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Thesis

Variation in number of vertebrae in populations of pike (*Esox lucius*) in the south-east of Sweden

Sammanfattning

Antalet ryggkotor varierar kraftigt mellan olika taxa, men också inom arter eller populationer. Omfattande forskning har visat att antalet ryggkotor hos fisk är resultatet av interaktioner mellan genetisk struktur och plastiska svar på miljöbetingelser under individens ontogeni. Ett vanligt mönster är tendensen för antalet ryggkotor att variera med kroppsform och/eller kroppslängd hos fisken. Gäddan (*Esox lucius*) i Östersjön har en komplex populationsstruktur, bestående av flera genetiskt distinkta subpopulationer, vilka utgörs av anadroma individer som uppvisar ett homing-beteende. Individer som tillhör dessa subpopulationer är sympatriska större delen av året och blir allopatriska endast kortvarigt under tiden för lek. Den här studien undersökte fördelningen av antalet ryggkotor hos tre anadroma sympatriska subpopulationer av gädda i Östersjön. Signifikanta skillnader i fördelningen av antalet ryggkotor hittades både inom och mellan alla undersökta subpopulationer och åldersklasser. Resultaten från ett "common-garden" experiment antydde att skillnader i antalet ryggkotor mellan olika subpopulationer delvis hade en genetisk basis, vilket indikerar möjligheten för selektion att verka på denna egenskap, och en potentiellt evolutionär förändring. En kvadratisk regression visade på ett kurvilinjärt samband mellan antalet ryggkotor och kroppslängd hos juveniler. Tillsammans tyfrt dessa resultat på att de kombinerade effekterna av stabiliserande och divergent selektion kan ha spelat en roll i fördelningen av antalet ryggkotor hos gäddan i Östersjön. Fördelningen av antalet ryggkotor inom subpopulationer verkar vara under inflytande av stabiliserande selektion. Skillnader mellan subpopulationer kan istället reflektera lokala anpassningar som drivs av divergent selektion. Dessa fynd signalerar behovet av att betrakta varje subpopulation som en egen enhet i bevarandebiologiska sammanhang.

Keywords

Ecology, *Esox lucius*, evolution, meristic characters, pleomerism, selection

Acknowledgements

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Abstract

Vertebral number (VN) is known to vary greatly across different taxa, but also within species or populations. Extensive research has shown that VN in fish is the result of interactions between genetic structure and plastic responses to environmental cues during ontogeny. A frequently reported pattern is the tendency for VN to vary with body shape and/or length of the fish. The pike (*Esox lucius*) of the Baltic Sea has a complex population structure, with genetically distinct subpopulations consisting of homing anadromous individuals. Individuals belonging to these subpopulations are sympatric for most of their lives and become allopatric briefly during spawning each year. This study examined the distribution of VN in three anadromous sympatric subpopulations of pike in the Baltic. Significant differences in VN were found between juveniles and adults belonging to different subpopulations, but also across life-stages within all three subpopulations. Results from a common-garden experiment indicated that differences in VN among subpopulations were in part the result of genetic differences, indicative of evolutionary change. Furthermore, a quadratic regression revealed a curvilinear relationship between VN and body length of juveniles. Taken together, these results suggest that the combined effects of stabilizing and divergent selection might have played a role in shaping the distribution of VN in pike of the Baltic. The distribution of VN within subpopulations seems to be under the influence of stabilizing selection. Differences among subpopulations might instead reflect local adaptations driven by divergent selection. These findings signal the need for conservationists to view these subpopulations as unique units of management.

Introduction

To understand the causes and consequences of phenotypic variation has since long been a key priority for research in evolutionary biology. Meristic characters such as vertebral number (henceforth VN) vary greatly between different groups of organisms and has been extensively studied (*e.g.* in snakes [1], salamanders [2] and fish [3]). The number of vertebrae is set during early ontogeny and is irreversible [3,4], making it a potential target for natural selection. As different environments impose different selective regimes, one could hypothesize VN to vary with the ecology and life-history traits of a species. This has been shown to be true for example in snakes, where traits such as foraging mode (constricting *vs.* non-constricting), habitat choice (burrowing *vs.* non-burrowing) and body size are associated with VN across species [1]. This same pattern is observed in fish, with VN being associated with body size [3], and with migration/spawning behavior [5,6].

In addition to varying between different genera or species, a great deal of intraspecific variation in VN also occurs [3,7-9]. Generally speaking, intraspecific variation in phenotypic traits can be considered as: *i*) the result of genetic differences, *ii*) a response to different environmental cues during ontogeny reflecting phenotypic plasticity, or *iii*) a combination of the two. In fish, studies have shown that several factors influence VN, including genetics, temperature during ontogeny and salinity [10-13]. It would then seem that complex interactions are what ultimately determine patterns of variation in VN. Other studies have focused on investigating how VN correlates with other phenotypic traits and how this may affect fitness. For instance, vertebral phenotype (*e.g.* the number of vertebrae, or the ratio of abdominal to caudal vertebrae) in larvae have been shown to be associated with survival (in peamouth *Mylochelious caurinus* [14]) and burst swimming performance (in sticklebacks *Gasterosteus aculeatus* [15]), which may in turn affect fitness by influencing foraging success and predator avoidance. It is possible then that individuals

possessing some specific number(s) of vertebrae may enjoy fitness advantages over their conspecifics. Natural selection would then shape the distribution of VN among individuals, possibly giving rise to quantifiable patterns both within and across populations.

Pike *Esox lucius* (also known as northern pike) is a large piscivorous fish with a Holarctic distribution inhabiting lakes, rivers and brackish waters [16]. During the last century, the species has suffered a large decline in population numbers over its entire distribution area due to habitat destruction and poor recruitment [17,18]. It has long been considered a keystone species in freshwater systems and used in the field of biomanipulation for its top-down control of freshwater communities [17]. More recently, pike is also becoming a new model organism for studies in evolutionary ecology [19]. A growing body of evidence have also shown that pike, much like salmonid fishes, employ natal-homing as a reproductive strategy [20,21]. In the Baltic Sea, pike seems to exist in two sympatric forms distinguished by the use of two different reproductive strategies: they either spawn along the coastline in the brackish seawater, or migrate up freshwater streams to spawn [22]. Evidence of reproductive barriers existing between these two forms of pike has been found, where eggs from freshwater females, although successfully fertilized, did not stay viable in salinities >6‰ [23]. In addition to this, the migrating form of pike in the Baltic has been shown to be anadromous and to display natal-homing, creating genetically differentiated subpopulations [24]. This differentiation is remarkable considering how these subpopulations are sympatric during most of the time, becoming allopatric only during spawning [22,24]. These subpopulations have been shown to differ in traits possibly related to fitness such as body size and growth rate [25], but differences in the distribution of VN - if any - are yet to be investigated.

The aim of this study was to investigate and quantify the variation in VN within and across three anadromous sympatric subpopulations of pike *Esox lucius* in the Baltic Sea. To this end, comparisons of VN among populations are reported based on data for individuals that were captured as juveniles or adults in the wild. Differences in VN among subpopulations could pertain to genetic differences, giving natural selection the potential to act. To assess whether this might be the case, the results from a common-garden experiment are reported. In order to investigate whether there is any signature of natural selection acting upon VN (or a trait associated with it) at some point during the individuals lifetime, tests for differences in VN across life-stages (juveniles and adults) within subpopulations are conducted. Finally, since VN has been reported to correlate with body size, a test for a relationship between these two traits is performed. Findings from this study could potentially serve as further evidence of evolutionary divergence between subpopulations of pike in the Baltic Sea.

Materials & methods

Locations, sampling and data sources

Individuals used in this study, both adults (individuals over 2 years of age, *N*=175) and juveniles (younger than six weeks, *N*=146), were sampled from three different streams with a discharge in the Baltic Sea (Fig. 1, table 1). These streams flow through similar environments on the Swedish mainland, and are known to be used for spawning and rearing of juveniles by genetically differentiated subpopulations of anadromous pike [24]. Juveniles were caught by using either hand-trawls in the streams or by larvae traps placed at the discharge of each stream during 4 weeks in the spring of 2014, whereas adults were caught using fyke-nets placed in the streams during their breeding migration over the course of four years (table 1). Data on the adults used in this study was collected within the framework of previously published studies [26] and is used in this study with the authors kind permission. These authors also performed a

common-garden experiment using juveniles with parental fish originating from streams Kronobäck and Lerviksbäcken.

Figure 1. A map showing the locations of the three different streams from where fish for this study were caught. The two streams that are the furthest from each other (Lerviksbäcken and Dunöbäcken) are 60 km apart.

A sub-set of data from this experiment was used in the present study to investigate a possible genetic basis of VN.

Table 1. Streams (subpopulations), sampling years and sample sizes of individuals used to study variation in vertebral number in pike.

Stream	Coordinates (lat., long.)	Age/project	Year	*N*
Kronobäck	N 57° 01.200' E 16° 26.700'	Adults Juveniles Common-garden	2011-2014 2014 2012	113 71 65
Dunöbäcken	N 56° 38.200' E 16° 18.800'	Adults Juveniles	2013-2014 2014	29 22
Lerviksbäcken	N 57° 04.400' E 16° 31.100'	Adults Juveniles Common-garden	2012-2013 2014 2012	33 53 59

Measuring, staining and counting of vertebrae
Staining and counting of vertebrae in adults and the individuals from the common-garden experiment was performed earlier, and the data was supplied for use in this study. The vertebral number of adult individuals was analyzed using radiographs and/or by dissection after tissues were removed by boiling, whereas juveniles were stained using Alizarin Red S (for details - see Tibblin [26]). Wild-caught juvenile individuals from this study's sampling efforts were placed in a petri-dish on top of a ruler and measured to the closest mm. Staining of vertebrae was performed using Alizarin Red S based on a protocol

procedure [27], followed by dissection. Stained individuals were then fixated between two slides using rubber bands, and placed on a backlit dissection table so that the vertebral column was visible (Fig. 2). Photography then followed using Olympus Digital camera UC 30 and subsequent counting of vertebrae was performed in Adobe Photoshop CS6.
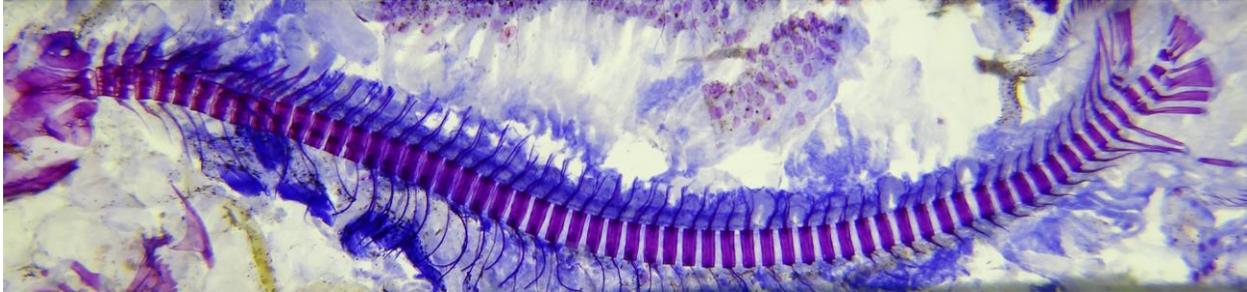

Figure 2. Vertebral column of a juvenile individual of pike *Esox lucius* after boiling, dissection and staining with Alizarin Red S.

Statistical analysis
Prior to statistical analyses the variance and normality in VN within and across subpopulations and life-stages, and histograms of relative frequency distribution (%) were visually inspected. The data was then tested using either Pearson's chi-squared test, or Fisher's exact test when expected counts within cells were low (i.e. <5 in >20 % of cells). This type of analysis was chosen since the data is considered categorical, *i.e.* there is a set interval for the number of vertebrae that is possible for an individual to have. This same testing procedure was also used when testing for differences between the two subpopulations used in the common-garden experiments. To minimize the frequency of cells with low expected counts, data on vertebrae were grouped into four categories: ≤ 59, 60, 61 or ≥ 62 vertebrae. The categorization of data into the four groups should not result in any major skewness of distributions, as 95 % of individuals had a VN matching one of the four categories. Comparisons of frequency distributions of VN among populations were performed separately for juveniles and adults.

To evaluate whether there was a relationship between body length and VN, a linear regression analysis was performed using data only for wild-caught juveniles from the stream Kronobäck. This subpopulation was chosen because it contained the highest number of sampled individuals (*N*=71; table 1), and adults were excluded from the analysis because they might differ in body size for reasons (*e.g.* age differences) other than VN.

A quadratic regression on the same data set for wild-caught juveniles was performed to examine whether there was a linear relationship (indicative of directional selection) or a curvilinear relationship (indicative of either stabilizing or disruptive selection) between body length and VN. These two analyses were performed on raw data. All tests and plots were performed in SPSS 23.

Results

Distribution of VN in different subpopulations and life-stages
In total, 445 individuals (175 adults and 270 juveniles) were used in this study. Across all subpopulations (including common-garden reared individuals) and life-stages VN ranged between 56-63 (Fig. 3). Collectively, there were only 20 individuals with a VN in the extremes of the range (i.e. < 59 or > 62 vertebrae), comprising less than 5 % of individuals in this study. Tests showed that the distribution of VN

differed significantly across all subpopulations, both in adults ($\chi^2 = 15.72$, $df = 6$, $P = 0.015$) and juveniles (Fisher's Exact test, $df = 6$, $P < 0.001$, Fig. 2). Significant differences were also evident between the two different life-stages within each subpopulation (Kronobäck: $\chi^2 = 17.18$, $df = 3$, $P = 0.001$; Dunöbäcken: Fisher's Exact test, $df = 2$, $P = 0.006$; Lerviksbäcken: Fisher's Exact Test, $df = 3$, $P < 0.001$). Overall, VN was lower in adults than in juveniles (Fig. 3). The comparison of VN in juveniles originating from the common-garden experiment revealed significant differences between the two subpopulations Kronobäck and Lerviksbäcken (Fisher's Exact Test, $df = 3$, $P < 0.001$).
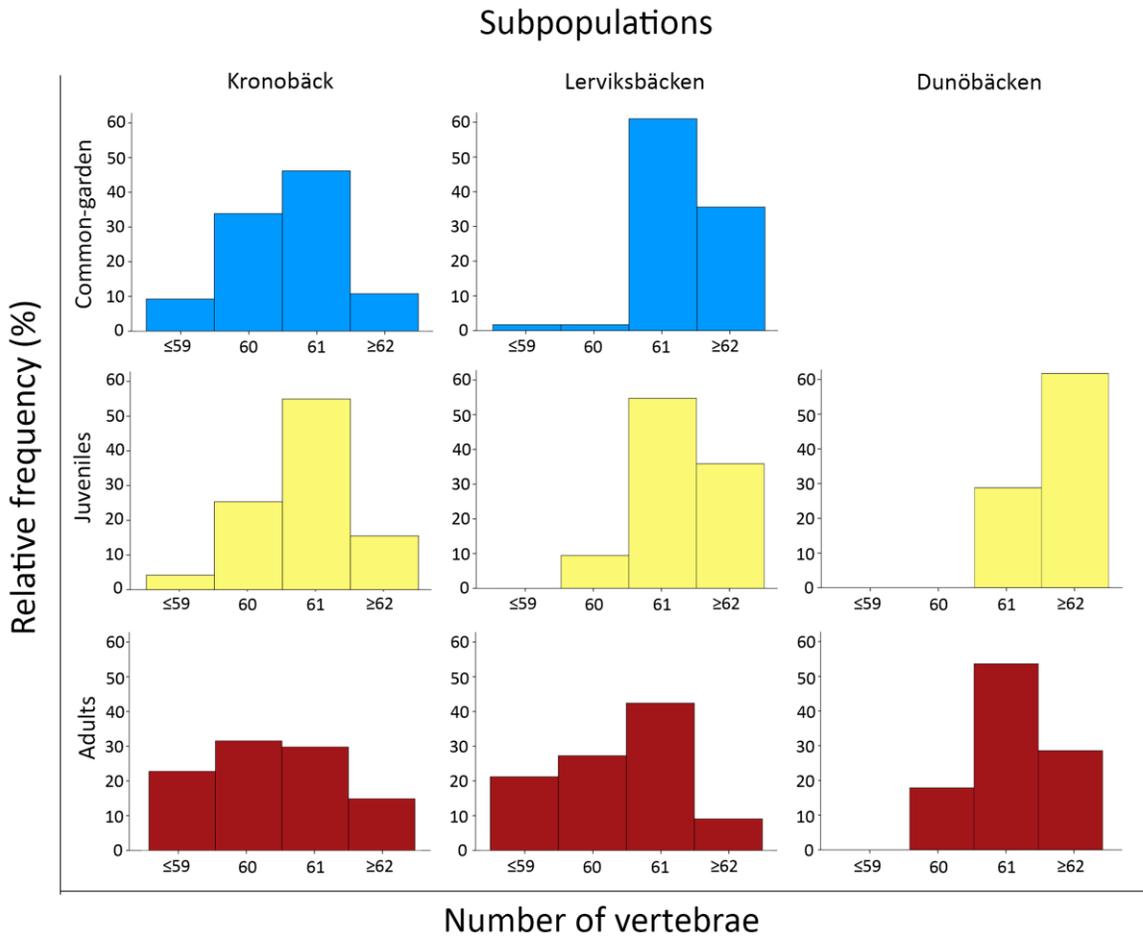

Figure 3. Frequency distributions of VN among the different subpopulations of pike *Esox lucius* used in this study. Figure shows data for common-garden reared juveniles (top panels, blue bars), wild-caught juveniles (middle panels, yellow bars) and wild-caught adults (bottom panels, red bars).

Relationship between body length and VN

Visual inspection of data suggested that individuals with a number of vertebrae in the extremes of the range were on average smaller than those possessing an intermediate number of vertebrae (Fig. 4). The results of a quadratic regression confirmed that there was a significant curvilinear relationship between body length and VN indicative of stabilizing selection (test of linear effect: $t = 2.42$, $P = 0.018$; test of quadratic effect: $t = -2.41$, $P = 0.019$; Fig. 4).
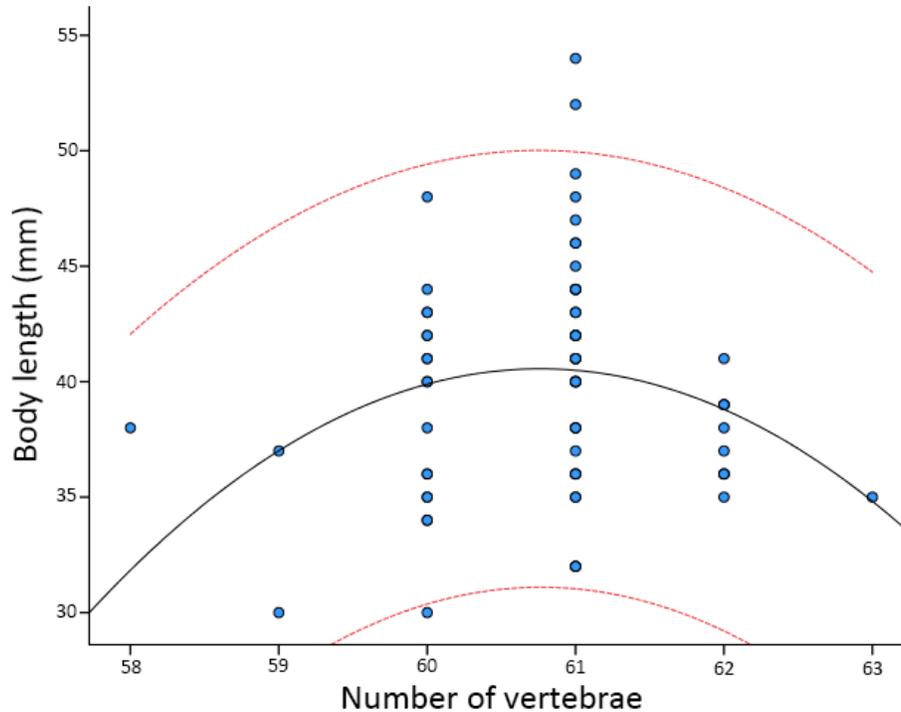
Figure 4. Results from a quadratic regression analysis performed on wild-caught juveniles originating from stream Kronobäck. Dashed red lines indicate 95 % CI. Please note that there is overlay in data points.

Discussion

In this study, variation in VN within and across three subpopulations of anadromous pike in the Baltic Sea was examined. When investigating the distribution of VN across subpopulations, all tests reported significant differences between them. Sampling efforts in this study did not control for the gender of individuals, and so these results could be influenced by sexual dimorphism with regards to the number of vertebrae. Some studies have indeed found a significant relationship between gender and the number of vertebrae [28], however, the majority of studies searching for such a relationship have not [3]. Another possibility is that since individuals from these subpopulations originated from different streams, one might argue that these results reflected in part phenotypic plasticity in response to differences in for example temperature conditions during ontogeny. While factors known to influence the development of vertebrae like temperature and salinity were not controlled for during this study (and may well have varied between streams and/or years), the results from the common-garden experiment might shed some light on this. Individuals originating from two different subpopulations that were raised under the same conditions still showed differences in the distribution of VN, indicating a genetic influence on the phenotypic expression of VN. This is in concurrence with results reported by other studies on the number of vertebrae in fish [10-13,29]. Furthermore, previous studies have found that rather large differences in temperatures (*e.g.* ± 5-10°C) are needed in order for the development of number of vertebrae to be affected [12], whereas others have found no relationship between temperature and the number of vertebrae [5,30]. In other words, it is not likely that the temperature in the different streams varied with this much, and even if it did it is not certain that it had any effect on the development of number of vertebrae.

Another aim of this study was to investigate the distribution of VN across life-stages to see whether differences associated with life-stages were present that might indicate that this trait is influenced by natural selection. Results showed that there were significant differences between juveniles and adults within all three subpopulations, with adults consistently having lower counts than juveniles. This suggests that natural selection plays a role in shaping the distribution of VN [7]. Since there are reports of selection against certain vertebral phenotypes [14,15], viability selection would seem a likely candidate. To explain the observed pattern, selection would have to occur at some point during or after the migration of juveniles to the sea, and before they return as adults to spawn. In some diadromous species of fish belonging to the family Galaxiidae, there is evidence of selection for VN occurring during the marine life-stage of juvenile fish, before they return to freshwater to spawn [6]. In the case of these galaxiids, the evidence is based on a strongly positive relationship between VN and the body size of individuals returning from the sea, which is a relationship not observed later on in their adult life. This tendency for individuals with a high number of vertebrae to have larger body sizes than individuals with fewer vertebrae (termed pleomerism) is widespread and reported in both fish and snakes [1,3]. To investigate whether such a relationship between VN and body size is present also in pike was the target of the final analysis in this study.

Testing performed on this material showed no significant linear relationship between VN and body size, i.e. pleomerism does not seem to exist in juveniles of pike. Instead, a curvilinear relationship with juveniles having an intermediate number of vertebrae being the largest was observed, indicating stabilizing selection. The peak of this curve might represent the point at which VN is most adaptive in relation to body size, granting a higher degree of hydrodynamic efficiency (e.g. burst-swimming performance) [3,31] which in turn may affect foraging success and survival. Considering that pike is a cannibalistic species [22] and size dependent in their choice of prey [32], this relationship between body length and VN might also have implications for the survival of individuals. In a laboratory experiment studying cannibalism in pike, the first individual to turn cannibalistic in each tank used was always the largest one [33]. After the onset of cannibalism, these individuals grew faster than their conspecifics. Assuming that the larger juvenile individuals from this study turn cannibalistic first and as a result enjoy a higher growth rate than their conspecifics, then these individuals should be more likely to survive into adulthood, at least if body size is the trait that selection primarily acts upon. However, there is a discrepancy between the VN at which the juvenile individuals are at their largest, and mean VN of adults returning (the largest juveniles from Kronobäck had a mean VN of 60.8, whereas mean VN in adults returning to this stream was 60.3). It therefore seems likely that some other selective force is at play, shifting VN towards a lower value. Further studies should be conducted in order to identify the selective mechanism(s) that cause this discrepancy.

In conclusion, the results of this study suggest that selection has influenced the phenotypic variation and evolution of VN within and among sympatric populations of pike that become allopatric only briefly during spawning. This has practical implications when considering conservational actions. The general goal of conservation is to maintain a healthy degree of genetic variation within a species or population, while at the same time maintaining unique genotypes and local adaptations. The subpopulations tested in this study are mostly sympatric, and only separated on a small geographical scale during spawning. Yet, they display a considerable amount of variation in characters that may be associated with fitness. Therefore, any enhancement stocking should be performed in such a way that genotypes are preserved and not "diluted" by breeding fish from one stream with fish from another stream [24,34]. The comparisons of VN between juveniles and adults, and the curvilinear association of VN with body size suggest that

differences in number of vertebrae might contribute to variation in relative fitness among individuals within populations. That the distribution of VN differed among subpopulations, and that these differences were evident also in common-garden reared juveniles, might be indicative of divergent selection and evolved (genetically based) local adaptations. The homing behavior of anadromous pike most likely contributes to uphold this pattern, and protecting the spawning streams of these subpopulations should be of key interest for conservation and fisheries management. Future studies should focus on disentangling the reasons (i.e. the possible selective forces) behind this variation and possible consequences if it is lost.

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