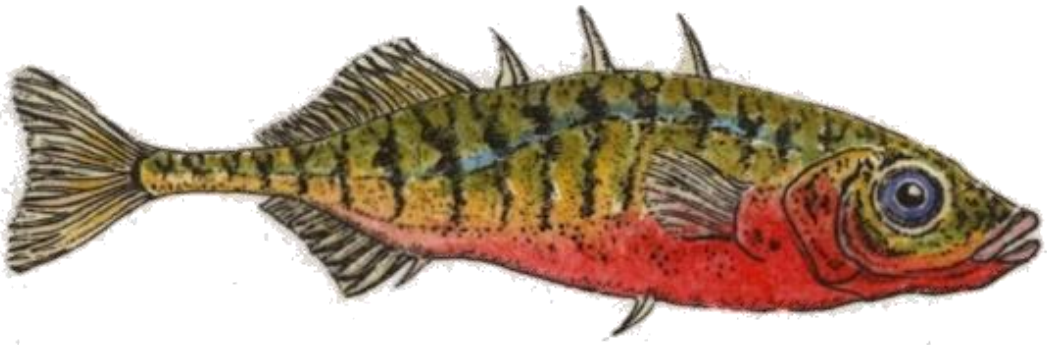
Molecular basis of ecological speciation in sticklebacks



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Peu d'observations et beaucoup de raisonnements conduisent à l'erreur.

Beaucoup d'observations et peu de raisonnements conduisent à la vérité.

Alexis Carrel

Summary:

Speciation, the concept of constantly adapting and diverging populations towards independent and distinct species under the influence of natural selection, has been of great scientific interest since the early works of Darwin (1859) and Cook (1906). Aiming to understand the motivation behind the immense biodiversity we see today has evoked a multitude of interlinked research fields, targeting the key aspects governing the evolutionary rise, persistence and fall of individual species.

A major factor driving this speciation is the environmental context the individuals are exposed to. The constant race to outcompete other organisms, either from the same or another species drives the continuous emergence of new and, better adapted individuals. As these new phenotypes become increasingly specialized to the specific environmental conditions they are exposed too, they grow progressively divergent from both their ancestors as well as their conspecifics in other environmental contexts. In fresh water three-spined sticklebacks (*Gasterosteus aculeatus*), this has resulted in a number of specific phenotypes, which have adapted different feeding regimes (e.g. limnetic-benthic), different reproductive strategies (anadromous-resident) or have evolved habitat specific growth and parasite resistance strategies (river-lake ecotypes). Each of which is characterized by specific adaptations to the environmental context they are exposed to and maintained through environmental pressures and sexual selection.

In my thesis, I investigate the environmental factors and underlying molecular mechanisms, driving the globally reoccurring distinction between river and lake three-spined sticklebacks. A key difference between rivers and lakes are the different parasite communities they harbor. Parasites impose strong negative selection pressures on their stickleback host by diverting resources, restricting reproduction, actively or passively promoting their host's predation risk and death or a combination of the former. Hence, it is crucial for sticklebacks to minimize their parasite load, resulting in a co-evolutionary arms race between parasite and host. As the parasite community varies between habitat types (i.e. river and lake) with regards to species abundance and composition, they demand different adaptations, resulting in increasing divergence between sticklebacks in their respective habitats. The globally reoccurring distinction of river and lake habitats implies an equally reoccurring habitat dependent differentiation into specific river respectively lake ecotypes.

Specializing to their environment bestows an ecological advantage onto the resident individuals, enabling them to outcompete potential migrants and sub optimally adapted conspecifics. This is

reinforced by sexual selection which privileges well adapted phenotypes by actively selecting for such individuals with low parasite burdens and optimal immune gene composition. Incidentally, this selects against potential migrants from other habitats as their mismatched (immune) genotype does most likely not comply with the habitat specific ideals, thus generating genetic isolation, a prerequisite for speciation. Hence, both habitat specific differences and the underlying sexual selection result in the parallel evolution of river respectively lake ecotypes on a global scale.

I demonstrate this in my thesis by exposing Canadian and German river-lake pairs to a common German lake parasite (*Diplostomum pseudospathaceum*) in a controlled laboratory experiment. Despite unexpectedly high parasite resistance by the German river fish, most likely the result of parasite specific local adaptation, the discrepancy between river and lake fish holds true. The accompanying gene expression analysis suggests differences in the underlying regulatory mechanisms of the immune genes. In a follow up experiment the same populations were exposed to either a natural river or lake environment for up to nine months. This allowed me to test the assumption of parallel evolution towards distinct ecotypes in a direct comparison under natural conditions. The preliminary results of which partially affirm the previously observed distinction. As expected, the German lake fish have, at least initially, significantly lower parasite burdens, and in general ecotypes seem to fair better in the matched as opposed to the mismatched habitat. However, due to overall rather low parasite pressures, the German lake fish are unable to profit from their high parasite resistance and fail to achieve comparable growth rates with the other populations, thus suffering from a mismatch between adapted phenotype and natural variation in parasitic pressure. The main ecotypic overlap was observed between both river populations. Having evolved under comparably low parasite pressures, they appear to have inherently higher growth rates, allowing them to outgrow the lake ecotypes in the low parasite environment they experienced during the exposure period.

Finally, I addressed the sexual selection for optimally adapted genotypes in controlled olfactory mate choice experiments. The main determining factor in olfactory mate choice of three-spined stickleback females are alleles of the major histocompatibility complex (MHC). These are a key component of the recognition machinery in the immune system and, consequently, a major contributor towards parasite resistance. Using artificially synthesized MHC peptides, I demonstrate that the females choose solely based on the number of different alleles in relation to their own as well as the population specific optimum, independently of the male's origin. In addition to the MHC signal, stickleback males produce a so-called male validation factor (MVF), signaling species identity and thus confirming the origin of the

perceived MHC signal. Using Canadian and German sticklebacks, I demonstrate that the MVF does not convey any habitat or population specific information and is evolutionary conserved between both populations, despite their large geographic and evolutionary distance.

In summary, I find strong evidence for parallel ecological speciation as a key driver towards distinct river or lake three-spined sticklebacks. These are characterized by a number of habitat specific adaptations, resulting in globally reoccurring ecotypes, independently of the genetic and evolutionary background of the respective population. Potentially, this will lead to the evolution of two distinct (sub)species in three-spined sticklebacks, which are characterized through their environment rather than ancestry.

Zusammenfassung:

Artbildung, das Konzept sich konstant anpassender und auseinanderstrebender Populationen zu unabhängigen und eigenständigen Arten unter dem Einfluss natürlicher Selektion, steht seit den frühen Arbeiten von Darwin (1859) und Cook (1906) im Fokus des wissenschaftlichen Interesses. Mit dem Ziel die zugrundeliegenden Mechanismen hinter der heute immensen Biodiversität zu entschlüsseln sind zahlreiche Forschungsbereiche entstanden welche sich mit den Schlüsselaspekten hinter dem evolutionären Entstehen, Etablieren und Zugrundegehens einzelner Arten befassen.
Die Umwelt der diese Arten ausgesetzt sind trägt maßgeblich zu deren Evolution bei. Im konstanten Wettstreit mit anderen Organismen, sowohl der eigenen als auch anderer Arten, entstehen kontinuierlich neue und potenziell besser angepasste Individuen. Mit zunehmender Spezialisierung dieser Phänotypen für ihre spezifische Umwelt, differenzieren sie sich zusehends sowohl von ihren Vorfahren als auch von Artgenossen in anderen Habitaten.

In Dreistachligen Stichlingen (Gasterosteus aculeatus) hat dies weltweit zu zahlreichen Phänotypen geführt welche unterschiedliche Anpassungen an die Nahrungsaufnahme (z.B. Limnisch-Bentisch), Fortpflanzungsstrategien (z.B. Anadrom-Ortstreu) oder Parasitenresistenzen etabliert haben (z.B. Fluss-Seeökotypen). Jeder dieser Differenzierungen wird durch spezifische Anpassungen an das Habitat charakterisiert und durch Umwelteinflüsse und sexuelle Selektion beibehalten.
In meiner Promotion untersuche ich die Umwelteinflüsse und grundlegenden molekularen Mechanismen welche die weltweite Differenzierung von Fluss- bzw. See Stichlingen vorantreiben und ermöglichen. Ein Hauptunterschied zwischen Flüssen und Seen sind die vorherrschende Parasitengemeinschaften. Diese üben einen starken negative Selektionsdruck auf ihre Wirte aus indem sie Nährstoffe entziehen, die Reproduktion beeinträchtigen, sowohl aktiv wie passiv den Prädationsdruck erhöhen und gegebenenfalls deren Tod herbeiführen. Demzufolge ist für Stichlinge die Minimierung der parasitären Belastung ein Grundanliegen, welches in einem evolutionären Wettrüsten zwischen Parasiten und Wirt mündet. Im selben Maße wie die Parasitengemeinschaften der verschiedenen Habitate variieren, stellen diese unterschiedlichen Anforderungen an die Stichlinge und fördern damit die Divergenz zwischen den Populationen der entsprechenden Habitate. Der weltweit widerkehrenden Unterschiede zwischen Fluss- und Seehabitat impliziert eine ebenso globale Differenzierung von spezifischen Fluss- respektive Seeökotypen.

Habitat spezifische Anpassungen verleihen den ortsansässigen Fischen einen ökologischen Vorteil und erlaubt es ihnen potentielle Migranten und suboptimal angepasste Individuen zu übertrumpfen. Dies

wird durch sexuelle Selektion verstärkt welche gut angepassten Phänotypen privilegiert indem diese Individuen mit niedrigem Parasitenbefall und optimalen Immungenen bevorzugt. Ein Nebeneffekt dieser Selektion ist die Benachteiligung von Habitat fremden Tieren da ihre Immungene höchst wahrscheinlich nicht mit dem Habitat spezifischen Idealen übereinstimmen, was wiederum zu genetisch Isolation führt, eine Grundvoraussetzung der Artbildung. Demzufolge führen sowohl die Habitats spezifischen Unterschiede als auch die zugrundeliegende sexuelle Selektion zu Weltweit wiederkehrender Differenzierung von Fluss- bzw. See Ökotypen.

Dies veranschauliche ich in meiner Promotion durch die experimentelle Infektion von kanadischen und deutschen Fluss- und Seefischen mit einem typischen deutschen Seeparasiten (Diplostomum pseudostpathaceum). Ungeachtet der unerwartet hohen Resistenz der deutschen Flusspopulation, wahrscheinlich aufgrund spezifischer lokaler Anpassung, bleibt der erwartete Unterschied zwischen den Fluss- und Seefischen bestehen. Die begleitende Genexpressionsanalyse suggeriert grundlegende Unterschiede in den regulatorischen Mechanismen als Grundlage der unterschiedlichen Parasitenresistenz.
In einem Nachfolgeexperiment wurden dieselben Populationen bis zu neun Monate lang einem natürlichen Fluss- bzw. Seehabitat ausgesetzt. Dies erlaubt es die vorherige Hypothese umweltabhängiger paralleler Evolution zu differenzierten Ökotypen in einem direkten Vergleich und unter natürlichen Bedingungen zu testen. Die vorläufigen Ergebnisse dieses Versuchs bestätigen, zumindest Teilweise, die vorherigen Beobachtungen. Wie erwartet, haben die deutschen Seefische, zumindest anfangs, eine deutlich niedrigere Parasitenbelastung. Im Allgemeinen scheint die Übereinstimmung von Ökotyp und Habitat einen Vorteil darzustellen. Aufgrund vergleichsweise niedrigem Parasitenaufkommens sind die deutschen Seefische allerdings nicht in der Lage von ihrer erhöhten Resistenz zu profitieren und wachsen deutlich schlechter als die Flussfische, unabhängig von deren Herkunft. Die Anpassung an ein Habitat mit vergleichsweise niedrigen Parasitendruck hat in diesen Populationen zu erhöhten Wachstumsraten geführt. Der geringe parasitäre Druck während des Versuchs erlaubt es ihnen grösser zu werden, ohne gleichzeitig einer erhöhten Parasitenbelastung anheim zu fallen.

Abschließend habe ich mich mit Hilfe von olfaktorischen Partnerwahlexperimenten mit der sexuellen Selektion von optimal angepassten Genotypen befasst. Der Hauptfaktor bei der olfaktorischen Partnerwahl in Stichlingen sind allele des sogenannten Haupthistokompatibilitätskomplexes (HHK). Diese sind eine Hauptkomponente der Erkennungsmaschinerie des Immunsystems und spielen demnach

eine ausschlaggebende Rolle in der Parasitenresistenz. Mit Hilfe synthetische hergestellter HHK Peptide bin ich in der Lage zu zeigen das Weibchen ihre Partnerwahl ausschließlich in Abhängigkeit der Anzahl unterschiedlicher Allele treffen. Hierzu werden diese sowohl mit den eigenen Allelen als auch mit dem populationsspezifischen Optimum verglichen, unabhängig von der Herkunft des einzelnen Männchens.

Zusätzlich zum HHK Signal scheiden männliche Stichlinge einen sogenannten Validierungsfaktor (VF) aus, dieser signalisiert Artspezifität und bestätigt dadurch die Herkunft der wahrgenommenen HHK Peptide. Mit Hilfe kanadischer und deutscher Stichlinge demonstriere ich das der VF keinerlei populationsspezifische Informationen vermittelt und evolutionär konserviert ist, ungeachtet des Großen geographischen und genetischen Abstandes zwischen den Populationen.

Zusammenfassend finde ich starke Beweise dafür das konvergente ökologische Faktoren, ins besondere Parasiten, maßgeblich an der Differenzierung zu Fluss- und Seestichlingen beteiligt sind. Diese werden durch Habitat spezifische Anpassungen charakterisiert welche, unabhängig von dem evolutionären und genetischen Hintergrund zur weltweit wiederkehrenden Differenzierung der einzelnen Ökotypen führt.

Durch sexuelle Selektion voneinander isoliert, könnte dies potenziell zur Evolution von differenzierten Unterarten führen, welche durch ihr Habitat nicht aber ihre Herkunft charakterisiert werden.

Introduction:

Evolution and ecology:

Ever since Darwin's 1859 "survival of the fittest" it has become increasingly evident that evolution is the driving force behind the appearance, establishment and disappearance of species and taxa. However, evolution of traits or characteristics can only be understood in the environmental context they evolved under. Hence, to understand the processes leading to individual divergence and ultimately speciation it is crucial to investigate them in the environmental or ecological setting leading up to their emergence and establishment. The unique circumstances and pressures of each environment forcing an individual to adapt or perish are the fundamental framework shaping the direction of evolutionary steps. Inheritable individual differences, caused either by random mutation events or selection, can be understood as a constant process following a "trial and error" approach where the better adapted phenotypes flourish and proliferate in a given environment. By outcompeting less beneficial mutations new phenotypes establish themselves in the population and, over time, differentiate themselves from different populations facing other, varying environmental challenges. In combination with other factors such as a lack of gene flow, either of geographic, genetic or behavioral origin, these different populations will drift further apart until they are no longer compatible (e.g. cannot produce fertile offspring) and are therefore, by definition, no longer the same species (Dobzhansky 1935). Understanding the mechanisms and underlying principles of these changes has led to numerous scientific studies and observation, all attempting to shed light on the evolutionary processes responsible for the incredible species diversity we see today.

Speciation, in its simplest form, can be understood as the separation of an initially homogenous population into two distinct sub-populations. Caused by geographical change (e.g. formation of a mountain range), different environmental factors, genetic drift and random mutations will, over time, lead to the divergence of the two sub-populations, potentially to such extends were they are reproductively isolated and no longer capable of genetic exchange (Mayr 1942, Dobzhansky 1937). A subtype of this so-called allopatric speciation, peripatric speciation, can be found following founder events, often linked to bottlenecks, were a small subset of the population becomes geographically isolated (e.g. on an island, Darwin 1859). Here, likely faced with new environmental circumstances, the population is more susceptible to genetic drift due to its small gene pool which also allows beneficial mutations to spread more quickly, thus accelerating the divergence.

However, alternatively to both the aforementioned allopatric and peripatric speciation, the segregation must not necessarily be of geographic nature but can be the result of different ecological niches (i.e. parapatric speciation, Maynard Smith 1966, Felsenstein 1981). Here, individuals from our initially homogenous population will exploit different ecological resources, gradually optimizing their phenotype and eventually genotype, enabling them to harness their specific niche more effectively. Besides potential spatial separation within the same environment, the different niches will demand varying adaptations which should, in theory, be select for during sexual selection, thus reinforcing both the niche dependent phenotype and the divergence within the population. Further, reinforcing selection will discriminate against hybridization if the crosses are either sterile or inferior with regards to niche exploitation. Thus, despite occupying the same habitat, parapatric speciation can drive the genesis of two, more or less distinct species within the same habitat. Finally, speciation can also occur alongside the ancestral population (eventually species) within the same habitat (i.e. sympatric speciation, Darwin 1859, Bush 1969). Following random genetic mutations, mutants within the population might start to interbreed, either due to incompatibility with the ancestral species or by actively selecting for the new mutant phenotype. Eventually, they will become reproductively isolated and thus form a new species, sharing the environment with the now distinct ancestral species. It is however important to note that the above mentioned forms of speciation are not mutually exclusive, in fact, they often interplay in the species formation process.

Another fascinating aspect of evolution and species divergence is the speed at which these processes occur. Often, drastic changes in environmental circumstances, for example due to a lack of competition (e.g. extinction of the dinosaurs) greatly increases the pace of speciation (Krug & Jablonski 2012). The availability of unused niches after such an extinction event or following the colonization of relatively empty environments (e.g. Darwin's finches, Grant & Grant 2011) allows for quick radiation from a common ancestor towards many different sub-species and finally species. In the absence of competition for resources, both with different as well as the ancestral species, new phenotypes can quickly establish themselves (Nosil 2012). However, the opposite also holds true as competition for resources can drive niche picking, allowing access to new, formally unused resources, consequently also favoring divergence and potentially speciation. Finally, the climate itself is a major factor influencing adaptation and evolution, usually changing at a slow but constant pace (e.g. glaciation, Barnosky & Kraatz 2007), resulting in the occurrence of equally slow but constant evolutionary adaptations. However, drastic weather events (e.g. hurricane) can radically shift the evolutionary direction and even the distribution of certain traits in an entire population (e.g. lizard body shape, Donihue et al., 2018). In the current light of

rapid climate change, likely a result of global warming, the pressure to adapt to new and unexpected environmental factors will increase and most likely increase in pace.

On a pre-speciation level, differences between or changes within the environmental circumstances and pressures can drive the divergence of populations into specific, habitat dependent ecotypes (e.g. clinal speciation as proposed by Fisher 1930). Such an ecotype encloses a subset of individuals from the same species which have evolved phenotypic adaptations to a specific environment (e.g. lake vs. river). Intriguing examples of speciation driving the evolution of distinct ecotypes occur in the presence of both host-parasite and predator-prey dynamics (e.g. Lenz et al., 2013). In contrast to classical ecological parameters (e.g. temperature) they themselves undergo directional adaptations (Schmid-Hempel 2011). Upon infection, the host immune system rages a war against the parasite, resulting in an evolutionary arms race between as well as within species, nick named “Red Queen Hypothesis” by Leigh van Valen (1973) based on Lewis Carols “Through the looking glass”. The context of which being that of an evolutionary competition were the parasite and the host have to run (i.e. adapt) as fast as possible to stay in place (head-to-head), just like Alice and the Red Queen in the story. Following the concept of a parasitic lifestyle, these organisms exploit their host for their own benefit, often at the expense of the host itself. The extend of this exploitation varies greatly between parasite species, ranging from commensalistic symbiosis in the form of simple hitchhikers in or on the host (e.g. Remoras (*Echeneidae sp.*)) over to invasive parasites which actively deprive their host of resources (e.g. Tapeworms (*Cestoda)*) all the way to manipulating the hosts phenotype to their own benefit (e.g. *Toxoplasma gondii*), with often detrimental and potentially lethal consequences for the parasitized individual. Consequently, it is in the host’s best interest to invest resources into (immunological) countermeasures, preventing or restricting the parasite’s grasp. Although costly, this investment greatly outweighs the negative consequences of unchecked parasite infections, especially in cases were parasitism results in the complete inhibition of reproductive output (e.g. castration of male crabs by Sacculina *sp.)* or death. In turn, the parasite will try to avoid or counteract the host’s defense measures, resulting in the previously mentioned Red Queen dynamic.

Parasitic lifestyles are as numerous as they are diverse, making up approximately 40% of all organisms described today (Dobson et al., 2008). Their life cycles range from rather simple direct transmission (from one host to another, e.g. fleas (*Siphonaptera*)) to complex networks of obligate and intermediate hosts. Finally, the different requirements of the various parasite species, especially with regards to (intermediate) host occurrence means that they vary between different habitats, similar to other

ecological factors such as Temperature. This makes them a crucial component of any ecosystem, broadening the concept of ecological factors, beyond that of simple environmental circumstances as a key driver of speciation.

Three-spined sticklebacks (*Gasterosteus aculeatus*) appear especially suited as a model organism to understand some of the key aspects of this inter-connected network of habitat dependent co-evolutionary dynamics, leading up to ecotypes (Eizaguirre et al., 2012, Feulner et al., 2015). Being targeted by a multitude of both specialized as well as generalist parasite species, the communal composition of which varies between habitats as well as over time; stickleback research helps to greatly increase our understanding of the evolutionary processes underlying differentiation and potentially speciation.

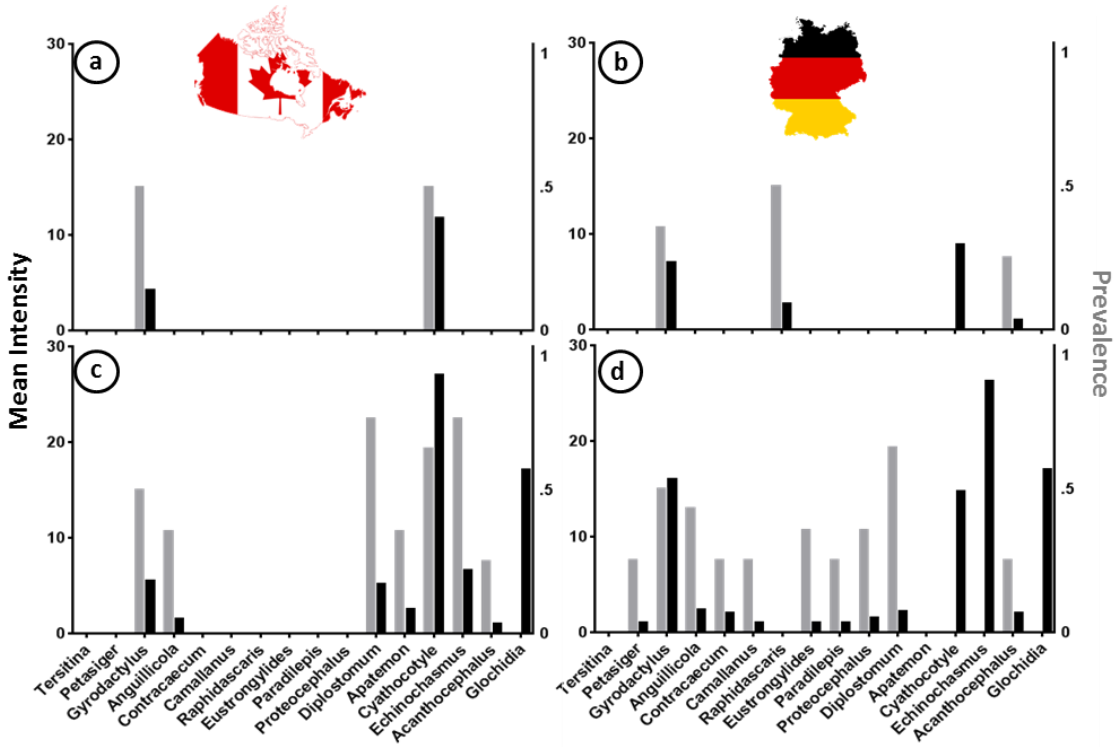
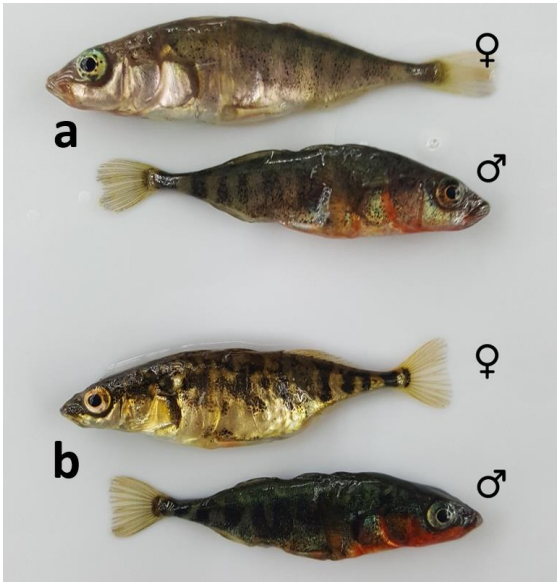


Figure 1: Mean intensity and prevalence of various parasite species from different river (a & b) and lake (c & d) of wild caught fish from Canada and Germany, collected in summer of 2015. For specific details on parameter calculation see Kalbet et al., 2006.

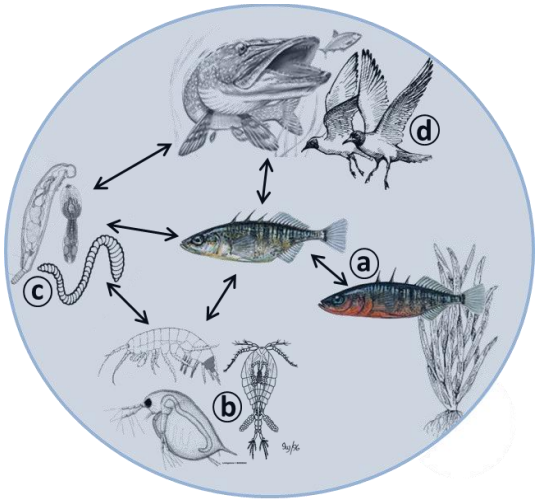
Three-spined sticklebacks:

First scientifically described by Carl Linnaeus in 1758, the three-spine stickleback (*Gasterosteus aculeatus*) is a small (approximately 3-5cm) teleost fish with a short life span averaging one year. Since the receding of the ice shelfs after the last glaciation period (approximately 12.000 years ago), marine sticklebacks have repeatedly colonized fresh water habitats across the entire northern hemisphere (North of 30˚ of latitude, Bell & Foster 1994; Jones et al. 2012). A process which is greatly facilitated by brackish and anadromous stickleback populations, migrating between marine and fresh water habitats. While all modern freshwater sticklebacks are characterized by a set of dorsal and pectoral spines (with a few exceptions, e.g. Giles 1983), serving predatory defense, marine sticklebacks are additionally covered in bony plates along their flanks. This external skeleton has been greatly reduced and even lost in fresh water sticklebacks, most probably due to a combination of different chemical properties of fresh water, making it difficult to accumulate enough resources (i.e. calcium ions) to develop and maintain the lateral plates (e.g. Bell 2001) as well as a shift in predation pressure. Consequently, stickleback populations are very polymorphic with regards to predatory defense as well as food (gill rakers, e.g. Peichel et al., 2001) or habitat dependent adaptations (e.g. body shape, Reimchen et al., 2013).

Sticklebacks are sexually dimorph; females are dull and usually larger than sexually mature male sticklebacks with their characteristic bright blue eyes and vividly red colored throats (Fig. 1). The latter results from carotenoids, which cannot be synthesized de-novo and are an essential part of the immune system's defense against pathogens (e.g. Chew & Park 2004), suggesting a trade-off between coloration and immune defense. Hence, the brightness of the male coloration is a good reflection of its body condition, associated with parasite load (Milinski & Bakker, 1990) and used by the females to determine mate quality, a text book example of the handicap theory of sexual selection.



<u>Figure 1:</u> Example of sexually mature river (a) and lake (b) three-spined sticklebacks from northern Germany.



*<u>Figure **2**:</u> Simplified network of the varying factors influencing and co-evolving with the Stickleback model system. Besides mate choice (**a**) and the environment itself (**blue circle**) these include predator-prey dynamics (**b** & **d**) and co-evolving parasite species (**c**).*

Mate choice in sticklebacks:

Three-spine sticklebacks are most commonly known for their elaborate courtship behaviors (Tinbergen 1951, Wootton 1976). In spring, male sticklebacks establish small territories were they build a nest out of sand and plant material, which they vigorously defend against conspecifics. The materials are glued together using so-called spiggin (Seear et al., 2015) produced from the hypertrophied body kidney of male sticklebacks in breeding condition. Male sticklebacks make use of a variety of different sensory signals to attract ripe (i.e. ready to spawn) females to their nest. After completion of the nest, the male alternates between so-called gluing behaviour during which he re-applies chemical cues onto the nest and fanning, a characteristic behaviour were the male stands above the nest and uses its fins to create a current, thus dispersing the chemical signal. By doing so he creates a scent trail in the water, advertising some of his genetic characteristics to surrounding female sticklebacks (see below for more detail). Once in the vicinity of the nest, female sticklebacks demonstrate their willingness to mate by standing in a 45˚ angle in the water, presenting their swollen abdomen (filled with eggs) to the male. This stimulus will cause the males to perform a ritualized zig-zag dance, while physically stimulating the female and guiding her to the entrance of the nest (Tinbergen, 1951; B. E. Kynard 1978, M. Reiss 1984). The female then proceeds to creep through the nest while laying her eggs, closely followed by the male who inseminates the clutch. After fertilization the male sticklebacks take care of the brood and defend the offspring until hatching whilst trying to attract more females to the nest.

Numerous publications have investigated the decision making of female sticklebacks resulting in a rather good understanding of the mechanisms and processes involved (e.g. Milinski & Bakker 1990, Rowland et al., 1995, Milinski et al., 2005, Andersson & Simmons 2006). The chemical signal, used to attract females to the nest is a combination of two components. The so-called male validation factor (MVF; Milinski et al., 2009) probably signaling the species (i.e. three-spine stickleback) as well as validating the second component; the male's major histocompatibility complex (MHC; Milinski et al., 2003 & 2005) profile. The MHC ligand peptides excreted by the male do not differ, as such, from those of other vertebrates, hence the MVF might signal the species identity. Comparing profiles of different suitors in relation to the females own profile as well as in relationship to the population specific optimum, allows to accurately predict the immunological compatibility with and suitability of the potential mate in the current environmental context. After assessing the males MHC alleles, a female will estimate the male's health status (e.g. through coloration, Milinski & Bakker 1990) to ensure that the presented MHC alleles convey resistance in the current environment. Finally, before entering the nest, the female will perform so-

called “nosing” were she probes the nest entrance, likely to ensure that the perceived MHC alleles originate from that particular nest and, by assessing the strength of the signal, further validating the perceived health status.

Due to their small size, availability and robustness, three-spine sticklebacks are relatively easy to keep in a laboratory setting and continuously gain in popularity as a model organism. This is further promoted by the increasing availability of genetic tools, giving rise to new fields of research in non-classical model organism such as three-spined sticklebacks (Kingsley et al., 2004, Gibson 2005).Varying environmental circumstances in different fresh water habitats drive the differentiation of stickleback populations into specifically adapted ecotypes. Either by differences in their habitat (river vs. lake, Tobi/Chris et al.,) or as a consequence of different niche picking within the same habitat (e.g. limnetic vs benthic, Schluter & McPhail 1992), these so-called ecotype pairs are, likely, on the brink of speciation. Accelerated by sexual selection for conspecifics and thus selecting against less optimally adapted phenotypes, the divergence increases in a variety of different traits ranging from body size and morphology (e.g. Zimmerman 2007) to different immune defense strategies (e.g. Eizaguirre et al., 20XX), where needed. These properties have led to the establishment of sticklebacks as a model organism for eco-evolutionary dynamics (e.g. Rundle et al., 2003), habitat specific adaptation (e.g. Schluter 1993) and host-parasite co-evolution research (e.g. Barber & Scharsack 2009).

Fish immuntiy and parasites:

Fish have a variety of different defense strategies against pathogens and parasites. Their body is covered with either scales or a dermis and coated in immunologically active mucus (e.g. Shephard 1994) serving as a first line of defense against intruders. Although a variety of parasite taxa are able to penetrate this outer defense to infect the fish (e.g. *Diplstomum sp.*, Niewiadomska 1984) or simply live on the outside of the animal (e.g. *Gyrodactylus sp.*, Nordmann 1832), most parasites enter their fish host via the gills or through the digestive system. Despite lacking the mechanical protection of scales or a dermis, the gills are still covered in the same mucus and thus protected to some degree. Consequently, a majority of parasites enter their fish host via the alimentary pathway. Usually hidden inside an intermediate host (e.g. Schistocephalus solidus (Müller 1776) inside a copepod) they either remain inside the digestive tract were they cling to the gut and leach nutrients from the fish (e.g. *Acanthocephalus sp., Müller 1780*) or, alternatively, cross the gut tissue to establish themselves in the body cavity or a variety of different tissues and organs (e.g. *Contracaecum sp.*, Railliet & Henry 1912). In general, sticklebacks are an intermediate host for most parasite species which require the stickleback to be eaten by a piscivorous

fish or bird to complete their life-cycle. To increase their odds, some parasites manipulate their host's behaviour actively (e.g. *Schistocephalus solidus*) or passively (e.g. *Diplostomum sp.*).

In contrast to non-vertebrates, fish can make use of two interwoven defense regimes, the innate and the adaptive immune system. The innate immune system forms the first line of immunologic defense using a variety of general mechanisms. The two main components of which are the complement system, a strongly developed system in fish whose role is the destruction of cell surface structures, thus counteracting foreign elements. The second major innate line of defense is inflammation, counteracting tissue damage and recruiting immune defense mechanisms to the inflammation site. Were as the innate immune system is very old from an evolutionary perspective and present in all animal species, the adaptive immune system only occurs in vertebrates. As fish are the oldest vertebrate class, they are becoming increasingly popular for investigation and understanding the evolution of the adaptive immune system and its interplay with innate immunity.

As its name suggests, the former can adapt to new, unknown threats and remembers previous immunological challenges. The main involved cell types are T- and B-lymphocytes which closely interact to recognize and counteract immunological challenges. B-lymphocytes, originating from the bone marrow (in mammals, Kondo 2010), the Bursa Fabricii (in birds, Glick et al., 1956) or the kidney in teleost fish (Zapata 1979), are the antibody producing cells of the immune system. To this end B-cells recognize and bind circulating antigens, migrate to the so-called "germinal centers" (in the spleen and lymphoid organs) and proliferate to greatly increase the antibody production. However, they also require a second signal from T_h cells, bound to peptides of the same antigens, for activation. This mechanism ensures protection from self-recognition and thus auto immune disease. (Surh & Sprent 1994)

Despite being present in fish, the adaptive immune system is rather rudimentary and slow compared to that of higher vertebrates and has been shown to need approximately 6 weeks to be functionally active after an initial immunological challenge (Whyte et al., 1987)

Immune system and the MHC

In general, the idea of mate choice aims at selecting the best suited mate to sire well adapted offspring. MHC plays a major role in immunity (see MHC paragraph for detail) and is therefore directly correlated with parasite resistance (e.g. Kurtz et al., 2004, Consuegra & de Leaniz 2008) and consequently survival and fitness. This direct link between non-random mating and functional relevance of habitat-adapted

alleles has been dubbed “magic trait” by Gavrilets (2004) and can be seen as a powerful accelerator of ecological speciation (Eizaguirre et al., 2009)

Cellular components are secreted from the body as a byproduct of cellular brake down. Among these components are membrane bound MHC molecules, most interestingly the anchor amino acids of the MHC peptide ligands, which are structural mirror images of the genetically encoded binding grooves of the MHC molecules. As these disperse from the body, they can be picked up through chemo-sensory receptors of conspecifics, effectively serving as pheromones. Olfactory receptors, located in the vomeronasal organ (e.g. mice, Leinders-Zufall et al., 2000), are able to recognize the differences between these molecules, allowing to precisely recognize specific differences between each individuals (unique) MHC profile. As MHC alleles differ in the immunity they convey, ideally one would aim at maximizing the amount of alleles to increase immune defenses. However, due to negative T-cell selection in the thymus, there is a direct trade of between an individual’s number of MHC alleles and the amount of mature T-cell clones. Resulting in an optimal amount of MHC alleles, correlating with the highest parasite resistance for that particular population (Wegner et al., 2003). As parasite prevalence and intensity varies between different habitats this optimum is shifted in a habitat dependent manner.

The MHC:

The major histocompatibility complex (MHC) and its human equivalent, the human leukocyte antigen (HLA) can be distinguished into two major clades, MHC I and II. MHC I is a mediator of so-called cellular immunity, responsible for the destruction of infected or malignant host cells (Janeway 2001). MHC II on the other hand functions as a peptide presenting cell surface marker, allowing the immune system (T-cells) to distinguish self from non-self (J. Klein, 1986) and consequently plays a major role in immune system effectivity and function of all vertebrate species (reviewed by C. Janeway 2001). Each individual MHC allele confers specific resistances against pathogens and parasites (Abbas, Lichtman & Pillai 2015). Based on varying parasite communities and changing immunological challenges, high allelic variation (i.e. polymorphism) in the MHC locus (on a population level) is a functional necessity (e.g. rare alleles and frequency dependent selection; Takahata & Nei 1990) driven by changing immunological pressures (i.e. pathogens; Paterson et al., 1998; Wegner et al., 2003, Meyer-Lucht & Sommer 2005) and maintained by sexual selection (Hamilton & Zuk, 1982; Hamilton et al., 1990). As maximizing MHC allele diversity would maximize both early pathogen recognition and defense (heterozygote advantage; Doherty & Zinkernagel 1975), mate choice should aim at maximizing offspring MHC heterozygosity (J. Brown, 1997; Apanius et al., 1997). However, negative T-cell selection in the thymus, preventing auto-

immune disease (Surh & Sprent 1994, see also Ed Palmer, 2003), inhibits infinite numbers of MHC alleles on an individual level. Hence, on the individual level, there are a finite or optimal number of MHC alleles which convey maximum immunity without jeopardizing the available T-cell count in the current environmental context. Because each MHC allele contributes different immune advantages, the MHC is highly polymorphic on a population level and varies tremendously between each individual. Further, co-evolution with pathogens increases the benefits of rare alleles and maintains these in the gene pool.

Thesis overview:

In my thesis I targeted some of the main questions of habitat dependent adaptations as a major factor leading to the divergence of separate ecotypes. Using the intricate network of environmental adaptation and host-parasite co-evolution found in the three-spined stickleback model system as a framework (Fig. 1). I raised the question whether analogous environments, including the occurring parasite pressures, lead to the parallel evolution and maintenance of diverging ecotypes, both on a phenotypic and molecular level.

To this end I performed several experiments aimed at understanding some of the key aspects of stickleback evolution. More precisely, the aim was to show how habitat dependent adaptations can lead to parallel evolution and consequently the formation of ecotypes, specialized to the type of environment they evolved in and independent of the genetic or geographical origin of the population.

Approaching the idea of habitat dependent ecotypes I exposed naïve lab reared stickleback offspring of distinct ecological (river and lake) and geographical (Canada and Germany) origin to a typical German lake parasite (*Diplostomum pseudospathaceum*), allowing me to demonstrate the global validity of this assumption. These different ecotypes varied with regards to parasite resistance as well as immune gene expression in a habitat dependent manner. Thus showing that habitat dependent ecological factors, mainly parasite pressure, have led to the establishment of distinct river and lake ecotypes.

By measuring the expression of several key immune genes, I am able to show that there are reoccurring parallels in the parasitic immune defense of river and lake sticklebacks, independently of the geographical distance between the ecotypes. Thus demonstrating the global validity of my assumptions in a controlled laboratory setting (see **Chapter I**).

Following up on these results, I tested the idea of global parallels between ecotypes in a common garden experiment by exposing the same fish populations to a river and lake system and monitoring

their survival, growth and parasite burden over several months**.** This allowed me to directly compare habitat dependent adaptations within ecotypes as well as between ecotypes of different origin in the same environmental background.

One prerequisite enabling differentiation or radiation into distinct ecotypes, potentially leading to speciation, is genetic isolation. Despite genetic compatibility in the lab, the different stickleback ecotypes seldom interbreed in the wild (e.g. Gow et al., 2007, Jones et al., 2006). This led me to investigate female mate preference in a habitat dependent manner, assuming that mate choice is habitat specific, discriminates against migration of other ecotypes as well as selecting against sub-optimally adapted individuals (see Chapter III).

In summary, these experiments show the potential of parasite driven local adaptation as a pathway to speciation. Habitat specific immunity shapes both the direction of ecotypic adaptation as well as strongly influencing sexual selection against migrants, effectively resulting in a reproductive barrier which provides the frame work for speciation through genetic isolation.

Thesis Outline:

In my thesis I investigate the environmental pressures and underlying mechanisms of environmental speciation in the three-spined stickleback (*Gasterosteus aculeatus*). It is divided into four chapters which are written as manuscripts, each with a separate abstract, introduction, material & methods, results and discussion section. Chapter III is published, chapter IV is currently under revision, chapter I & II will be submitted shortly. My individual contribution to each Chapter can be found in the "author contribution" section of this thesis.

Chapter I:

In this experiment I tested whether the adaptation to a river or lake habitat by three-spined sticklebacks results in a similar parasite resistance phenotype, independently of the fish or parasite's origin. To this end I exposed lab reared naïve sticklebacks originating from Canadian and German lakes and rivers to a typical German lake parasite (*Diplostomum pseudospathaceum*). This experiment was conducted over two consecutive years to control for natural variation in both the parasite as well as the fish. Each treated fish was exposed to 100 cercariae (i.e. infectious stage of the parasite) for 24h before been sacrificed. Parasite numbers were recorded and the head kidneys extracted, processed and analyzed for the expression of pre-selected target genes, associated with the immune system and related processes.

Chapter II:

Following up on the previous experiment (i.e. Chapter I), the aim was to replicate my findings in a common garden experiment. By exposing the same Canadian and German populations to a river and lake environment, I can test the general validity of the previous findings under the influence of natural conditions. To this end a total of n=1248 fish were exposed to either the river or lake habitat for up to 9 months, spanning nearly the entire natural life cycle of a wild stickleback. Sampling a subset of these individuals at three distinct time points, reflective of the different seasons and life history stages of wild sticklebacks, allows me to analyze how well the phenotypes fair and develop over the course of the exposure. A small subset of the experimental fish remained in the lab for the entirety of the study, serving as negative or base line controls they were kept at matching temperatures and light regimes with the exterior conditions. To deepen our understanding of the underlying biological mechanisms and processes, we performed full transcriptome analysis on a representative subset of the individuals.

Chapter III:

One prerequisite of speciation is genetic isolation, for example through sexual selection. Performing olfactory mate choice trials, I investigate how females evaluate one of the key olfactory cues, MHC peptides. By using artificially synthesized MHC peptides in combination with the natural male validation factor signal, I can manipulate what the female perceives. Using fish from two distinct ecotypes with differing MHC optima allows me to show the selection criteria the females apply during the decision making process of sexual selection.

Chapter IV:

To date, we know that the male validation factor does not convey any population or habitat specific information in connected rivers and lakes. To test whether it is also evolutionary conserved on a global scale, I performed mate choice trials with a Canadian and German stickleback population. Testing female preference in a flow channel solely based on the male's validation factor and in the absence of MHC signaling, allows me to investigate the information content of the male validation factor.

Chapter I:

Habitat dependent (parallel) evolution of parasite resistance

in geographically distinct river-lake sticklebacks (*Gasterosteus aculeatus*)

Unsubmitted manuscript

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Abstract:

Contrasting environmental pressures can drive species divergence via adaptive evolution. Populations from different habitat types should show increased divergence whereas similar environmental requirements would be expect to drive parallel evolution. A major environmental factor imposing differential adaptations across habitats types is parasite-mediated selection. As the diversity and abundance of parasites vary between but not within habitat types, theory predicts parallel evolution of resistance. In a controlled single infection experiment, we tested the resistance of river and lake three-spined stickleback populations from Canada and Germany to a typical lake parasite from northern Germany, *Diplostomum pseudospathaceum*. We found significant differences in the susceptibility to the parasite in a habitat dependent manner with the lake habitat suffering lower infection rates than its river counterpart. Furthermore, investigating patterns of genes expression for 27 candidate genes, we could demonstrate that parallel patterns of infection phenotypes are also reflected in parallel patterns of gene expression between fish ecotypes. In general, habitat specific adaptations to high or low parasite pressures seems to be transferable to comparable environmental circumstances but does not necessarily overweigh local adaptation to a specific parasite species.

Introduction:

Varying local environmental pressures have the potential to drive the evolution of locally adapted populations through divergent selection (Savolainen et al., 2013). Local adaptation then can form the basis for population genetic structure to emerge culminating in speciation in certain cases (Kirkpatick & Barton 2006, Eizaguirre et al., 2009, Lenormand et al., 2012, Picq et al., 2016). The environmental changes underlying this so-called ecological speciation (Schluter 2009) can be influenced both by climate change and colonization events (reviewed by Rundle & Nosil 2005). Reversely, similar environmental circumstances are likely to impose similar pressures, resulting in habitat dependent parallel evolution towards similar phenotypes with underlying genotypes (Schluter & Nagel 1995, Fraser et al., 2011). A famous example can be found in the Anolis lizards who, isolated on several islands of the greater Antilles, have radiated into a multitude of habitat dependent phenotypes (Rand and Williams 1969, Williams 1972 & 1983, Losos 2011). The evolutionary history of the three-spined stickleback Gasterosteus aculeatus offers a further example for local adaptation as the key driver for parallel or convergent evolution, in a habitat dependent manner (Feulner et al., 2015, Berner et al., 2009).

Since the receding of the ice shelf at the end of the last glaciation period (approximately 12000 years ago,) marine sticklebacks have repeatedly colonized freshwater lakes and rivers of the Northern Hemisphere (Mäkinen et al., 2006, Bell & Foster 1994, McKinnon & Rundle 2002). The changes in environmental condition have driven the adaptation of these fish towards habitat specific optima, resulting in different "ecotype pairs" along the speciation continuum (Schluter & McPhail 1992, Mäkinen et al., 2006; Jones et al., 2012, Feulner et al., 2015). One such specific ecotype pair has evolved in rivers and lakes across the entire Northern Hemisphere (e.g. Berner et al., 2008, Eizaguirre et al., 2011). Populations from these two habitats cluster into clades (river and lake, Feulner et al., 2015) independently of their migratory background (Reusch et al., 2001), shown for northern Germany. Besides ecological factors such as salinity, water flow and food availability, the variation in parasite communities is a major factor shaping local adaptation of stickleback river-lake ecotypes (Wegner et al., 2003; Eizaguirre et al., 2009, Eizaguirre et al., 2012). Their major fitness impacts reduces reproduction, growth and survival (Pennycuick 1971, Wootton 1976) but parasites also influence the outcome of sexual selection and combined with local adaptation result in assortative mating (Eizaguirre et al., 2011, Andreou et al., 2017, Gahr et al., 2018).

In contrast to lake three-spined sticklebacks, witch harbour a heavy parasite load composed of a large parasite richness, river sticklebacks harbor a reduce parasite richness, where each taxon typically occurs in large numbers (e.g. *Gyrodactylus sp.*, Johnson & Jenser 1991, Eizaguirre et al., 2012). Following data obtained in field surveys, this disparity between parasite species and abundance appear to be a reoccurring phenomenon in lake-river comparisons (Feulner et al., 2015). These re-occurring environmental pressures impose similar challenges on the exposed populations, potentially leading to comparable phenotypic solutions. Hence, we hypothesized that local resistance genotypes of sticklebacks are habitat dependent, based on the parasite communities and consistent within habitat types, independently of geographical distance between the populations (Eizaguirre et al., 2009). If parasites are indeed the major selective force driving local adaptation towards lake and river ecotypes, comparable parasite pressures should have resulted in similar gene expression patterns of genes associated to parasite resistance and immunity. Ultimately, comparable habitats might then result in parallel evolution of similar phenotypes and thus genotypes (e.g. Eizaguirre et al., 2012, Feulner et al., 2015).

To investigate this "parallel adaptation hypothesis" in an experimental approach, we exposed naïve laboratory reared sticklebacks from German and Canadian river-lake pairs to a typical lake parasite

(Diplostomum pseudospathaceum). D. pseudospathaceum is a generalist parasite with regards to host preference and typically occurs in large numbers in slow flowing (i.e. lake) habitats in northern Germany, due to the requirements of its first intermediate snail host Limnea stagnalis (Niewiadomska 1984). By establishing itself in the immunologically privileged eye lenses it does not only evade the host's immune defenses but also greatly increases the fish's predation risk by piscivorous birds, the final host for D. pseudospathaceum. Besides their different geographic origin, these stickleback populations also differ in their genetic background, descending from either an Atlantic (N. Europe) or a Pacific (Canada) marine stickleback population, which have diverged approximately 31-59 thousand years ago (Fang et al., 2018). Assuming habitat specific adaptations, we expected the lake fish to have higher resistance phenotypes against the typical lake parasite than the river ecotypes. As D. pseudospathaceum is a common lake parasite in northern Germany (Kalbe & Kurtz 2006), the German populations are expected to have co-evolved with the local parasite species and should therefore be more resistant that the Canadian fish. However, the Canadian lake habitat harbors comparable parasite species diversity which should have led to the evolution of defensive strategies against generalist parasites in these fish. They might therefore have the potential to resist the parasitic infection in a similar fashion to the German populations.

Material & Methods:

Experimental Animals:

The three-spined stickleback populations originated from McCreight lake (50˚28'12.4''N, 125˚65'31.7''W) and Amour de Cosmos creek (50˚23'54.3''N, 125˚63'62.9''W) on Vancouver Island, BC, Canada, and from the Großer Plöner See (54˚14'61.0''N, 10˚40'86.9''E) and Westensee (54˚26'89.8''N, 9˚96'09.2''E) lakes in Schleswig-Holstein, Germany and their connected rivers Trave (54˚05'16.7''N, 10˚51'63.1''E) and Eider (54˚16'65.5''N, 10˚07'60.1''E). All fish used in this experiment were in-vitro fertilized offspring of wild fish, caught during the breeding season of 2014 and 2015. After fertilization, the Canadian clutches were cleaned using Acriflavine (Dajana) and Methylene Blue (King British) and shipped to Germany at a constant temperature of 4˚C. To mimic similar conditions, the German clutches were kept in the fridge for 4 days after cleaning; simulating the transport conditions that Canadian clutches had been exposed to. Fish were then incubated and raised in the lab at 18 ˚C, 18:6 Light:Dark with a constant water flow and fed *ad libidum*, until the start of the experiment.

Parasite collection and experimental exposure:

All parasites of *Diplostomum pseudospathaceum (*Niewiadomska 1984) cercariae, originated from wild caught naturally infected snails (*Limnea stagnalis*) collected in the Kleiner Plöner See lake (54˚10'17.6''N, 10˚37'74.4''E). As described in Kalbe & Kurtz (2006), snails were individually placed in small plastic cups with fresh water under a bright light, in order to trigger the *D. pseudospathaceum* cercariae to shed from their snail host. As each snail harbors different *D. pseudospathaceum* clones, we pooled the water from 10 different snails. To standardize parasite exposure, we manually sorted parasites from the mixed pool into petri dishes (100 cercariae/dish).

Experiments took place in October and November of 2014 and 2015 when the fish were approximately six months old. At this age, the fish have not yet reached sexual maturity. Fish were placed individually into 1 liter plastic tanks 24h prior to the parasite exposure to allow for acclimatization. Petri dishes filled with water and 100 cercariae each were then randomly distributed among half the individuals. After 24h of exposure, fish were sacrificed by decapitation and, with exception of the eyes, stored in RNAlater (Sigma) for further gene expression analysis. The eyes were dissected and *D. pseudospathaceum* within the lenses were counted to assess the infection rate. A total of 108 fish were exposed to the parasite in the first cohort (2014) and 187 in the second cohort (2015) with the same amount serving as un-exposed controls. All animal experiments described were approved by the Ministry of Agriculture, Environment and Rural areas in the State of Schleswig-Holstein, Germany.

Gene expression:

For the second cohort of fish (2015) cohort, in addition to repeating the previous exposure experiment, we analyzed the underlying gene expression profiles of fish to understand whether phenotypes of infection match parallel gene expression responses. The head kidneys are the immunologically active organs in fish (reviewed by Gallo & Civinini, 2003), hence we extracted RNA from these using the RNAeasy 96 Kit (Qiagen) following the manufacturer's protocol. RNA concentration was measured by spectrophotometry (NandoDrop, ThermoFisher). Samples were then diluted to 10ng and reversely transcribed into cDNA via Omniscirpt Reverse transcription kit (Qiagen). The obtain cDNA was stored at -80˚C until further analyses. Gene expression was then assessed with the Fluidigm-BioMark™ system using a 96.96 dynamicarray (GE-chip).

First, the target cDNA was pre-amplified with 2.5 µL TaqMan PreAmp MasterMix (Applied Bioscience®) and 0.5 µL STA Primer mix (includes all 32 primer pairs 50 µM diluted in low EDTA-TE buffer) under the following PCR conditions: 10min at 95°C, 14 cycles: 15s at 95°C, 4min at 60°C. PCR products were diluted 1:5 with EDTA-TE buffer. Before loading the chip, an assay mix was prepared containing 0.7 µL of 50 µM primer pair mix, 3.5 µL 2xAssay loading reagent (Fluidigm) and 3.15 µL 1xlow EDTA-TE buffer. A sample mix containing 3.3 µL preamplified cDNA, 3.5 µL 2xSsoFast EvaGreen Supermix with low Rox (BioRAD) and 0.35 µL 20xDNA Binding Dye Sample loading reagent (Fluidigm) was prepared. The chip was primed with control line fluid after which 5 µL assay mix and 5 µL sample mix were loaded onto the chip and measured with Fluidigm-BioMark™ system applying GE-fast 96.96 PCR+Melt v2 protocol according to Fluidigm protocols. Each chip contained a dilution series (1:2, 1:5, 1:10, 1:100, 1:500) of pooled cDNA as a standard, a negative "no template control" (NTC) and a gDNA contamination control (-RT). Technical triplicates and random sample distribution across the chip allowed for technical bias control. We analyzed the gene expression of a total of 27 different genes (Appendix. Table A1) related to innate and adaptive immune functions, oxidative stress, the complementary system and housekeeping genes. Primer sequences have previously been optimized for three-spined sticklebacks by Brunner et al. (2017, Appendix. Table A1).

Statistical analyses:

All statistical analyses of the parasite infection data were performed in RStudio (Version 1.0.136) with mixed effect models using the lmer function (lme4) followed by Tukey post-hoc test and false discovery rate correction for multiple testing. Date of infection and fish family were set as random factors. To characterize differences in gene expression between treatment groups and fish origin, we performed a permutation analysis of variance (PERMANOVA) including all genes and all fish as well as focusing within treatment groups (i.e. exposed and unexposed fish). Furthermore, we investigated the different gene categories (innate immune genes, adaptive immune genes and complement system) separately for difference between treatments (Table 2). As we did not find any differences with regards to both the infection rate and gene expression between the two German Lake populations (here after G.L., Figure 1), these were combined into a single group (G.L.). The same held true for the German River (here after G.R.) populations, thus treating them in identical fashion.

Fluidigm real-time PCR analysis software (Fluidigm) was used to access the amplification profiles of the data and calculate mean cycle time (Ct), standard deviation (SD) and the coefficient of variation (CV) for all technical triplicates. CV is used as an indicator of measurement precision, a CV >0.04 will falsify the

measurement and results in a measurement error (Bookout & Mangelsdorf 2003). Missing data points (0.13% of sample data) were replaced with the mean expression value of the respective gene for that individual's population of origin and treatment group. Subsequently, all analyses were performed with the relative gene expression values (–δCt) computed by the qbase+ software (Biogazelle). Multivariate approaches using PERMANOVA (permutation 10000) from the vegan package (Oksanen et al. 2016) were performed on the entire data set and with the different gene groups (innate and adaptive immune functions, oxidative stress and the complementary), detecting main and interaction effects between fish ecotype (i.e. river or lake), country of origin (i.e. Canada or Germany) and exposure treatment. Finally, we used the fviz_pca function out of the factoextra package (Kassambara & Mundt 2017) for graphical representation of the gene expression data (Fig. 3). Single gene expression variation were evaluated between populations for both treatments separately as well as between treatments within population using mixed effect models (lmer, lme4 package (Bates et al., 2018)) with sex and date of infection set as random factors followed by a Tukey post-hoc test and false discovery rate correction for multiple testing.

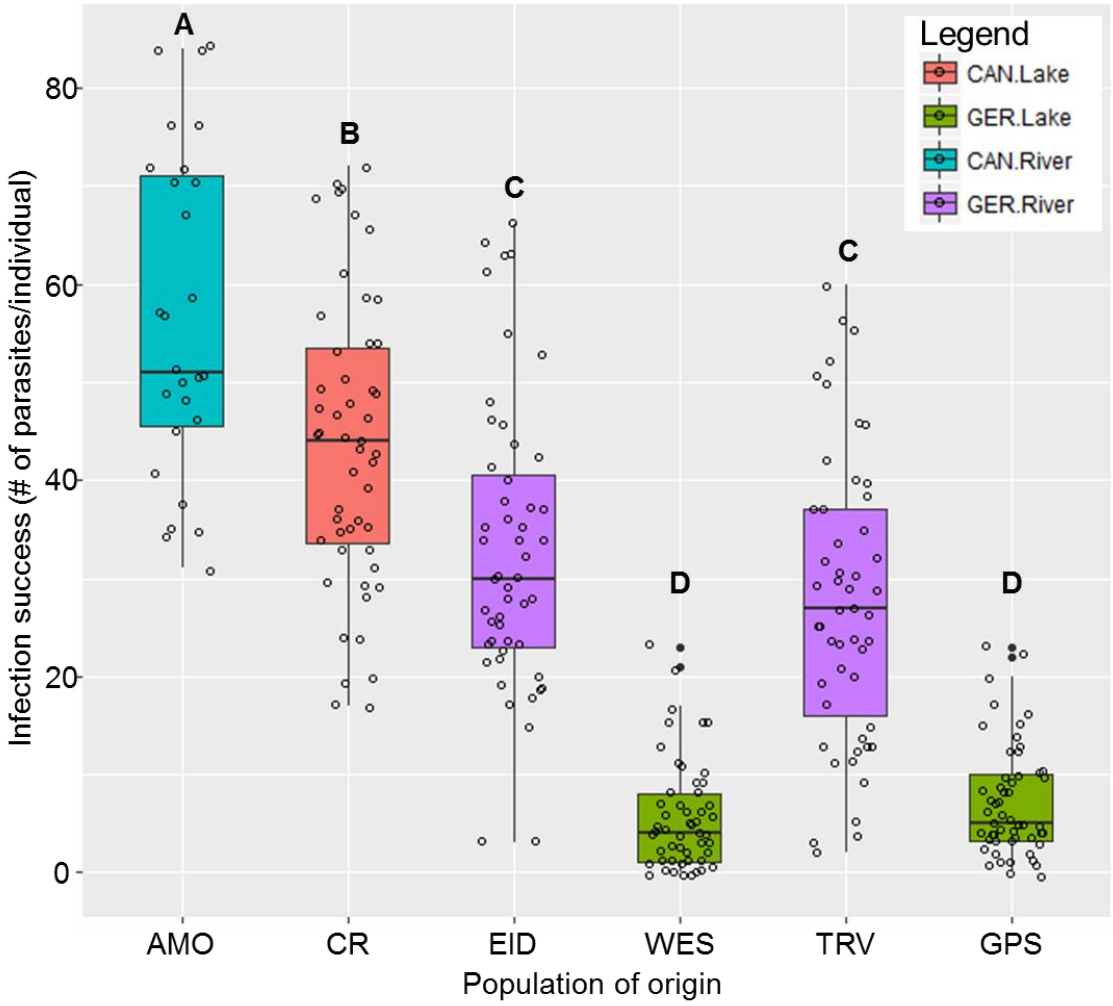
Results:

Survival and infection rate:

After 24 h of exposure to the *D. pseudospathaceum* parasite, we found a significantly higher number of successfully established parasites in the eye lenses of the Canadian as opposed to the German fish ($z=-7.501$, $p<0.001$) as well as in the river when compared with the lake ecotypes ($z=3.674$, $p<0.001$). Furthermore, we found a significant interaction of fish ecotype and country in the susceptibility to the eye fluke ($F=6.066$, $p=0.018$). In general, German fish were more resistant than the Canadian populations and lake fish withstood the parasite infection better than river fish. A post-hoc test revealed all pairwise differences between the different populations to be significant except for the comparisons between the two German river populations as well as the comparison between the two German lakes populations (Table 1, Figure 1). The parasite infection data (Table 1, Figure 1) is based on the pooled data from both the 2014 and 2015 cohort. Due to natural variation in the specific Diplostomum cercariae, we find differences in the infection success between the time points which we corrected for by exposing equal numbers of each population to the parasite at any given time point.

Comparison	Estimate	Std. Error	z	P	significance
CR-AMO	-11.183	3.162	-3.537	0.005	**
EID-AMO	-25.513	3.146	-7.474	<0.001	***
GPS-AMO	-48.629	3.131	-15.529	<0.001	***
TRV-AMO	-27.157	3.146	-8.632	<0.001	***
WES-AMO	-50.047	3.139	-15.946	<0.001	***
EID-CR	-12.331	2.607	-4.731	<0.001	***
GPS-CR	-37.446	2.586	-14.481	<0.001	***
TRV-CR	-15.974	2.607	-6.129	<0.001	***
WES-CR	-38.864	2.596	-14.971	<0.001	***
GPS-EID	-25.115	2.563	-9.801	<0.001	***
TRV-EID	-3.644	2.584	-1.41	0.719	
WES-EID	-26.533	2.573	-10.314	<0.001	***
TRV-GPS	21.472	2.563	8.379	<0.001	***
WES-GPS	-1.418	2.551	-0.556	0.9936	
WES-TRV	-22.89	2.573	-8.896	<0.001	***

Table 1: *Pairwise difference in parasite susceptibility between the different fish populations (AMO=Can. River, CR=Can. Lake, EID & TRV=Ger. River and WES & GPS = Ger. Lake). Results were obtained using a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr).*



<u>Figure 1:</u> The different ecotypes significantly differ from each other in their susceptibility to the D. pseudospathaceum parasite. For visual representation the infection success is shown with medians ± 1st and 2nd quartiles, each dot represents a single individual. The x-axis shows the populations: Canadian river (AMO), Canadian lake (CR), the German rivers (EID & TRV) and the German lakes (GPS & WES) sorted according to watershed.

On a family level, the general pattern of parasite resistance between ecotypes seen in Fig. 1 holds true. Despite the large variation between the different families, these do not significantly differ from each other within habitat type. The overlap between the G.R. and C.L. families is, at least partially, an artifact of random in-vitro fertilization, thus increasing the variation between families

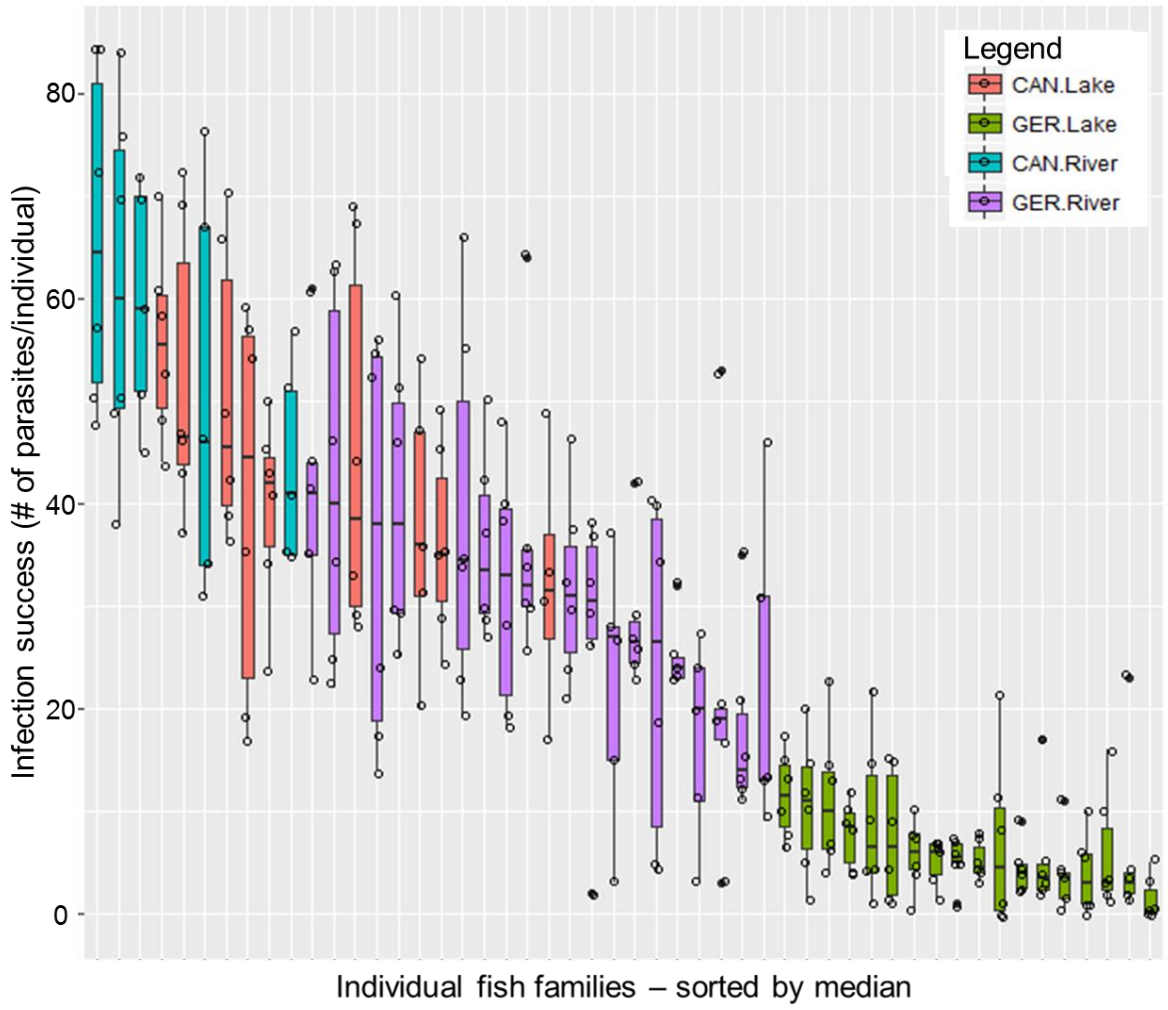


Figure 2. Susceptibility of the individual fish families to the D. pseudospathaceum parasite varies strongly. Yet, the clustering according to ecotype generally holds true. The infection success is shown with medians ± 1st and 2nd quartiles, each dot represents a single individual. For clearer visual representation the families are sorted by their median.

Gene expression:

We found significant differences in the overall gene expression patterns between country, ecotype and their interaction (C:E, Table 2). Further, the gene expression profiles seem to diverge between the two treatment groups (i.e. control and exposed). Looking at the different parts of the immune system in more detail, we find the main changes in gene expression in the innate and adaptive immune system, not so however in genes associated with the complement system.

	All				**Exposed**				**Control**			
					All genes							
	DF	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*
Country	1	10.216	0.026	**0.002**	1	4.578	0.025	**0.035**	1	6.498	0.033	**0.001**
Ecotype	1	2.197	0.006	0.057	1	0.746	0.004	0.413	1	2.049	0.010	0.058
Treat	1	5.072	0.013	**0.001**								
C:E	1	3.547	0.009	**0.019**	1	2.121	0.012	0.187	1	2.307	0.012	0.056
Res	368		0.946		177		0.96		188		0.945	
Total	372		1		180		1		191		1	
					Innate immune system							
	DF	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*
Country	1	4.003	0.01	**0.007**	1	2.445	0.013	0.072	1	2.66	0.014	**0.044**
Ecotype	1	4.005	0.01	**0.011**	1	1.771	0.01	0.091	1	3.063	0.157	0.257
Treat	1	7.370	0.019	**0.001**								
C:E	1	3.159	0.008	**0.026**	1	4.069	0.022	**0.008**	1	1.439	0.007	0.138
Res	368		0.952		177		0.955		188		0.963	
Total	372		1		180		1		191		1	
					Adaptive immune system							
	DF	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*
Country	1	34.830	0.081	**<0.001**	1	16.2	0.808	**0.002**	1	19.833	0.909	**<0.001**
Ecotype	1	4.294	0.01	**0.001**	1	1.726	0.009	0.089	1	2.773	0.127	**0.009**
Treat	1	10.917	0.025	**<0.001**								
C:E	1	12.597	0.029	**<0.001**	1	5.615	0.28	**0.002**	1	7.477	0.034	**0.002**
Res	368		0.855		177		0.883		188		0.862	
Total	372		1		180		1		191		1	
					Complement system							
	DF	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*
Country	1	7.005	0.019	**0.004**	1	2.796	0.0155	0.210	1	4.935	0.253	**0.012**
Ecotype	1	0.436	0.001	0.512	1	0.014	0.0008	0.982	1	0.9143	0.005	0.309
Treat	1	1.906	0.005	0.154				0.697				
C:E	1	1.159	0.003	0.302	1	0.002	0.002		1	1.1689	0.006	0.295
Res	368		0.972		177	0.983	0.983		188		0.964	
Total	372		1		180	1	1		191		1	

*Table 2: Analysis of the gene expression in the different treatment groups as well as for the different gene categories, analyzed using permutational analysis of variance (PERMANOVA) with country (**C**), ecotype (**E**) and the interaction (**C:E**) as factors*

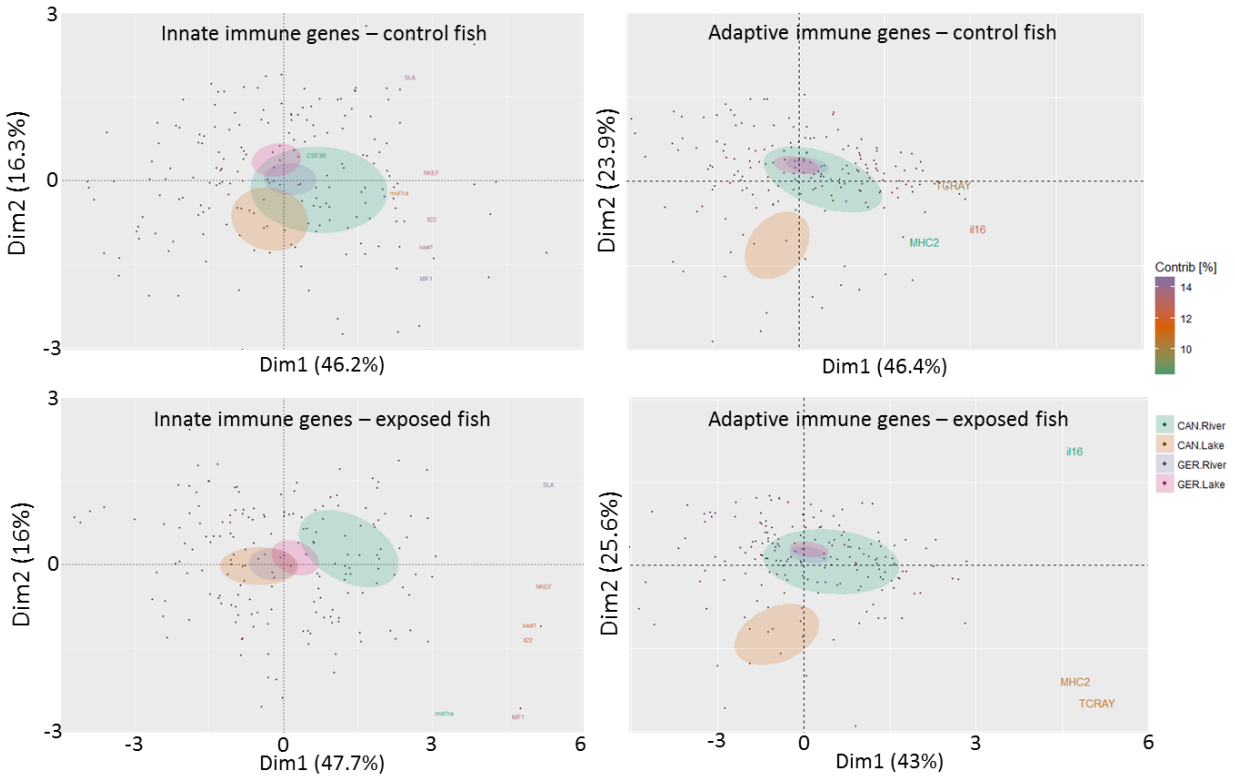
Based on this initial discrepancy between immune gene categories, we analyzed the gene expression differences for each gene separately within gene category (e.g. innate immune genes) between biologically relevant comparisons (i.e. between habitats with the same treatment or between treatments within the same habitat) in more detail (Table 3, general liner model (glm)). Demonstrating that the majority of significant difference between populations occurred in the innate immune system (Table 3, Appendix Fig. A1). With the exception of the Canadian river population, which hardly showed any change in gene expression between treatments. In general, the parasite exposure seems to provoke an upregulation of innate immunity while, simultaneously, downregulating the adaptive immune gene function. Between populations, the number of differently expressed genes seems to parallel the increase of parasite susceptibility between the populations, with the exception of the aforementioned Canadian river fish.

Compared Groups		Estimate	Std. Error	z value	p value	Direction of effect
Innate immune system						
csf3r						
G.R.Ctrl	G.L.Ctrl	0.271676	0.073054	3.719	<0.01	G.R. > G.L.
Il22						
G.R.Exp	G.R.Ctrl	-0.2891137	0.0591945	-4.884	< 0.001	Exp < Ctrl
C.R.Exp	C.L.Exp	0.40536	0.12682	3.196	0.027	C.R. > C.L.
G.R.Exp	G.L.Exp	-0.2219066	0.0593966	-3.736	0.004	G.R. < G.L.
G.R.Exp	C.R.Exp	-0.4551321	0.118558	-3.839	0.003	G.R. < C.R.
saal1						
G.L.Exp	G.L.Ctrl	0.1808448	0.0409709	4.414	< 0.001	Exp > Ctrl
mst1ra						
G.L.Ctrl	C.L.Ctrl	-0.2090431	0.046991	-4.449	<0.001	G.L. < C.L.
G.R.Ctrl	C.L.Ctrl	-0.1479639	0.046991	-3.149	0.031	G.R. < C.L.
G.L.Exp	C.L.Exp	-0.1634396	0.0490157	-3.334	0.017	G.L. < C.L.
G.R.Exp	C.L.Exp	-0.1607831	0.0493504	-3.258	0.022	G.R. < C.L.
p22phox						
G.L.Ctrl	C.L.Ctrl	0.143874	0.037064	3.882	0.002	G.L. > C.L.
G.L.Exp	C.L.Exp	0.159279	0.038663	4.12	< 0.001	G.L. > C.L.
G.R.Exp	G.L.Exp	-0.107778	0.03085	-3.494	0.01	G.R. > G.L.
nkef-ß						
G.R.Ctrl	C.L.Ctrl	0.140601	0.039164	3.59	0.007	G.R. > C.L.
C.R.Exp	C.L.Exp	0.247663	0.069527	3.562	0.008	C.R. > C.L.

sla1						
G.L.Ctrl	C.L.Ctrl	0.125489	0.040062	3.132	0.033	G.L. > C.L.
C.L.Exp	C.L.Ctrl	-0.216943	0.047683	-4.55	<0.001	Exp < Ctrl
G.L.Exp	G.L.Ctrl	-0.151378	0.032826	-4.612	<0.001	Exp < Ctrl
G.R.Exp	G.R.Ctrl	-0.141535	0.033188	-4.265	<0.001	Exp < Ctrl
G.L.Exp	C.L.Exp	0.191054	0.041788	4.572	<0.001	G.L. > C.L.
C.R.Exp	C.L.Exp	0.213097	0.071103	2.997	0.049	C.R. > C.L.
G.R.Exp	C.L.Exp	0.188774	0.042074	4.487	<0.001	G.R. > C.L.
mif1						
G.R.Exp	G.R.Ctrl	0.0767922	0.0235918	3.255	0.022	Exp > Ctrl
Adaptive immune system						
igm						
G.L.Ctrl	C.L.Ctrl	0.696301	0.079369	8.773	<0.001	G.L. > C.L.
C.R.Ctrl	C.L.Ctrl	0.645425	0.129609	4.98	<0.001	C.R. > C.L.
G.R.Ctrl	C.L.Ctrl	0.648646	0.079369	8.173	<0.001	G.R. > C.L.
G.L.Exp	C.L.Exp	0.645365	0.082789	7.795	<0.001	G.L. > C.L.
C.R.Exp	C.L.Exp	0.593532	0.140866	4.213	<0.001	C.R. > C.L.
G.R.Exp	C.L.Exp	0.642267	0.083354	7.705	<0.001	G.R. > C.L.
Il16						
C.L.Exp	C.L.Ctrl	-0.186769	0.051449	-3.63	0.006	Exp < Ctrl
G.L.Exp	C.L.Exp	0.168481	0.045106	3.735	0.004	G.L. > C.L.
G.R.Exp	C.L.Exp	0.152626	0.045402	3.362	0.015	G.R. > C.L.
MHC II						
C.L.Exp	C.L.Ctrl	-0.19465	0.05859	-3.323	0.018	Exp < Ctrl
G.L.Exp	G.L.Ctrl	-0.15075	0.04033	-3.738	0.004	Exp < Ctrl
G.R.Exp	G.R.Ctrl	-0.1664	0.04078	-4.081	< 0.001	Exp < Ctrl
tcr-β						
G.R.Ctrl	G.L.Ctrl	0.1200597	0.031569	3.803	0.003	G.R. > G.L.
G.R.Exp	G.R.Ctrl	-0.1326254	0.0320299	-4.141	< 0.001	Exp < Ctrl
Complement system						
c7						
G.L.Exp	C.L.Exp	0.134196	0.039069	3.435	0.012	G.L. > C.L.
G.R.Exp	C.L.Exp	0.165532	0.039336	4.208	< 0.001	G.R. > C.L.

Table 3: Differences in the gene expression of individual genes between the biologically relevant groups. Only significantly different results are shown. P-values were fdr corrected for multiple testing.

Using only genes which significantly differed for at least one comparison (Table 3), we computed PCA's for the innate and adaptive gene groups, separately for both treatments



<u>Figure 3:</u> PCA analysis of innate and adaptive immune genes by treatment for the four different ecotypes (Canadian river (green), Canadian lake (orange), German river (purple) and German lake (pink)). Principal component 1 and 2 explain 62.5% - 70.3% of the variation in the data. With exception of the Canadian river fish, the treatment seems to have a strong effect on innate immune genes, reducing the difference between the other three habitat types. Genes associated with the adaptive immune genes appear generally unaffected.

Discussion:

Exposed to a large number of *D. pseudospathaceum* cercariae, we find significant differences in the resistance phenotype between the populations were lake fish are more resistant than there connected river counterpart. Surprisingly, the German river (G.R.) populations were able to withstand the infection better than the Canadian lake (C.L.) fish, suggesting local adaptation to the specific parasite species. With the exception of the Canadian river (C.R.) individuals, we also find significant differences in the underlying gene expression, especially in genes associated with innate immunity. As expected, the German lake (G.L.) fish had significantly lower infection rates than any other population, reflecting their co-evolution with the local *D. pseudospathaceum* parasite and the generally higher immunological requirements of high parasite pressures typically associated with the lake habitat.

Comparable ecological circumstances are expected to drive the parallel evolution of similar immunological defense mechanisms in stickleback ecotypes, based on their habitat, rather than genetic or historical background. It is therefore rather surprising that the German river (G.R.) fish displayed higher parasite resistance then the Canadian lake (C.L.) population. Apparently, the G.R. fish benefit from the maintenance of *D. pseudospathaceum* resistance despite its comparably low occurrence in wild river habitats. Again, the similarities between the two G.R. populations support the theory of parallel immunological adaptation between ecotypes. Finally, the significantly lower infection rate in the C.L. compared to the Canadian river (C.R.) fish falls in line with the assumption of divergent evolution towards river and lake ecotypes. This, in turn, suggest that they have evolved similar general parasite defenses as the G.L. fish, which reflects the comparable parasite load found in lakes of both countries. On a family level (Fig. 2), the parasite resistance pattern between habitats holds true, despite genetic variation between the families resulting in the apparent overlap of the C.L. and G.R. families.

An effect which was potentially reinforced by the absence of potential immunologically beneficial mate choice, due to random in-vitro fertilization, between mating pairs (Kurtz 2004, Milinski 2006), artificially increasing the genetic variation within habitat types. Despite this possible in-vitro artifact, the general pattern of habitat dependent parasite resistance persists between ecotype pairs, implying a strong genetic background for the resistance phenotype.

Among populations, parasite exposure had a significant effect on several genes associated with the immune system which appears to appear to diverge in a treatment dependent manner. Whereas innate immune genes are upregulated, genes associated with the adaptive immune system appear to be downregulated in response to the parasite treatment (Table 2 & 3, Appendix Fig. A1). This falls in line with previous work by Whyte et al., (1987), showing that naive fish require approximately six weeks to establish a functional adaptive immune response to parasite exposure.

Interestingly, several genes of the innate immune system are also downregulated following the parasite treatment. All three of these (csf3r, il-22, sla1) play a role in granulocyte function, generally associated with bacterial and viral immune defense, they should convey no immunological benefit against macro-parasites. In contrast, the upregulated genes are associated with either macrophage functioning, inflammation or oxidative stress. Assuming that the G.L. fish's approach is the optimal one, supported by the infection data, suggest that the interplay of significantly downregulating sla1 and mhc II while upregulating saal1 results in the optimal resistance strategy. Surprisingly, the C.L. fish, although not significant, downregulate saal1. In contrast, the G.R. fish did not, offering a possible explanation for the elevated infection rate in the C.L. cohort. Despite having the highest infection rates, the C.R. fish did not significantly differ from the other habitat types in any of the measured genes.

Within the G.L. fish, parasite exposure significantly decreased the expression of both mhc II and sla1 while simultaneously increasing the expression of saal1. Saal1 is associated with cell proliferation in

response to proinflammatory stimuli (Harder et al., 2013) while sla1 is an inhibitor of Tcell-Receptor signaling (Marton et al., 2015). Thus, both expression changes can be seen as an upregulation of the (innate) immune system. The fact that most genes did not change in the G.L. fish in response to parasite exposure suggest that they are inherently well prepared for any potential immunological challenge, which we find reflected in the low parasite infection success in these fish.

In general, genes significantly affected by the parasite treatment were downregulated (Appendix Fig. A1) in the G.R. fish with the exception of the upregulated macrophage migration inhibitory factor-like protein (MIF1). While MIF1 plays a crucial role in pro-inflammatory innate immune response, the downregulated genes (e.g. sla1, tcr-β, mhc II) are related to the adaptive immune response and signaling. Thus, over all, the G.R. habitats appear to downregulate their adaptive immunity, which should have no functional benefit in this single infection event of immunologically naïve fish, for the benefit of an elevated inflammatory immune response. Parasite exposure did however decrease il-22 expression in G.R. fish. Associated with the activation of the innate immune response, this potentially explains the increased infection rate compared to the G.L. fish.

As the parasite establishes itself in the eye lenses within 24 hours, the immune system has to act within that time window (Rauch et al., 2006). Thus, variation in the speed at which the immune defense is mounted as well as inherent differences in the standing immune defenses contributes to the different parasite resistance phenotypes of the population. In similar fashion to the German habitats, the C.L. fish also downregulate genes associated with the adaptive immune system (sla1, mhc II, il-16) in response to the parasite exposure whereas treatment did not significantly alter gene expression in the C.R. cohort.

Although shifted in the Canadian fish, the differences between river and lake populations with regard to parasite resistance appear to be comparable within country. The discrepancy between Canada and Germany can probably be pinned to the German origin of our particular parasite. This, in turn, suggests

specific immunity against this particular Diplostomum species in the G.R. fish rather than the elevated base immunity seen in the German lake.

Surprisingly, however, these fish also did not show any significant change in their gene expression in response to the parasite exposure with the exception of nkef-β which was strongly upregulated compared to the other ecotypes in the exposed cohort. Most likely the C.R. fish either failed to detect the parasite all together or were simply overwhelmed by the simultaneous exposure to a large number of *Diplostomum* cercariae which has previously been shown to overwhelm the immune system, thus increasing infectivity (Karvonen et al., 2012).

In contrast to the C.R. population, the other three habitat types did show significant differences in the gene expression patterns of the control fish. Most of these differences however are in relation to the C.L. population, which appear to have generally lower expression levels compared to the two German habitats. Most likely, these baseline differences are, at least partially, responsible for the unexpectedly high infection rate (compared to the G.R.) in the C.L. population.

Whereas the German *D. pseudospathaceum* parasite establishes itself in the immunologically privileged eye lenses (Chapell et al., 1995), prevalent Canadian *Diplostomum sp.* are mainly found in the vitreous humour of the eye, within reach of the host's immune system (Scharsack & Kalbe 2014). Therefore, there might have been less pressure for Canadian sticklebacks to evolve a continuously high level of base immunity to immediately counteract *Diplostomum sp.* infection. Nevertheless, the higher resistance of C.L. fish compared to their river counterpart supports the general idea of habitat dependent local adaptation towards higher base immunity in lake over river fish. Admittedly, the immunological defense mechanisms mounted by the Canadian fish might simply be ineffective against the German *Diplostomum pseudospathaceum* parasite.

Supported by the gene expression differences, we are able to show that the discrepancy between river and lake sticklebacks is consistent across continents. Higher base immunity levels are beneficial in a high parasite environment (i.e. lake) and probably too costly to maintain in a river setting, with a typically lower parasite diversity. The reoccurring parallels of resistance patterns between river and lake sticklebacks in Canada and Germany support the idea of habitat dependent parallel evolution. Consequently, specialization to the given habitat allows residents to outcompete potential migrant fish originating from another environmental background. Paired with selective mate choice (Scharsack et al., 2007, Andreou et al., 2017) these advantages should lead to the isolation of the two ecotypes (river and lake) from each other and ultimately speciation. Potentially, similarities between the Canadian and German habitats could lead to the separate evolution of distinct river and lake three-spine sticklebacks, which are more similar based on their habitat rather than on their geographic or genetic origin.

Chapter II:

Habitat dependent evolution of river-lake ecotypes in geographically three-spined stickleback (*Gasterosteus aculeatus*) populations; experimentally proven in the field

Unsubmitted manuscript

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Abstract:

Local or habitat dependent adaptation is a key driver of speciation. By benefiting better adapted individuals, the environmental pressures in different habitats shape the divergence of a homogenous population towards specifically adapted phenotypes. These phenotypes are continuously refined through ecological and sexual selection, eventually resulting in distinct ecotypes.

In three-spined sticklebacks (*Gasterosteus aculeatus*) a major ecological pressures is exerted by the parasite communities of the stickleback population in any particular habitat. As the parasites richness varies based on the type of environment (e.g. river and lake), but is comparable within habitat type, the various stickleback populations diverge towards optimally adapted habitat specific phenotypes. The overlap within habitat types, such as lakes, with regards to environmental pressures implies the formation of reoccurring or parallel phenotypes which are characterized by the adaptation to their local habitat.

Using both a Canadian and German river and lake population, we hypothesized that the acquired adaptations of the respective ecotypes are transferable to similar environmental conditions, were they should convey comparable benefits. Therefore, we exposed these four populations to both a natural river and lake environment, assuming that matching ecotypes and habitats result in a lower parasite burden as opposed to the mismatched combinations. Once recovered, we performed full transcriptome analysis on all four populations to unravel the underlying regulatory mechanisms, especially with regards to the immune system, which convey these adaptive benefits. Due to surprisingly low parasite numbers we find the river populations, which have co-evolved with low parasite diversities, at an advantage in both environments. These are able to outgrow the lake ecotypes in the majority of measured physiological parameters without suffering from increased parasite pressures.

Introduction:

Varying environmental pressures, such as can be found in different habitats, can drive the divergent evolution (J. T. Gulick 1888) of different populations towards distinct, habitat dependent ecotypes through adaptive radiation (D. Schluter 2000) and divergent selection (Fraser et al., 2011). These adapted phenotypes should, over time, become increasingly divergent as long as geographic (S. Wright 1943), behavioral (Ringo & Hodosh 1978) or genetic segregation (Dobzhansky 1937, Mayr 1942 is maintained. Equally, similar selective pressures might demand comparable adaptations, resulting in phenotypic (and potentially genotypic) parallels between the distinct populations (Schluter & Nagel 1995). Consequently, these populations can then be characterized by their ecotypes rather than through ancestry. Each of these specific ecotypes should have the highest phenotypic (and genetic) resemblance with populations, which have evolved and adapted under equivalent environmental pressures. The habitat dependent specialization will lead to increasing isolation from other different habitats, e.g. by outcompeting potential migrants, thus accelerating the differences and laying the frame work for speciation through isolation (J. Hereford 2008). In contrast, if migration was to occur between populations but within habitat types, the acquired (phenotypic) benefits from the habitat of origin should convey comparable benefits in the new environment.

Famous examples for this habitat dependent convergence can be found in the Anolis lizards of the greater Antilles (Rand & Williams 1969, Williams 1972 & 1983, Ogde & Thorpe 2002) which have independently developed similar physical adaptations (i.e. body shapes) in a habitat dependent manner. On a broader scale the evolutionary adaptations to an aquatic lifestyle seen in several mammalian taxa (Reidenberg 2007) is a great example for environment dependent convergence. More recently, the three-spine stickleback (Gasterosteus aculeatus) has emerged as a model for ecotype evolution. Originating from the pacific basin, these marine fish first dispersed into the Atlantic, approximately 31-59 thousand years ago (Fang et al. 2018), before colonizing fresh water habitats across the entire extend of their distribution at the end of the last glaciation period (McPhail 1994, McKinnon & Rundle 2002). Here, faced with new environmental pressures, habitat specific ecotypes have repeatedly evolved along the speciation continuum (Mäkinen et al., 2006, Jones et al., 2012, Feulner et al., 2015). In northern Germany, these ecotypes have been shown to cluster according to their habitat of origin rather than genetic ancestry (Chain et al., 2015), resulting in distinct river and lake ecotypes. Beyond environmental factors such as water flow or eutrophication, differences in parasite pressure are considered a major driving force dictating this divergence (Wegner et al., 2003, Eizaguirre et al., 2009, 2010 & 2012). Indeed, within habitat type, there are many reoccurring parallels between the river or lake habitat with regards to parasite diversity and pressure across the entire geographical extent of the stickleback species (Kalbet et al., 2002, Scharsack et al., 2007). Consequently, we expect distinct river and lake ecotypes, each with considerable parallels in their adaptations to lake, respectively river habitats from various regions of the stickleback's distribution range. This leads us to hypothesis that habitat specific adaptations are equally beneficial in a similar environmental setting, independent of the genetic or evolutionary background the population originates from. To test this, in a common garden experiment, we exposed lab bred three-spine sticklebacks from either a river or lake habitat as well as of Canadian or German origin to a river or lake habitat in northern Germany. Using wire mesh cages, the four populations (i.e. Canadian river, Canadian lake, German river and German lake) were exposed to either of the two environments for up to 9 months. Sampling three distinct time points allowed us to investigate both seasonal differences as well as accounting for the different life-history stages over the stickleback's course of life. Besides physiological measurements and parasite load, we performed full transcriptome analysis in a subset of individuals to investigate the underlying gene expression between the ecotypes in the different habitats.

Material & Methods:

Animals, Breeding & Housing:

The stickleback populations originate from McCreight lake (50˚28'12.4''N, 125˚65'31.7''W) and Amour de Cosmos creek (50˚23'54.3''N, 125˚63'62.9''W) on Vancouver Island, BC, Canada, from the Westensee (54˚26'89.8''N, 9˚96'09.2''E) lake in Schleswig-Holstein, Germany and its connected stream Eider (54˚16'65.5''N, 10˚07'60.1''E). The initial fish were caught in the wild during the breeding season of 2014 and 2015. F1's were generated by in-vitro fertilization from these wild caught fish. After fertilization, the Canadian clutches were cleaned using Acriflavine (Dajana) and Methylene Blue (King British) and shipped to Germany at a constant temperature of 4˚C. To avoid confounding factors the German

clutches were treated in the same fashion and kept in the fridge for 4 days after cleaning, simulating the transport conditions. Fish were then incubated and raised in the lab at 18 ˚C, 18:6 Light:Dark with a constant water flow and fed ad libidum. The resulting F1 was cycled through autumn (12 ˚C, 12:12 L:D), winter (6 ˚C, 18:6 L:D) and spring (12 ˚C, 18:6 L:D) to initiate sexual maturation upon return to summer (18 ˚C, 18:6 L:D) conditions. Here, sexually mature males were placed in single tanks and allowed to build a nest. Females were also singled out and, once ripe, randomly mated with an un-related male from the same population of origin. Once established, mating pairs were allowed to mate multiple times to produce large families of identical genetic origin. The resulting F2 was kept in "family" tanks and fed *ad libidum* until the start of the experiment. To avoid confounding factors we did not cross fishes from the 2014 and 2015 cohort and used three unrelated families per habitat from each (i.e. 2014 and 2015) cohort.

Experimental Set-up:

To investigate the effect of a natural environment on different fish populations we placed a total of 48 wire mesh cages (Fig. 1, see Eizaguirre et al., 2012 for details), into the Grosse Plöner See (54˚14'61.0''N, 10˚40'86.9''E) lake and the Malenter Au ((54˚19'62.7''N, 10˚55'65.9''E) in northern Germany. The cages were divided in the middle with plexiglas, effectively dividing each cage into two halves. Each half cage contained 12 same sex individuals from either Canada or Germany. In detail, each cage contained 6 river males, 6 river females, 6 lake males and 6 lake females either of Canadian or German origin. The fish were brought out to the filed in the first week of October 2016.

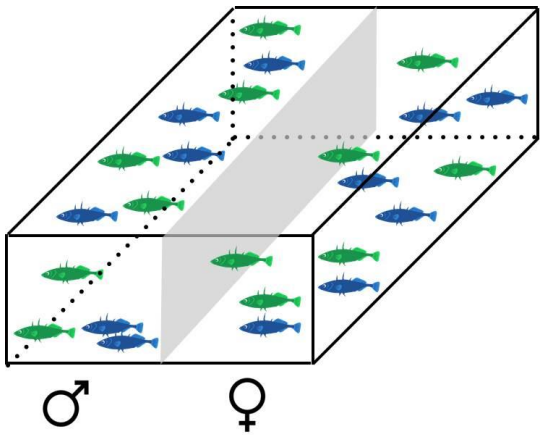

Figure 1: Illsutration of an experimental cage. Males and females are sepparaed in the middle with a plexiglas sheet (grey) to prohibit an influx of foreign genetic material into the natural habitat. Each male or female half contained six river and six lake sticklebacks from genetically sepparate families (represented by the green and blue fish).

Sextyping and fish selection:

In September of 2016 fish were sex typed and placed in same sex tanks, separated by family until the start of the experiment. We excluded fish with a standard length (SL) of less than 24mm to prevent escape through the cage's wire mesh. From these tanks, one random individual was allocated to one of the cages following the previously described allocation design.

Cage maintenance:

Cages were controlled weekly to detect any damage and, in the river habitat, ensure that they remained submerged. Additionally, 4 cages (2 for each Country) were replicated in the lab were each half cage was kept in a separate fish tank. These served as negative controls with temperature and light conditions following the outdoor conditions.

Collection time points:

Fish were collected at three different time points, following the different life history stages of natural stickleback populations. The first one at the onset of winter (December 2016), the other two in spring (May 2017) and summer (July 2017) corresponding to the onset and end of the breeding season, respectively. Predicting increasing mortality rates over the course of the experiment, we dedicated one quarter of the cages to either the December or May time point, were as the remaining 50% of cages was to be dissected at the final (July) time point.

Data collection:

During collection days, fish from 3 randomly selected cages were extracted from the cages and transported to the lab, where they were kept overnight in the water from their exposed habitat, at the corresponding temperatures and oxygenated to prevent mortality. The following day, the fish were measured, weight and checked for external parasites. After which the fish were sacrificed with tricaine methansulfonate (MS222, 200mg/l, Sigma) according to animal welfare policies and dissected to extract the head-kidneys, which were transferred to RNAlater (Sigma). The remaining material was frozen in saline solution and dissected at a later stage, weighing the organs and determining parasite load. In a small pilot study, I tested whether transferring and holding the fish in the lab overnight influences the gene expression profiles. Showing that the effects of long term environmental exposure overweigh the short term effects of handling and transferring the fish to the lab. Detailed results for this can be found in the Appendix of this thesis.

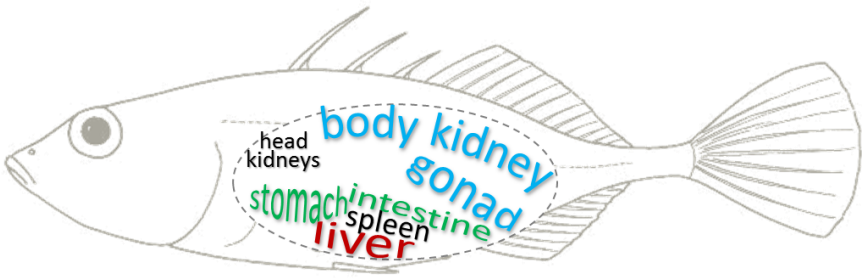

Figure 2: Illsutration of the different organs which were measured and checked for parasite infection. The head kidneys were stored in RNAlater for transcriptome analysis. The organs are colour coded according to their main role in the fish's body; Immunity (black), metabolism(red), reproduction (blue) and digestion (green).

Microsatellites:

The DNA acquired during sextyping in September was used to match recovered fish from the field using 14 microsatellites (Kalbe et al., 2009, Eizaguirre et al., 2012). Allowing us to accurately allocate each surviving individual and analyze the indivdual change in physiology (i.e. length and weight) since the onset of the experiment.

Statistics:

All statistical analysis were performed in RStudio (version Version 1.0.136). Using the build in general liner models (glm's) with family and cage as random factors, we compared the physiological measurements in a habitat and time dependent manner, separately for each sex.

Results:

Survival:

From the 1200 individuals used in this study a total of 994 were recovered at the end of the experiment. Mortality strongly varied between habitats, populations and dramatically increased over the course of the experiment (Appendix Table A2). We observed the highest mortality rate (>90%) amongst the two Canadian populations exposed to the lake habitat in the July cohort. Unfortunately, all recovered remains were in advanced states of decomposition, making it impossible to determine cause of death or accurately measure physiological data (e.g. weight) and parasite load. Additionally, a small "heat wave" days prior to the final collection time point raised the water temperature in the lake habitat to 24°C, accelerating the decomposition process and contributing, or potentially causing, the high mortality rate. Consequently, all comparisons with the Canadian fish in the lake from the July time point have to be performed with caution, taking into account the difference in sample size.

Size & length:

Using microsatellites the recovered individuals were matched with the data collected prior to the habitat exposure (i.e. in September). With the exception of 6 individuals, who could not be definitively matched with the pre-experimental data and consequently excluded, I am able to report individual growth rates for all recovered individuals which were calculated using general linear models (glm's with both Family and Cage as random factors). Despite slight differences in size and weight between the populations in September, these were statistically not significant for weight (glm; $F=1.4991$, $P=0.2453$) or length (glm; $F=0.61$, $p=0.6165$).

The December cohort showed nearly no weight growth as weight was statistically indifferent to the September measurements (glm; $F=0.99841$, $P=0.9895$). This was to be expected as they were only exposed to the habitat for approximately 2 months, during which temperatures decreased, thus slowing down metabolism and overall growth in poikilothermic organisms such as fish. In the lake, fish did however grow significantly larger in length compared to the September measurements (glm; $F=1.9218$, $P<0.001$), but not in the river (glm; $F=1.145$, $P=0.431$, Fig. 3). Between populations, the German lake fish grew significantly less than the other populations both for weight and length, in both males and females (Fig. 3, Appendix Table A3 & A4). This trend largely held true across all three sampling time points and re-occurred in both habitats. In the river habitat, both river populations (i.e. Canadian river (C.R.) and German river (G.R.)) grew significantly more than the lake populations (i.e. Canadian lake (C.L.) and German lake (G.L.)) for both length and weight respectively. A trend which can be found in both the May and July cohort (Fig. 3, Sup. Table A3 &A4**)**.

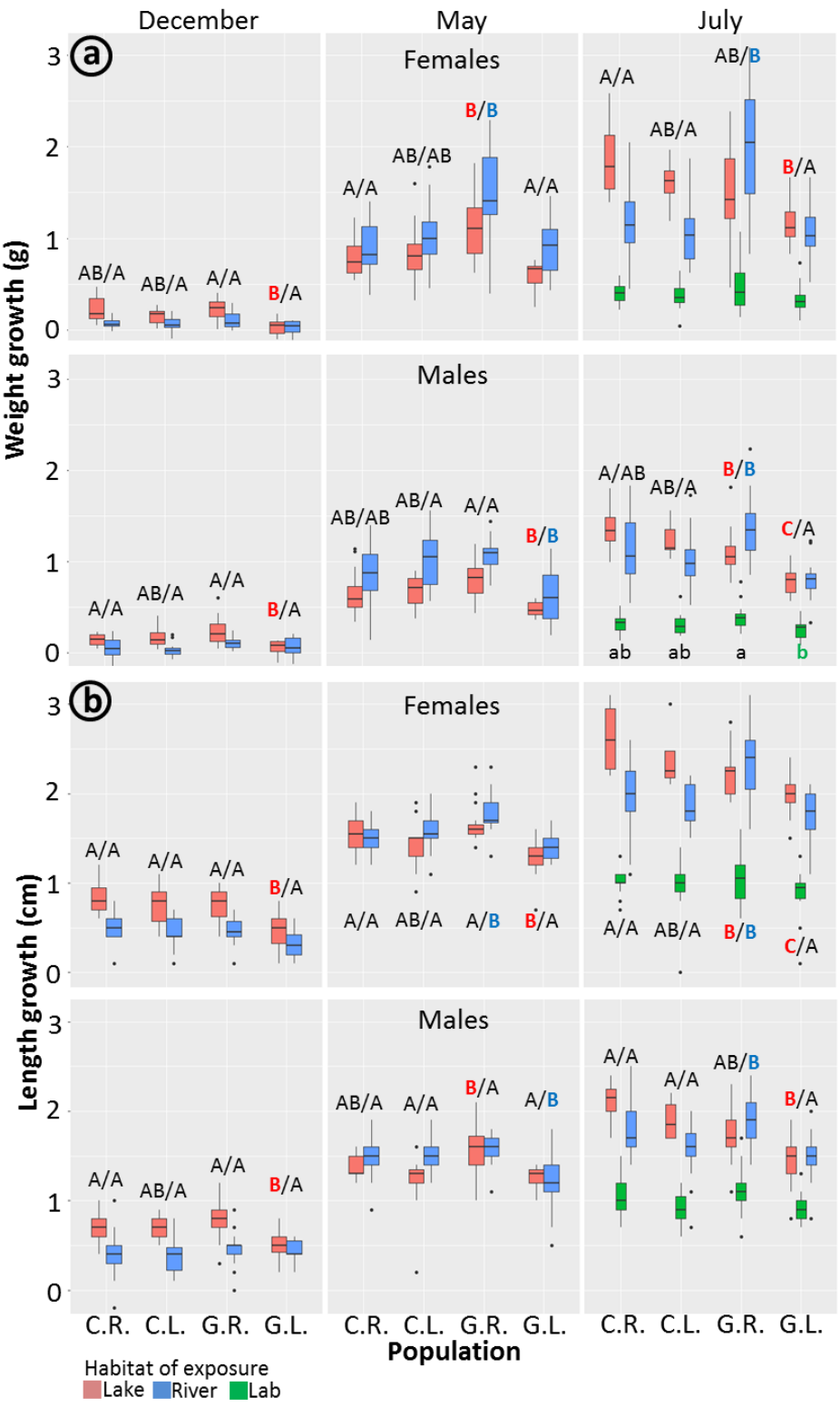

Figure 3: *Net weight (**a**) and length (**b**) gain since September, separated by sampling time point, sex and population (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.); boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model*

(glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table ***A3 & A4****.*

Compared to previous work in the same lake and river (e.g. Eizaguirre et al., 2012), we experienced extremely low over-all parasite numbers, especially towards the summer. Over the course of the experiment we were able to recover a total of 10 different parasite species from the river and 19 from the lake habitat. Most of these were too low in number to allow for reliable statistical analysis, and did (where applicable) also not differ significantly between the populations. Thus, following the approach by Eiaguirre et al., 2012, we calculated the Shannon Index as a grouping factor for parasite burden. The Shannon index did not statistically differ between the sexes (F=1.1196, P=0.29) and was hence calculated for both genders combined. Here, we did find a significantly lower parasite burden in the G.L. population compared to the other three populations in December (general linear model; G.L. – C.R., F=-5.824, P<0.0001; G.L. – C.L., F=-5.094, P<0.0001; G.L. – G.R., F=-6.366, P<0.0001) as well as May (general linear model; G.L. – C.R., F=-7.291, P<0.0001; G.L. – C.L., F=-5.907, P<0.0001; G.L. – G.R., F=-6.557, P<0.0001) in the lake habitat (Fig. 4). This discrepancy is lacking in the July cohort were all four populations have statistically indifferent Shannon indices.

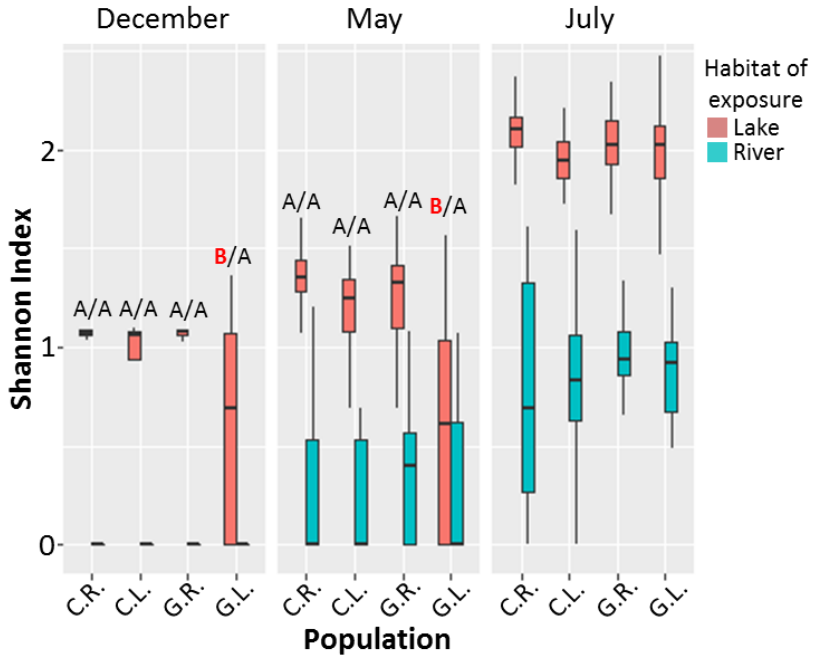

Figure 4: *Shannon index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.) at the different sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Specific statistical differences can be found in the appendix table* ***A5***

Organ weights:

Besides parasite load and size, we also measured individual organ weights (see. Fig. **2**, Methods section) of several organs. These were chosen based on their involvement in different physiological processes in fish, under the assumption that their size is a good approximation of their functional importance in each individual. As we generally find that organ weights vary in a size dependent manner between the populations, we will focus our analysis on the somatic indices (i.e. their % contribution to the total body mass) to account for the difference in size between the populations.

The weight of both gonads (in females) as well as the body kidney (in males) is a good reflection of the reproductive potential in three-spined sticklebacks. However, their size is dependent on the reproductive state each individual fish is in and, as we are unaware of that state, the resulting differences might be inconclusive and require cautious interpretation. These are therefore not discussed in detail at this stage but can be found in the supplementary information of this Chapter (see Appendix p. 119 for details).

The liver is the main metabolic organ in fish, generally involved in all nutrition related processes and should therefore be reflective of the differences in growth rate between the populations. Indeed, liver size closely followed the difference in body mass between the populations (see Appendix p. 119 for details). However, these are not discussed in detail at this point as metabolic differences were not the main focus of this study.

The two main immunologically relevant organs in sticklebacks are the spleen and head kidneys. Their size is believed to be a good reflection of the immune system activity in fish. The spleen somatic index (SSI) significantly varied between the populations and habitats for both sexes, especially in the July cohort. Here, both Canadian populations had a significantly smaller SSI than either of the German populations (Fig. 5**,** Appendix Table A10). Interestingly, spleen size varied in a habitat dependent manner between the German ecotypes in a matched habitat-ecotype fashion. In the river, the G.R. fish had higher SSI than the G.L. fish (significant only in the males) were as the opposite held true in the lake habitat, although this was not statistically different. This is especially interesting regarding the laboratory control fish, were the G.L. population has a significantly higher SSI than the G.R. population (in females only) despite not encountering any parasites. Thus, there appears to be an inherent difference in spleen size between the different populations.

The head kidneys roughly follow the same pattern as the spleen. The main difference however is that the head kidney index (HKI) is already significantly higher in May in the G.L. population (compared to all other populations). In July, the main significant differences can be found between the Canadian and German populations. Interestingly, and opposite to the previous SSI results, we do not observe the matched habitat-ecotype pattern as the HKI does not significantly differ between the German populations (Fig. 6, Appendix Table A8).

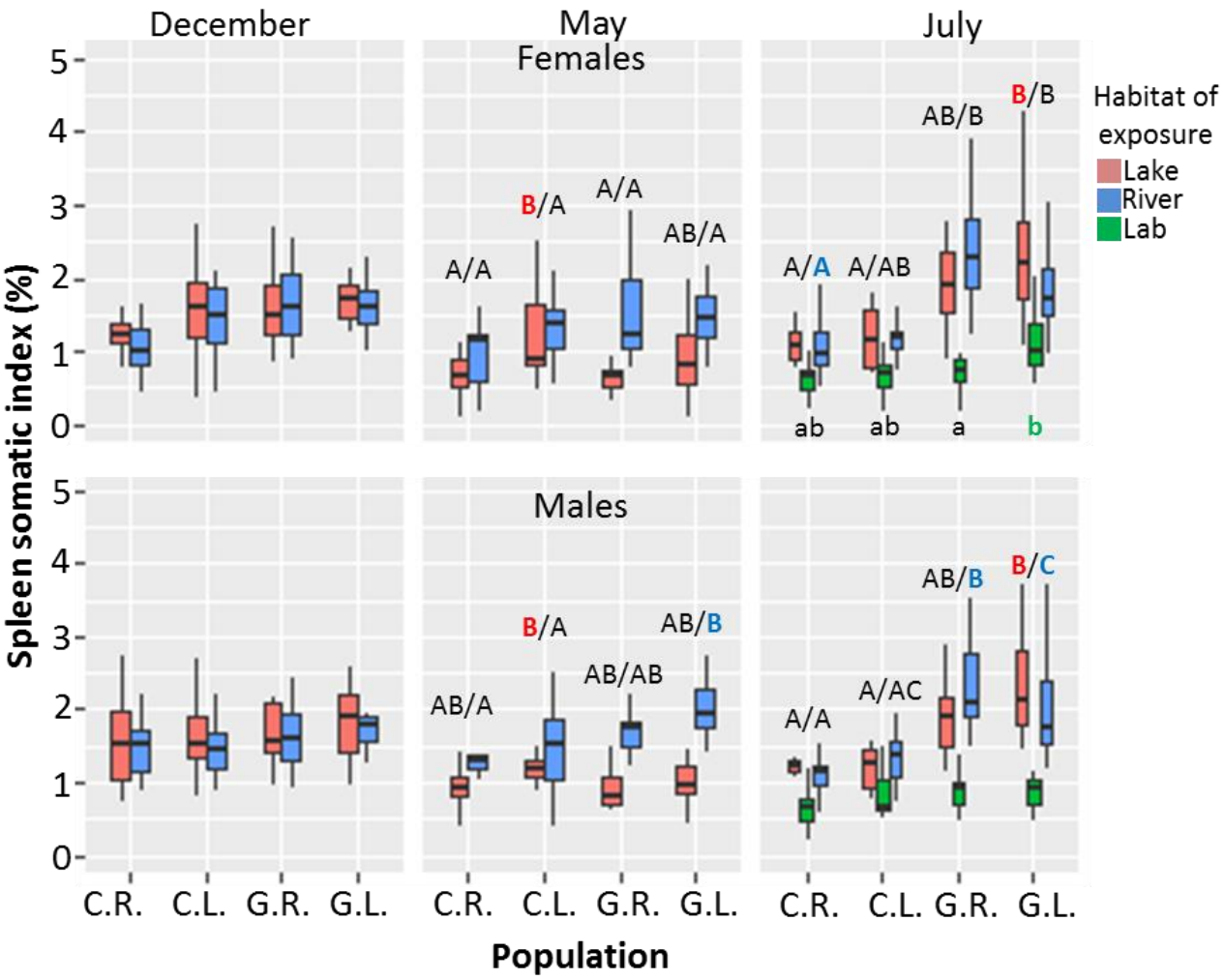

Figure 5: *Spleen somatic index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.), separated by sex and sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table A10.*

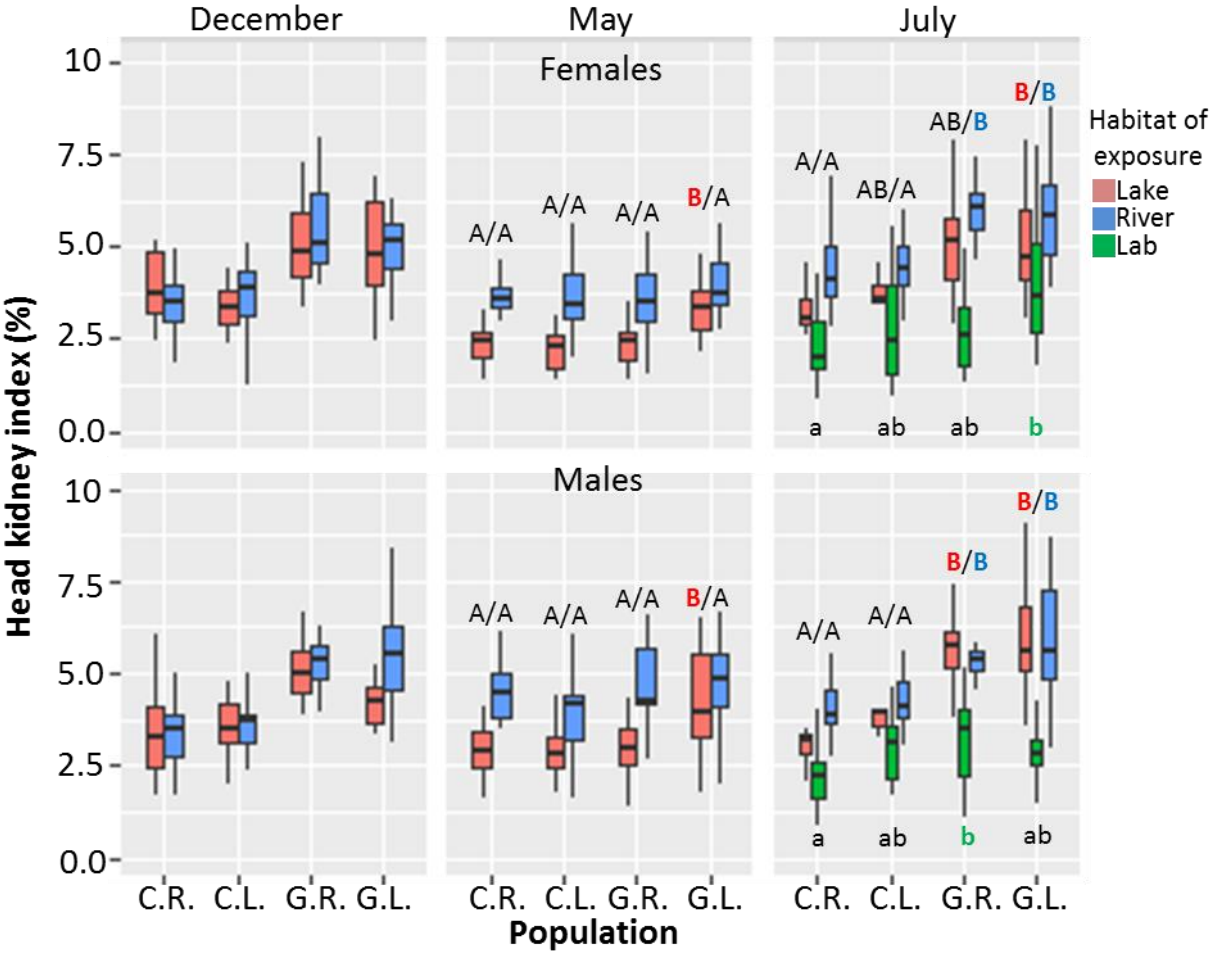

Figure 6: *Head kidney index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.), separated by sex and sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table A8.*

Discussion:

Habitat dependent (local) adaptation is a major factor driving the differentiation of three-spine sticklebacks towards distinct river and lake ecotypes on a globally reoccurring scale (Feulner et al., 2015). These acquired adaptations are expected to convey benefits in all comparable habitats and should, in theory, enable transplantations between similar habitats without negative consequences for that respective ecotype. To test this, we exposed lab reared sticklebacks from two distinct lake and river populations to an unfamiliar river and lake environment in northern Germany. Expecting the matched combinations of population and habitat (e.g. lake fish in lake) to outcompete the mismatched combinations (e.g. river fish in lake), irrespective of the Canadian or German origin of the specific population. If, however, the German fish were to surpass those of Canadian origin, irrespectively of the population's habitat of origin, it would hint towards local adaptations as a major driving factor towards ecotypic divergence. Indeed, the discrepancy in mortality rate in the July lake fish is most likely an effect of local adaptation since both Canadian populations had dramatically higher mortality rates than either of the German populations did. As the parasite pressures were unusually low compared to other studies (e.g. see Eizaguirre et al., 2012), other environmental factors are most likely to blame for this. The water temperatures reached 24˚C in the lake prior to sampling which is a multitude of the average water temperatures of 8 ˚C found in either of the Canadian fish's habitat of origin (Lough et al., 1999). In theory, an argument can be made that this is evidence for specific local adaptation to the lake habitat in northern Germany. However, the German river fish did not differ from the German lake population regarding mortality, despite originating from a river with an average water temperature of 15˚C (pers. comm. Landesamt für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein) in the summer months. Hence, the susceptibility to succumb and perish under higher water temperatures appears to be a discrepancy between the Canadian and German origin of the fish, rather than an adaptation to summer conditions in German lakes.

We also found temporal differences in the growth rates between the two habitats. Most likely attributed to the different environmental properties of the respective habitats. After winter, the shallow river will heat up more quickly, thus increasing metabolism and food resources, in turn leading to increased growth compared to the lake habitat. However, lower water flow and larger surface area of the lake allow for increasing temperatures towards the summer (i.e. July) sampling time point, in turn enabling the lake residents to outgrow individuals in the river habitat. Thus explaining the switch in growth rates between the lake and river habitat between the May and July time point within populations, exempt the German lake cohort which grew equally poorly in both habitats.

In line with our expectations of habitat dependent adaptation, both the German river and lake population grew better in their respective habitat than they did in the unfamiliar one. In support of habitat dependent specialization towards distinct river, respectively lake ecotypes in three-spined sticklebacks. Surprisingly however, the German river fish outgrew the German lake population in both habitats, which we find mirrored in the Canadian river-lake pair. River fish seem to have repeatedly higher growth rates than lake fish do. This becomes especially apparent when the control fish from the lab are taken into account. Despite poor growth rates (compared to the "outdoor" cohorts), the German river fish grew to significantly larger lengths, but not weights, than the German lake population. The

same trend can be observed, although not statistically significant, in the Canadian river fish, which also outgrew the Canadian lake population in the lab. Hence, there seems to be a strong genetic basis for increased growth rates in river fish. From an environmental standpoint, large and elongated body shapes are advantageous in flowing water, increasing maneuverability. Simultaneously, this requires the allocation of potentially limited resources to metabolism and growth, possibly at the expense of other biological processes.

Typically, river habitats are associated with comparably low parasite pressures, creating an evolutionary basis for increased growth rates as resources can be liberated from superficial immune defenses. In turn, this reduction of immunity for the benefit of increased growth rates would be costly if the parasite pressure were to rise to atypical levels as a result of fluctuations in the natural parasite community or change of habitat (e.g. through migration). In either case, lake fish have the advantage over river fish in high parasite environments as they already invest more into "standing" immune defenses whereas river fish have to "catch-up", which has previously been shown by Lenz et al., (2012), allowing lake fish to effectively outcompete fish of river origin. Unfortunately, the parasite pressures were unusually low over the course of our experiment in either habitat, neglecting the potential disadvantage of river fish in the lake habitat. Especially regarding *Gyrodactylus sp*., the leading river parasite in northern Germany (Eizaguirre et al., 2012) which was missing in both the December and May cohort as well as being only present in comparably low numbers in the July cohort. Statistically, the only significant difference can be found in the Shannon index of the German lake fish which were less infected at the December and May time points, a discrepancy which did not carry through to the final July sampling. Consequently, the low parasite pressure enabled the German river fish to maintain their high growth-rate phenotype without the simultaneous disadvantage of a higher parasite burden.

Large size is a desirable attribute in fish as it enables the production of larger clutches in females, thereby increasing reproductive output. This correlation of size and fitness is reflected in male three-spine sticklebacks, which have an affinity for larger females during mate choice (Kraak & Bakker 1998). However, in the absence of potential male partners, female sticklebacks will eventually spawn on their own to avoid so-called "stone eggs" which can prevent further clutch formation and be potentially lethal. Implying that the state of clutch "maturation" for each female is unknown and highly variable, demanding cautious interpretation of the measurements. Comparable problems arise from the eutrophicated body kidney in males. Therefore, these are not discussed at this point, but can be found in the appendix of this thesis.

As we are currently awaiting the transcriptome analysis, we must use physiological measurements as proxy for immune system activity. Both the spleen and the head kidneys are considered major immunologically active organs in fish, involved in lymphocyte production and development, their size can be used as an approximation for immune activity (Zapata et al., 2006). In line with the expectation of increased immunity, the German lake fish allocated significantly more percentage of their total body mass to both the spleen and head kidneys in the lake habitat. Large spleen and head kidneys appear to be an inherent trait in the German lake fish as we found the same in the control (i.e. lab exposed) fish, providing further support for the assumption of high base level immunity in lake fish. Reflected in the significantly lower Shannon index of the German lake populations, both for the December and May time

point. Despite increasing parasite pressures towards July, the advantage in parasite resistance of the German lake fish did not carry through, albeit maintaining high organ weights. Outside of the German lake fish, we did not find any significant difference in the general parasite burden (i.e. Shannon index) between the populations. Surprisingly, both Canadian populations had significantly smaller spleens and head kidneys than the Germans did, despite not differing from the German river ecotype with regards to parasite burden, thus contradicting the correlation in organ size with immunological activity. Alternatively, the parasite pressure might not have been sufficiently elevated for the other populations to be at a disadvantage, effectively resulting in an overinvestment into costly immune defenses of the German lake population.

To date, the differences we see point at habitat dependent adaptation towards specific ecotypes. Especially with regards to the German lake and river fish, which clearly differ in their phenotypes with regards to parasite resistance and growth. However, the discrepancy between the German river respectively lake habitat does not directly convert to the Canadian populations. Here, the differences are subtler and especially the Canadian lake ecotype diverges strongly from the German lake fish. However, the low parasitic pressures, especially in the lake habitat, might be to blame for the lack of clear segregation into ecotypes. At the moment, it appears as though both river ecotypes follow the expected path of lower immune system activity to the benefit of increased growth. The same holds true for the German lake fish, which also behave in the expected manner of lower growth rates and higher immune defenses. The Canadian lake population, on the other hand, appears to either downregulate its immune system activity due to the low parasite pressure or, inherently, never “intended” to express higher immune functions in the first place. Certainly, this perception will remain entirely speculative until the transcriptome analysis are completed.

Solely based on the measured physiological parameters, we find that the assumption of habitat dependent divergence towards adapted ecotypes hold true in the German river-lake pair, but not so in the Canadian populations. Here, only minor differences between the ecotypes can be observed which do not overlap sufficiently with the corresponding German ecotype to make a general statement towards the global validity of our assumption. This suggest **a/the** lack of distinct ecological speciation in the Canadian river lake pair, potentially due to briefer genetic isolation between the two habitats. Alternatively, the difference in environmental pressures between the two exposed habitats were not strong enough to unravel the phenotypic differences, both between the two Canadian ecotypes as well as between the matched and mismatched environment.

Awaiting the transcriptome analysis, we can only speculate on the underlying molecular mechanisms, responsible for the innate and acquired differences between the populations. Based on the observed phenotypic differences, we assume to find large discrepancies in both the metabolic processes and immune system gene expression between the populations.

Chapter III:

Female assortative mate choice functionally validates synthesized male odours of evolving stickleback river–lake ecotypes

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Abstract:

During mate choice decisions, females of many vertebrates use male olfactory cues to achieve immunogenetic optimality of their offspring. Three-spined sticklebacks (Gasterosteus aculeatus) populating habitats that differ in their parasite communities evolve locally adapted combinations of genetic variants encoded at the Major Histocompatibility Complex (MHC). Such adaptation confers optimal resistance to the local parasite fauna. Immunogenetic signatures co-evolved with local parasites favor population-specific assortative mate choice behaviour. Previous studies have shown that female sticklebacks evaluate male MHC-associated olfactory cues during the process of mate choice, but how habitat-specific information is exchanged between males and females has remained elusive. Here, we directly demonstrate the molecular nature of the olfactory cue providing habitat-specific information. Under controlled laboratory conditions females that are ready to mate prefer mixtures of synthetic MHC peptide ligands mimicking the optimal allele number of their original population. These results imply that female sticklebacks can determine the number of MHC alleles of their prospective mates, compare it to their own immunogenetic status, and, if optimal with respect to the immunogenetic complementarity, accept the male as mate. Our results suggest a potentially common mechanism of ecological speciation in vertebrates that is based on the olfactory assessment of habitat-specific immunogenetic diversity.

Introduction:

Eco-evolutionary dynamics shape the genotypic and phenotypic characteristics of populations and individuals. Since survival of populations ultimately depends on the maintenance of genotypes optimally adapted to the specific habitat, mate choice is critical for producing high quality offspring (Andersson & Simmons 2006). Of particular value for mate choice decisions are physiologically costly signals, such as bright colors (Milinski & Bakker 1990) or elaborate bird songs (Catchpole 1987), since they honestly reveal physical health and thus potential resistance against prevailing parasites (Hamilton & Zuk 1982).

Sexually mature three-spined stickleback males seek out and defend a territory, build a nest out of plant material and create a scent trail to attract females. Apart from the breeding coloration, the most intriguing signal assessed by choosing females is the odor signal created by the male (Reusch et al., 2001). The odour contains two major components (Fig.1): a so-called male validation factor (Sommerfeld et al., 2008; Milinski et al., 2010; Andreou et al., 2017) signals the presence of a male

'stickleback' and thus validates the second odor component, which is associated with an individual male's MHC profile (Reusch et al., 2001; Aeschlimann et al., 2003). MHC molecules inform the immune system of the composition of intracellular proteins, and thus are critical mediators of an immune response that is initiated when a cell contains foreign protein(s), for instance after an infection (Janeway 2001). MHC genes are the most polymorphic genes in vertebrates and their high allelic diversity – which is maintained by both parasite (Eizaguirre et al., 2012) and sexual selection (Hamilton & Zuk 1982; Milinski & Bakker 1990) is advantageous when dealing with complex parasite communities and changing immunological challenges (Takahata & Nei 1990; Paterson et al., 1998; Wegner et al., 2003).

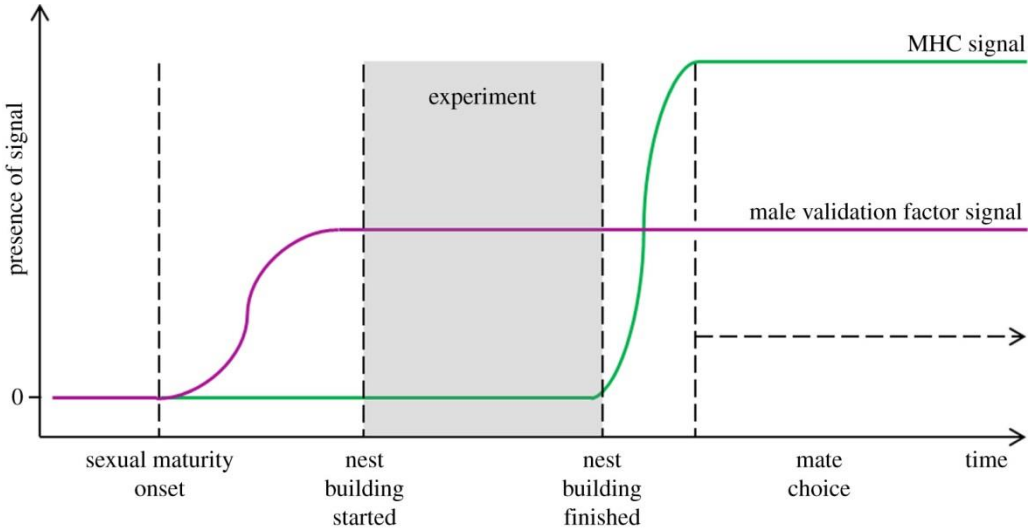


Figure 1: Presence of MHC signal and male validation factor signal during the breeding cycle (adapted from Sommerfeld et al., 2008; Milinski et al., 2010). Only males were used that produced the male validation factor but not yet the MHC signal.

MHC molecules bind different subsets of intracellular peptides (including those that are produced by pathogens) and present them at the cell surface to the antigen receptors of T cells. The greater the number of structurally distinct MHC molecules an individual possesses, the greater the sequence space of intracellular proteins that becomes detectable by the immune system. However, since the repertoire of T cell receptors must be purged from overly self-reactive versions to avoid inadvertent self-destruction, a trade-off exists between the ability to recognize foreign antigens and the avoidance of autoimmunity; as a result, each individual expresses only a small subset of MHC alleles present in the population (Nowak et al., 1992; Woelfing et al., 2009). This optimal number of MHC alleles varies among populations exposed to different parasite faunas, but is considered to be an ecotype-specific parameter

of local adaptation: the optimum increases with the number of parasite species in a population (Milinski 2006; Eizaguirre et al., 2011; Andreou et al., 2017).

Sensory evaluation of MHC genotype focuses on anchor amino acids of the peptide ligands, which are structural mirror images of the genetically encoded binding grooves of the MHC molecules. MHC peptide ligands are liberated from the peptide-MHC complexes and appear in bodily fluids; in this way, they become available for olfactory assessment. Studies in mice (Leinders-Zufall et al., 2004), fish (Milinski et al., 2005), and humans (Milinski et al., 2013) suggest that MHC peptides are widely employed as olfactory cues that influence vertebrate social behaviour including mate choice. Indeed, supplementing water from male sticklebacks' nests with synthetic versions of MHC ligand peptides altered the male's perceived MHC profile and predictively changed female choice (Milinski et al., 2005).

During mate choice decisions, female sticklebacks choose males optimally complementing their own set of MHC alleles in such a way that the offspring are close to the population-specific optimal number (Andreou et al., 2017). A direct link between non-random mating and functional relevance of habitat-adapted alleles has been dubbed "magic trait" by Gavrilets (2004) and can be seen as a powerful accelerator of ecological speciation (Thibert-Plante & Gavrilets 2013). However, although our previous experiments based on the outcome of interference with natural odors strongly suggested the possibility that MHC peptide ligands function as a mechanistic underpinning of MHC-associated behavior, this hypothesis remained so far untested. Here, using sticklebacks from two physically connected populations that are ecologically separated by different parasite faunas we identify the molecular nature of the population-specific MHC-associated odour signal.

Materials and Methods:

Animal origin and housing:

Wild-caught three-spined sticklebacks (Gasterosteus aculeatus) originated from a lake and a connected river in northern Germany. The fish were caught in December 2017 and cycled through winter (6˚C, 12:12 L:D), spring (12 ˚C, 12:12 L:D) and finally summer (18 ˚C 18:6 L:D) conditions in the laboratory. Fish were housed individually until the experiments. Males were provided with standardized nesting material consisting of green polyester threads. Nest (Wootton 1976) progression was monitored daily. Male sticklebacks will not produce the MHC signal until their nest is finished when they start 'creeping

through the nest' (Milinski et al., 2010). However, the male validation factor is present from the onset of nest building (Milinski et al., 2010, Fig. 1); this offers the possibility to expose females to the male validation factor without the natural male-derived MHC component of the signal peptides.

All animal experiments described were approved by the Ministry of Nature, Environment and Country Development, Schleswig Holstein, Germany (project number: 1096).

Experimental design:

Gravid female sticklebacks were placed in a flow chamber fed by two columns with laminar water flow (Reusch et al., 2001; Aeschlimann et al., 2003) and video-recorded when choosing between the two front quarters of the chamber for two periods of 300s each, with spatial reversal of the water source after the first 300s period. The remaining space was regarded as neutral and not scored. Determining odor preference in this setup has been shown to reliably predict mate choice (see supporting text of Milinski et al., 2005, supporting text), which allows for testing the effect of synthetic peptides on female preference. To this end, for each run, we took two 1 litre water samples from the tank of a single male (containing male validation factor but no MHC-associated component (Milinski et al., 2010; see above)) and spike each of these with either 2 or 4 synthetic peptides as described (Milinski et al., 2005). As each female was presented with water from two different males in two separate runs, and each male was used twice with two different females, we sampled the male tank twice, once for each run. When used as river-like stimulus, two peptides in solvent were continuously added to one half of the flow channel; when used as a lake-like stimulus, four peptides in solvent were continuously added to the other half of the flow channel. Each female was tested using water taken from the tank of a sympatric and an allopatric male, within a one-hour interval. All experiments were performed in double-blinded fashion in the Plön laboratory. Each female-male combination was used only once to avoid pseudo replication and thus is a single independent statistical unit.

MHC-analysis:

DNA was extracted from clipped spines using the DNeasy 96 Blood & Tissue Kit (Qiagen), MHC allele numbers were measured using Reference Strand-mediated Conformation Analysis (RSCA) as described (Lenz et al., 2009).

Peptides:

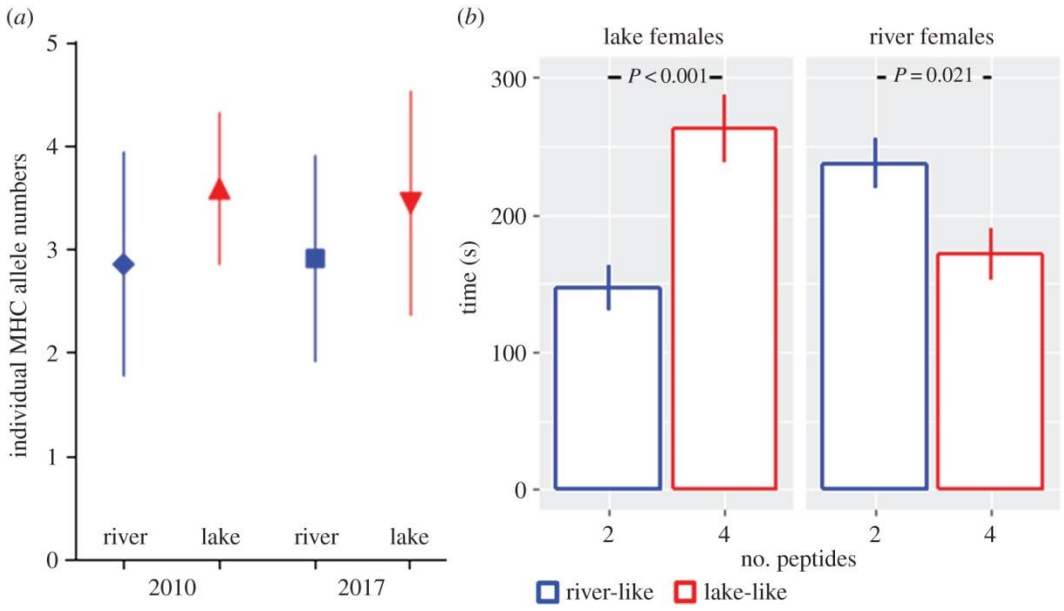
The four different MHC-ligand peptides used in this study were: SYIPSAEKI, SFVDTRTLL, ASNENMETM, and AAPDNRETF (Milinski et al., 2005 & 2010). Peptides were chemically synthesized, purified, verified by mass spectroscopy (MALDI-TOF), and dissolved in phosphate-buffered saline (PBS), as described (Milinski et al., 2005).

Statistical analysis:

All statistical analysis were done in RStudio (version 1.0.136).

Results:

We first determined the population-specific optima of MHC class IIB allele numbers in wild-caught fish of lake (Großer Plöner See (GPS); *n=53* fish) and river (Söhrener Au (SAU); *n=49* fish) populations sampled in 2017. Consistent with previous work, we find that the MHC optimum is higher in lake 3.453 ± 1.084, mean ± SE, than in river (2.918 ± 0.997, River2017-Lake2017, P=0.011, t= -2.588, df=100, two-tailed t-test) fish. Remarkably, the population-specific MHC optima determined in 2010 (lake: 3.595 ± 0.735, n=42; river: 2.865 ± 1.084, n=37, River2010-Lake2010: P=0.0007, t=3.54, df=77) do not differ significantly from those from 2017 (difference between River2017-River2010: P=0.85, t=0.2351, df=84; Lake2017-Lake2010: P=0.47, t=0.7266, df=93), indicating immunogenetic stability of these two populations (Fig. 2a). Previous findings showed that the mean individual number of MHC alleles (Reusch et al., 2001) corresponds with the local optimum (Wegner et al., 2003).



<u>Figure 2:</u> Mean (+SE) MHC allele numbers from the wild caught sticklebacks from river and lake populations (a). Despite being caught 7 years apart, the wild population averages remain almost identical between the fish from this study (caught in 2017) and the ones used by Andreou et al. (2017), caught in 2010. Habitat specific female preference for synthesized male MHC profiles (b). Time (s) (mean + SE) of 600 s the female spent in the quarter of the test chamber where either two or four peptides arrived with the current.

Mice (Leinders-Zufall et al., 2004) and fish (Milinski et al., 2005) decode MHC allelic complexity by olfactory evaluation of the typically two anchor residues of the peptide ligands, after they have become liberated from the binding pockets of the MHC molecules (Boehm & Zufall 2006), whereas the other residues in the peptides are of minor importance (Leinders-Zufall et al., 2004). For sticklebacks, exposure to conditioned water in a flow chamber reliably predicts mate choice (see the supplementary information of Milinski et al., 2005). In order to mimic the habitat-specific number of MHC alleles in a mate choice experiment, we exploited the observation that lake sticklebacks rarely exhibit only two or less alleles, and, correspondingly, that river sticklebacks rarely carry four or more alleles (Fig. 2a). Therefore, when a female is confronted with a male odour containing only two different kinds of peptides, she would be rarely mistaken in assuming that this signal comes from an individual of the river ecotype; an odour with three peptides would be ambiguous in origin, whereas an odor containing four peptides would most of the time come from an individual of the lake ecotype. Hence, our synthetic river-specific and lake-specific MHC mimics consisted of two and four peptides, respectively.

In the next set of experiments, we confirmed that the allele-specific combination of anchor residues is the functionally relevant property of the MHC peptide mimics. The comparatively small number of MHC alleles per individual ensures that the discriminatory power of the olfactory system is rarely challenged. According to the 2-anchor site rule of ligand binding, one anchor pocket can accommodate one out of the 20 amino acids, resulting in a maximum of (20x20=) 400 decodable alleles. This proved to be the case. For the 2-peptide signal, two random exclusive combinations from the four peptides were created; the outcome of choices by both river and lake females tested with one or the other 2-peptide combination did not differ significantly (n=45, t(29) =1.2402, P=0.23; two-tailed t-test).

When females of the lake ecotype were confronted with a choice of 4 (the “lake mimic”) or 2 (the ”river mimic”) peptide cocktails in the flow chamber experiment, lake females spent significantly more time in front of the 4-peptide inlet compared to the one with 2 peptides (Fig. 2b; n=12, t(18) = -3.9659, P=0.00091, two-tailed paired t-test). The river females, by contrast, significantly preferred the 2-peptide signal (Fig. 2b; n=14, t(25) =2.4822, P=0.021). Females thus showed a significant preference for their habitat specific synthetic MHC allele number. Control experiments corroborated that the male validation factor, which is required for accurate interpretation of the MHC-based signal by female sticklebacks (Milinski et al., 2010) is invariant between lake and river males (Andreou et al., 2017). Indeed, the preference for either 2-peptide or 4-peptide mimics was independent of the male’s population of origin, both for the river (n=12, t(22) = -1.0063, P=0.33) or the lake (n=12, t(21) = -0.16543, P=0.87), i.e. when the male providing the validation factor was either a river or a lake male for both the 2- and the 4-peptide inlet.

Discussion:

The sexual selection strategy used by sticklebacks incorporates an odor-based assessment of MHC diversity (Reusch et al., 2001; Aeschlimann et al., 2003). Here we have shown that a habitat-specific male odor signal that determines the outcome of assortative mate choice can be reduced to synthetic peptide combinations, whose anchor sequence diversities reflect the numbers of MHC alleles possessed by sticklebacks of a typical river ecotype or of a typical lake ecotype.

The exceptional diversity of MHC alleles in a population and their defining combinations of anchor residues explain why during olfactory assessment of MHC diversity individual female sticklebacks count each synthetic peptide ligand as representing one allele (Milinski et al., 2005), if accompanied by the

stickleback validation factor. During mate choice, female sticklebacks need to distinguish between the number and the quality of MHC alleles, whereas their quality in terms of their immunoprotective capacity becomes important during the second part of mate choice (Aeschlimann et al., 2003). After a female has approached a male that offers the habitat-specific optimal number of MHC alleles, she spawns only if the male is brightly red (Milinski & Bakker 1990), indicating that the male's alleles also confer resistance against the current parasite fauna.

The parasite faunas of lakes and rivers are largely distinct, with lakes exhibiting a greater diversity of parasites (Eizaguirre et al. 2011; Feulner et al., 2015, 27), reflected in a greater number of MHC IIB alleles in populations of lake sticklebacks (Eizaguirre et al., 2011; Andreou et al., 2017); correspondingly, river sticklebacks carry fewer MHC IIB alleles. Ecological speciation has set in to separate the two ecotypes through pre-zygotic mating barriers, as a consequence of the fact that lakes and rivers are connected with each other (Eizaguirre et al., 2011). During mate choice, female sticklebacks evaluate MHC diversity of prospective males in order to be able to maintain the different optima in the number of individual MHC alleles (Wegner et al., 2003; Milinski 2006). Because MHC alleles of lake fish provide resistance to lake parasites, and the river MHC composition to river parasites (28), the evaluation of their MHC genes possesses the quality of a magic trait that links habitat-specific adaptation with assortative mate choice (Gavrilets 2004; Thibert-Plante & Gavrilets 2013).

Because different natural habitats can be assumed to harbor different parasite communities, mate choice employing olfactory assessment of habitat-specific immunogenetic diversity may represent a common mechanism of ecological speciation in vertebrates.

Acknowledgements:

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Data accessibility:

Data available from the Dryad Digital Repository: doi:10.5061/dryad.16qk7vf

Chapter IV:

Evolutionary conservation of stickleback male validation factor

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Abstract:

Background: During mate choice, stickleback females make use of male olfactory cues to determine the suitability of potential mates in a habitat-dependent manner. This signal consists of peptides reflecting the individual male's MHC profile and a so-called validation factor, to indicate the correct species.
Hypothesis: As a result of long-term genetic isolation, the male validation factor is no longer compatible between sticklebacks from two geographically distinct populations.
System: Three-spined stickleback (*Gasterosteus aculeatus*) from a Canadian and German lake population.
Methods: Using a laminar flow channel, we exposed gravid female sticklebacks to olfactory male odor cues to determine their mate preference.
Results: In the absence of MHC signals, females do not distinguish between the male validation factors of males from two distinct populations.
Conclusion: The stickleback male validation factor is an ancient evolutionarily conserved signal that helps identifying the sender species but plays no role in habitat-dependent female mate choice decisions between males of the same species.

Introduction

Mate choice, ultimately, strives at establishing and maintaining genotypic optimality in the current environmental circumstances, resulting in high quality offspring (Anderson & Simmons 2006), thus playing an important role in environment dependent ecotype differentiation and potentially speciation. Consequently, females employ an intricate selection procedure, comprising costly male signals, such as bright colors (Milinski & Bakker 1990) or elaborate courtship rituals (Friberg et al., 2008), which honestly reflect the male's physical and health status to determine the quality of suitors.

One such signal can be found in the form of Major Histocompatibility Complex (MHC) peptide ligands, which directly reflect an individual's genetic MHC profile in his body odor (Milinski 2006, Boehm & Zufall 2006). The MHC is a crucial component of the vertebrate immune system involved in the control of immune responses (Janeway 2001). As such it is highly polymorphic (e.g. >1000 alleles in humans, Mack et al., 2012), a polymorphism, which is maintained by parasite-mediated selection (Takahata & Nei 1990, Eizaguirre et al., 2012) and amplified by sexual selection (Hamilton & Zuk 1982). Upon liberation from the peptide-MHC complex at cell surfaces (Boehm Zufall 2006), the MHC peptide ligands are secreted through bodily fluids and can be picked up by chemosensory receptors (i.e. olfaction). Their implication as behavioral modulators, including mate choice, has been demonstrated in a number of different

species (e.g. mice (Leinders-Zufall et al., 2004), fish (Milinski et al., 2005) and humans (Milinski et al., 2013).

In sticklebacks, females choose their partner in a population-dependent manner (Eizaguirre et al., 2011, Andreou et al., 2017), selecting for complementary MHC genes and healthy males. To this end, female sticklebacks make use of a number of different criteria such as male coloration (correlated with health, Milinski & Bakker 1990) and male odor cues, reflecting the male's individual MHC profile (Reusch et al. 2001, Aeschlimann et al., 2003).

The female is able to compare the male's MHC profile to her own set of alleles, selecting for optimal allelic diversity for her offspring (Aeschlimann et al., 2003, Milinski et al., 2005). By comparing the combined profiles of her own and the male´s MHC with the population-specific optimal number of alleles per individual, the female aims at approaching optimality of the MHC allele count for her offspring. Finally, discriminating complexions for redness increases the probability that the male's MHC alleles are beneficial and relevant in the current environmental context. MHC plays an important role in parasite resistance (Janeway 2001), and, as the parasite communities differ between habitats (Eizaguirre et al., 2011), so does the optimal individual number of MHC alleles (Wegner et al., 2003, Milinski 2006). Consequently, habitat-dependent mate choice contributes to the divergence and maintenance of sticklebacks into distinct river and lake ecotypes across the northern hemisphere (Chain et al., 2014, Feulner et al., 2015).

These distinct populations date back to the end of the last glaciation period, after which marine sticklebacks have repeatedly colonized fresh water habitats, often differentiating into ecotypes. By favoring males with optimally adapted MHC genes, population-dependent mate choice effectively selects against migrants, facilitating ecotype differentiation and potentially leading to speciation through genetic isolation. However, as the MHC is present and conserved in all jawed vertebrate species, three-spined sticklebacks make use of an additional "maleness" signal to validate the perceived olfactory cue as being derived from own species and thus relevant to the female.

Milinski et al., (2010) showed the necessity of this additional signal, the so-called male validation factor (MVF) in olfactory mate choice of three-spined sticklebacks. In its absence, females did not differentiate the presented MHC peptides from plain water. Andreou et al., (2017) demonstrated that females did not distinguish between the MVF from river and lake stickleback populations originating from adjacent habitats in Northern Germany. Since the MVF does not contribute to population-dependent mate choice

it seems to merely help to identify a species and thus avoids inadvertent inter-species hybridizations. Following colonization events of the Atlantic basin, different stickleback populations across the northern hemisphere became genetically isolated from each other for 31-59 thousand years (Fang et al., 2018), possibly also leading to divergence of the MVF signal across continents. We therefore considered the possibility that females from geographically distinct populations, i.e. Canada and Europe, might be able to differentiate between male validation factors of stickleback males originating from another continent and consequently genetic clade (i.e. Pacific vs. Atlantic ancestry). To test this hypothesis, we examined whether female three-spined sticklebacks originating from either a Canadian or a German lake population preferred their local MVF in an olfactory mate choice assay.

Males do not emit MHC signals before completion of the nest, which can be determined both through nest appearance and male final nest building behavior (Milinski et al., 2010). By contrast, the MVF is produced independently of the nest´s status (Milinski et al., 2010). Hence, using tank water from males with unfinished nests, female sticklebacks could be exposed to the male's MVF without his MHC signal; importantly, our previously described flow channel design (Reusch et al., 2001, Aeschlimann et al., 2003, Milinski et al., 2005, Milinski et al., 2010, Eizaguirre et al., 2011, Andreou et al. 2017) excludes male visual and behavioral signals in the female´s mate choice decision. The odour of a male could be completed by the addition of chemically synthesized MHC peptides resulting in a functionally complete olfactory mimic of a male.

Materials and Methods:

Animal origin and housing:

All fish used in this study were F1 in-vitro offspring from wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) originating from the Grosser Plöner See lake (n = 30, 54˚14'61.0''N, 10˚40'86.9''E) in northern Germany and the McCreight lake (n=31, 50˚16'46.5''N, 125˚39'03.1''W) from Vancouver Island, BC, Canada. Fish were caught during the 2015 breeding season and after in-vitro fertilization, eggs were shipped to our lab in Germany. To increase survivability, the eggs were cleaned using Acriflavine and Methylene Blue (SigmaAldrich) before being transported at 4°C. This treatment was replicated with the German in-vitro clutches to minimize potential effects of differential handling. To initiate breeding condition, seasonal changes were simulated in the laboratory by cycling the fish through winter (6˚C, 12:12 L:D), spring (12 ˚C, 12:12 L:D) and finally summer (18 ˚C 18:6 L:D) conditions.

Fish were housed individually upon transfer to the summer conditions, fed *ad libitum* with live *Chironomidae spec.* larvae and were spine-clipped for sex-typing and MHC-allele analysis. Once individually housed, males were provided each with standardized nesting material consisting of green polyester threads (cut to a length of ~10cm) and a sand-filled petri dish. Nest progression was monitored daily and nest status was determined based on nest appearance and male behavior (see Wootton 1976 for details), using only males with an incomplete nest for the experiment.

All animal experiments described were approved by the Ministry of Nature, Environment and Country Development, Schleswig Holstein, Germany.

Experimental design:

Gravid female sticklebacks were placed in a flow chamber fed by two columns with constant laminar water flow of filtered lake water (Reusch et al., 2001, Aeschlimann et al., 2003, Milinski et al., 2005, Eizaguirre et al., 2011, Andreou et al., 2017). Females were able to freely investigate the water composition in the chamber for two periods of 300s each, with spatial reversal of the water source after the first 300s period. Determining odor preference in this setup has been shown to reliably predict mate choice (Milinski et al., 2005, *supporting text*), which provides the opportunity to test the effect of male derived MVF signaling on female mate choice. To this end, water was taken from the tank of a single Canadian and German male (containing male validation factor but no MHC-associated component (Milinski et al. 2010) per trial, and presented for choice to either a German or a Canadian gravid female. Females were then tested for their preference for either of the two male validation factors in a direct comparison.

In consecutive control trials, each female underwent three further preference tests with one hour brakes in between trials, presented with the MVF from either the sympatric or the allopatric male against plane water, respectively. Finally the water from the sympatric male was spiked with either synthesized MHC peptides solved in PBS or plain PBS. This allowed for validating the absence of the male's own MHC signal. If present, the male's MHC signal in combination with the synthetic peptides on the peptide side would simulate a super-optimal male, which is avoided. In this case, the un-spiked side would be preferred and the trial discarded.

All experiments were performed in double-blinded fashion in the Plön laboratory. Each female-male combination was used only once to avoid pseudo replication and thus is a single independent statistical unit. Each fish was only used once.

Run validation:

To validate a female's readiness to spawn during the trial, she had to spontaneously spawn in her home tank in the absence of a male within 24h after her final test (Milinski et al. 2005). Otherwise all her trials of that day were discarded. Further, females that remained on the same side in the 1st and 2nd run of a trial and thus revealed an unwillingness of exploration were designated as 'no choice' and excluded. Two males and their associated trials were completely excluded because the female showed a clear preference for the non-peptide PBS side, thus suggesting the presence of the male's own MHC peptides in these trials.

MHC-analysis**:**

DNA was extracted from clipped spines using the DNeasy 96 Blood & Tissue Kit (Qiagen), following the manufacturer´s protocol. MHC allele numbers were measured using Reference Strand-mediated Conformation Analysis (RSCA) as described (Lenz et al. 2009). The resulting measurements were analyzed using the GeneMarker software (Version 2.4.2, Softgenetics). This analysis was necessary to allow for testing whether the females prefer the MHC subsided side in the final control trial only in the absence of the male's own MHC peptides. To this end female-male combinations were chosen as to always combine into an at least optimal MHC allele number. If the male's own MHC signal is also included in addition to the synthesized peptides, the male would appear to be super-optimal, which is to be avoided. If the spiked side is preferred, the male's own MHC signal was lacking from the tested water samples

Peptides:

The four different MHC-ligand peptides used in this study were: SYIPSAEKI, SFVDTRTLL, ASNENMETM, and AAPDNRETF (Milinski et al. 2005 & 2010). Peptides were chemically synthesized, purified, verified by mass spectroscopy (MALDI-TOF), and dissolved in phosphate-buffered saline (PBS), as described (Milinski et al. 2005). Using four peptides allows us to clearly determine whether the male MHC signal was present (see above).

Statistical analysis:

All statistical analyses were done in RStudio (version 1.0.136) using the build in packages for statistical analysis (two-tailed t-test) and the ggplot2 package for graphical representation. Times from the 1st and

2nd run of each female-male combination were added up and regarded as one variable (controlling for weak side preference). The data did not significantly differ from a normal distribution for either the MVF comparison (Shapiro-Wilk, P=0.4535), the sympatric control runs (Shapiro-Wilk, P=0.3803), the allopatric control runs (Shapiro-Wilk, P=0.463) or the final MHC peptide trial (Shapiro-Wilk, P=0.7183), allowing the use of parametric tests (two-tailed paired t-test) for data analysis.

Results:

We directly compared the ability of females to distinguish between the MVF of Canadian and German males. Unexpectedly, females did not distinguish between the MVFs of their sympatric and foreign males (Fig. 1, n=8, t(7)= -0.14008, p=0.8925, two-tailed paired t-test). However, females were capable of identifying the presence of foreign MVF. When given the choice between plain water and water containing either sympatric or allopatric MVF in the flow channel, females always preferred the MVF-containing sources. The results for sympatric males are shown in Fig. 2a (n=8, t(7)= -5.5579, p<0.001, two-tailed paired t-test), those for allopatric males in Fig. 2b (Fig. 2b, n=8, t(7)=3.1268, p=0.017, two-tailed paired t-test). To exclude the possibility that the results reflect the presence of a complete (MVF plus MHC peptides) male signal, we combined the test males´ water sources with exogenous MHC peptides. We have previously shown that through the addition of exogenous peptides to a complete male signal the MHC peptide complexity is perceived as reflecting a super-optimal MHC genotype, which is invariably rejected by the choosing female. In the present situation, the preference of females for the water source containing additional MHC peptides (Fig. 3; n=8, t(7)= -3.0992, p=0.02114, , two-tailed paired t-test) confirms that no endogenous MHC peptides were present in the male water source.

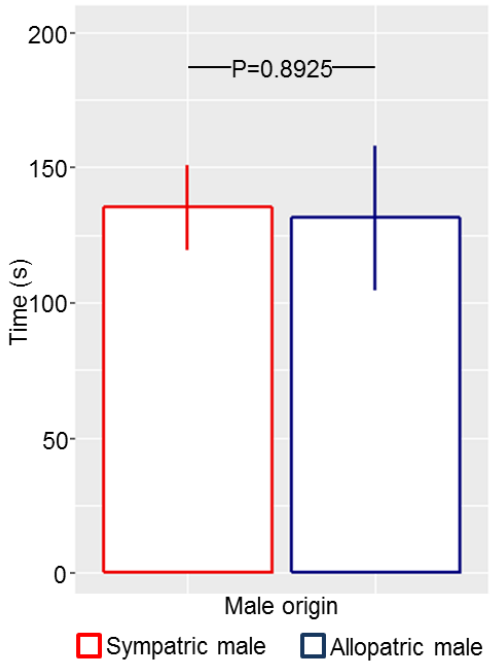

Figure 1: Comparison of allopatric and sympatric male validation factors (MVF). Time (s) mean ± SE of 600s the female spent in the quarter of the test chamber where either own continent or foreign continent MVF arrived with the current.

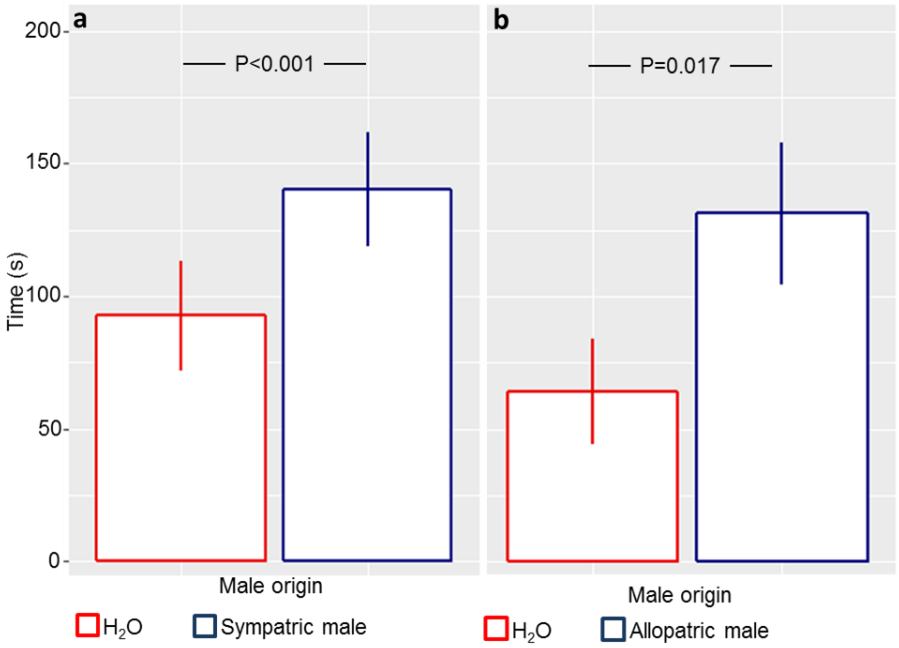

*Figure 2: Female preference for the MVF compared to plain water, both for the sympatric (**a**) and allopatric (**b**) MVF origin. Time (s) mean ± SE of 600s the female spent in the quarter of the test chamber where either MVF or no MVF arrived with the current.*

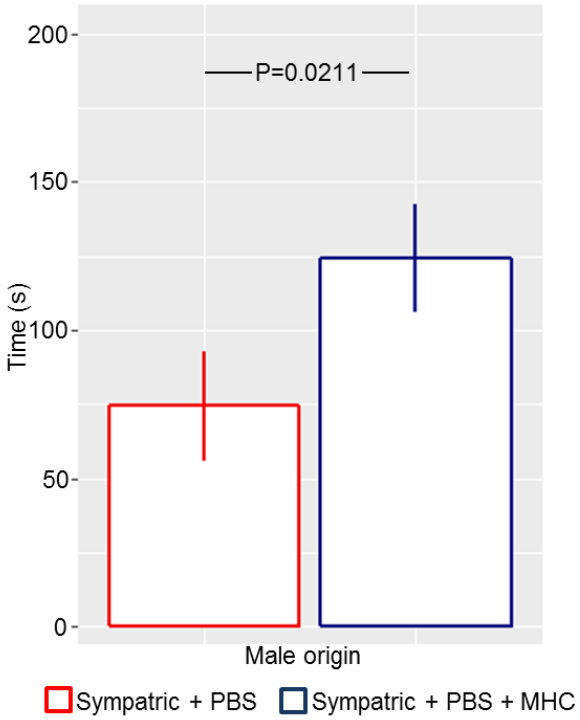

Figure 3: Female preference for the complete male odour. Time (s) mean ± SE of 600s the female spent in the quarter of the test chamber where MVF with either synthesized MHC peptides in PBS or only PBS arrived.

Discussion:

It is likely that the geographic separation of the two stickleback populations in Germany and Canada is associated with considerable genetic differences. Despite this, females of either continent are indifferent to the origin of the male validation factor (MVF) that accompanies the male MHC odor signal. This finding adds to our previous result that females do not distinguish between river or lake MVFs from adjacent populations in Germany (Andreou et al. 2017). Hence, the MVF signal seems to be evolutionarily conserved in three-spined sticklebacks. Within the context of mate choice, the MVF serves as a species identifier, allowing females to determine that a male three-spined stickleback is the source of a perceived MHC signal. Since MHC-based mate choice appears to be a general strategy of vertebrates (Milinski 2006, Boehm & Zufall 2006), it is likely that sticklebacks are constantly exposed to MHC signals of other fish in their environment. The presence of a species-specific validation factor thus prevents potential hybridization.

Our finding of a conserved MVF requires that the genes producing the MVF in males are not linked with the genes involved in the perception of the MVF in females. Any change on one side would produce a mismatch between signal and signal perception and consequently lead to unsuccessful mate recognition, ultimately purging any deviating signal or signal-perception from the population. Thus, it is unlikely that the MVF of three-spined sticklebacks from either Canada or Germany has been changed by genetic drift. The genetic basis of the MVF and its perception is unknown.
In conclusion, we demonstrate that the MVF is evolutionary conserved on a global scale and probably solely signals species identity. Our finding that the stickleback MVF appears to lack population-specific features is biologically important, since it suggests that information about populations and/or individuals primarily rests with the polymorphic MHC systems, supporting the unique status of MHC genes as magic traits (Gavrilets 2004, Andreou et al. 2017).

Acknowledgments: We thank D. Martens for help with maintaining the fish, S. Liedtke and T.L. Lenz for help with the RSCA, T. Henrich and M. Kalbe for help with catching the fish and in-vitro fertilizations.

This study was supported by the Max Planck Society for the Advancement of Science; C.L.G. received funding through the International Max Planck Research School for Evolutionary Biology.

General discussion & outlook:

Habitat dependent divergence towards distinct river and lake ecotypes is a globally reoccurring phenomenon. The underlying evolutionary pressures, driving this distinction, can most likely be attributed to the particular properties of the different habitats. Especially the diverse parasite communities, which greatly vary between the river and lake habitat, are a key driver of this divergence. These different properties drive the evolution towards optimally adapted phenotypes, specifically tailored to suit the environmental needs. Especially with regards to morphology and immunological parasite resistance.

The desirability of these traits becomes apparent during sexual selection, were the choosy females select their mate based on his congruence with the optimal, habitat specific phenotype. By relating the male's health status to his immune genes, the female ensures an optimal immunological foundation for her offspring's while selecting for beneficial genes. This optimization however, becomes a handicap on an individual level when the habitat changes, either through migration or naturally. Here, the mismatch will most likely result in a disadvantage towards resident fish as well as a potential reproductive dead end through adverse sexual selection.

In my thesis I demonstrate the strength of habitat dependent evolution on a global scale. Despite the geographic and genetic distance between the Canadian and German fish, we find reoccurring habitat specific adaptations towards river and lake ecotypes. As I show in Chapter I the different stickleback ecotypes vary in their affinity to succumb to the simultaneous infection with a large number of parasites under defined laboratory conditions. As these fish were naïve regarding parasite exposure, this demonstrates an inherent genetic basis for parasite resistance, which differs in a population dependent manner.

The surprisingly high resistance in the German river fish, compared to the Canadian lake, appears to be a local adaptation to the specific parasite species *Diplostomum pseudospathaceum*. Despite being a typical lake parasite, it does (albeit rarely) occur in German rivers at sufficiently high numbers to justify the maintenance of specific immune defenses in these fish. Nonetheless, we find the same discrepancy towards parasite resistance between both river and lake pairs. Exempt of the Canadian river fish, which show hardly any immunological reaction to the infection, all populations respond in similar fashion to the parasite. Thus, despite differences in the gene expression of the control fish, which hint at different initial immune defenses, the populations convergence towards similar gene expression profiles This

overlap in immune reaction between the populations suggests that ecotypic differentiation does not necessarily occur on a genetic, but rather on a regulatory level. Hence, from an immunological standpoint, the habitat dependent adaptation seems to mostly act on the regulatory mechanisms, governing appropriate immune responses. The distinction into river and lake ecotypes reoccurs across the entire extend of the sticklebacks distribution range. Driven by environmental factors, especially parasite pressures, the varying stickleback populations undergo environment dependent selection towards distinct, habitat dependent, ecotypes. The different immunological defense strategies of these ecotypes are specific to their habitat of origin, yet the acquired immunological benefits appear to be transposable between similar ecological contexts.

Ideally, I would have repeated the experiment using a Canadian *Diplstomum* species or, alternatively, with a parasite all populations are unfamiliar with (e.g. if the parasite where of Scandinavian origin). This proof of principle would have enabled me to weed out the discrepancies between local adaptation to a specific parasite species or lineage and the divergence towards habitat dependent ecotypes. Unfortunately, it was impossible to conciliate this experiment with both Canadian and European customs and environmental protection laws.

Following up on the previously described discrepancy between ecotypes in the laboratory, I exposed the same stickleback populations to a river and lake environment in a common garden experiment in the wild (Chapter II). To this end, lab reared fish of German or Canadian origin were distributed across cages into the respective habitats and segregated by sex to avoid confounding factors. I found that both river populations have inherently high growth rates, whereas the German lake population appears to invest strongly into its immune defense, at comparably detrimental cost to the their growth rate. This led to a significantly lower parasite burden during the first two measurement time points, but did not carry through to the final measurements. Most likely due to poor over all parasite numbers, the river fish were able to outgrow the lake fish in most relevant physiological measurements without suffering from excruciatingly high parasite burdens. There seems to be a tradeoff in resource allocation between growth and immunity. In the background of their original river habitat, with low parasite diversity, these are beneficial adaptations, allowing for higher fitness as size translates to reproductive output in sticklebacks. In the atypical lake environment the expected negative consequences of the river adaptations lacked, due to the comparably low parasite pressures. Most likely, the lake fish overinvested into their standing immune defense without benefiting from lower parasite burdens.

Consequently, the river fish were at an advantage in both habitats, despite their potentially higher susceptibility to parasitic infection.

Regarding the underlying molecular mechanisms, I am currently awaiting the analysis of the transcriptome data, hence I can currently only speculate on the mechanisms. In general, I expect a higher base line activity of immune relevant factors in the German lake population at the expense of a lower metabolism, compared to the German river fish. This pattern should be similar between the Canadian river and lake ecotypes. Further, based on the results from the lab infection (Chapter I), I would assume that the discrepancies in immune gene expression decrease over time, respectively between the different sampling time points. Due to the low parasite numbers, the initial disadvantage regarding immune defenses was not severe enough to have a long lasting negative impact on the (river) ecotypes. In contrary, this would allow the river fish to regulate or adapt their immune defenses to the occurring parasite pressures, eventually eliminating the initial differences with regards to parasite susceptibility.

Similar to the Chapter I experiment, it would be interesting to expose the different ecotypes to rivers and lakes in such a way that it excludes any local adaptation, e.g. by repeating the experiment in a geographically distant environment. This should further validate the habitat dependent evolution of distinct ecotypes, and allow for more direct comparison between the later. A further, maybe more interesting approach, would be to introduce the same ecotypes into a “semi” natural but enclosed environment (e.g. artificial lake) bare of any wild three-spined sticklebacks. By closely monitoring this mixed population over several generations, we should find that the difference between the ecotypes gradually decreases towards a single, specific phenotype, optimally adapted to the specific environmental pressures of that lake. Initially the original lake ecotypes will constitute the majority of the population, both due to parasite pressures and sexual selection. Over time, the remaining (former) river ecotypes will become virtually indistinguishable from the former lake ecotypes, resulting in a rather homogenous lake population. Besides validating all previous findings, it would show the speed (i.e. number of generations) at which these processes occur, contributing towards the understanding of ecological speciation in general.

Finally, for ecotype differentiation and potentially speciation to occur, we require mating barriers, either of behavioral, genetic or physical nature, to isolate the different populations (in this case ecotypes) from each other. In sticklebacks, the genetic basis for mate choice are so-called major histocompatibility complex (MHC) peptides, which are an integral part of vertebrae immunity. Female three-spined

sticklebacks will associate these MHC peptides, excreted by sexually reproductive males, with the health status of that respective male. I demonstrate that stickleback females assess the MHC allele diversity by counting the number of different alleles, and relate these to their own as well as the optimal number of alleles specific to that population (Chapter III). Indeed, I was able to show that both river and lake females choose solely based on the number of alleles, independently of the male's habitat of origin (i.e. river or lake).

Secreted MHC peptides are virtually identical between all vertebrae species, which has led to the establishment of a second factor, validating the species identity in sticklebacks. The absence of this so-called male validation factor (MVF) leads to an indifference towards any MHC signal during female mate choice decisions (Milinski et al., 2009). Previous work by Andreou et al., (2017) has demonstrated the MVF does not differ between adjacent river and lake populations of northern Germany, thus not carrying any population specific information. Using a Canadian and German river population (Chapter IV), I recorded female preference in the absence of the MHC signal, thus solely comparing the information content in the MVF signal. The conclusive results show that female three-spined sticklebacks do not differentiate between the MVF signal of these two, geographically and evolutionary distinct populations. Hence, I was able to show that the MVF does not convey any information past that of species identity and is thus evolutionary conserved on a global scale.

The remaining key question of stickleback mate choice is devoted to the nature of the MVF. From an evolutionary perspective, MHC based mate choice was most likely able to evolve on the basis of already present MVF signaling. As, contrary to MHC signaling, it is imaginable that the MVF might play a functional role outside of mate choice (e.g. schooling with conspecifics). The MVF must either be the glue-like protein spiggin itself, secreted by the body kidney or excreted from the urinary system (e.g. bladder). Unraveling the chemical nature of the validation factor might allow for synthetic production of the later. If successful, one could conduct mate choice trials in three-spined sticklebacks without any male participation whatsoever. Thereby proving that both the MVF and MHC are sufficient to accurately predict female mate choice decisions based on the individual's habitat of origin. Further, one could now test if both the use of MHC alleles and MVF signaling are equally valid in the ancestral marine populations. This has hitherto been difficult to achieve based on the different salinity levels the populations require, potentially influencing the female preference independently of the MHC and MVF signal. If achieved, it would demonstrate that signaling of species identity and subsequently MHC based

mate choice have been evolutionary conserved **since** the very beginning of the three-spined stickleback lineage.

One step further would be to conduct mate choice trials between three-spined sticklebacks and their closest relative, the nine-spined stickleback (*Pungitius pungitius*). As both are often found in the same habitat, I expect the three-spined stickleback MVF to play no functional role in sexual selection of nine-spined sticklebacks. Besides finalizing the clear demonstration of the MVF as a signal of species identity, this would also allow to pinpoint the evolutionary time point at which the species identity first appeared. Potentially, the initial divergence of these two species was facilitated by the three-spined stickleback MVF as we understand it today.

In summary, my thesis contributes to the further understanding of ecological speciation in general and in three-spined sticklebacks in particular. Shaped by environmental pressures, especially host-parasite dynamics, these ecotypes diverge in a habitat dependent manner on a globally reoccurring scale. The resulting differences are maintained and consolidated both by sexual and natural selection. The numerous different factors such as temperature, predation pressure, resource availability and immune challenges influencing theses divergences are highly variable, both between habitats as well as due to natural fluctuations, demanding phenotypic flexibility on an individual level. This flexibility allows mismatched individuals (e.g. through migration or displacement e.g. flooding) to acclimatize to new environmental pressures and eventually catch up with the resident population.

Author contribution:

Chapter I:

Gahr CL, Henrich T, Eizaguirre C, Reusch TBH, Milinski M, Kalbe M: Habitat dependent (parallel) evolution of parasite resistance in geographically distinct river-lake sticklebacks (Gasterosteus aculeatus)
MK conceived the study**.** MK, TH and CLG performed the experiments. CLG analyzed the data and wrote the manuscript. All authors* discussed the results and read and approved the final version of the manuscript.

Chapter II:

Gahr CL, Henrich T, Eizaguirre C, Reusch TBH, Milinski M, Kalbe M: Habitat dependent evolution of river-lake ecotypes in geographically three-spined stickleback (Gasterosteus aculeatus) populations; experimentally proven in the field
MM, TBHR and CE conceived the study. MK, TH and CLG performed the experiments, CLG analyzed the data with the help of CE. CLG wrote the manuscript. MM, CE, TBHR, TH and CLG discussed the results. and read and approved the final version of the manuscript.

Chapter III:

Gahr CL, Boem T, Milinski M: Female assortative mate choice functionally validates synthesized male odors of evolving stickleback river-lake ecotypes. Biol. Lett. 14: 20180730.
doi:http://dx.doi.org/10.1098/rsbl.2018.0730
MM and CLG conceived the study, CLG performed the experiments and analyzed the data. TB provided the synthetic peptides. CLG wrote the manuscript. All authors read, revised and approved the final version of the manuscript.

Chapter IV:

Gahr CL, Boem T, Milinski M: Evolutionary conservation of stickleback male validation factor.
Evolutionary ecology research, in review
MM and CLG conceived the study, CLG performed the experiments and analyzed the data. TB provided the synthetic peptides. CLG wrote the manuscript. All authors read, revised and approved the final version of the manuscript.

** To our greatest regret Martin Kalbe passed away before the finalization of the manuscripts, and was not able to approve the final version.*

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Eidesstattliche Erklärung:

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel:

Molecular basis of ecological speciation in sticklebacks

selbstständig, mit der Unterstützung meiner Betreuer, verfasst habe. Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein bisher erstes und einziges Promotionsverfahren.

Kapitel III dieser Arbeit wurde in der wissenschaftlichen Fachzeitschrift “Biology Letters” veröffentlicht. Die Koautoren aller Kapitel finden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Beitrag der Autoren zu den einzelnen Manuskripten wird im Abschnitt “Author contributions” erläutert.

Plön, im Dezember 2018 Christoph L. Gahr

Appendix:

Chapter I:

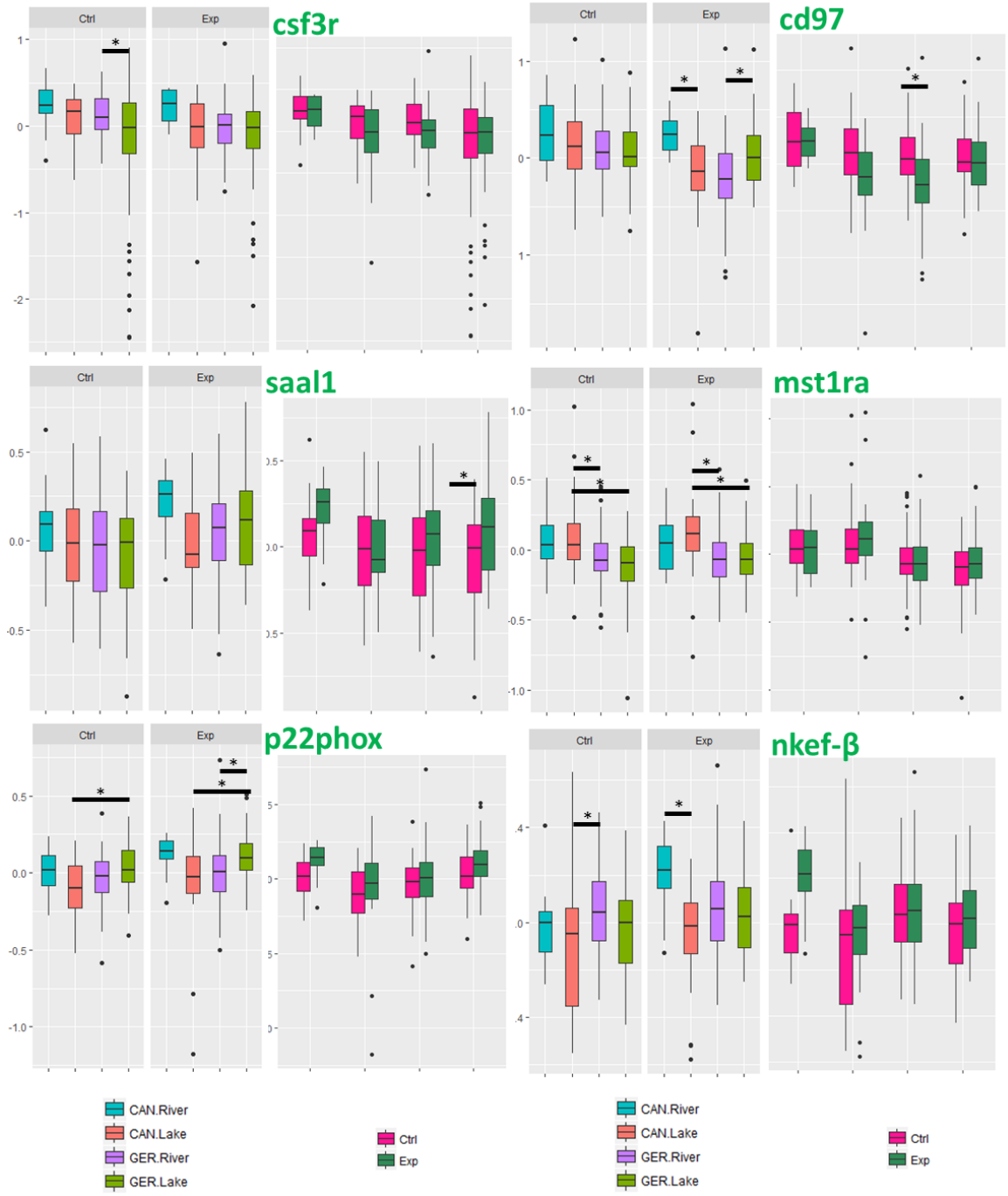
Appendix Table A1: Gene expression target, gene references and primer sequences used in the gene expression analysis of the study

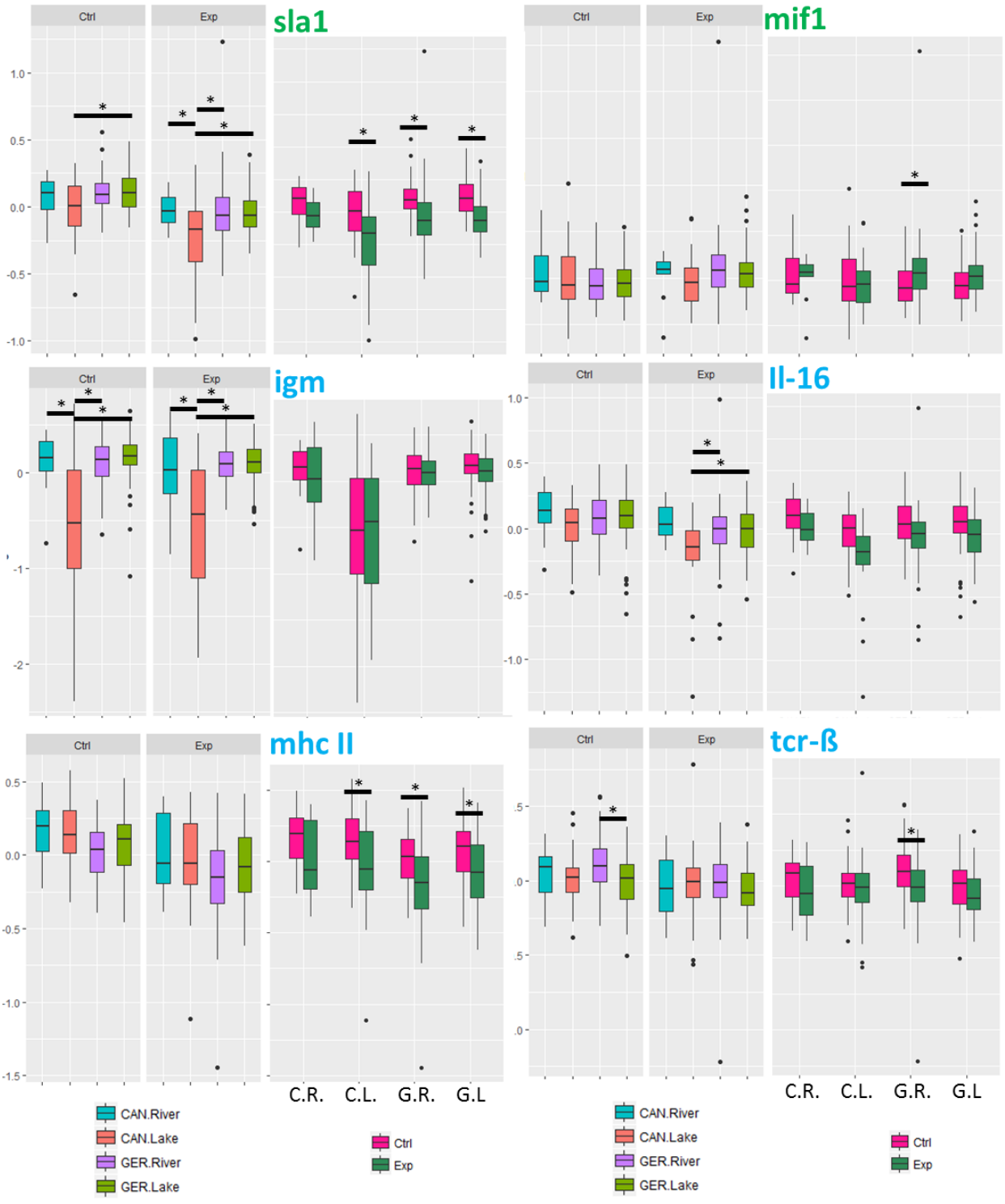
Gene	Function	Refernces	Forward / Reverse primer	Ensemble ID
Reference genes				
b2m	Beta-2-microglobulin	Hibbeler et al., 2008	GAAGATGTGTTGAATAGAAGCTGG AGACTATGCCTGGGAATCAAAC	ENSGACT00000025537
ef1a	Elongation factor 1α	Hibbeler et al., 2008	CCACCGTTGCCTTTGTCC TGGGACTGTTCCAATACCTCC	ENSGACT00000002833
Rpl13a	L13A ribosomal binding protein	Hibbeler et al., 2008	CACCTTGGTCAACTTGAACAGTG TCCCTCCGCCCTACGAC	ENSGACT00000012319
ubc	Ubiquitin	Hibbeler et al., 2008	AGACGGGCATAGCACTTGC CAGGACAAGGAAGGCATCC	ENSGACT00000010662
Innate immune genes				
cd97	Promoter of granulocyte and neutrophil migration, required for activation of the innate immune response	Leemans et al., 2004 Rhodes et al., 2009	CTCGTGGCACTCTACGACATGAAG CAGCCCTATCTTGGTGACCAGTTG	ENSGACT00000024871
csf3r	Granulocyte colony-stimulating factor 3 receptor; role in differentiation and proliferation of granulocytes	Tabbara 1993 Birrer et al., 2012 Maxson et al., 2013 Brunner et al., 2016	TCGGGATTCGTCCTCTTCTCAG TGGGTCAAACTTGGCTGCAC	ENSGACT00000018254
il-1β	Interleukin 1 β; cytokine with function in early response proinflammatory signaling	Zhu et al., 2012 Brunner et al., 2017	TGACGATGAAGCAGGTGGTCAAC ACAGCGTCACGATCTCCTCTTC	ENSGACT00000019325
marco, RON	Macrophage receptor with collagenous structure; mediates macrophage recognition and clearance of pathogens	Kraal et al., 2000 Kissick et al., 2014	CCCTTTCGACCTTCACTGCC TGTTTACCCCAACCCCTCCA	ENSGACG00000001509
mif1	Macrophage migration inhibitory factor; stops random macrophage migration through tissue, proinflammatory mediator of the innate immune system	Calandra & Roger 2003 Brunner et al., 2017	ATCAGCGGAGCTCACAACAAGC TCAGGAGAGATGCTCAGGTGTTTG	ENSGACT00000023656
mst1ra	Macrophage stimulating 1 receptor a; plays an important role in macrophage regulation	Wang et al., 2002 Huang et al., 2016	ATGGCCATCGAAAGCTTGCA TGATGTCGTACGGGTCCACA	ENSGACG00000010551
nkef- β, peroxiredoxin 1	Natural killer cell enhancing factor; Enhances cytotoxicity of NK cells, Also protects cells against	Shau et al., 1993 Stutz et al., 2015	ACTTCTCCCACTTTGCATGG CAATGCCTTCATCCTCCTTC	ENSGACG00000021380

	oxidative damage.			
p22phox	NADPH oxidase component p22phox; part of the reactive oxygen species production machinery	Matute et al., 2005 Mayumi et al., 2008	GCCTCGGGACTCATTCTCCT TGGCCCTCTTGCTTCTTGGA	ENSGACG00000021084
saal1	Serum amyloid A; acute phase protein during inflammation response, mediates release of TNF-α and il-1β	Haarder et al., 2013 Kovacevic et al., 2015 Brunner et al., 2017	TCGCAGTGAGGCCAAAGATGAG AAATCTGCCACCGTGTCCTTGG	ENSGACT00000007599
sla1	Src-like-adaptor, necessary for maturation and activation of monocytic and dendritic cells, functions in T-cell singaling and B-cell development and function	Marton et al., 2015 Brunner et al., 2017	ACAGAGTCGGCTCCTTCATGATAC TCACAGAGAGCGAATACAGACCTC	ENSGACT00000007895
tnfr1	Tumor necrosis factor receptor 1; functions in regulation of inflammation, mediates cellular apoptosis and differentiation	Zhu et al., 2012 Brunner et al., 2017	AACTACTACAGAGCCAAGGGCAAG ACGGCACTCAGCGGTACAATTC	ENSGACG00000013502
		Adaptive immune genes		
cd83	Marker for mature dendritic cells; expressed on activated B- and T-cells, costimulatory to activate naïve and memory T-cells	Aerts-Toegaert et al., 2007 Stutz et al., 2015	AGGACCCAGCGTATAAATGG CCCTGGTGATTTTCCTCATC	ENSGACG000000
foxp3; forkhead box N2b	Transcription factor; regulates functions important for the establishment of the T-reg lineage, key mediator of T-cell activation	Rao & Naqvi 2011 Robertson et al., 2016 Kasheta et al., 2017	GTTGACCCATGCAATTCCGA CTGCTGTAGTTGTGGTCCTG	ENSGACG00000012777
igm	Immunoglobulin heavy constant mu (IgM); antibody molecule, part of the humoral immune response	Hibbeler *unpublished* Rønneseth et al., 2015 Zhu et al., 2012	TGTTGAGGTTGACCAGGTTG AGCAGTGGCAAAAGAACGAC	ENSGACG00000016907
il-16	Interleukin 16; cytokine with function in T-cell migration and expansion, chemoattractant for monocytes and eosinophils	Wen et al., 2006 Zhu et al., 2012 Brunner et al., 2017	CTGGTCTGGGCTTCAGTATTGC CTGGGAAACACTCTGTGGACTG	ENSGACT00000016499
mhc II	Major histocompatibility complex class IIb exon 2; pathogen recognizing protein of the adaptive immune response	Lenz et al., 2009	GTCTTTAACTCCACGGAGCTGAAGG ACTCACCGGACTTAGTCAG	ENSGACG00000000425
stat4	Signal transducer and activator of transcription 4; required for TH1-cell differentiation, opposes TH2 and TH17 like responses	Kaplan 2005 Premachandra et al., 2013 Wang & Secombes 2013	CTCTCAGTTTCGAGGCTTGCTT GGCAGTTGGCTCACATTGG	ENSGACG00000002684
stat6	Signal transducer and activator of transcription 6;	Wang & Secombes	CTCAGCCACAGTTCCAACCGTTC GTCGGATGTTCTGGACCTCGAGT	ENSGACG00000008477

	required for TH2-cell differentiation, regulates expression of TH2 relevant cytokine IL-4	2013 Robertson et al., 2016		
tC.L.-β	T-cell receptor β-chain; function in binding of MHC-peptide ligands to initiate adaptive immune response	Yanagi et al., 1984 Smith-Garvin et al., 2009 Stutz et al., 2015	GAGGGCAAAAACTTCACCTG TAGGAGAATCTGGCCGTTTG	ENSGACG00000016457
tgf-β	Transforming growth factor β; cytokine with functions in cell growth, migration, differentiation and proliferation of T- and B-cells	Zhu et al., 2012 Robertson et al., 2016	TCCCGCTTCGTCACCAACCA ACGTCTGTCTGGCCACATTCAC	ENSGACT00000016968
		Complement system		
c7	Complement component 7; initializing function in the membrane attack complex of the complement system	Zhu et al., 2012 Haase et al., 2014 Brunner et al., 2017	TGGCTCAAGCTCAGCACAACAG AGCGACACGTGTTTGTTTGATCG	ENSGACT00000009181
c9	Complement component 9; structural part of the memebrane attack complex of the complement system	Zhu et al., 2012 Haase et al., 2014 Brunner et al., 2017	CCGTGACGAACAAAGACTCAGTTG TCTGACCGATGTCAGCACCTTG	ENSGACT00000020968
cfb	Complement factor B; activating complement component of the alternative pathway	Zhu et al., 2012 Haase et al., 2014 Brunner et al., 2017	GAGCGTCGCACAATACAGGTTG TACCACCGGAAGCGCACAAATC	ENSGACT00000027346

Appendix. Fig. A1: Gene expression difference (ΔCt, y-axis) for the individual genes used in the study. The gene expression is shown separately for the two treatment groups (control (ctrl) and exposed (exp)) between populations as well as in a direct comparison within population. Only genes with significant differences are shown and colour coded according to functional groups; green=innate immune genes, blue=adaptive immune genes. The box plots represent the medians ± 1st and 2nd quartiles. Statistical analysis where performed for each plot individually using linear models with Tukey post-hoc test and fdr corrected for multiple testing.




Ctrl
Exp
sla1
mif1
igm
Il-16
mhc II
tcr-ß
C.R.
C.L.
G.R.
G.L
CAN.River
CAN.Lake
GER.River
GER.Lake
Ctrl
Exp

Chapter II:

Appendix Table A2: Mortality rate of the different populations, separated by time point and habitat of exposure.

Date	Location	Population	%Female	%Male	%Total
DEZ	Lake	CAN River	83.33	94.44	88.89
MAY	Lake	CAN River	100.00	72.22	86.11
JUL	Lake	CAN River	11.11	11.11	11.11
DEZ	Lake	CAN Lake	88.89	88.89	88.89
MAY	Lake	CAN Lake	72.22	83.33	77.78
JUL	Lake	CAN Lake	8.33	19.44	13.89
DEZ	Lake	GER River	100.00	100.00	100.00
MAY	Lake	GER River	100.00	100.00	100.00
JUL	Lake	GER River	94.44	69.44	81.94
DEZ	Lake	GER Lake	100.00	100.00	100.00
MAY	Lake	GER Lake	77.78	66.67	72.22
JUL	Lake	GER Lake	91.67	61.11	76.39
DEZ	River	CAN River	88.89	94.44	91.67
MAY	River	CAN River	83.33	94.44	88.89
JUL	River	CAN River	72.22	55.56	63.89
DEZ	River	CAN Lake	100.00	94.44	97.22
MAY	River	CAN Lake	100.00	94.44	97.22
JUL	River	CAN Lake	61.11	77.78	69.44
DEZ	River	GER River	100.00	94.44	97.22
MAY	River	GER River	88.89	94.44	91.67
JUL	River	GER River	72.22	86.11	79.17
DEZ	River	GER Lake	88.89	83.33	86.11
MAY	River	GER Lake	88.89	94.44	91.67
JUL	River	GER Lake	77.78	80.56	79.17
JUL	LAB	CAN River	100.00	94.44	97.22
JUL	LAB	CAN Lake	94.44	94.44	94.44
JUL	LAB	GER River	100.00	77.78	88.89
JUL	LAB	GER Lake	77.78	72.22	75.00

Appendix Table A3: Statistically significant difference in weight growth between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Habitat	Sex	factor	Est.	Std. Error	z	p	direction of effect
body mass growth since September								
December	Lake	m	Population	-0.17376	-0.04845	-3.586	0.00197	G.L. - G.R.
December	Lake	f	Population	-0.17632	0.0537	-3.284	0.00533	G.L.- C.R.
December	Lake	f	Population	-0.18749	0.05325	-3.521	0.00244	G.L. - G.R.
July	Lake	m	Population	-0.28307	0.10709	-2.643	0.038	G.R. - C.R.
July	Lake	m	Population	-0.58315	0.10849	-5.375	<0.001	G.L. - C.R.
July	Lake	m	Population	-0.45368	0.09206	-4.928	<0.001	G.L. - C.L.
July	Lake	m	Population	-0.30008	0.05887	-5.098	<0.001	G.L. - G.R.
July	Lake	f	Population	-0.72371	0.22471	-3.221	0.00674	G.L. - C.R.
May	Lake	m	Population	-0.32399	0.08816	-3.675	0.00138	G.L. - G.R.
May	Lake	f	Population	0.35156	0.11723	2.999	0.0144	G.R. - C.R.
May	Lake	f	Population	-0.5731	0.12211	-4.693	<0.001	G.L. - G.R.
July	River	m	Population	0.34566	0.12947	2.67	0.0382	G.R. - C.L.
July	River	m	Population	-0.56458	0.12917	-4.371	<0.001	G.L. - G.R.
July	River	f	Population	0.81755	0.17888	4.57	<0.0001	G.R. - C.R.
July	River	f	Population	1.00351	0.18257	5.496	<0.0001	G.R. - C.L.
July	River	f	Population	-0.93364	0.1778	-5.251	<0.0001	G.L. - G.R.
May	River	m	Population	-0.42293	0.12285	-3.443	0.00318	G.L. - C.L.
May	River	m	Population	-0.44841	0.12285	-3.65	0.00137	G.L. - G.R.
May	River	f	Population	0.5997	0.19109	3.138	0.00909	G.R. - C.R.
May	River	f	Population	-0.62134	0.19109	-3.252	0.00649	G.L. - G.R.

Appendix Table A4: Statistically significant difference in length gain between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
length gain since September								
December	Lake	m	Population	-0.200	0.072	-2.778	0.028	G.L.-C.R.
December	Lake	m	Population	-0.244	0.071	-3.420	0.003	G.L.-G.R.
December	Lake	f	Population	-0.375	0.082	-3.581	<0.001	G.L.-C.R.
December	Lake	f	Population	-0.297	0.082	-3.631	0.002	G.L.-C.L.
December	Lake	f	Population	-0.289	0.080	-3.594	0.002	G.L.-G.R.
July	Lake	m	Population	-0.622	0.153	-4.075	<0.001	G.L.-C.R.
July	Lake	m	Population	-0.430	0.143	-3.004	0.014	G.L.-C.L.
July	Lake	f	Population	-0.410	0.136	-3.018	0.012	G.R.-C.R.
July	Lake	f	Population	-0.633	0.136	-4.658	<0.001	G.L.-C.R.

July	Lake	f	Population	-0.408	0.136	-3.004	0.013	G.L.-C.L.
July	Lake	f	Population	-0.224	0.080	-2.812	0.023	G.L.-G.R.
May	Lake	m	Population	0.347	0.105	3.295	0.005	G.R.-C.L.
May	Lake	m	Population	-0.335	0.114	-2.947	0.017	G.L.-G.R.
May	Lake	f	Population	-0.222	0.086	-2.577	0.049	G.L.-C.R.
May	Lake	f	Population	-0.360	0.090	-4.003	<0.001	G.L._G.R.
July	River	m	Population	-0.296	0.108	-2.746	0.031	G.L.-C.R.
July	River	m	Population	0.345	0.103	3.364	0.004	G.R.-C.L.
July	River	m	Population	-0.481	0.102	-4.714	<0.001	G.L.-G.R.
July	River	f	Population	0.359	0.091	3.924	<0.001	G.R.-C.R.
July	River	f	Population	0.484	0.095	5.080	<0.001	G.R.-C.L.
July	River	f	Population	-0.591	0.091	-6.525	<0.001	G.L.-G.R.
May	River	m	Population	-0.304	0.091	-3.325	0.005	G.L.-C.R.
May	River	m	Population	-0.309	0.091	-3.377	0.004	G.L.-C.L.
May	River	m	Population	-0.369	0.091	-4.036	<0.001	G.L.-G.R.
May	River	f	Population	0.281	0.087	3.235	0.006	G.R.-C.R.
May	River	f	Population	0.228	0.085	2.673	0.038	G.R.-C.L.
May	River	f	Population	-0.382	0.087	-4.400	<0.001	G.L.-G.R.

Appendix Table A5: Statistically significant difference in Shannon Index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Shannon Index								
December	Lake	m/f	Population	-0.43013	0.07385	-5.824	<0.0001	G.L.-C.R.
December	Lake	m/f	Population	-0.3762	0.07385	-5.094	<0.0001	G.L.-C.L.
December	Lake	m/f	Population	-0.45695	0.07178	-6.366	<0.0001	G.L.-G.R.
May	Lake	m/f	Population	-0.80068	0.10981	-7.291	<0.0001	G.L.-C.R.
May	Lake	m/f	Population	-0.65922	0.1116	-5.907	<0.0001	G.L.-C.L.
May	Lake	m/f	Population	-0.70829	0.10802	-6.557	<0.0001	G.L.-G.R.

Appendix Table A6: Statistically significant difference in body kidney weight and index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Body kidney								
July	Lake	m	Population	-0.040	0.012	-3.345	0.004	G.L.-C.R.
May	Lake	m	Population	0.028	0.008	3.326	0.005	G.R.-C.R.
May	Lake	m	Population	0.025	0.008	3.005	0.014	G.R.-C.L.
May	River	m	Population	0.053	0.013	4.037	<0.001	G.R.-C.R.
May	River	m	Population	0.043	0.013	3.334	0.005	G.R.-C.L.

May	River	m	Population	-0.041	0.013	-3.172	0.008	G.L.-G.R.
Body kidney Index (%/total body mass)								
July	Lab	m	Population	1.010	0.344	2.937	0.017	G.R.-C.R.
July	Lake	f	Population	0.312	0.109	2.860	0.020	G.L.-C.R.
July	Lake	f	Population	0.311	0.109	2.852	0.020	G.L.-C.L.
May	Lake	m	Population	2.569	0.641	4.006	<0.001	G.R.-C.R.
May	Lake	m	Population	2.105	0.630	3.340	0.005	G.R.-C.L.
May	Lake	f	Population	0.298	0.098	3.036	0.013	G.L.-C.R.
May	Lake	f	Population	0.338	0.106	3.181	0.008	G.L.-C.L.
May	Lake	f	Population	0.293	0.102	2.864	0.021	G.L.-G.R.
July	River	m	Population	1.791	0.601	2.978	0.015	G.R.-C.R.
July	River	m	Population	1.563	0.605	2.585	0.048	G.L.-C.R.
May	River	m	Population	3.389	0.659	5.145	<0.001	G.R.-C.R.
May	River	m	Population	3.001	0.659	4.556	<0.001	G.R.-C.L.
May	River	m	Population	-2.258	0.659	-3.428	0.004	G.L.-G.R.

Appendix Table A7: Statistically significant difference in gonad weight and index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Gonads								
July	Lake	m	Population	-0.063	0.023	-2.783	0.026	G.R.-C.L.
July	Lake	m	Population	-0.064	0.023	-2.807	0.024	G.L.-C.L.
May	Lake	f	Population	0.307	0.060	5.071	<0.001	G.R.-C.R.
May	Lake	f	Population	0.231	0.065	3.535	0.002	G.R.-C.L.
May	Lake	f	Population	-0.215	0.064	-3.362	0.004	G.L.-G.R.
July	River	f	Population	0.260	0.054	4.821	<0.001	G.R.-C.R.
July	River	f	Population	0.230	0.056	4.137	<0.001	G.R.-C.L.
July	River	f	Population	-0.160	0.053	-2.996	0.014	G.L.-G.R.
May	River	f	Population	0.302	0.063	4.798	<0.001	G.R.-C.R.
May	River	f	Population	0.224	0.062	3.621	0.002	G.R.-C.L.
Gonad Index (%/total body mass)								
July	Lab	f	Population	5.834	1.445	4.037	<0.001	G.R.-C.R.
July	Lab	f	Population	4.178	1.542	2.71	0.034	G.L.-C.R.
July	Lab	f	Population	4.823	1.487	3.243	0.007	G.R.-C.L.
July	Lake	m	Population	-3.321	1.206	-2.753	0.028	G.R.-C.L.
July	Lake	m	Population	-3.296	1.228	-2.683	0.034	G.L.-C.L.
May	Lake	f	Population	17.051	3.235	5.271	<0.001	G.R.-C.R.
May	Lake	f	Population	11.793	3.297	3.577	0.002	G.L.-C.R.
May	Lake	f	Population	10.932	3.506	3.118	0.010	G.R.-C.L.
July	River	f	Population	7.868	1.921	4.095	<0.001	G.R.-C.R.
July	River	f	Population	7.927	1.905	4.162	<0.001	G.L.-C.R.

May	River	f	Population	13.983	2.807	4.982	<0.001	G.R.-C.R.
May	River	f	Population	12.102	2.807	4.311	<0.001	G.L.-C.R.
May	River	f	Population	8.634	2.745	3.146	0.009	G.R.-C.L.

Appendix Table A8: Statistically significant difference in head kidney weight and index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Head kidney								
July	Lake	m	Population	0.032	0.010	3.054	0.011	G.R.-C.R.
July	Lake	m	Population	0.025	0.009	2.596	0.044	G.R.-C.L.
July	River	m	Population	0.035	0.009	3.988	<0.001	G.R.-C.R.
July	River	m	Population	0.033	0.009	3.818	<0.001	G.R.-C.L.
July	River	m	Population	-0.025	0.009	-2.942	0.017	G.L.-G.R.
July	River	f	Population	0.078	0.013	5.915	<0.0001	G.R.-C.R.
July	River	f	Population	0.081	0.013	6.038	<0.0001	G.R.-C.L.
July	River	f	Population	-0.057	0.013	-4.328	0.000	G.L.-G.R.
May	River	f	Population	0.019	0.006	2.937	0.017	G.R.-C.L.
May	River	f	Population	-0.021	0.007	-3.126	0.009	G.L.-G.R.
Head kidney Index (%/total body mass)								
July	Lab	m	Population	1.010	0.344	2.937	0.017	G.R.-C.R.
July	Lab	f	Population	1.772	0.513	3.451	0.003	G.L.-C.R.
July	Lake	m	Population	2.807	0.735	3.820	<0.001	G.R.-C.R.
July	Lake	m	Population	3.085	0.743	4.152	<0.001	G.L.-C.R.
July	Lake	m	Population	1.869	0.658	2.839	0.002	G.R.-C.L.
July	Lake	m	Population	2.147	0.667	3.217	0.007	G.L.-C.L.
July	Lake	f	Population	1.826	0.687	2.656	0.035	G.R.-C.R.
May	Lake	m	Population	1.391	0.451	3.083	0.014	G.L.-C.R.
May	Lake	m	Population	1.449	0.441	3.285	0.006	G.L.-C.L.
May	Lake	m	Population	1.309	0.427	3.070	0.012	G.L.-G.R.
May	Lake	f	Population	1.240	0.325	3.811	0.001	WS-C.R.
May	Lake	f	Population	1.434	0.352	4.080	0.000	G.L.-C.L.
May	Lake	f	Population	1.328	0.339	3.915	0.001	G.L.-G.R.
July	River	m	Population	1.492	0.366	4.077	<0.001	G.R.-C.R.
July	River	m	Population	1.892	0.368	5.139	<0.001	G.L.-C.R.
July	River	m	Population	1.193	0.342	3.491	0.003	G.R.-C.L.
July	River	m	Population	1.593	0.344	4.629	<0.001	G.L.-C.L.
July	River	f	Population	1.650	0.498	3.316	0.005	G.R.-C.R.
July	River	f	Population	1.779	0.496	3.590	0.002	G.L.-C.R.
July	River	f	Population	1.390	0.508	2.733	0.032	G.R.-C.L.
July	River	f	Population	1.519	0.507	2.998	0.014	G.L.-C.L.

Appendix Table A9: Statistically significant difference in liver weight and index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Liver								
July	Lab	f	Population	0.019	0.003	5.888	<0.001	G.R.-C.R.
July	Lab	f	Population	0.011	0.003	3.233	0.007	G.L.-C.R.
July	Lab	f	Population	0.019	0.003	5.632	<0.001	G.R.-C.L.
July	Lab	f	Population	0.011	0.004	3.071	0.011	G.L.-C.L.
July	Lake	m	Population	-0.017	0.007	-2.558	0.048	G.L.-C.R.
July	Lake	m	Population	-0.016	0.006	-2.799	0.024	G.R.-C.L.
July	Lake	m	Population	-0.214	0.006	-3.756	<0.001	G.L.-C.L.
May	Lake	f	Population	0.058	0.012	4.762	<0.001	G.R.-C.R.
May	Lake	f	Population	0.052	0.013	4.078	<0.001	G.R.-C.L.
May	Lake	f	Population	-0.044	0.013	-3.470	0.003	G.L.-G.R.
May	Lake	m	Population	0.014	0.005	2.669	0.038	G.R.-C.R.
May	Lake	m	Population	-0.016	0.006	-2.860	0.023	G.L.-G.R.
July	River	f	Population	0.121	0.013	9.498	<0.001	G.R.-C.R.
July	River	f	Population	0.038	0.013	3.022	0.013	G.L.-C.R.
July	River	f	Population	0.122	0.013	9.262	<0.001	G.R.-C.L.
July	River	f	Population	0.040	0.013	3.016	0.014	G.L.-C.L.
July	River	f	Population	-0.083	0.013	-6.544	<0.001	G.L.-G.R.
July	River	m	Population	0.029	0.008	3.620	0.002	G.R.-C.R.
July	River	m	Population	0.023	0.008	2.975	0.153	G.R.-C.L.
July	River	m	Population	-0.027	0.008	-3.590	0.002	G.L.-G.R.
May	River	f	Population	0.072	0.020	3.527	0.002	G.R.-C.R.
May	River	f	Population	0.062	0.020	3.068	0.012	G.R.-C.L.
May	River	f	Population	-0.056	0.020	-2.755	0.030	G.L.-G.R.
Liver Index (%/total body mass)								
July	Lab	m	Population	0.799	0.264	3.025	0.013	G.R.-C.R.
July	Lab	m	Population	0.732	0.267	2.742	0.031	G.R.-C.L.
July	Lab	f	Population	2.221	0.330	6.728	<0.0001	G.R.-C.R.
July	Lab	f	Population	2.003	0.353	5.674	<0.0001	G.L.-C.R.
July	Lab	f	Population	2.219	0.340	6.519	<0.0001	G.R.-C.L.
July	Lab	f	Population	2.000	0.362	5.518	<0.0001	G.L.-C.L.
July	Lake	f	Population	1.785	0.464	3.849	<0.001	G.R.-C.R.
July	Lake	f	Population	2.279	0.464	4.908	<0.001	G.L.-C.R.
July	Lake	f	Population	1.413	0.464	3.042	0.011	G.L.-C.L.
July	Lake	m	Population	-0.923	0.328	-2.811	0.024	G.R.-C.L.
May	Lake	f	Population	3.011	0.643	4.684	<0.001	G.R.-C.R.
May	Lake	f	Population	2.580	0.651	3.966	<0.001	G.L.-C.R.
May	Lake	f	Population	2.630	0.668	3.938	<0.001	G.R.-C.L.

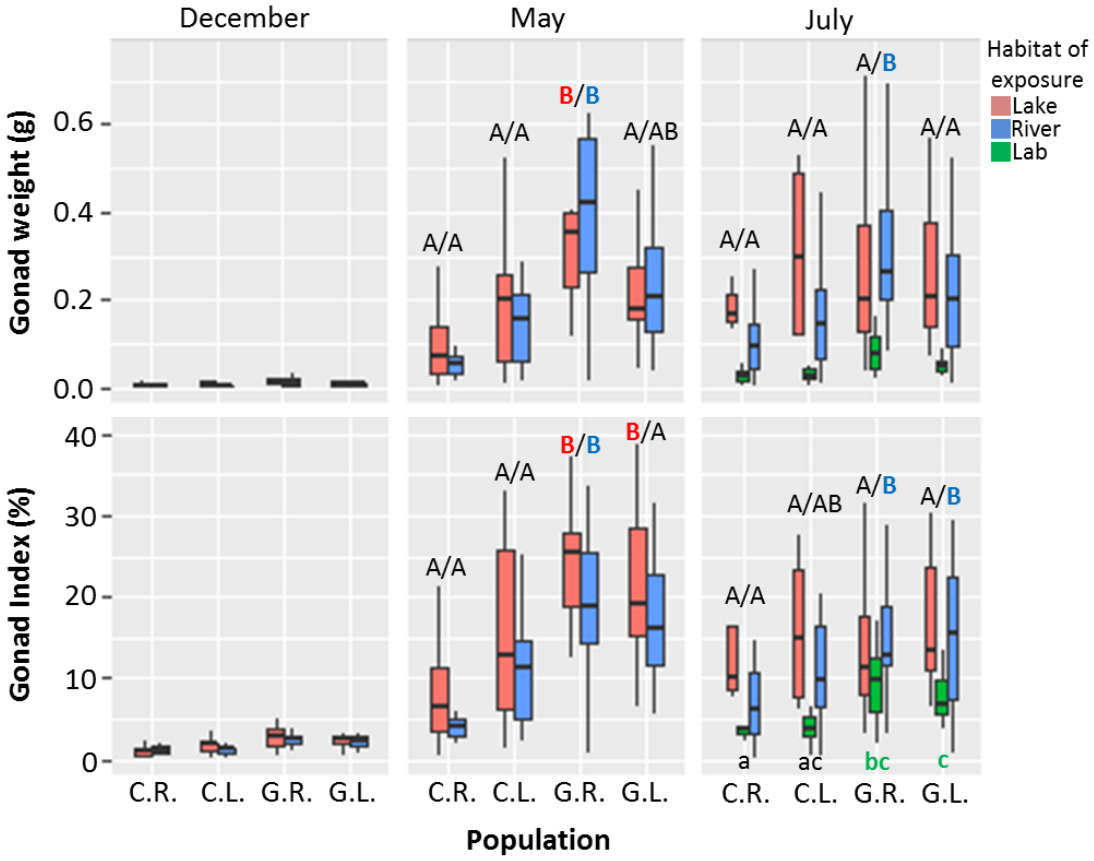
May	Lake	f	Population	2.198	0.675	3.256	0.006	G.L.-C.L.
May	Lake	m	Population	1.105	0.377	2.931	0.018	G.R.-C.R.
July	River	f	Population	3.769	0.318	11.872	<0.001	G.R.-C.R.
July	River	f	Population	3.045	0.315	9.678	<0.001	G.L.-C.R.
July	River	f	Population	3.329	0.335	9.937	<0.001	G.R.-C.L.
July	River	f	Population	2.605	0.332	7.838	<0.001	G.L.-C.L.
July	River	m	Population	1.391	0.328	4.243	<0.001	G.R.-C.R.
July	River	m	Population	1.125	0.330	3.410	0.004	G.L.-C.R.
May	River	f	Population	1.895	0.709	2.675	0.038	G.R.-C.R.

<u>Appendix Table A10:</u> Statistically significant difference in spleen weight and index between the populations, calculated separately for sex and time point (i.e. Date) using general linear models (glm's) with Tukey post-hoc test and fdr correction for multiple testing.

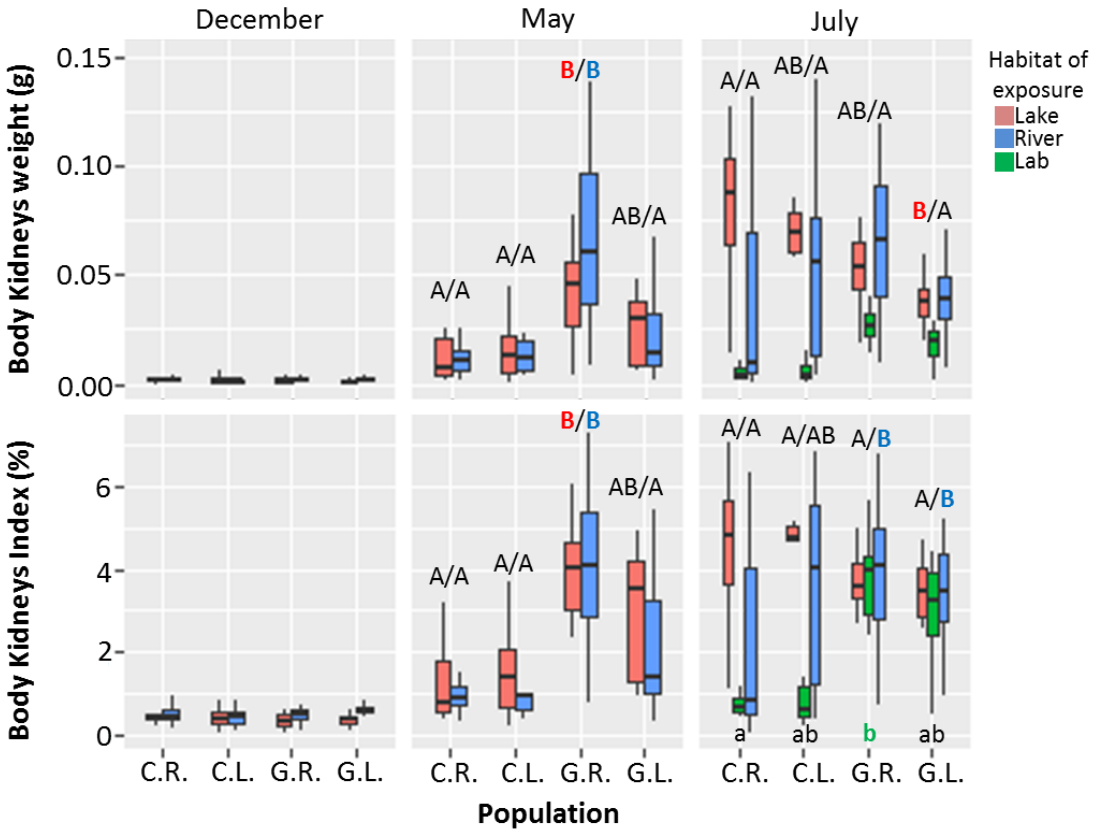
Date	Location	Sex	factor	Est.	Std. Error	z	p	direction of effect
Spleen								
July	Lab	f	Population	0.003	0.001	3.289	0.006	G.L.-C.R.
July	Lab	f	Population	0.002	0.001	2.675	0.038	G.L.-C.L.
July	River	f	Population	0.039	0.008	4.668	<0.001	G.R.-C.R.
July	River	f	Population	0.031	0.008	3.682	0.001	G.R.-C.L.
July	River	f	Population	-0.027	0.008	-3.271	0.006	G.L.-G.R.
July	River	m	Population	0.023	0.005	5.051	<0.001	G.R.-C.R.
July	River	m	Population	0.021	0.004	4.640	<0.001	G.R.-C.L.
July	River	m	Population	-0.014	0.004	-3.185	0.008	G.L.-G.R.
May	River	f	Population	0.011	0.004	2.941	0.017	G.R.-C.R.
Spleen Index (%/total body mass)								
July	Lab	f	Population	0.473	0.114	4.154	<0.001	G.L.-C.R.
July	Lab	f	Population	0.435	0.117	3.721	0.001	G.L.-C.L.
July	Lab	f	Population	0.320	0.114	2.809	0.026	G.L.-G.R.
July	Lake	f	Population	1.275	0.455	2.800	0.025	G.L.-C.R.
July	Lake	f	Population	1.178	0.455	2.587	0.045	G.L.-C.L.
July	Lake	m	Population	1.011	0.355	2.848	0.022	G.L.-C.R.
July	Lake	m	Population	1.074	0.329	3.267	0.006	G.L.-C.L.
May	Lake	f	Population	0.526	0.180	2.922	0.019	C.L.-C.R.
May	Lake	f	Population	-0.554	0.185	-2.989	0.015	G.R.-C.L.
May	Lake	m	Population	-0.265	0.100	-2.634	0.042	G.R.-C.L.
July	River	f	Population	1.250	0.341	3.671	0.001	G.R.-C.R.
July	River	f	Population	0.915	0.339	2.697	0.036	G.L.-C.R.
July	River	m	Population	1.182	0.277	4.276	<0.001	G.R.-C.R.
July	River	m	Population	1.012	0.277	3.650	0.001	G.L.-C.R.
July	River	m	Population	0.982	0.269	3.650	0.002	G.R.-C.L.
July	River	m	Population	0.811	0.270	3.008	0.014	G.L.-C.L.
May	River	m	Population	0.539	0.170	3.178	0.008	G.L.-C.R.

May	River	m	Population	0.468	0.170	2.762	0.030	G.L.-C.L.

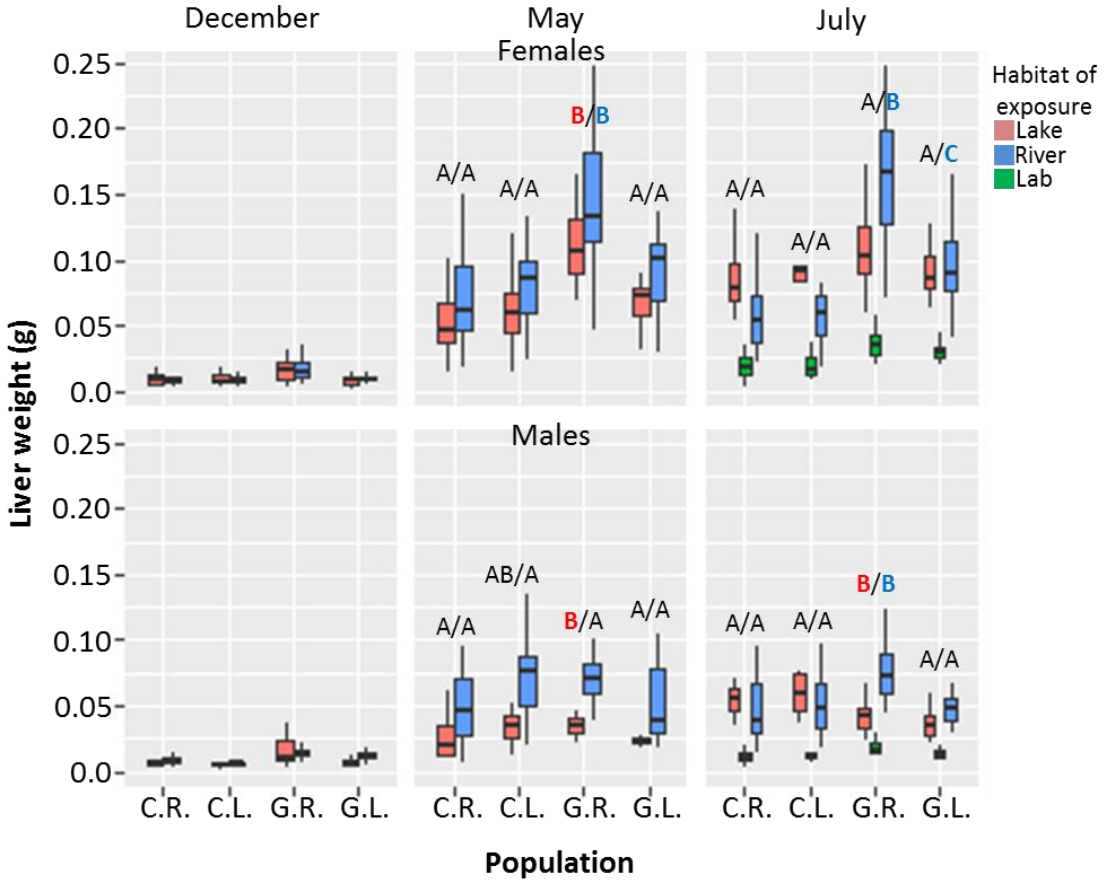
<u>Figure A2:</u> Female gonad weight and index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.), separated by sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table A7.

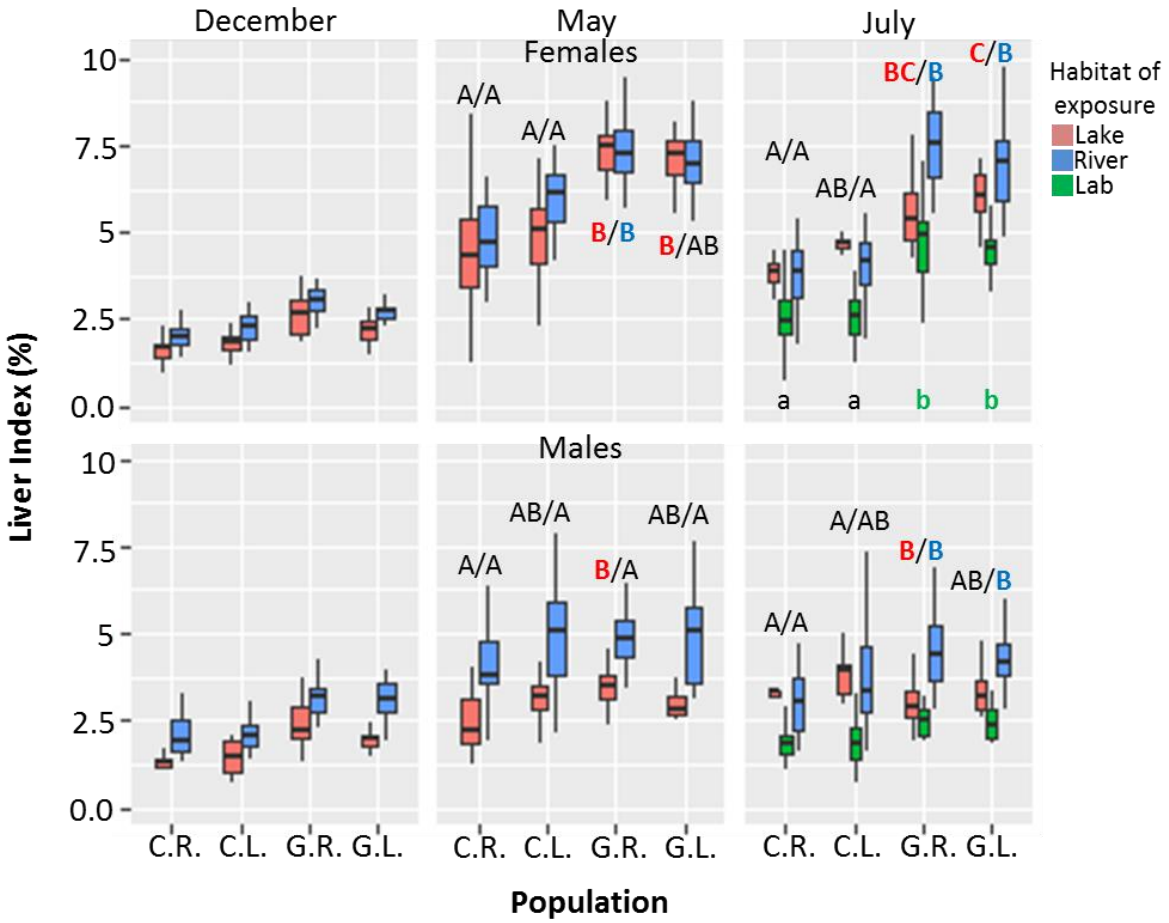


<u>Figure A3:</u> Male body kidney weight and index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.), separated by sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table A6



<u>Figure A4 & A5:</u> Liver weight and index of the different populations (Canadian river = C.R.; Canadian lake = C.L.; German river = G.R.; German lake = G.L.), separated by sex and sampling time points; boxplots represent medians ± 1st and 2nd quartiles. Results were obtained with a general liner model (glm) followed by a "Tukey" post-hoc test, corrected for multiple testing (fdr); significant differences are calculated by habitat of exposure, marked by different letters and highlighted in the corresponding colors. Lowercase letters show statistical differences for the control (i.e. lab) individuals, were applicable. Specific statistical differences can be found in the appendix table A9.





Extended discussion of organ weights:

Large size is a desirable attribute in fish as it enables the production of larger clutches in females, thereby increasing reproductive output. This correlation of size and fitness is reflected in male three-spine sticklebacks, which have an affinity for larger females during mate choice (Kraak & Bakker 1998). Consequently, it is in the female's best interest to attribute as many resources as possible to the gonads. Indeed, we found that only the larger German river females had significantly higher gonad weights in the river (May and July) as well as in the lake habitat in May. These did however not differ between the two habitats despite growing significantly larger in the river, hinting at a possible limit in gonad size. In May, despite growing to smaller sizes in the lake as opposed to the river, all females attributed a larger percentage of their total body mass (Fig. A2, GOI) to gonads, resulting in an indifference in gonad weight between the lake and river habitat for each population. This increased investment into the reproductive organ might have been facilitated by higher resource availability in the lake habitat. In July, despite over all smaller organ weights and attributing a lower percentage of total body mass to the gonads, the trend of increased gonad weight in the lake habitat **is** maintained.

In the absence of potential male partners, female sticklebacks will eventually spawn on their own to avoid so-called "stone eggs" which can prevent further clutch formation and be potentially lethal. It is hence likely that the decrease in gonad weight from May to July, despite sexual segregation of the fish,

is a reflection of this behaviour. However, it also implies that the state of clutch “maturation” for each female is unknown and highly variable, demanding cautious interpretation of the measurements. Male testes, on the other hand, are largely identical in size between the populations, despite significant variation in body size between the males. As testis size and reproductive output do not necessarily correlate, no benefit can be expected from an increase in testis size past a functional state. The opposite holds true for the body kidney, were only the males did significantly vary in size in a population dependent manner.

The body kidney is responsible for the production of spiggin (Seear et al., 2015) in sexually mature male three-spine sticklebacks. This glue-like protein is secreted during nest construction and used to bind together plant material. It plays a further role in sexual communication, contributing to the olfactory attraction of ripe females towards the finished nest (Milinski 2006). Thus, one could speculate that the body kidney is a secondary sexual organ in male three-spine stickleback whose size, similar to the female gonads, correlates with fitness. In contrast to the females however, the larger German river males had both larger organs as well as allocating a higher percentage of total body mass to the later. It appears as though they were able to invest more into their sexual reproduction than the other populations, converting their advantageous “large-growth” phenotype into a direct fitness advantage, without suffering the consequences of higher parasite burdens.

The German lake and especially the German river population had significantly larger livers than either of the Canadian populations. Due to its involvement in metabolism, this would suggest higher metabolic rates in the German over the Canadian fish. Surprisingly however, despite growing to larger sizes, the German river fish did not significantly differ in liver size from the German lake population, in either habitat. We might thus assume that both German populations have comparably high metabolic rates. This should, in turn, translate to similar resource availability between the populations. Paired with the difference in size, this again supports the assumption of different resource allocation, either to growth (German river) or immunity (German lake), as the underlying reason for the variation in physiological measurements. Both Canadian populations had consistently smaller livers, irrespective of time point or habitat. On the contrary to the previous observation in the German fish, this did however not convert to generally smaller growth rates. Assuming identical resource availability for all fish in the same habitat, this might be indicative of more efficient liver function in the Canadian fish, allowing for comparable growth rates whilst allocating fewer resources towards the liver.

Chapter III:

Materials and Methods – extended:

Animal origin and housing:

All fish used in this study were wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) originating from the Grosser Plöner See lake (n = 53, 54˚14'61.0''N, 10˚40'86.9''E) and the Sörener Au river (n = 53, 54˚22'50.1''N, 10˚60'91.5''E) in northern Germany. The fish were caught in December 2017 and cycled through winter (6˚C, 12:12 L:D), spring (12 ˚C, 12:12 L:D) and finally summer (18 ˚C 18:6 L:D) conditions in the laboratory. Fish were housed individually upon transfer to the summer conditions, fed *ad libidum* with live *Chironomidae spec.* larvae and were spine-clipped for sex-typing and MHC-allele analysis.

Males were provided with standardized nesting material consisting of green polyester threads (cut to a length of ~10cm), half a petri dish half-filled with sand and a small rock. Nest progression was monitored daily and nest status was determined based on appearance and male behavior (see Wootton 1976 for details), using only males with unfinished nest for the experiment.

Male sticklebacks will not produce the MHC signal until their nest is finished when they start 'fanning' and 'creeping through the nest' (Fig. 1), suggesting that it is costly for the male to produce the MHC-associated signal and/or to attract females to the nest too early (Milinski et al., 2010). As MHC molecules are shed from the cell surface (Singh et al., 1987), it is assumed that this process changes the conformation of the peptide binding groove, causing faster liberation of peptides which then become available for assessment via other sensory modalities, such as the vomeronasal system (Leinders-Zufall et al., 2009). However, the male validation factor is present from the onset of nest building (Milinski et al., 2010, Fig. 1); this offers the possibility to expose females to the male validation factor without the natural male-derived MHC component of the signal peptides (Milinski et al., 2010, Fig.1).

All animal experiments described were approved by the Ministry of Nature, Environment and Country Development, Schleswig Holstein, Germany.

Experimental design:

Gravid female sticklebacks were placed in a flow chamber fed by two columns with laminar water flow (Reusch et al., 2001; Aeschlimann et al., 2003). Females were able to freely investigate the water composition in the chamber for two periods of 300s each, with spatial reversal of the water source after the first 300s period. Determining odor preference in this setup has been shown to reliably predict mate choice (ref. Leinders-Zufall et al., 2004, *supporting text*), which provides the opportunity to test the effect of synthetic peptides on female preference. To this end, water was taken from the tank of a single male (containing male validation factor but no MHC-associated component (6; see above) per trial and converted to fully functional stimulus water by addition of synthetic peptides. When used as river-like stimulus, two peptides in solvent were continuously added to one half; when used as a lake-like stimulus, four peptides in solvent were continuously added to the other half of the flow channel. The concentration of peptides in solvent and the volume of supplement added to the water columns were identical on both sides in all experiments. Each female was tested using water taken from the tank of a sympatric and an allopatric male, within a one-hour interval. All experiments were performed in double-blinded fashion in the Plön laboratory. Each female-male combination was used only once to avoid pseudo replication and thus is a single independent statistical unit. Each fish was only used once with the exception of two males that were allowed to build a new nest and then tested again with two different females.

Run validation:

To validate a female's principal readiness to spawn during the trial, she had to spontaneously spawn in her home tank in the absence of a male within 24h after her second test (Milinski et al., 2005). Further, females that remained on the same side in the 1st and 2nd run of a trial and thus revealed an unwillingness of exploration were designated as 'no choice' and excluded. Three runs were excluded because one of the two males showed clear fanning behavior in their home tank between tests indicating the start of producing the MHC signal on the trial day; these males might have produced the MHC signal already before the start of the trial.

MHC-analysis:

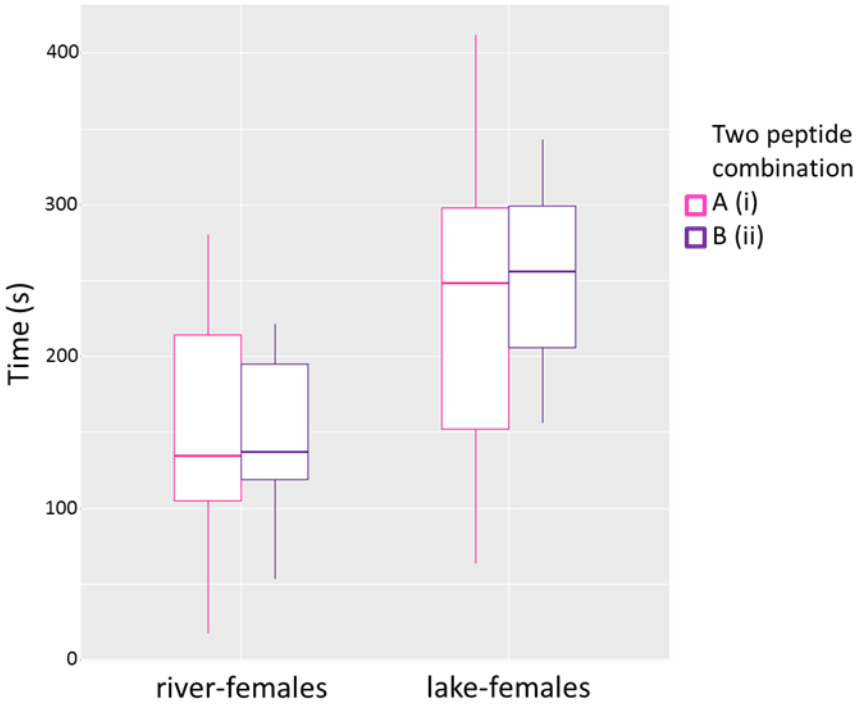
DNA was extracted from clipped spines using the DNeasy 96 Blood & Tissue Kit (Qiagen), following the manufacturer´s protocol. MHC allele numbers were measured using Reference Strand-mediated

Conformation Analysis (RSCA) as described (25). The resulting measurements were analysed using the GeneMarker software (Version 2.4.2, Softgenetics).

Peptides:

The four different MHC-ligand peptides used in this study were: SYIPSAEKI, SFVDTRTLL, ASNENMETM, and AAPDNRETF (Milinski et al., 2005 & 2010). Peptides were chemically synthesized, purified, verified by mass spectroscopy (MALDI-TOF), and dissolved in phosphate-buffered saline (PBS), as described (Milinski et al., 2005). Two or four peptides were chosen as to represent two distinct allele combinations mimicking either a definite river or a definite lake male MHC genotype; as can be seen from Fig. 2a, it is extremely unlikely that a river male has 4 alleles and that a lake male has 2 alleles. For the 2-peptide signal, two random exclusive combinations from the four peptides were created. Females tested with one (SYIPSAEKI and SFVDTRTLL**,** n=32) and females tested with the other (ASNENMETM and AAPDNRETF**,** n=13) combination did not differ significantly in their choice (n=45, t(29) =1.2402, P=0.2247, two-tailed t-test). The discrepancy between the "n" for each two-peptide combination is due to unsuccessful runs (e.g. female did not spawn within 24h after the final run) which happened to be more common in the 2nd (ii) combination and to fewer ripe females available towards the end. Females did however not differentiate between either of the two peptide combinations (P=0.7481, for river females and P=0.9024 for lake females, Mann-Whitney U-test, two-tailed (see Figure Si1).

Figure A5: Both river and lake females show no significant preference for either of the two random two peptide combinations used in the study. Time (s) (median ± first and second quartiles) of 600 s the female spent in the quarter of the test chamber where the two-peptide combination arrived.



Statistical analysis:

All statistical analysis were done in RStudio (version 1.0.136) using the build in packages for statistical analysis (two-tailed t-test) and the ggplot2 package for graphical representation. Times from the 1st and 2nd run of each female-male combination were added up and regarded as one variable (controlling for weak side preference). The data did not significantly differ from a normal distribution (Shapiro-Wilk, P=0.384), hence allowing the use of parametric tests for data analysis.

Chapter V:

Short-term changes of the environmental context does not increase expression of immune system related genes in the three-spine stickleback

Unsubmitted manuscript

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Abstract:

Long term experimental effects often run the risk of being distorted by short term effects resulting from animal handling and animal transfer into a laboratory environments for data acquisition. In an attempt to evaluate such an impact on gene expression of immune system related genes in three-spined sticklebacks (*Gasterosteus aculeatus*) we handled these animals in two different ways after long term exposure to a natural lake environment: The fish of these two cohorts were either dissected directly in the field or after being transferred to the laboratory aquaria overnight. We find no significant difference in the overall expression level of a pre-selected group of genes, or on the level of individual genes between the two groups. These results suggest no negative consequences of transferring the individuals from their long-term "natural" environment to the lab for standardized data acquisition in a controlled laboratory setting.

Introduction:

Long-term exposure to experimental conditions are generally a good way to determine environmental influences on the immune system and physiology of vertebrate animal models (e.g. harbor seals; De Swart et al. 1996, Zebrafish; Nash et al. 2004). In the majority of cases, experimental animals are exposed to a variety of different environmental conditions, influencing their development, survival or reproduction (e.g. Riddington & Gosler 1995). These studies are of great ecological and evolutionary interest as their findings can be directly placed in a relevant environmental context. However, short-term effects associated with moving the animals from the field to the laboratory for sampling such as handling related stress, new sensory environments, or, in case of fish different water conditions are possible sources of noise in the data, confounding the measurement of conditions related to the environment in which the animals were living previously. This holds especially true with regards to rather dynamic biological processes such as metabolism, endocrine (e.g. stress hormones in birds change within minutes, Sheriff et al. 2011) or immune system and to the related gene expression (e.g. immediate early genes in response to stress, Cullinan et al 1995).

To obtain realtime measurements of these fast and plastic physiological processes, extended measurements and analysis are often performed directly in the field. However, varying and unstable environmental conditions during data acquisition potentially alter the resulting measurements (e.g. unstable scale for weight measurements). In contrast, measurements performed in a standardized

laboratory setting provide more reliable and comparable data, possibly at the expense of gene expression accuracy and relevance.

This study therefore aimed at demonstrating the accuracy of lab based gene expression measurements, ensuring replicable results obtained under standardized conditions.

Evolutionary ecology research in sticklebacks is largely focused on understanding the emergences and maintenance of distinct phenotypes and genotypes in different habitats (e.g. lake and river: Berner et al. 2008). These so-called ecotypes are a result of varying parasite communities and pressures, distinguishing the habitats (Thompson 1994). Consequently, immune relevant genes are under strong selective pressure, highly adaptive and variable between individuals (e.g. MHC IIb, Kurtz et al. 2004). Like all vertebrates, fish immunity consists of two interwoven components, the innate and the adaptive immune system (reviewed in Pilar Alvarez-Pellitero, 2008). In fish, as is the case in other vertebrates (Dantzer & Kelley 1989, Padgett & Glaser 2003), gene expression of the immune system is most likely influenced by stress, increasing the risk of skewed and false measurements as a result of catching and handling.

With this study we aimed at demonstrating that handling and transferring the animals to standardized lab conditions did not alter the gene expression measurements resulting from the long term exposure to a natural lake habitat. To this end we mainly focused on genes, which have previously been shown to vary due to parasite load or between fish populations and are associated to the immune function in sticklebacks (Hibbeler et al., 2008, Lenz et al. 2009, Robertson et al. 2016, Brunner et al. 2017). Besides genes directly linked to the innate or adaptive immune system and the complementary system, we used real-time qPCR to measure genes associated to oxidative stress as well as housekeeping in order to control for random/unrelated expression changes. We expected these genes to respond to short-term influences like handling stress (Padgett & Glaser 2003).

Methods:

Experimental Animals:

Three-spined sticklebacks (*Gasterosteus aculeatus*) were chosen for this experiment, since they are a widespread model in evolutionary ecology research (Lescak & Milligan-Myhre 2017) and provide ample possibilities for gene expression analysis (e.g. Shapiro et al., 2004, Lenz et al., 2013). All fish were naïve *in vitro* fertilized F1 offspring of wild caught fish, originating from the Groβer Plöner See (54˚14’61.0’’N, 10˚40’86.9’’E) in Schleswig-Holstein, Germany. After fertilization, clutches were incubated at a constant

18 ˚C, 18:6 L:D with daily water changes and continuous oxygen supply until hatching. Hatchlings were then transferred to 40x20x20 plastic tanks with constant water flow, oxygen supply and fed *ad libitum* until the start of the experiment.

Experimental design:

After growing in the lab for nine months, the fish (n=96) were equally distributed across 8 cages (for details see Eizaguirre et al. 2012) and exposed to a natural lake environment for three months before being dissected. One half of the fish (n=39) were dissected in the field immediately after extraction from the cages. The remaining animals (n=35) were collected and transferred to the lab where they were kept in lake water at 12˚C (=lake temperature) and dissected the following day. The remaining 22 fish could not be recovered from the cages and probably died during the 3 month exposure period.

Dissection:

The animals were killed using an overdose of tricaine methanesulfonate (MS222, 200 mg l^{-1}, Sigma) followed by decapitation, according to animal welfare policies. Subsequently, the body cavity of the fish was opened; both head kidneys and half the gills (left side) were extracted and immediately stored in RNAlater (Sigma) for further analysis. Head kidneys are the main immunologically active organ in fish (Tort et al. 2003) and are as such of great interest for immune system analysis. The gills on the other hand are directly exposed to the environment, covered in immunologically active mucous, forming the first line of defense against exterior pathogens (Press & Evensen 1999).

Gene expression:

RNA was extracted from the two organs using an RNAeasy 96 Kit (Qiagen) following manufacturer protocol. RNA concentration was measured by spectrophotometry (NandoDrop, ThermoFisher). Samples were then diluted and reversely transcribed into cDNA via Omniscript Reverse transcription kit (Qiagen). The obtain cDNA was stored at -80˚C until further analyses. Gene expression was then assessed with the Fluidigm-BioMark™ system using a 96.96 dynamicarray (GE-chip).

First, the target cDNA was pre-amplified with 2.5 µL TaqMan PreAmp MasterMix (Applied Bioscience®) and 0.5 µL STA Primer mix (includes all 32 primer pairs 50 µM diluted in low EDTA-TE buffer) under the following PCR conditions: 10min at 95°C, 14 cycles: 15s at 95°C, 4min at 60°C. PCR products were diluted 1:5 with EDTA-TE buffer. Before loading the chip, an assay mix was prepared containing 0.7 µL of 50 µM

primer pair mix, 3.5 µL 2xAssay loading reagent (Fluidigm) and 3.15 µL 1xlow EDTA-TE buffer. A sample mix containing 3.3 µL preamplified cDNA, 3.5 µL 2xSsoFast EvaGreen Supermix with low Rox (BioRAD) and 0.35 µL 20xDNA Binding Dye Sample loading reagent (Fluidigm) was prepared. The chip was primed with control line fluid after which 5 µL assay mix and 5 µL sample mix were loaded onto the chip and measured with Fluidigm-BioMark™ system applying GE-fast 96.96 PCR+Melt v2 protocol according to Fluidigm protocols. Each chip contained a dilution series (1:2, 1:5, 1:10, 1:100, 1:500) of pooled cDNA as a standard, a negative "no template control" (NTC) and a gDNA contamination control (-RT). Technical triplicates and random sample distribution across the chip allowed for technical bias control.

We analyzed the gene expression of a total of 27 different genes (Sup. Table 1) related to innate and adaptive immune functions, oxidative stress, the complementary system and housekeeping genes. Primer sequences have previously been optimized for three-spined sticklebacks by Brunner et al. (2017, Appendix Table A1).

Gene expression analysis:

Fluidigm real-time PCR analysis software (Fluidigm) was used to access the amplification profiles of the data and calculate mean cycle time (Ct), standard deviation (SD) and the coefficient of variation (CV) for all technical triplicates. CV is used as an indicator of measurement precision, a CV >0.04 will falsify the measurement and results in a measurement error (Bookout & Mangelsdorf 2003).

Two samples from the Gill data set and one from the Head Kidneys were excluded due to poor sample quality, leading to mostly undetectable amounts of RNA. Further, several primers (TLR2, LY75, TCRA, $p40^{phox}$, vegfa, TLR2) were excluded due to double peak formation (multiple targets), faulty primer design or self-binding.

Statistics:

Missing data points (0.13% of sample data) were replaced with the mean expression value of the respective gene. All further analyses were performed with the relative gene expression values (–δCt) computed by the qbase+ software (Biogazelle). After controlling for normality with Shapiro-Wilk test and log-transforming, normality was assumed. Multivariate approaches using PERMANOVA (permutation 9999) from the Vegan package (Oksanen et al. 2016) were performed on the entire data set and to the different gene groups (innate immune system, adaptive immune system, and complementary system), detecting main and interaction effects with the relevant factors (treatment & Sex) and Cages as random factor. Univariate linear mixed models were carried out using the lmer

function (lme4 package (Bates et al., 2018)). The gene expression data was further analyzed by means of a principal component analysis (PCA) using the ade4 and BCA packages in RStudio (Version 1.0.136). The resulting p-values were then corrected for multiple testing according to Benjamini-Yekutieli (Benjamini & Yekuteli 2001).

Ethics:

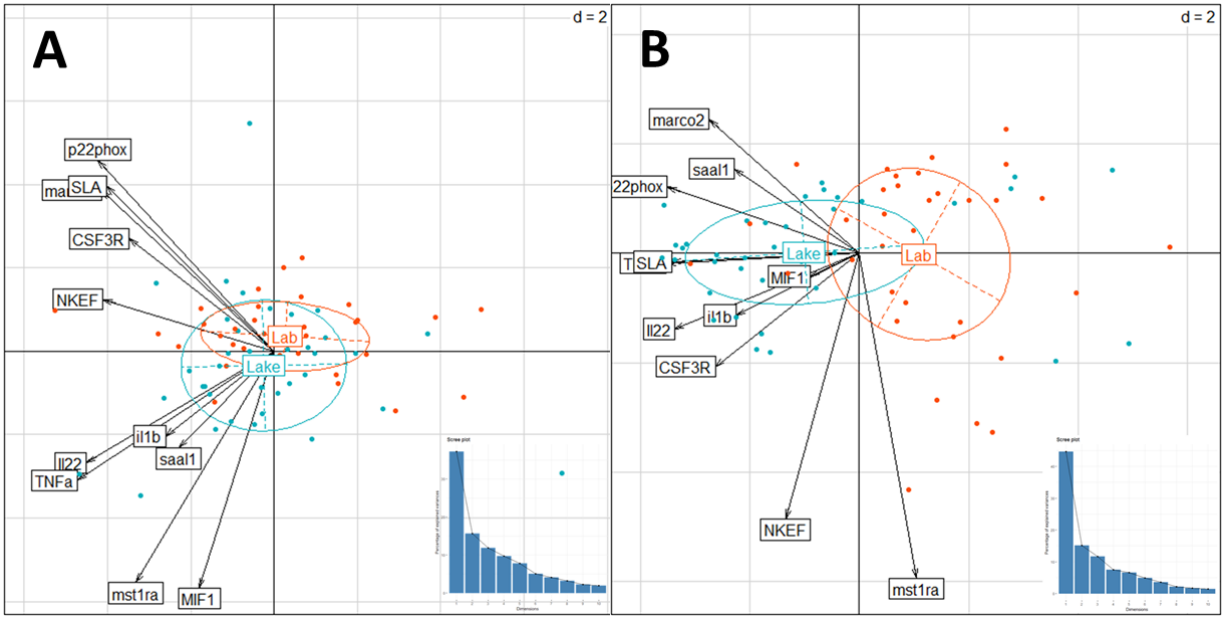
All animal experiments in this study were approved by the 'Ministry of Energy, Agriculture, the Environment and Rural Areas' of the state of Schleswig-Holstein, Germany (reference number: V 313–72241.123-34).

Results:

Due to their different role in immune function, as expected, we found significant differences between the head kidney and the gills (head kidneys: Zapata et al. 2006; gill: Press & Evensen 1998). In the scope of the present study, further gene expression analysis was performed separately for the two organs. From the 27 analyzed genes, there was no significant effect of treatment in the overall comparisons for the Gills ($F_{1,68}$=0.0132, p=0.212). Gene expression of the head kidneys showed overall differences ($F_{1,69}$=0.0552, p=0.0174) which were however not significant after false discovery rate (fdr) correction following Benjamin-Yekutieli. In combination with the low effect sizes (Table1) we refrained from performing in depth analysis of individual genes.

Assigning the different genes to functional groups before statistical analysis showed no significant difference for the genes related to adaptive immunity or the complementary system (Table1). There were differences for innate immune related genes in the gills ($F_{1,68}$=0.038, p=0.0031), which remained significant after fdr correction. In the head-kidneys, only sex was found to have a significant effect on genes associated with the innate immune system ($F_{1,69}$=0.0487, p=0.004). Despite low effect sizes (R^2 Table1) in both cases, we further performed univariate LMMs to test the effect of treatment on an individual gene level (Table2).

On this individual gene level, treatment only significantly affected the mif1 gene expression in the Gills (p=0.0006). The saal1 gene was significantly affected by sex in both head kidney and gills. In all significant cases, gene expression appeared to be upregulated in the fish dissected directly at the lake.



<u>Figure1:</u> PCA analysis of innate immune genes for the Gills (A) and Head-Kidneys (B). The Eigenvalues explaining the contribution of the individual PC can be found in the right bottom corner. PC1 and PC2 contribute 52% in the Gills and 59% in the HK. The two treatment groups "Lake" (blue) and "Lab" (red) and the individuals associated with them are shown.

<u>Discussion:</u>

Gene expression is the functional bridge between genotype and phenotype, regulating everything from cellular processes to behaviour and biological processes in general. Rapidly changing expression profiles allow for quick response to environmental shifts and stimuli. Consequently, changes of the environment such as transferring sticklebacks for 1 day to the laboratory could alter the gene expression patterns, and might effectively disturb the phenotype, in particular the transcriptomes that are related to the animals environment of origin, in our case the lake.

The effects of bringing the fish into the lab over night before dissecting did not tamper with the long term effects of habitat exposure. Differences in the over-all expression of innate immune relevant genes (Table 1) might suggest a small unwanted influence of handling stress (i.e. short term effects), tampering with the results of the long term exposure. The expression appears to be higher in field dissected fish,

suggesting that handling stress had a greater influence on the innate immune genes in these animals. This would be in line with previous findings (Demers and Bayne 1997; *Krasnov et al.* 2005), which showed increased innate immune system activity in response to handling stress in rainbow trout. Further, since the differences in gene expression between the two groups is very small, these might as well represent stochastic differences.

In summary, if anything, lab based dissections might be a better representation of innate immune gene expression profiles resulting from the long-term lake-water environment.

On a single gene level; treatment (i.e. transferring fish vs direct dissections) only affected the expression of mif1 in the Gills. Mif1 is a constitutively expressed gene involved in inflammatory response and gram-bacteria recognition (Calandra & Roger 2003). Mif1 has however also been shown to respond to stress and plays a role in stress dependent insulin release (Waeber et al. 1999) which might offer an explanation to the increased expression in the gill tissue, a proxy for the periphery, and not the head-kidneys.

The sex dependent expression differences of saal1, an inflammatory response mediator, appear to be independent of treatment. Saal1 has however been shown to have increased expression in the reproductive organs of mice and humans (especially in the testis, proteinatlas & NCBI database). Offering an explanation for the higher effect sizes in the gill tissue (periphery) compared to the head-kidney expression results.

The lack of over-all differences between the two groups underlines the expected stability of long term environmental effects such as lake-water exposure, strongly outweighing the short term effects of catching, handling and transferring the fish. As their appears to be no or only a very weak downside of lab transfer, the impact of natural environments such as living in lake-water on gene expression can be quantified from short-term laboratory housed sticklebacks.

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Supplementary Material Appendix Chapter V:

Table 1: Multivariate PERMANOVAs testing the effect of experimental treatment on gene expression in the two organs. We tested all genes as well as functional gene groups separately. We tested the effects of treatment (T), sex (S) as well as their interaction (T:S). Significant p-values after false discovery rate correction according to Benjamini-Yekutieli and are highlighted in bold.

	Head kidneys				**Gills**			
	DF	*F*	R^2	*p-value*	*DF*	*F*	R^2	*p-value*
	All genes							
T	1	4.3318	0.05518	0.0174	1	0.9475	0.01323	0.212
S	1	2.1860	0.02785	0.0704	1	2.12275	0.02964	0.0737
T:S	1	2.9876	0.03806	0.0388	1	0.55277	0.00772	0.7772
Residuals	69		0.87892		68		0.94942	
Total	72		1		71		1	
		Innate immunity						
T	1	8.9852	0.10680	0.0102	1	2.9478	0.03809	**0.0031**
S	1	4.0944	0.04867	**0.0040**	1	5.4409	0.07030	0.0007
T:S	1	2.0498	0.02436	0.1338	1	1.0023	0.01295	0.5142
Residuals	69		0.82017		68		0.87866	
Total	72		1		71		1	
		Adaptive immunity						
T	1	2.20501	0.03019	0.4554	1	0.68824	0.00977	0.5091
S	1	0.74051	0.01014	0.4974	1	0.78502	0.01114	0.4533
T:S	1	1.09064	0.01493	0.3379	1	0.99311	0.01409	0.4383
Residuals	69		0.94474		68		0.965	
Total	72		1		71		1	
		Complement system						
T	1	0.26835	0.00382	0.3835	1	0.26835	0.00382	0.3813
S	1	1.77804	0.02528	0.1825	1	1.77804	0.02528	0.1861
T:S	1	0.29375	0.00418	0.7516	1	0.29375	0.00418	0.7513
Residuals	69		0.96673		68		0.96673	

Total	72		1		71		1	

Table 2: Univariate LMMs testing the effects of treatment on individual genes. The two organs were tested separately for effects of treatment (T) and Sex (S). P-values which remained significant after false discovery rate correction are highlighted in bold.

	Head kidneys				***Gills***			
	numDF	*denDF*	*F-value*	*p-value*	*numDF*	*denDF*	*F-value*	*p-value*
marco								
T	1	6	3.7136	0.1023	1	6	0.0053	0.9441
Sex	1	64	7.5491	0.0078	1	63	2.8265	0.0977
mst1ra								
T	1	6	0.9043	0.3784	1	6	0.4002	0.5503
Sex	1	64	5.7902	0.0119	1	63	0.0004	0.9838
mif1								
T	1	6	12.965	0.0114	1	6	41.950	**0.0006**
Sex	1	64	1.1985	0.2777	1	63	0.4923	0.4855
il-1β								
T	1	6	11.417	0.0149	1	6	1.4776	0.2698
Sex	1	64	0.2273	0.6352	1	63	4.7975	0.0322
tnfr1								
T	1	6	9.6469	0.021	1	6	0.0461	0.8371
Sex	1	64	2.5626	0.1143	1	63	0.0032	0.9550
saal1								
T	1	6	21.339	0.0036	1	6	6.4185	0.0445
Sex	1	64	18.202	**0.0001**	1	63	42.080	**<.0001**
csf3r								
T	1	6	4.0477	0.0909	1	6	0.0150	0.9064
Sex	1	64	2.2343	0.1399	1	63	2.0150	0.1607
p22^{phox}								
T	1	6	12.318	0.0127	1	6	0.5838	0.4738

Sex	1	64	4.3910	0.0401	1	63	0.0388	0.8445
nkef-β								
T	1	6	0.1521	0.71	1	6	0.0257	0.878
Sex	1	64	0.4587	0.5007	1	63	0.0205	0.8867
sla1								
T	1	6	7.3030	0.0355	1	6	0.388158	0.5562
Sex	1	64	4.6485	0.0348	1	63	0.430308	0.5142
cd97								
T	1	6	7.2857	0.0356	1	6	0.5934	0.4704
Sex	1	64	2.8286	0.0975	1	63	0.0617	0.8047