacks (Koizumi et al. 2007; Meguro et al. 2009; Shapiro et al. 2009; Shikano et al. 2010b). Overall, these markers should provide a useful resource for a better understanding of the genetic basis of behaviour in sticklebacks.

In the second chapter these and additional markers were used to investigate whether there is a relationship between individual heterozygosity and behaviour in the four populations. With the laboratory settings I made sure that the inbreeding coefficient was constant, thus enabling me to have more power in detecting heterozygosity-behaviour correlations. No associations were detected with any behavioural trait in any population or over all populations when genetic variability was measured using all the markers combined. This suggests that the number of markers is not necessarily a guarantee for detecting a correlation between heterozygosity and behaviour – an observation that contrasts previous studies (Aparicio et al. 2007; Balloux et al. 2004; Slate et al. 2004; Slate and Pemberton 2002). It seems that rather the functional constraint the marker is experiencing and the population-specific effects will determine the correlation to be detected. When I separated the markers into three functional categories (behavioural, physiological and random), several significant associations were observed, but only in one of the four populations assessed, in the Baltic Sea population from Helsinki, Finland (Figure 4). In this population, the strongest correlation existed between markers of physiological importance and of complex behaviour, with less aggressive, less bold and less active feeder fish being more heterozygous. This indicates a direct link between the measured behavioural traits and the physiological mechanisms controlling behaviour (Sneddon 2003; Wilson and McLaughlin 2007), such as the correlations between metabolic rate and behaviour (Biro and Stamps 2010; Careau et al. 2008).

Furthermore when dividing the physiological gene markers into further subcategories based on their specific physiological functions, a strong relationship between the heterozygosity of markers linked to osmoregulation-related genes and behaviour was unravelled. Osmoregulation is a highly energy-consuming process (Tseng and Hwang 2008) and it has been noted that appropriate behaviour can be an important component of the osmoregulatory process (Wolcott and Wolcott 2001). Yet, another observation described in the study performed by Lesbarrères et al. (2005) showed that heterozygosity-fitness correlations were most pronounced in stressful environments. In this regard, the fact that the strongest association was observed with markers linked to genes with an osmoregulatory function may suggest that the brackish water environment could contribute to the observed association. The salinities observed in the Baltic Sea are in fact quite variable, and annual fluctuations are also observed regionally (Alenius et al. 1998). Thus, local variation in salinity could pose osmoregulatory challenges specific to this population compared with the others included in the study. The fact that the observed heterozygosity-behaviour correlation was negative (i.e. less genetically variable fish were bolder) can be explained if adaptation to local conditions is also important (Emlen 1991; Purcell et al. 2010; Purcell et al. 2008).

In addition to physiological markers I also saw correlations between random markers and behaviour, but the effect was the opposite: bold fish being more heterozygous (Figure 4.). As mentioned above, in a recent study on birds a negative association was observed with several fitness traits for functional markers, whereas a positive association was observed with neutral marker heterozygosity (Olano-Marin *et al*. 2011b). The authors proposed that these different results could be distinguishing local (functional marker) and global (neutral marker) effects. This is also a plausible explanation for the results observed in my study. My random and supposedly neutral loci may reflect variation across the genome (inbreeding depression), and functional loci may reflect local effects through loci linked to behaviour. The negative correlation between physiological markers and both boldness and complex behaviour in the Baltic Sea population could therefore be attributed to outbreeding depression associated with local adaptations (Emlen 1991). Physiologically important genes and their function are crucial for an individual's survival in its local habitat, so we can assume that introgression of non-locally adapted genes (or alleles) could affect viability in the local environment. This could be particularly true for osmoregulatory functions in primarily freshwater species, such as the nine-spined stickleback, in a brackish water environment, such as the Baltic Sea (Papakostas et al. 2012). Directional selection has been shown to affect physiological gene markers more frequently than randomly distributed ones in three-spined sticklebacks, which provides additional evidence that local effects are important (Shimada et al. 2011b).

The Baltic Sea population exhibited the highest level of genetic variability and was therefore more sensitive with respect to the detection of inbreeding depression (Szulkin and David 2011). The environmental conditions experienced by the two pond populations, as well as the extremely low levels of genetic diversity observed in these populations, would suggest that they are much more likely to be affected by inbreeding than the more genetically variable marine populations. However, the very low level of genetic variability may complicate the detection of heterozygosity-behaviour correlations in these cases.

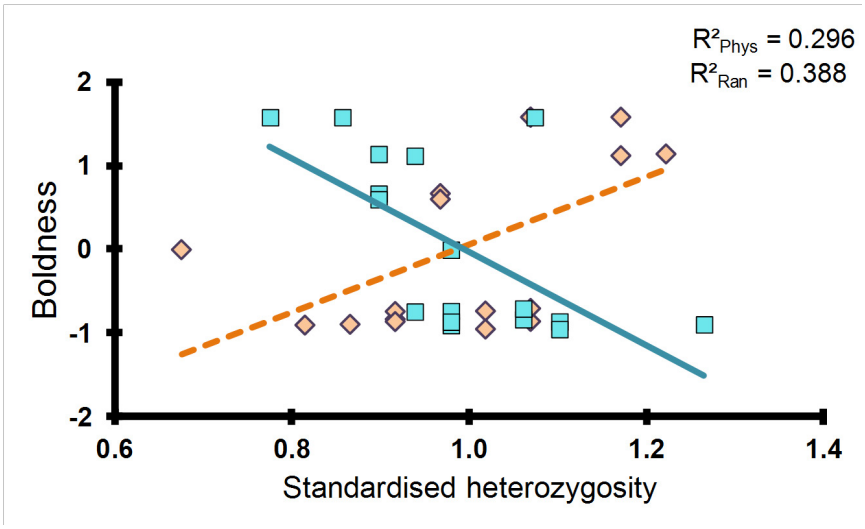


Figure 4. An example of contrasting effects. The relationship between standardized heterozygosity and boldness in Baltic sea population. Standardised heterozygosity was estimated using either physiologically important gene (◊) or randomly selected (□) microsatellite markers. The lines represent the linear relationship between standardised heterozygosity and boldness (dashed line for physiological markers, solid line for random markers). Within population coefficients of determination (R^2) are given in the figure; R^2_{Phys} for physiological markers and R^2_{Ran} for random markers.

4.2 Linkage map and quantitative trait loci analysis (III-V)

4.2.1 Linkage map and QTL for pelvic reduction (III)

I constructed a linkage map for the Northern European nine-spined sticklebacks utilizing 226 microsatellite markers (Figure 5). When comparing this map to the three-spined stickleback genome a segment corresponding to LG 7 in three-spined sticklebacks was linked to LG 12 in Northern European nine-spined sticklebacks. None of the loci located in this region show linkage to the markers mapped to LG 7. Thus, the rearrangement of genetic linkage patterns is likely due to a chromosomal

rearrangement that has occurred after the divergence between three-spined and nine-spined sticklebacks. In the previous North American nine-spined stickleback linkage map no linkage was detected between this segmental part and LG 12 (Shapiro et al. 2009). It remains unclear if the arrangement of linkage patterns has occurred before or after the split between Northern European and North American populations. I further detected potential chromosomal inversions in several linkage groups as compared to the three-spined stickleback genome but, however, could not rule out errors in the genome sequences of three-spined stickleback (Natri et al. 2013; Ross and Peichel 2008). I also mapped the sex-determining gene to LG 12 which is the same location as in North American fish (Shapiro et al. 2009). Although it is not certain whether the linkage between LG 12 and the segment of LG 7 is caused by physical or pseudo linkage, it appears that the segmental region of LG 7 co-segregates with the sex-determining locus in Northern European nine-spined sticklebacks. Theoretical studies have shown that the formation of linkage between the sex-determining locus and autosomal genes under sexually antagonistic selection has significant consequences on both population fitness and sex chromosome evolution (Charlesworth and Charlesworth 1980; van Doorn and Kirkpatrick 2007). Thus, it would be interesting to investigate in future studies whether the genes affecting sexually dimorphic traits and mating behaviour reside in the chromosomal region newly linked to the sex-determining locus.

In this study pelvic spine and girdle length were mapped (Table 1) and I found that the location for pelvis reduction was different compared to North American nine-spined sticklebacks, where pelvic spine and girdle lengths were located in LG 4 (Shapiro et al. 2009). In my study, QTL for pelvic spine and girdle lengths were identified in the region of the *Pitx1* gene in LG 7 which is the same region responsible for pelvic reduction in three-spined sticklebacks. These results have two possible explanations: either there has evolved a distinct genetic mechanism for pelvic reduction in nine-spined sticklebacks possibly within the last 1.6 million years after the split between Northern European and North American populations, or the mapping conditions in the North American study were not sufficient to locate this QTL.

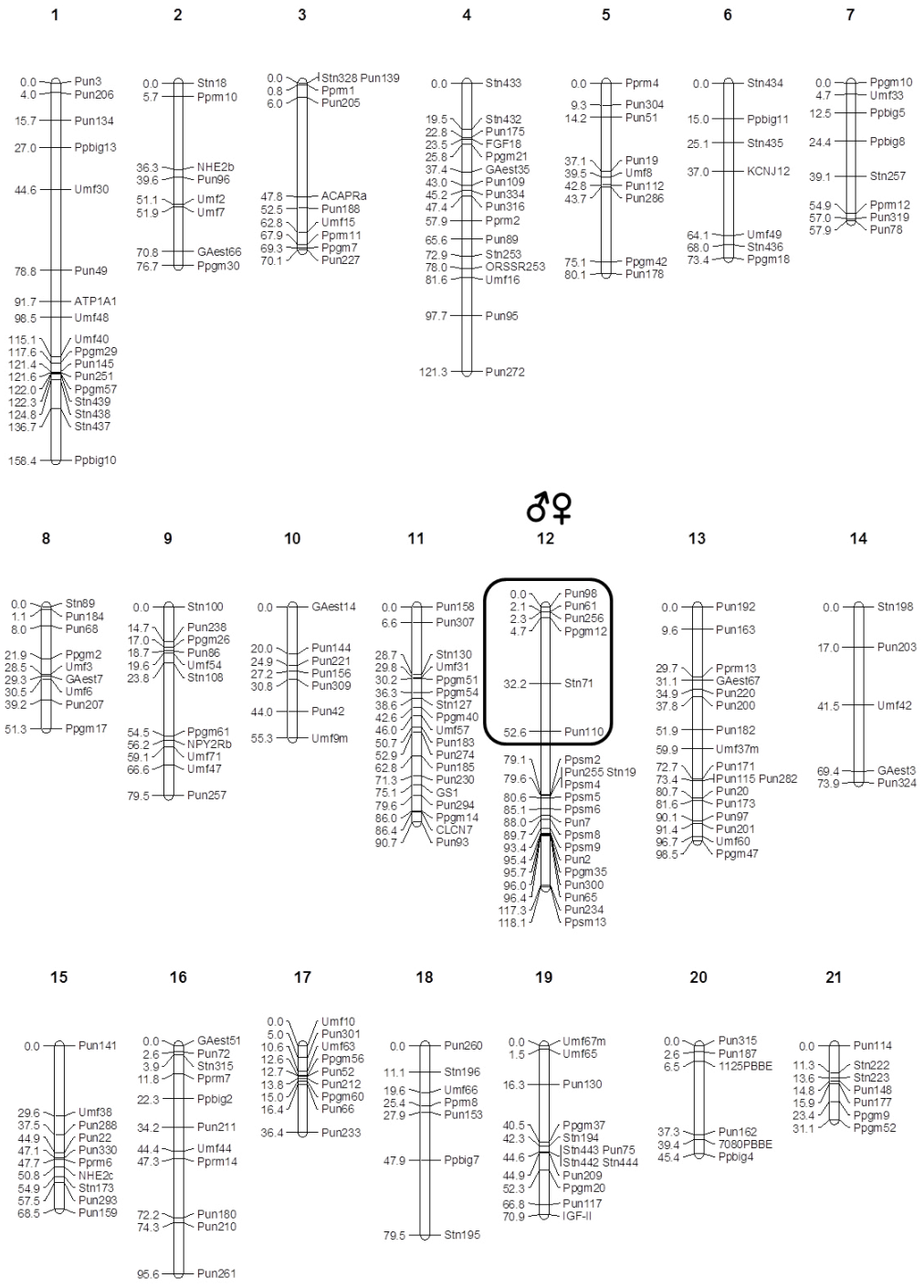


Figure 5. Sex-averaged linkage map of the northern European nine-spined stickleback. Marker positions are given in cM. The black square corresponds to the chromosomal rearrangement originated from LG7.

4.2.2 QTL for behaviour (IV)

In chapter **IV** three behavioural measurements were QTL mapped; feeding activity, risk-taking and exploration, in addition to the principal components (PC1 = the common behavioural component placing individuals along the shyness-boldness continuum; PC2 = an independent exploration component) calculated from these measurements to grasp the complex behavioural types. Behaviour is most likely polygenically regulated and thus many QTL are expected (Flint 2003), as was indeed the case in this study (Table 1). Two significant (experiment-wide) and six suggestive (chromosome-wide) QTL regions were found altogether. Together these QTL accounted for 4% to 20% of the total phenotypic variance in each of the behavioural variables.

In LG 3 I found QTL influencing different behavioural traits. This may suggest that the genetic factors influencing one behaviour may have pleiotropic effects on other behaviours, or that genetic factors influencing different behaviours cluster into this linkage group. The resolution of my linkage map does not allow me to differentiate between these alternatives. In addition, it seems that in the two measured main behavioural components, PC1 and PC2, were different from each other to map to different parts of nine-spined stickleback genome whereas the correlated traits (exploration and PC2) mapped to similar locations. This suggests that different genetic factors underlie different behaviours.

In addition to QTL amount, it was expected that pond alleles would be associated with an increase in the feeding activity, risk-taking and explorative behaviour of nine-spined stickleback compared to alleles originating from marine populations, because of the population differences found from previous studies. In many cases, this was what I observed. However, there were also cases where pond population decreased behavioural activity especially in exploration and PC2 in contrast to the predictions. The explanation for these “cryptic” results might be found from chapter **II**. Variation (heterozygosity) in the wild has been probably maintained at the QTL loci affecting exploration due to the general effect result found from the random markers in chapter **II**. Therefore, these QTL loci are not behaving according to the expectation (that the loci affecting the characteristic traits of the grandparental populations would be fixed to different alleles in the populations). Due to this variation, alleles with “cryptic” effects are found in our study because the QTL genotypes of the picked grandparents are not known. Other explanation for these differing effects could be the new allele combinations in the offspring. Because different behaviours are commonly results of many genes acting together, combining the alleles from two populations in the offspring might lead to different behaviours than in grandparents because of the interactions between the new alleles.

In this study risk-taking was mapped to the same location where the pelvic reduction QTL is situated (chapter **III**). *Pitx1* might also be a potential candidate gene for behaviour due to findings in recent studies done with humans. It has been shown that *Pitx1* is linked with the behavioural aspects of autism, such as failure in stress/anxiety control (Philippi et al. 2007). Feeding can be stressful due to competition and predators which makes the pituitary-hypothalamic-adrenal (or interrenal in fishes) axis (HPA) system activated. Because *Pitx1* is a regulatory factor for the expression of hormones of HPA, which is known to be especially involved in stress response (Lamonerie et al. 1996), it can also affect the feeding activity and risk taking behaviour. In addition to *Pitx1*, many other possible candidate genes were identified with the help of gene linked microsatellite markers from both behavioural and physiological gene marker groups. More studies are needed to confirm the linkage between these genes and behaviour in nine-spined sticklebacks in order to investigate its similarity to other behavioural studies.

4.2.3 Body size and growth QTL (V)

In the final chapter the aim was to map body size and growth related traits which also show population differences similarly to the behavioural traits in Fennoscandian nine-spined sticklebacks (Shimada et al. 2011a). Body size is an ecologically important trait, the genetic basis of which is thought to be highly polygenic in many organisms (Falconer and Mackay 1996). However, comparisons of growth patterns in different populations of nine-spined sticklebacks (Herczeg et al. 2012; Shimada et al. 2011a) suggest that size differences among populations could have been achieved by relatively simple genetic mechanisms. Namely, at the point in time when the growth of the fish from normal sized marine population levels off, the pond fish destined to continue their growth (Herczeg et al. 2012; Shimada et al. 2011a). Hence, it seemed possible that a simple 'genetic switch' – rather than complex polygenic inheritance – underlies the population differences in body size.

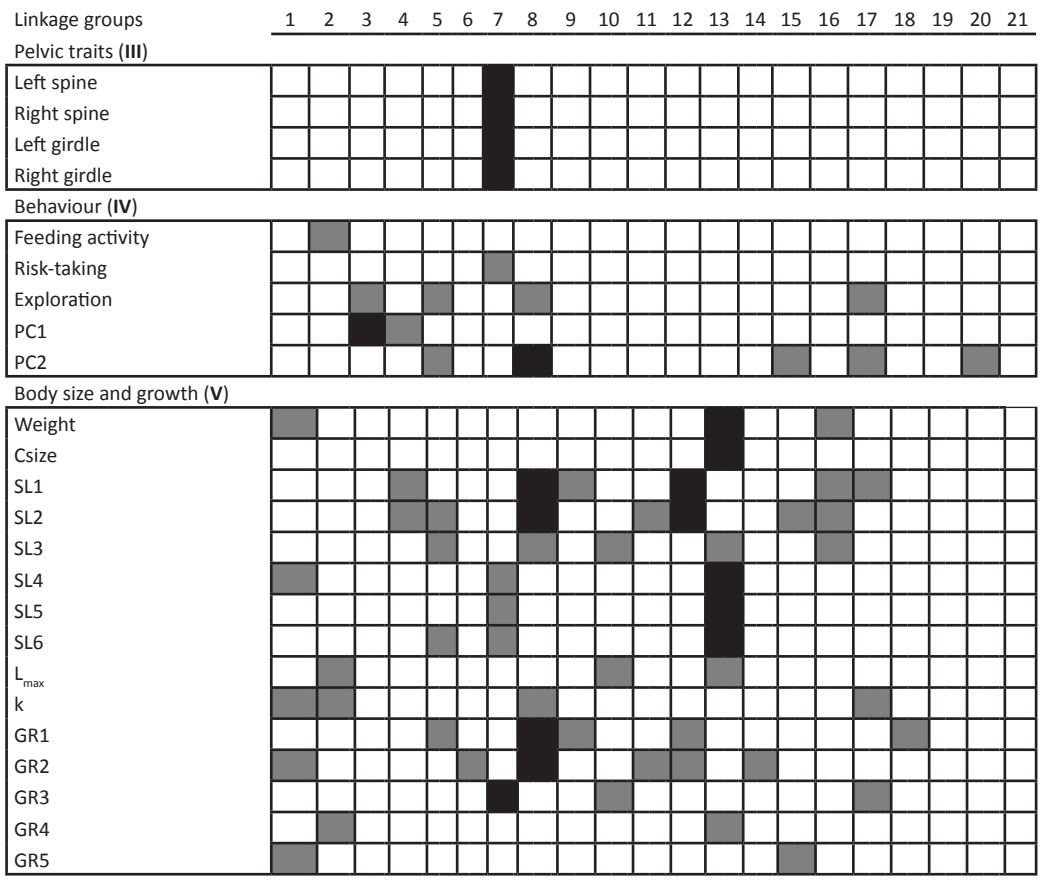
In this study I have identified several significant and suggestive QTL -influencing variations in body size and growth related traits. As opposed to the hypothesis of a simple major gene based "switch"-like genetic architecture of body size control in this species, the results support the idea that many genes with small effects underlie variability in body size. In total, 17 QTL locations were detected for body size and growth (Table 1). The proportion of phenotypic variation explained by individual body size-related QTL ranged from 3% to 12%, and those related to growth parameters and increments ranged from 3% to 10%. When comparing the amount of QTL found to be related to growth and body size in other fish, it is possible to divide the obtained results into two groups: fish with single major QTL for size or many QTL with small or intermediate effects (Hosoya et al. 2012). My result falls into the former group by having body size mapped to LG

13. Earlier studies done with three-spined stickleback suggest that this species has also a single QTL for size (Albert et al. 2008; Colosimo et al. 2004). However, although these two three-spined stickleback studies had a similar study setting (similar population cross design with comparable number individuals and markers) they mapped size to different locations. Hence, it seems that experimental designs are affecting the results instead of different evolutionary mechanisms. Furthermore, the main study questions in Albert et al. (2008) and in Colosimo et al. (2004) were about body shape and armour, respectively, and they chose the study populations irrespective of size possibly resulting in small size variation.

In addition size was measured in different time points during development. Early and late stage measurements were mapped to different locations (Table 1). Hence my results suggest distinct gene sets for early and later stage growth. Additive effects were generally more frequent in my study and this result is consistent with the findings of an earlier quantitative genetic study of body size divergence in Fennoscandian nine-spined stickleback populations (Ab Ghani et al. 2012). Similarly from conclusions of Ab Ghani *et al.* (2012) study, alleles from the paternal grandparent pond population increased body size both at the early and late stages of development.

As mentioned before, behaviour can have a direct impact on growth. When comparing these results with QTL analyses done with behavioural traits (chapter **IV**) I noticed that some of the QTL areas were similar. This indicates that genetic linkage or pleiotropic effects of single QTL may be the genetic mechanisms behind body size/growth and behaviour. The biological link can be straightforward also in nine-spined sticklebacks: more active/bold/explorative individuals are likely to gather more food, translating to higher energy input aiding faster growth and larger size. This is a very promising avenue for future studies, because it could provide direct proof that genetic variation in growth rates and patterns may be mediated by genetic factors influencing behaviour. In this study also many possible candidate genes for future studies were found.

Table 1. Distribution of QTL related to pelvic traits, behaviour, body size and growth across the 21 linkage groups indicating the chromosome- (grey blocks) and experiment- (black blocks) wide significance levels. Csize = centroid size; SLnumber = standard length measured at specific time point; Lmax = asymptotic length; k = growth constant; GRnumber = growth increment. For more details about the variables, see Materials and methods



5. IMPLICATIONS AND FUTURE DIRECTIONS

The main findings of my PhD project were twofold. First, I found heterozygosity-behaviour correlations that varied between populations and the type of genetic markers (random *vs.* functionally important) used in nine-spined sticklebacks (chapter **II**), partly based on new, behavioural gene-linked microsatellite markers developed for my PhD project (chapter **I**). Second, I discovered links between adaptive phenotypic variation in fundamental traits like behaviour, growth and antipredatory morphology and variation in certain sections of the genome of nine-spined sticklebacks (chapters **III-V**). Given that nine-spined stickleback is a recently emerging evolutionary model (Merilä 2013), my results are not only interesting as they are, but they have the potential to stimulate lot of research in the near-future. In the following, I will first discuss the potential implications of my results on behaviour – heterozygosity correlations and second, of QTL mapping.

5.1 Variation in behaviour-fitness correlations

I have shown that heterozygosity correlates with behaviour in one of the marine nine-spined stickleback populations but with contrasting effects depending on the marker function. From the different functional marker categories a strong correlation was found between osmoregulation- related physiological gene markers and behaviour. Because the marine habitat presents a high predation risk for sticklebacks as well as variable brackish water salinity levels, these characteristics could place pressure on osmoregulation processes and thus require adaptations specific to this local environment. This hypothesis is still speculative at this stage; hence analysing additional populations and/or more specific tests of the effects of predation threats are required before any firm conclusions can be made. Furthermore, it would be interesting to correlate physiological and morphological traits with heterozygosity to see which of the marker groups associate with the traits and whether some of the results are similar with behavioural ones.

5.2 QTL mapping of traits with high ecological and evolutionary relevance

Thus far, there has only been one linkage map for nine-spined sticklebacks and this was now aided by a second linkage map with more markers constructed in this thesis. I observed genomic rearrangements between autosomal (LG7) and sex-determing (LG12) linkage groups. It is possible that phenotypes with sexual dimorphism are residing in this newly formed part in LG12. Further cytogenetic analyses would clarify whether the linkage between LG12 and the segment of LG7 has formed via physical or pseudo linkage, as well as verify the occurrence of intrachromosomal rearrangements in

Northern European nine-spined sticklebacks, which also has implications for a better understanding of chromosome evolution in general.

This thesis brought more light to population differences/convergent evolution and these insights were gained by studying divergent nine-spined stickleback populations. In addition to behaviour I also studied pelvic structures and body size/growth related traits as these also show large variation between nine-spined stickleback populations in northern Europe. One major QTL was found for pelvic structures and *Pitx1* was related to these traits as was predicted from three-spined stickleback studies, but this was in contrast to earlier nine-spined stickleback study. My thesis studies revealed also that behaviour and body size/growth were genetically more complex traits by having more QTL than pelvic traits. In many cases pelvic structure, body size/growth and behaviour were linked to similar map locations indicating possible pleiotropic effects of genes locating in these QTL regions. In this thesis a candidate gene approach was used to see if some of the earlier studied behaviour related genes are linked to nine-spined stickleback behaviour, but the linkage was not obvious in many cases. Instead I found potential candidate genes from the physiology group for behaviour also in the QTL studies. In the future, studying the possible candidate genes found from QTL -studies by using for example targeted sequencing would show if there are sequences or some other mechanisms affecting these traits. In addition, improving the contrast of the QTL results by adding more individuals and making a denser map with more markers would reveal additional QTL for these traits.

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