Genomic Response to Selection for Predatory Behavior in a Mammalian Model of Adaptive Radiation

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Abstract

If genetic architectures of various quantitative traits are similar, as studies on model organisms suggest, comparable selection pressures should produce similar molecular patterns for various traits. To test this prediction, we used a laboratory model of vertebrate adaptive radiation to investigate the genetic basis of the response to selection for predatory behavior and compare it with evolution of aerobic capacity reported in an earlier work. After 13 generations of selection, the proportion of bank voles (*Myodes [=Clethrionomys] glareolus*) showing predatory behavior was five times higher in selected lines than in controls. We analyzed the hippocampus and liver transcriptomes and found repeatable changes in allele frequencies and gene expression. Genes with the largest differences between predatory and control lines are associated with hunger, aggression, biological rhythms, and functioning of the nervous system. Evolution of predatory behavior could be meaningfully compared with evolution of high aerobic capacity, because the experiments and analyses were performed in the same methodological framework. The number of genes that changed expression was much smaller in predatory lines, and allele frequencies changed repeatably in predatory but not in aerobic lines. This suggests that more variants of smaller effects underlie variation in aerobic performance, whereas fewer variants of larger effects underlie variation in predatory behavior. Our results thus contradict the view that comparable selection pressures for different quantitative traits produce similar molecular patterns. Therefore, to gain knowledge about molecular-level response to selection for complex traits, we need to investigate not only multiple replicate populations but also multiple quantitative traits.

Key words: **selection experiment, genetic architecture, RNA-Seq, quantitative traits, predatory behavior, mammals.**

Introduction

Genetic basis of adaptive evolutionary change has intrigued researchers for decades (Lewontin 1974; Radwan and Babik 2012), but general patterns of response to selection, especially in sexually reproducing species, are poorly understood. It has recently been proposed that, as a remedy, two strategies—identification of the loci that contribute to variation in traits influencing fitness and examination of the trajectories of polymorphisms during adaptation—should be combined in the evolve and resequence approach (Turner et al. 2011; Kofler and Schlötterer 2014). In evolve and resequence studies, experimental evolution is combined with high throughput sequencing to identify the genotype–phenotype link, to test the role of selection in shaping genetic variation, and to study evolution in real time (Garland and Rose 2009; Kawecki et al. 2012; Schlötterer et al. 2015). In our previous work, we used this strategy to investigate initial molecular-level response to selection for high aerobic capacity in bank voles (*Myodes [=Clethrionomys] glareolus*) (Konczal et al. 2015). Studying four independent replicates in both the selected and unselected groups (line types) allowed an effective control for the effects of drift, but similarly to other selection experiments, our conclusions were based on analyses of a single phenotype. The bank voles selected for high aerobic capacity are, however, a part of larger project—a laboratory model of adaptive radiation (Sadowska et al. 2008)—including also lines selected for other ecologically important traits. It is thus worthwhile to ask whether directional selection imposed under identical conditions on different quantitative traits will produce similar patterns at the genomic level.

Experimental studies showed that evolution can be rapid: significant changes in phenotype have been observed for many species after only several generations (Garland and Rose 2009; Johansson et al. 2010; Orozco-terWengel et al. 2012). Trajectories of selected variants are, however, complex.

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Instead of going to fixation, variants may sometimes stabilize at intermediate frequencies, which reduces power to distinguish them from variants affected mainly by drift (Burke et al. 2010; Orozco-terWengel et al. 2012). General patterns of response to selection, such as repeatability of allele frequency changes, magnitude of change, number of loci under selection, or proportion of adaptive changes in regulatory sequences remain still a matter of uncertainty.

The patterns of response to selection depend on population size and selection protocol, but it is less clear whether they vary depending on the trait under selection. The available evidence from flies, mice, and humans (Flint and Mackay 2009) suggests that most quantitative, fitness-related traits have complex genetic basis, with large number of loci of small effect underlying their variation. Flint and Mackay (2009) argued that the distributions of effect sizes of common variants for most phenotypes and species are similar, and discrepancies can be explained by differences in experimental design. This uniform genetic architecture would imply that selection for various quantitative traits performed under identical conditions starting from the same base population should produce similar patterns at the genomic level (i.e., number of responding genes, magnitude of allele frequency changes, repeatability at the molecular level). On the other hand, theory suggests that genetic architecture underlying quantitative traits should evolve and differ between phenotypes (Hansen 2006; Rajon and Plotkin 2013; Remington 2015). For example, a theoretical model proposed by Rajon and Plotkin (2013) predicts that traits under moderate selection pressure should be encoded by many genes, whereas traits under either weak or strong selection should be encoded by relatively few genes. Some light may be shed on this controversy by answering the empirical question whether directional selection imposed under identical conditions on different quantitative traits will produce similar patterns at the genomic level.

To study general patterns of molecular-level response to selection for quantitative traits, we performed transcriptomic analyses on animals from a unique laboratory model of mammalian adaptive radiation (fig. 1; Sadowska et al. 2008): 12 lines derived from a natural, outbred population of a wild rodent, the bank vole (*Myodes [=Clethrionomys] glareolus*). Four independent lines were selected for increased predatory behavior (time to catch a cricket; P/predatory lines), four for high aerobic exercise metabolism (1-minute maximum rate of oxygen consumption achieved during swimming; A/aerobic lines), and four were not intentionally selected (C/control lines). We sequenced pooled RNA samples (pooled RNA-Seq; Konczal et al. 2014) to compare both gene expression and allele frequencies in transcribed parts of the genome (De Wit et al. 2015). In our previous work, we reported the results for a comparison of the aerobic and control lines (Konczal et al. 2015). The main aims of this work were to 1) investigate the transcriptome-wide response to the selection for predatory behavior, and 2) compare it with the response to selection for high aerobic metabolism.

Predation is an ecological factor of almost universal importance for regulating ecosystems and sustaining biodiversity (Curio 1976; Ritchie and Johnson 2009; Ishii and Shimada 2010; Ritchie et al. 2012) and has serious consequences for survival and reproductive success (Curio 1976). Yet, little is known about potential of species to evolve predatory behavior, not to mention its genetic basis. According to our knowledge, only one other experiment focused on response to artificial selection for predatory behavior (Polsky 1978), and only few others concerned predatory behavior in rodents as a

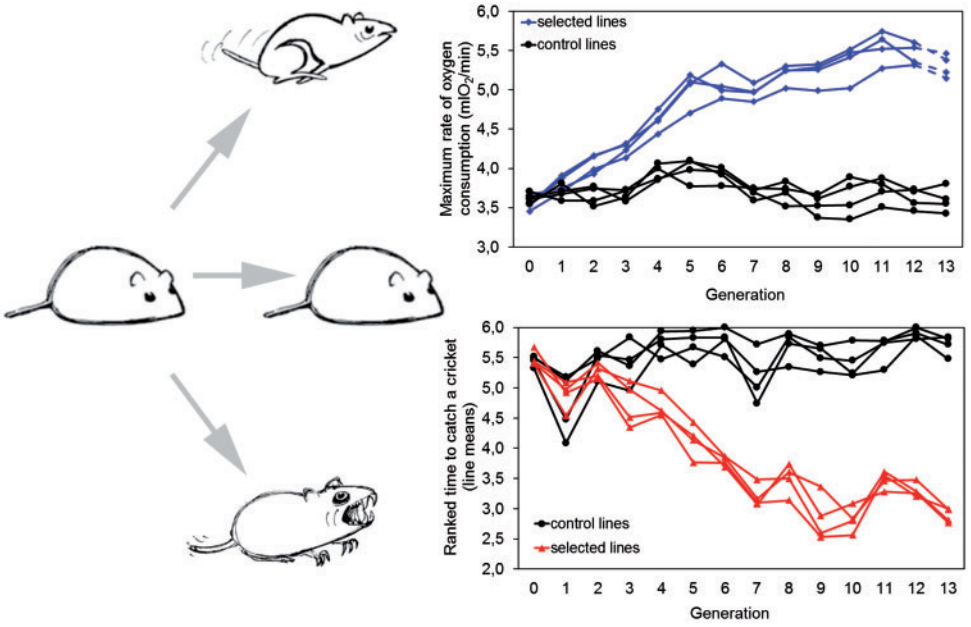

Fig. 1. Phenotypic response to selection in the experiment (mean values for replicate lines). Four independent lines were selected for increased predatory behavior (triangles), four lines were selected for high aerobic exercise metabolism (diamonds; the selection was relaxed in generation 12), and four lines were maintained as controls (circles). (Cartoon drawings by January Weiner, adapted from Sadowska et al. 2008.)

possible correlated response to selection for other forms of aggression (Sandnabba 1995) or high wheel-running activity (Gammie et al. 2003), or in insects in response to divergent selection for developmental rate (Siddiqui et al. 2015) or to laboratory natural selection driven by low quality food (Vijendravarma et al. 2013). The potential to evolve predatory behavior may be, however, substantial itself if genes involved in interindividual aggressive behaviors can be co-opted to produce predatory aggression. Identification of 'predation genes' may shed light on the possibility of shared genetic basis of intra- and interspecific aggression. At the neuronal level, predatory behavior is recognized by higher aggression, minimal arousal, and limited social communication (Tulogdi et al. 2015). Hippocampus, as associated with many of these attributes, is studied here by transcriptomic analyses.

In most evolve and resequence studies, researchers focus on a single phenotype, which is selected in replicate populations (Swallow et al. 1998; Burke et al. 2010; Turner et al. 2011; Orozco-terWengel et al. 2012). Identical conditions and experimental setup allow us to compare response to selection between two quantitative traits—aerobic exercise performance and predatory behavior—in liver transcriptomes. In the lines selected for aerobic capacity, response occurs through changes in expression of many genes, but no evidence for repeatable changes of allele frequencies in transcribed regions was found (Konczal et al. 2015). However, this pattern of response to selection is primarily driven by changes in regulatory sequences and lack of repeatable allele frequencies shifts may not be universal for all traits. By comparing genetic response to selection on various traits within the same experiment, we control for biases, which could be associated with different methodologies, genetic composition of selected populations, population sizes, or selection regimes.

Results

Transcriptome Assembly and Annotation

We used 80.2 million (M) of paired-end reads (2 × 100 bp) from one control line (C3) to reconstruct the hippocampus transcriptome. *De novo* assembly resulted in 219,886 transcripts, which were then reduced to 153,677 transcriptome-based gene models (putative isoforms were merged into a single consensus sequence and referred to as contigs; table 1). Of these 28,743 were identified as putatively protein coding, and 21,407 (74.5%) were successfully annotated to 13,305 known genes using SwissProt, showing that some genes were fragmented in the transcriptome assembly.

For the remaining seven lines (three C lines and four P lines), we obtained altogether 250 M of 100-bp single-end reads from hippocampus transcriptomes (35.6 M ± [SD] 15.3 M per sample). These reads, together with subsampled sequences from the C3 line (35 M single-end reads) were used to compare hippocampus transcriptomes between the selected and control lines.

For analyses of liver transcriptomes, we used the previously published liver reference transcriptome (Konczal et al. 2014). We sequenced liver transcriptomes of P lines (127 M single-end 100 bp reads, 31.9 M ± [SD] 3.7 per sample) and compared them with transcriptomes of control lines from the same generation of the selection experiment (Konczal et al. 2015).

Table 1. Basic Statistics of the Hippocampus Reference Transcriptome.

No. of contigs	**153,677**
No. of contigs >1 kb	**29,267**
N50 contig length (bp)	**1,598**
No. of contigs within N50	**18,518**
No. of putative protein coding contigs	**28,743**
Total length (Mb)	**122**

NOTE.—contigs (transcriptome-based gene models) contain both coding and non-coding sequences;
N50, 50% of the assembly length is in contigs of the length of N50 bp or longer.

Table 2. Number of SNPs Identified in the Liver and Hippocampus Transcriptomes of Bank Voles Derived from Predatory and Control Lines.

No. of SNPs	**179,468**
No. of contigs with SNPs	**15,580**
No. of nonsynonymous SNPs	**21,708**
No. of synonymous SNPs	**44,102**
No. of UTR-located SNPs	**82,422**
No. of SNPs in noncoding contigs	**31,236**

Polymorphism

Using reads from the liver and hippocampus, we identified 179,468 single-nucleotide polymorphisms (SNPs), which were grouped into four classes: nonsynonymous, synonymous, UTR-located, and noncoding (table 2). The SNPs were localized in 15,580 contigs, 11,076 of which were putatively protein coding. In accordance with expectations, allele frequency spectra differed between SNP classes. Spectrum of nonsynonymous variants was the most skewed, indicating purifying selection (supplementary fig. S1, table S1, Supplementary Material online). To test the effects of selection and effective population size (N_e, calculated from pedigree ranged from 51 to 74, supplementary fig. S2, Supplementary Material online) on variation within lines we counted the number of polymorphic sites (minor allele frequency > 0.05) within each line. Polymorphism was affected by N_e (generalized linear-mixed model [GLMM], $F(1,6)=9.9$, $P = 0.02$), while the effect of treatment (C vs. P) was not significant (GLMM, $F(1,6)=1.6$, $P = 0.25$).

Repeatability of Allele Frequency Changes in Predatory and Aerobic Lines

To test whether selection resulted in repeatable changes of allele frequencies, we investigated pairwise F_{ST} distances between predatory and control lines for all SNPs. Ordination of the pairwise F_{ST} matrix did not reveal clustering of selected and control lines ($P = 0.20$; permutation test; supplementary fig. S3, Supplementary Material online), similarly as in the case of the previous comparison between aerobic and control lines ($P = 0.64$; permutation test; supplementary fig. S3, Supplementary Material online), although the difference between predatory and control lines appears to be higher than

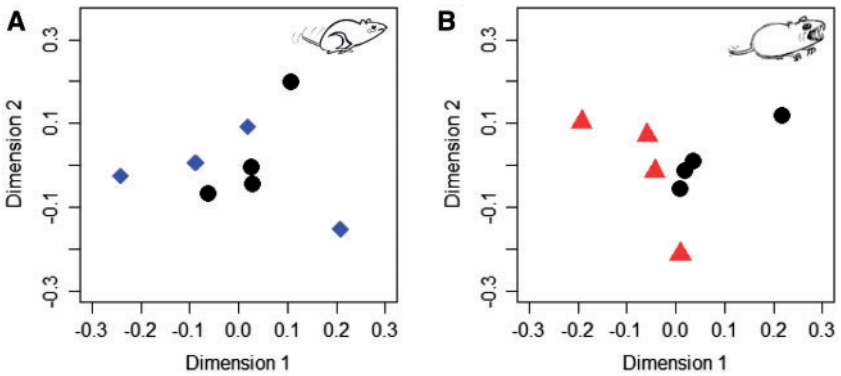

FIG. 2. Genetic differentiation of (*A*) aerobic-selected (diamonds) versus control lines (circles) and (*B*) predatory-selected (triangles) versus control lines, in the bank vole selection experiment. Multidimensional scaling (MDS) was performed on the matrices of pairwise F_{ST} distances between lines calculated for top 500 SNPs with the highest mean pairwise F_{ST}, that is, showing the most overall differentiation among lines (separately for the two comparisons).

Table 3. Number of SNPs with Nonoverlapping Allele Frequencies (Differentiated SNPs) between Selected Lines and Controls.

	Predatory Lines		Aerobic Lines	
	Observed	Expected	Observed	Expected
No of differentiated SNPs	3,342	2,565–3,198	2,882	2,349–3,009
No of SNPs	163,646	164,400–168,720	156,452	154,760–159,120

that between aerobic and control ones. However, when we sampled 500 SNPs with the highest mean pairwise F_{ST} (i.e., showing the highest overall differentiation), the clustering was not observed for aerobic lines (fig. 2A; $P = 0.61$; permutation test), whereas the predatory lines clustered apart from controls (fig. 2B; $P = 0.06$; permutation test).

We further tested whether the number of SNPs differentiating predatory lines from controls exceeded neutral expectations obtained from pedigree-based simulations. To this end, we investigated SNPs with allele frequencies nonoverlapping between predatory and control lines (3,715 SNPs, 2.07% of all SNPs, located in 2,050 contigs). Within this set of differentiated SNPs, 419 were nonsynonymous (1.93% of all nonsynonymous SNPs, in 338 contigs), 965 (2.19%, in 696 contigs) synonymous, 1,682 (2.04%, in 962 contigs) UTR-located, and 649 (2.08%, in 407 contigs) noncoding. The observed number of differentiated SNPs was significantly higher than expected ($P = 0.02$). In separate tests for various SNP classes, the excess of differentiated SNPs was found for synonymous polymorphisms ($P = 0.01$), while the number of differentiated nonsynonymous ($P = 0.52$), UTR-located ($P = 0.16$) and noncoding ($P = 0.25$) SNPs did not depart from neutral expectations. This result again differs from that for aerobic lines, in which the number of differentiated SNPs did not exceed drift expectations either for all SNPs or for any particular class (Konczal et al. 2015). However, in both cases the relatively small population sizes result in low population recombination rate, which may cause entire long haplotypes to drift. To control for the effect of linkage, additional analyses were performed.

For each contig, variation in the base population was estimated using polymorphism data from control lines corrected for the loss of variation during the experiment. A set of haplotypes in the base population was then obtained using coalescent simulations (see Materials and Methods). These haplotypes were used for pedigree-based simulations to estimate the expected number of differentiated SNPs. Also this analysis confirmed an excess of differentiated SNPs in predatory lines, whereas for aerobic lines the observed and simulated data did not differ (table 3). The excess of differentiation in the predatory lines was observed mostly in synonymous and UTR-located SNPs (table 4).

Table 4. Number of Differentiated SNPs in Predatory Lines Grouped According to the SNP Class.

SNP type	Observed	Expected
Nonsynonymous	380	296–395
Synonymous	849	634–780
UTR	1527	1196–1430
Noncoding	586	439–593

Candidate Loci for Predatory Behavior

To identify loci most differentiated between predatory and control lines, we applied two strategies based on differences in allele frequencies or differences in read counts. First, we sorted SNPs with nonoverlapping allele frequencies according to their diffStat value (Turner et al. 2011). DiffStat is the difference in allele frequency between a selected line with the highest frequency and a control line with the lowest (or vice versa). To select the loci most differentiated between treatments, we considered SNPs with diffStat > 0.2 (94 SNPs; probability of obtaining such diffStat by chance in pedigree based

simulations $< 4.5 \times 10^{-4}$). For this group, we carried out manual verification and investigation of their molecular functions. The full list of these genes is provided in supplementary table S2 (Supplementary Material online). Second, following the strategy applied by Jha et al. (2015), we used GLMM to compare allele frequency differentiation between predatory and control lines. To do so, for each SNP we compared the number of sequencing reads with reference and alternative variants between treatments. We narrowed down the list to 114 SNPs with P value $< 10^{-10}$ (probability of obtaining such P value by chance in simulations $< 5.98 \times 10^{-4}$) and listed these SNPs in supplementary table S3 (Supplementary Material online).

Results of both analyses were consistent, showing a significant overrepresentation of SNPs with allele frequencies highly differentiated between treatments, although in both cases false discovery rate (FDR) assessed by simulations was substantial (0.86 and 0.94, respectively). Nevertheless, SNPs detected by both approaches (38 SNPs; FDR = 0.69) are the strongest candidates for being targets of selection and some genes harboring such SNPs are discussed below (supplementary table S4, Supplementary Material online).

Changes in Gene Expression

To determine differences in gene expression between predatory and control lines, we mapped reads from the liver and hippocampus to the respective transcriptomes, and compared expression between the P and C lines. Multidimensional scaling separated selected lines from controls for the hippocampus ($P = 0.012$) but not for the liver ($P = 0.286$). The same pattern was observed in analyses limited to 500 contigs with expression most differentiated among all samples (hippocampus: $P = 0.016$; liver: $P = 0.289$, fig. 3). On the other hand, the number of contigs with statistically significant differences in expression between the P and C lines (FDR < 0.05) was higher in the liver (90; 0.10% of all contigs expressed in liver) than in the hippocampus (59; 0.04% of expressed hippocampus contigs) ($\chi^2 = 29.8$, $P < 0.001$; supplementary tables S5 and S6, Supplementary Material online). Candidate loci potentially associated with predatory behavior are described in Discussion section.

Discussion

Molecular Basis of Variation in Predatory Behavior

We employed the evolve and resequence approach to provide the first genome-wide dataset on genetic variation associated with predatory behavior in a mammalian species. The analysis revealed both highly differentiated allele frequencies in transcribed portions of the genome and differences in expression of several genes, which function suggests plausible role in the increased predatory behavior (supplementary tables S4–S6, Supplementary Material online).

The two SNPs with the highest differences (diffStat values 0.47) and with the same allele fixed in all predatory lines were localized next to each other in a noncoding transcript, which was manually annotated as 3'UTR region of cAMP-specific 3',5'-cyclic phosphodiesterase 4D gene (*PDE4D*). *PDE4D* is one of four known cAMP-specific genes in the mammalian genome, expressed in the cerebellum, thalamus, habenula, hippocampus, and cerebral cortex (Iona et al. 1998; Zhang et al. 1999). Inhibition of PDE4D produces antidepressant-like effects in both animals and humans via reduction of cAMP signaling in the brain (Zhang 2009). Interestingly, Wang et al. (2015) found an association between presence of PDE4D isoforms and changes in behavioral tests in mice. They reported 85.9% increase of the latency to feed in the Novelty-Suppressed Feeding test after the chronic unpredictable stress procedure. This tendency (together with other behaviors specific for animal models of depression) was, however, reversed, if long isoforms of *PDE4D* gene were blocked. Although hippocampal expression of *PDE4D* did not differ between P and C lines, these results might suggest that changes in alternative splicing of *PDE4D* played an important role in response to selection for predatory behavior.

Another intriguing example is presented by the contig over-expressed in the predators' hippocampus that encodes 3'UTR of gamma-aminobutyric acid B receptor 1 (*GABBR1*). Interestingly, SNP with one of the highest

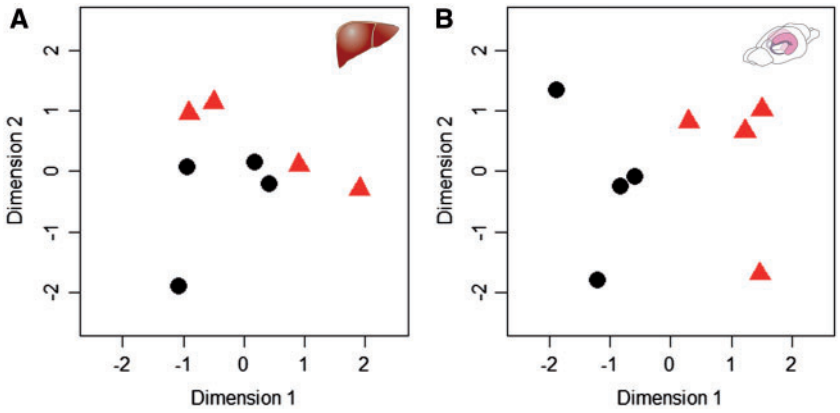

FIG. 3. Expression differentiation in liver (*A*) and hippocampus (*B*) samples from predatory (triangles) versus control (circles) lines of the bank vole selection experiment. Multidimensional scaling plots were drawn from top 500 contigs with the largest variation in expression, treating all lines as one group.

diffStat values is localized in gene that encodes pyridoxine-5′-phosphate oxidase. This gene catalyzes the rate-limiting step in the synthesis of pyridoxal 5′-phosphate, an important cofactor in biosynthesis of many neurotransmitters including dopamine, serotonin, and gamma-aminobutyric acid (GABA). Imbalance between glutamatergic and GABAergic activity leads to the hyperactivity of the limbic regions and aggressive behavior (for review see Siever 2008). GABA is the main inhibitory neurotransmitter in the central nervous system, acting through ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. The latter is responsible for the neuronal activity modulation, synaptic plasticity, and neurogenesis (Pinard et al. 2010; Hensler et al. 2012). It was shown that GABA signaling through $GABA_{B1}$ receptors inhibits proliferation in the hippocampus and reduces neurogenesis (Giachino et al. 2014). We thus suspect that changes associated with GABAergic signaling may be responsible for the evolution of predatory behavior. It is also worth to mention that both serotoninergic and dopaminergic systems regulate arousal, mood, attention, and cognitive functions, thus changes in their activations may be associated with aggressive disorders (Kudryavtseva 2000).

The analysis revealed also several other highly differentiated genes encoding proteins involved in functions of the central nervous system, such as proline dehydrogenase 1 (*PRODH*; the highest *P* value in GLMM test), associated with cognitive dysfunctions in humans (Kempf et al. 2008), Phospholipase A2 (*PLA2*), linked to schizophrenia and autism (Bell et al. 2004), or CB1 cannabinoid receptor-interacting protein 1 (*CNRIP1*), involved in the mechanism of reward-related eating (Harrold et al. 2002; Guggenhuber et al. 2010).

Expression of testosterone 17-beta-dehydrogenase 3 (*HSD17B3*), a gene encoding the enzyme involved in the reduction of androstenedione to testosterone, was increased in livers of P-line voles. Increased testosterone level results in increased intraspecific aggression (Nelson and Chiavegatto 2001), and the question whether intraspecific aggression shares a partly common control mechanism with the interspecific predatory aggression is subject to debate (Sandnabba 1995; Weinshenker and Siegel 2002). Upregulated expression of a few other genes in liver indicates that the increased readiness of P voles to attack and eat the crickets could be due to increased sensitivity to hunger: both G0/G1 switch gene 2 (*G0S2*) and protein phosphatase 1 regulatory subunit 3G (*PPP1R3G*) were reported to be upregulated during fasting (Zandbergen et al. 2005; Luo et al. 2011), whereas leptin receptor is involved in the main regulatory mechanism of feeding and energy balance (e.g., Ahima and Flier 2000; Otte et al. 2004). Finally, differentiated expression of aryl hydrocarbon receptor nuclear translocator 2 (*ARNT2*) in the liver, and differentiated allele frequencies in delta-aminolevulinate synthase 1 (*ALAS1*) intron SNPs, indicate plausible changes in the general activity and circadian rhythm pattern, which could contribute do difference in predatory behavior observed at a particular time of day.

Overall, the selection experiment showed considerable genetic potential for evolution of predatory behavior in the bank vole, which was here related to genes differentiated between predatory and control lines. All the genes presented briefly above are interesting targets for future research not only in the context of possible role in evolution of predatory aggression but also using the selected lines of voles as prospective models of mental and metabolic disorders.

Patterns of Response to Selection for Quantitative Traits

In this study, we used replicate lines derived from a natural population of an omnivore rodent to experimentally quantify molecular level response to selection for predatory behavior and to compare it with response to selection for aerobic capacity. In case of both traits, the overall pattern of gene expression separated selected lines from controls, but expression of twice as many contigs was differentiated in aerobic as in predatory lines.

Differentiation of allele frequencies between selected and control lines exceeded neutral expectations for predatory but not for aerobic lines. This conclusion is supported by two findings in predatory lines: 1) separate clustering of predatory lines based on pairwise F_{ST} and 2) an excess of SNPs with repeatable allele frequency differences compared to neutral expectations obtained by pedigree-based simulations. Interestingly, in predatory lines, regardless of the simulation method (independent SNPs vs. entire haplotypes) an excess of differentiated SNPs was found among synonymous polymorphisms, but we did not observe higher than expected differentiation at nonsynonymous sites. This might be associated with high false discovery rate but might also suggest that changes other than amino acid substitutions dominate in evolution of predatory lines. The second hypothesis is supported by the trend observed in genes containing candidate SNPs with highly differentiated allele frequencies. Such genes selected by either diffStat or GLMM approach tend to show higher differences in expression between predatory and control lines than other genes. This trend is visible both in the comparison of the nominal *P* values ($P = 0.10$ and $P = 0.005$ for diffStat and GLMM, respectively; Mann–Whitney *U* test; supplementary figs. S4 and S5 and tables S3 and S4, Supplementary Material online) and in the expression fold change ($P = 0.07$, $P = 0.08$ for diffStat and GLMM, respectively; Mann–Whitney *U* test; supplementary figs. S6 and S7, Supplementary Material online). We thus argue that, as in the case of previously reported results for aerobic lines, the response to selection for predatory behavior occurs mostly via changes in gene expression. (Konczal et al. 2015). On the other hand, repeatable changes at synonymous and UTR-located sites (the latter supported only by comparison with simulated haplotypes)—potentially associated with regulation of gene expression, alternative splicing, and mRNA stability (Kuersten and Goodwin 2003; Chamary et al. 2006; Plotkin and Kudla 2011) were observed for predatory but not for aerobic lines. This difference suggests that selection for various quantitative traits conducted under the same conditions can produce different genomic patterns. Below we discuss potential causes and consequences of this observation.

Nature of genome-level response to selection may depend on several factors: amount of standing genetic variation, effective population size, selection pressure, or genetic architecture of selected trait (Kofler and Schlötterer 2014; Kessner and Novembre 2015). In our experiment, most of these factors did not differ between lines selected for aerobic metabolism and those selected for predatory behavior. All lines were derived from the same base population (Sadowska et al. 2008), thus voles share both population history before the onset of selection and the pool of standing genetic variation. Effective population sizes do not differ between aerobic and predatory lines ($P = 0.51$, t-test). Selection regimes are comparable between treatments as is response to selection at the phenotypic level (fig. 1; Chrzascik et al. 2014; Sadowska et al. 2015). All lines are kept in the same laboratory under identical standard conditions, so domestication, if occurs, should affect them in a similar way (Sadowska et al. 2015). Finally, the same methodology was applied: number of individuals sequenced, data filtering and quality control, transcriptome reconstruction, SNP calling, and allele frequency estimation were all identical for predatory, aerobic, and control lines (Sadowska et al. 2008; Konczal et al. 2015). Therefore, in our opinion, the most likely explanation for the observed difference between predatory and aerobic lines is the difference in genetic architectures between selected traits.

Genetic architecture refers to the pattern of genetic effects that build and control a given phenotypic character and its variation properties (Hansen 2006). Full characterization of genetic architecture requires information about the number of genes and alleles, the distribution of allelic and mutational effects, the distribution of allele frequencies in population, and patterns of pleiotropy, dominance, and epistasis (Mackay 2001). Such full characterization remains beyond reach for quantitative traits in any system. However, our results provide insight into some aspects of differences in genetic architecture between traits.

The probability of obtaining differentiated allele frequencies between selected and control lines depends on the strength of selection acting on particular variants. Weakly selected SNPs will have less differentiated allele frequencies than SNPs strongly influencing the trait under selection. If many variants, each of small effect, underlie aerobic performance, dynamics of allele frequency changes will be dominated by stochastic processes. Hence, we would not observe repeatable allele frequency changes, despite repeatable evolution of phenotypes. More complex genetic architecture in terms of the number of genes involved is also capable of generating greater correlated responses through pleiotropy (Falconer and Mackay 1996). Higher number of differentially expressed genes in aerobic lines compared to predatory lines is consistent with this scenario. Thus, the difference in the number of genes responding to selection probably contributes to the observed difference between predatory and aerobic lines.

The contrast between aerobic and predatory lines could in principle result from differences in initial frequencies of selected variants (Konczal et al. 2015) or differences in frequencies of variants that form epistatic interactions with selected variants (Flint and Mackay 2009; Mackay 2014). In both cases, variants of large effect might have been selected in aerobic lines, but different SNPs responded in different replicated lines. Yet another scenario might explain the observed pattern: large effect variants in nontranscribed part of genome are selected in aerobic lines but could not be detected with transcriptomic surveys. While these effects cannot be tested with our data and thus we cannot exclude their contribution to the differences between aerobic and predatory lines, future work on the genome scale is required to confirm the findings presented here. However, neither of the alternative hypotheses explains the higher number of differentially expressed genes in aerobic lines, so currently the most likely explanation is that a smaller number of larger effect variants underlie variation in predatory behavior, whereas a larger number of smaller effect variants affect aerobic performance.

The distribution of effect sizes of common variants appears similar for most quantitative traits and the model of large number of loci, each of small effect was postulated as an adequate description of genetic variation in fitness-related traits (Flint and Mackay 2009). According to this model, response to selection for various quantitative traits should generate similar genomic patterns. Our results, however, suggest differences in distribution of effect sizes between traits sufficient to produce dissimilar patters of response to selection at the molecular level. This result is in line with suggestions that genetic architectures may differ between complex traits (Hansen 2006). For instance, some theoretical studies showed that genetic architecture should evolve according to selection pressure acting on a trait (Hansen 2006; Rajon and Plotkin 2013).

As we demonstrated here, evolve and resequence approach performed on selection lines derived from a natural population can reveal differences in genetic architecture of different traits. We caution, however, against an excessive optimism and unrealistic expectations—the statistical power to detect loci under selection is relatively low and strongly depends on effective population size, number of replicates, and genetic architecture of selected traits (Kofler and Schlötterer 2014). Various types of analytical approaches differ also in power and may result in very high rates of false positives. For example, the diffStat statistic is not sensitive to differences in coverage across loci and thus may produce many false positives in low expressed transcripts (Turner et al. 2011; Kofler and Schlötterer 2014). An alternative method (GLMM on read counts) may result in excess of false positives because coverage for many genes is much higher than the number of chromosomes sampled from a population. Yet another problem—high overdispersion of allele frequencies resulting from multiple generations of drift—may rend the standard distributions used for hypothesis testing too narrow (i.e., if distributions derived from drift simulations are not applied). Indeed, we found a large overrepresentation of SNPs with differentiated allele frequencies (supplementary fig. S8, Supplementary Material online) when the false discovery rate was calculated with the standard method (q value). However, when we compared these results with simulated data, this high overrepresentation of SNPs differentiated between treatments was strongly reduced. Thus, in our opinion

drawing general conclusions about the molecular basis of adaptive evolutionary change from selection experiments might be difficult, especially when the population sizes and numbers of replicates are small. Such experiments can, however, constitute an important step in understanding genomic basis of variation in traits of interest, especially when multiple populations show repeatable genomic response to selection. Selection experiments also shed light on the longstanding questions concerning genomics of complex traits, such as those about differences in genetic architecture of different traits, as addressing them does not necessarily depend on the identification of specific genes.

Materials and Methods

Selection Experiment

This study was performed using individuals from the 13th generation of the bank vole (*Myodes (=Clethrionomys) glareolus*) selection experiment. Samples sequenced for this study were obtained from control lines and from lines selected for predatory behavior. The additional analyses were performed on previously reported data for lines selected for aerobic performance (Konczal et al. 2015). Detailed information about the animal maintenance and welfare, selection protocols, and direct effects of selection are presented elsewhere (Sadowska et al. 2008, 2015; Babik et al. 2010; Chrzascik et al. 2014; Stawski et al. 2015). Briefly, the colony was established from about 320 voles captured in the Niepołomice Forest in southern Poland (Sadowska et al. 2008). For six to seven generations, the animals were bred randomly, and then multidimensional selection experiment was established. In P lines analyzed here (four independent lines), the selection criterion was a time to catch a live cricket (*Gryllus assimilis*), according to the protocol of the predatory test (Polsky 1978; Gammie et al. 2003). The voles were fasten for 10–12 h, next crickets were placed in each cage and their presence was verified after 0.5, 1, 3, 6, and 10 min. The results were scored as ranks (1–5: cricket caught in 0.5, 1, 3, 6, or 10 min, respectively; rank 6: cricket not caught). Tests were repeated two to four times (mostly four) for each individual, depending on generation and human resources. After 13 generations of selection, the proportion of voles attacking crickets was five times higher in the selected P lines than in unselected control C lines (fig. 1; Sadowska et al. 2015). The main difference is that about 80% of voles from the unselected C lines do not attack the cricket at all in any of the replicated trials whereas about 70% of voles from the P-selected lines attack the cricket already in the first trial. Thus, the major difference appears already in the first trial, which is also the first contact with a cricket in the vole's life. In the A lines, the selection criterion was the maximum mass-independent 1-min rate of oxygen consumption achieved during 18 min swimming (Konczal et al. 2015). After 13 generations of selection swim-induced maximum rate of oxygen consumption was 48% higher in aerobic lines than in C lines (fig. 1).

It could be argued that the difference in the selected trait between the P and C lines resulted not from increased motivation or ability to catch a live prey (predatory behavior per se) but from increased motivation to get any food (i.e., the increased sensitivity to hunger). To test for this possibility, we performed behavioral trials designed in the same way as the predatory trials, except that the animals received a small pellet of standard food rather than a cricket. The tests were performed on 32 P-line voles and 32 C-line voles (eight from each of the four P-lines and four C- lines) from generation 21. Unlike in the trials with crickets, all individuals started to eat the food pellet after it was offered, and most of them did it nearly instantly, within 30 s. The latency was slightly longer in the control lines, but the difference was small. In the first trial, performed after 6 h of fasting, in C lines 27 voles took the pellet within 30 s, 1 within 1 min, and 4 within 3 min, whereas in P lines 29 within 30 s, 2 within 3 min, and 1 within 6 min. In the second trial, performed on the same animals after additional 3 h of fasting, in C lines 26 voles took the pellet after 30 s, 3 within 1 min, 1 within 3 min, and 1 within 10 min, whereas in P lines all individuals took the pellet within 30 s. Thus, a selection for an increased motivation to take any kind of food (sensitivity to hunger) may partly explain the observation that P-line voles attack the cricket faster than those from C lines but not the observation that most of C-line voles do not attack the cricket at all, whereas most of P-line voles do that.

All the breeding and experimental protocols have been approved by the Polish State and Local Ethical Committee for Ethics in Animal Research in Krakow (decisions No. 99/2006, 21/2010 and 22/2010).

P versus C Lines comparison—Sampling, Sequencing, and Quality Control

Five females and five males of 75–80 days in age were sampled from each of eight lines; each individual came from a different family. The individuals were previously used only for routine body mass measurements. Voles were euthanized by being placed individually in a jar containing isofulrane (Aerane) fumes. Small part of the left liver lobe and entire hippocampus were excised and immediately placed in RNAlater (Sigma-Aldrich). Tissues were collected between 8.00 AM and 2.00 PM. Samples were stored overnight at 4 °C and then frozen at −20 °C.

We analyzed here the hippocampus and liver transcriptomes to understand genetic basis of pedatory behavior. The studied behavior is likely controlled by the limbic system consisting of the prefrontal cortex, amygdala, hypothalamus, and hippocampus. The interaction between the amygdala and the hippocampus coordinates the emotional and reward-related modulation of behavior (Terada et al. 2013). Moreover, the hippocampus was shown to be engaged in episodic memory processing and spatial learning (for review see Bannerman et al. 2014), as well as involved in feeding-related (Tracy et al. 2001) and goal-directed (Kennedy and Shapiro 2009) behaviors. In the light of above facts, we have chosen the hippocampus as the candidate organ in which many genetic changes may occur.

Liver transcriptomes were analyzed because many genes expressed in this organ provide information about allele

frequency changes in genes which are not expressed in the hippocampus. Moreover, if evolution of predatory behavior is accompanied by changes in overall physiology (e.g., hunger level, metabolism, or stress) then gene expression changes may also occur in the liver.

Total RNA was extracted with RNAzol (Molecular Research Center); RNA concentration and quality were measured with Nanodrop and Agilent 2100 Bioanalyzer. All samples had RNA Integrity Number higher than 7.0. Then, for each organ, we prepared one pooled sample per line using equal amounts of total RNA from each individual. Residual DNA was removed from pooled samples using DNA-free Kit (Ambion).

Preparation of barcoded cDNA libraries with TrueSeq RNA kit was performed by Georgia Genomic Facility, USA. Hippocampus sample from one control line (C3) was paired-end sequenced (2 × 100 bp) and used for reference transcriptome reconstruction. For the remaining seven pools, single-end (1 × 100 bp) sequencing was performed. Four pools of liver transcriptomes predatory lines were also single-end sequenced (1 × 100 bp) and compared with previously reported liver samples from control lines (Konczal et al. 2015).

Hippocampus Reference Transcriptome Reconstruction and Annotation

Pair-end reads were trimmed with DynamicTrim (Cox and Peterson 2010) and used for de novo hippocampus transcriptome assembly with Trinity (version 2013-02-15 with –REDUCE option; Grabherr et al. 2011). We then processed the Trinity output by merging transcripts that were probably derived from the same genomic location and subsequently produced transcriptome-based gene models, which we refer to as "contigs" (Stuglik et al. 2014).

Putative coding sequences were identified using the pipeline distributed with Trinity and annotated using Trinotate software and homology search to the Swissprot database. For candidate contigs that could not be annotated automatically, we attempted manual annotation using blastn searches against the mouse genome. We found that many of these sequences represent 3'-untranslated regions (3'UTRs) or regions immediately downstream of genes, probably extended 3'UTRs in bank voles or unannotated transcribed regions in mouse (supplementary table S1, Supplementary Material online). Existing assembly strategies often fragment long 3'UTRs (Shenker et al. 2015) and some 3'UTRs may be transcribed separately from the associated protein coding sequences (Mercer et al. 2011). Allele frequency changes in such sequences may be caused by either linkage to causative variants in noncoding regions (coding nonsynonymous changes were investigated) or may be functionally important per se. The 3'UTRs and downstream sequences affect the expression of eukaryotic genes by regulation of mRNA translation, stability, and subcellular localization (Kuersten and Goodwin 2003).

SNP Analyses

Single-end reads were trimmed with DynamicTrim (Cox 2010) and adaptors were removed with Cutadapt (Martin 2011). We subsampled also single-end reads from pair-end reads, to obtain comparable amount of data for all lines and organs. Reads were mapped to the reference transcriptomes using Bowtie2 (Langmead and Salzberg 2012) and we considered only reads with mapping quality > 20 and positions with base quality > 20 phred. SNP calling was performed with samtools (Li et al. 2009), Popoolation2 (Kofler et al. 2011), and custom scripts as described in detail elsewhere (Konczal et al. 2015). Genome-wide F_{ST} estimates were calculated from average number of pairwise differences between and within population across all analyzed SNPs with custom scripts. To select most differentiated SNPs, F_{ST} distances were calculated for each SNP with PoPoolation2 and SNPs with the largest average distance were subjected for downstream analyses. To test for separate clustering of selected and control lines, we calculated the ratio of between treatment to within treatment variance using R package vegan (Oksanen et al. 2013).

Candidate SNPs were identified by two approaches. First, similar to the analyses presented for voles selected for aerobic capacity (Konczal et al. 2015), we calculated minimum absolute difference in allele frequency between the predatory and control lines, as the diffStat score (Turner et al. 2011). To narrow down the list, we selected SNPs with diffStat > 0.2. Additionally, we applied generalized linear-mixed model GLMM, similar to approach presented by Jha et al. (2015). We used R package lme4, and reads counts (reference $= 0$, variant $= 1$) were used as an outcome variable. To avoid technical problems during calculations, zero read counts were changed to one, which should not significantly affect results. The list of candidate SNPs was constructed for SNPs with P value $< 10^{-10}$.

Simulations of Allele Frequency Differentiation under Drift

To obtain expectations of allele frequency differentiation under drift, we performed forward drift simulations on known pedigrees. The initial allele frequencies were estimated from four control lines and used to randomly assign alleles to individuals at the beginning of the experiment. Genetic drift was then simulated by random pass of alleles from parents to offspring. Finally, the results expected from sequencing were obtained by adding pooling and sequencing variation as explained in detail elsewhere (Konczal el al. 2015). To obtain read counts expected from simulations, information about coverage was sampled from the empirical distribution, and such number of reads was sampled randomly with probability of obtaining the reference variant given by the simulated frequency. Simulations were performed separately for allele frequency spectra derived from all, synonymous, nonsynonymous, UTR-located, and noncoding SNPs. Additionally, to control for linkage of SNPs within contigs, haplotype-based drift simulations were performed in three steps: haplotypes in the base population were simulated using information about genetic variation from the control lines and some assumptions about natural population of bank voles (1); these haplotypes were used for pedigree-based simulations and estimation of allele frequency (2); and the results were compared between the observed data and simulations (3).

In the first step, we estimated for each contig variation in the base population. Nucleotide diversity calculated for each control line (π_t) was corrected for the loss of variation during the experiment according to the formula:

$$\pi_0 = \frac{ie^{-\frac{t}{2N}}}{(i-1)\pi_t},$$

where i is the number of individuals sequenced, t is the number of generations (13), and N is the effective population size estimated from pedigree (Charlesworth and Charlesworth 2010). Mean π_0 calculated from four control lines was then used as the estimate of the population mutation rate (θ) in the base population. Values of θ together with information about contig length were used to simulate haplotypes in the base population. To introduce recombination in the genealogical process producing these haplotypes, we assumed effective population size $N_e = 10^5$ and recombination rate of $r = 5.8 \times 10^{-9}$ per bp as estimated for the mouse (Jensen-Seaman et al. 2004). Both N_e and r values are only very crude estimates or even educated guesses, but details should not matter too much as long as some recombination is allowed. For each contig 1,000 sets of haplotypes were simulated with ms (Hudson 2002).

In the second step, haplotypes were randomly assigned to individuals at the beginning of the experiment, and pedigree-based simulations were performed as described above for independent SNPs. Forward drift simulations were performed 1,000 times, each time with different set of haplotypes. This approach assumes no recombination within contigs during the experiment, which appears reasonable given its time scale and effective population sizes.

Finally, the total number of SNPs and number of SNPs with nonoverlapping allele frequencies were compared between the observed data and simulations. To minimize the effect of rare variants which are rarely called from pooled data, we removed all SNPs with mean minor allele frequency in four control lines < 0.0125 (singletons).

Expression Analyses

To identify differentially expressed genes, we mapped reads to the reference transcriptomes with bowtie and used Trinity pipeline with EdgeR Bioconductor and RSEM (Gentleman et al. 2004; Grabherr et al. 2011; Li and Dewey 2011). Only contigs for which the sum of expected counts over all samples was higher than 10 were used for analyses.

We found that expression of twice as many genes was differentiated in aerobic as in predatory lines. This difference may be partially explained by the newer release of software (edgeR) used for analyses of predatory lines, but even when older version was applied, sequenced in both cases liver transcriptomes had much more contigs differentially expressed in aerobic (278) than in predatory lines (178).

To statistically test for separate clustering of transcriptional profiles of selected and control lines, we used similar strategy to that for F_{ST}. We used the table of expression values (FPKM [Fragments Per Kilobase of exon per Million fragments mapped], TMM normalized) and calculated distance matrix (dist() function) followed by calculation of the ratio of between treatment to within treatment variance. The statistical significance of this ratio was assessed through 1,000 randomizations. Differences between lines in genome-wide transcriptional profiles were visualized with multidimensional scaling (plotMDS {edgeR}).

Data Accessibility

Raw sequencing reads are available in the NCBI BioProject PRJNA296483. The reference transcriptome, its annotation, entire datasets about allele frequencies and expression, and used in this project scripts are available at the Dryad Digital Repository (doi:10.5061/dryad.13t00).

Supplementary Material

Supplementary figures S1–S8 and tables S1–S6 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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