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Positive selection of digestive proteases in *Daphnia*: A mechanism for local adaptation to cyanobacterial protease inhibitors

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Abstract

Due to the combined effects of global warming and eutrophication, the frequency of deleterious cyanobacterial blooms in freshwater ecosystems has increased. In line with this, local adaptation of the aquatic keystone herbivore *Daphnia* to cyanobacteria has received major attention. Besides microcystins, the most frequent cyanobacterial secondary metabolites in such blooms are protease inhibitors (PIs). Recently, it has been shown that a protease gene showed copy number variation between four *D. magna* populations that differed in tolerance to PIs. From that study, we chose two distinct populations of *D. magna* which had or had not coexisted with cyanobacteria in the past. By calculating F_{ST} values, we found that the two populations were genetically more distant in the protease loci than in neutral loci. Population genetic tests applied to the tolerant population revealed that positive selection was most probably acting on the gene loci of the digestive protease CT448 and CT802. We conclude that the selection of digestive proteases and subsequent reduction in copy number is the molecular basis of evolutionary changes leading to local adaptation to PIs.

KEYWORDS

adaptation, cyanobacteria, *Daphnia magna*, macroevolution, population genetics, protease inhibitor, tolerance

1 | INTRODUCTION

Adaptations – locally and over time – have been observed in both terrestrial and aquatic ecosystems. In contrast to studies on adaptations over time, for which recent populations are ideally compared with ancestral populations, the study of local adaptations offers the possibility of investigating adaptation by comparison between recent local populations. These populations should have evolved under different conditions. The process leading to local adaptation is the ongoing (or very recent) natural selection of traits responsible for higher tolerance of a population in its local environmental (Kawecki & Ebert, 2004). As local adaptation is a very recent event, it is often possible to identify the selective forces at work (Kawecki & Ebert, 2004).

However, the search for the target genes that had been selected remains difficult. Many studies have aimed at elucidating the molecular basis of local adaptation, e.g., by identifying differences in gene expression patterns or SNPs (Brown et al., 2013) between adapted and nonadapted populations. Although the functional relevance of these patterns often remains unclear, a few studies were

able to draw a clear link between distinct candidate genes and the adaptive traits of tolerant populations. For example, an indication for the genetic basis of local adaptation has been found by Xia, Camus-Kulandaivelu, Stephan, Tellier, and Zhang (2010) who have reported differences in nucleotide diversity patterns between drought-related candidate genes and reference genes of three populations of a tomato species. Storz et al. (2007) demonstrated that haplotypes of α-globulin with high oxygen-binding affinity predominated in high-altitude samples of deer mice which frequently experience high-altitude hypoxia, whereas α-globulins with low oxygen-binding affinity predominated in low-altitude mice.

In the case of the aquatic herbivore *Daphnia*, it has been shown that populations can locally adapt to a variety of biotic (e.g., cyanobacteria and predators: Cousyn et al., 2001; Sarnelle & Wilson, 2005) and abiotic stressors (e.g., temperature: Yampolsky, Schaer, & Ebert, 2013). However, only a few target genes in combination with their functional role in local adaptation have been identified. One study connecting target genes with the origin of *Daphnia* clones was from Schwarzenberger et al. (2014). Here, four *D. magna* genotypes from ponds with or without microcystin-producing cyanobacteria differed in the expression of transporter genes which was associated with differences in tolerance to microcystin. However, to our knowledge, the only study that found a clear connection between candidate gene expression and local adaptation of *Daphnia* populations was a study by Scoville and Pfrender (2010) in which invariantly increased upregulation of *Ddc* and *ebony* went along with reduced melanization and thus adaptation to a newly introduced fish predator.

One very important stressor of *Daphnia* is cyanobacterial protease inhibitors. The production of protease inhibitors as an anti-herbivore defence of cyanobacteria against *Daphnia* has received increasing attention. This is due to the fact that instances of elevated cyanobacterial biomass have increased in lakes over the last few decades. This increased frequency of cyanobacterial blooms is attributed to the combined effects of nutrient input and global warming (Smith & Schindler, 2009). Although cyanobacterial blooms have been shown to decrease the abundance of *Daphnia* (Ghadouani, Pinel-Alloul, & Prepas, 2003), the increasing frequency, duration and intensity of such blooms should select for more tolerant zooplankton genotypes (Ger, Hansson, & Lürling, 2014). In fact, Blom, Baumann, Codd, and Jüttner (2006) showed local adaptation of *Daphnia* to Oscillapeptin J, a strong protease inhibitor (Blom et al., 2003), in an in vitro study. Similarly, Schwarzenberger, Keith, Jackson, and Von Elert (2017) have demonstrated that four distinct *D. magna* populations differed in tolerance to dietary chymotrypsin inhibitors, and that this difference in tolerance corresponded with the cyanobacterial history of the populations' lakes of origin which hints at local adaptation.

In *Daphnia*, digestive serine proteases have been identified as targets of cyanobacterial protease inhibitors (Agrawal et al., 2005). These protease inhibitors affect *Daphnia* by decreasing their somatic growth rate and influencing expression and activity of serine proteases in *Daphnia's* gut (Schwarzenberger, Zitt, Kroth, Mueller, & Von Elert, 2010; Appendix S1). The most important digestive serine proteases in the gut of *Daphnia magna* are chymotrypsins and trypsins (Von Elert et al., 2004). Schwerin et al. (2009) have found a surprisingly high number of serine protease genes in the genome of *D. pulex* (Colbourne et al., 2011), which can also be observed in the genome of *D. magna* (www.wflebase.org). Interestingly, only three trypsin and three chymotrypsin genes (i.e., *CT383*, *CT448* and *CT802*) were assigned to the proteases active in the gut of *D. magna* (Schwarzenberger et al., 2010). One of those chymotrypsin genes, i.e., *CT448*, was demonstrated to show copy number variation with fewer copies in a more tolerant population (Schwarzenberger et al., 2017). This indicates that fewer but more stable isoforms of *CT448* were maintained. Therefore, the three chymotrypsin genes and especially *CT448* are likely to be the targets of selection, and might therefore constitute the genetic basis underlying local adaptation of *Daphnia* populations to chymotrypsin inhibitors.

Here, we chose two populations from the study of Schwarzenberger et al. (2017): One population was sensitive and the other tolerant to a cyanobacterial strain that originated from the tolerant population's lake of origin. We hypothesized that (a) the three chymotrypsin genes and especially *CT448* showed signs of positive selection, and that (b) the tolerance of *Daphnia magna* to chymotrypsin inhibitors could be explained by genetic variation in chymotrypsin gene loci. For this we sequenced alleles of three chymotrypsins and, for comparison, six putative neutral loci, i.e., single-copy genes, of several *D. magna* genotypes of both populations. In a population genetic approach, we calculated the genetic distances between the two populations and investigated whether the chymotrypsin genes of the tolerant population had been selected.

2 | MATERIALS AND METHODS

2.1 | Animals and cultures

Daphnia magna clones stemmed either from Lake Bysjön (22 clones, Southern Scania, Sweden) or from a pond near Warsaw (12 clones, Kampinoski National Park, Poland). Lake Bysjön is known to frequently develop cyanobacterial blooms (Schwarzenberger, D'Hondt, Vyverman, & Von Elert, 2013), whereas no cyanobacteria were observed in the Polish pond in the sampling year. Furthermore, the state of the Polish pond (peaty sediments, transparent water, location next to a wet meadow in a national park without farming) suggests that *D. magna* from this pond have not experienced cyanobacteria in the past (Thomas Brzezinski, personal communication). *Daphnia magna* were cultivated at 20°C in membrane-filtered (0.2 µm), aged tap water. From each clone, 15 animals per litre were kept under nonlimiting food concentrations (2 mg C L^{-1}) with *Chlamydomonas klinobasis*, originating from Lake Constance, as the control food. *C. klinobasis* was cultivated semicontinuously in cyanophycean medium (Von Elert & Jüttner, 1997) at 20°C under constant light (130 µE m^{-2} s^{-1}), with 20% of the medium exchanged every other day. The cyanobacterium *Microcystis* sp. BM25, originating from

Lake Bysjön, Sweden (Schwarzenberger, D'Hondt, et al., 2013), was cultivated in a chemostat (dilution rate 0.23/day) on cyanophyceanmedium at 20°C and constant light (50 µE m^{-2} s^{-1}). This cyanobacterium contains neither trypsin inhibitors nor microcystin but shows a high chymotrypsin inhibition (Schwarzenberger, Sadler, & Von Elert, 2013). Carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (470 nm) and from carbon extinction equations previously determined and described in detail by Schwarzenberger et al. (2017).

2.2 | Cloning and sequencing of chymotrypsins and reference genes

Genomic DNA from each genotype (clonal lineages were differentiated with microsatellites; data not shown) was extracted with the peqGold DNA Tissue Kit (peqlab). Primers for the single-copy genes (*ATP synthase gamma chain*, *guanyl-nucleotide exchange factor*, *vitamin k-dependent γ-carboxylase*, *pyridoxal kinase*, *YEATS domain containing protein 4* and *smad anchor for receptor activation*, Appendix S1) were designed based on information from the *D. magna* genome data base (www.wfleabase.org) with the program NETPRIMER (http://www.premierbiosoft.com/netprimer/). Primers for the chymotrypsin genes were taken from Schwarzenberger et al. (2010). Each primer pair is specific to one gene locus and was not found to bind at other positions, i.e., other paralogs, within the genome. After PCR amplification, the ORFs of three chymotrypsin genes (Schwarzenberger et al., 2010) and six single-copy genes were cloned (TOPO TA cloning Kit, LifeTechnologies, TZ101 alpha chemically competent cells, Genaxxon). A total of 12 haplotypes from each population were sequenced except for *CT383* (five haplotypes per each populations), *CT802* (five haplotypes of the Polish population), and single-copy gene *guanyl-nucleotide exchange factor* (24 haplotypes of the Swedish population). Each clone used in the analyses was either homozygous (one haplotype) or heterozygous (two or three haplotypes). The sequences were aligned with the program BioEdit (Hall, 1999). Intron and exon sequences of the single-copy genes were determined with EST-data from the *D. magna* genome database (www.wfleabase.org). Consensus sequences of the protease genes - generated with BioEdit from three nonrelated *D. magna* clones (Schwarzenberger, Kuster, & Von Elert, 2012) – and single-copy gene sequences from the *D. magna* genome were used for outgroup comparisons. Maximum likelihood trees using Jukes-Cantor as a substitution model with a bootstrap test of phylogeny (500 replicates) were constructed with Mega v6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

2.3 | Population genetic tests

Population genetic tests, i.e., frequency spectrum neutrality tests (Tajima's *D*: Tajima, 1989), Fu & Li's *D* and *D** (Fu & Li, 1993), Fu's F_s (Fu, 1997)), nucleotide diversity (Pi), statistics for population differentiation (F_{ST}: Lewontin & Krakauer, 1973) and statistics based on the mismatch distribution (R_2: Ramos-Onsins & Rozas, 2002) were calculated with DNASP v5 (Librado & Rozas, 2009). The F_{ST} of microsatellite data (Schwarzenberger, D'Hondt, et al., 2013) were calculated with Arlequin (Schneider, Roessli, & Excoffier, 2000), and the UPMGA microsatellite tree (D_A distance) with a bootstrap test of phylogeny (500 replicates) was constructed using the web version of POPTREE (Takezaki, Nei, & Tamura, 2014). Clones from a geographically distinct population (Lake Westveld, Ghent, Belgium) served as the outgroup population for the microsatellite tree.

The K_a/K_s ratio at each site of the proteases and the exon sequences of the single-copy genes was calculated with Selecton (Stern et al., 2007). The K_a/K_s ratio is calculated as the ratio of the number of nonsynonymous substitutions per nonsynonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s) to provide insights into the form of selection acting on a gene. A likelihood ratio test was performed between the two models M8 and M8a that are implemented on this platform. M8 considers sites under purifying selection versus. a category of sites experiencing positive or neutral selection. M8a, which is nested in the M8 model, is restricted to purifying and neutral selection only.

3 | RESULTS

3.1 | Genetic distance between populations

As a measure of genetic distance between the two populations, F_{ST} values were calculated for microsatellites (Schwarzenberger, D'Hondt, et al., 2013), the exon sequences of the single-copy genes and the three chymotrypsin genes. The single-copy genes showed F_{ST} values between 0.04 and 0.20, and the analysis of the microsatellites resulted in an F_{ST} value of 0.29 (*cf.* also UPMGA microsatellite tree of the two populations: $p < .01$; Figure 1a, Appendix S2), whereas the three chymotrypsin genes showed F_{ST} values of between 0.34 and 0.52. The markedly greater genetic differentiation of the protease genes than the single-copy genes between the two *D. magna* populations is also demonstrated by the shape and scale of the corresponding genealogies (Figure 1b,c, Appendix S3).

3.2 | Selection of proteases

Positive selection on particular coding sites may lead to a reduction in the level of (linked) polymorphism in the nearby region (Smith & Haigh, 1974). Non-coding sequences on the other hand should show a neutral level of polymorphism, since selective forces usually do not directly work on them. Therefore, in order to test whether the Swedish *D. magna* population follows the neutral expectation of mutation-drift equilibrium, we conducted population genetic tests using the intron sequences of the single-copy and protease genes. Nucleotide diversity of all intron sequences was not different from zero, indicating a neutral level of polymorphism of the Swedish population in these regions. To exclude population expansion after a

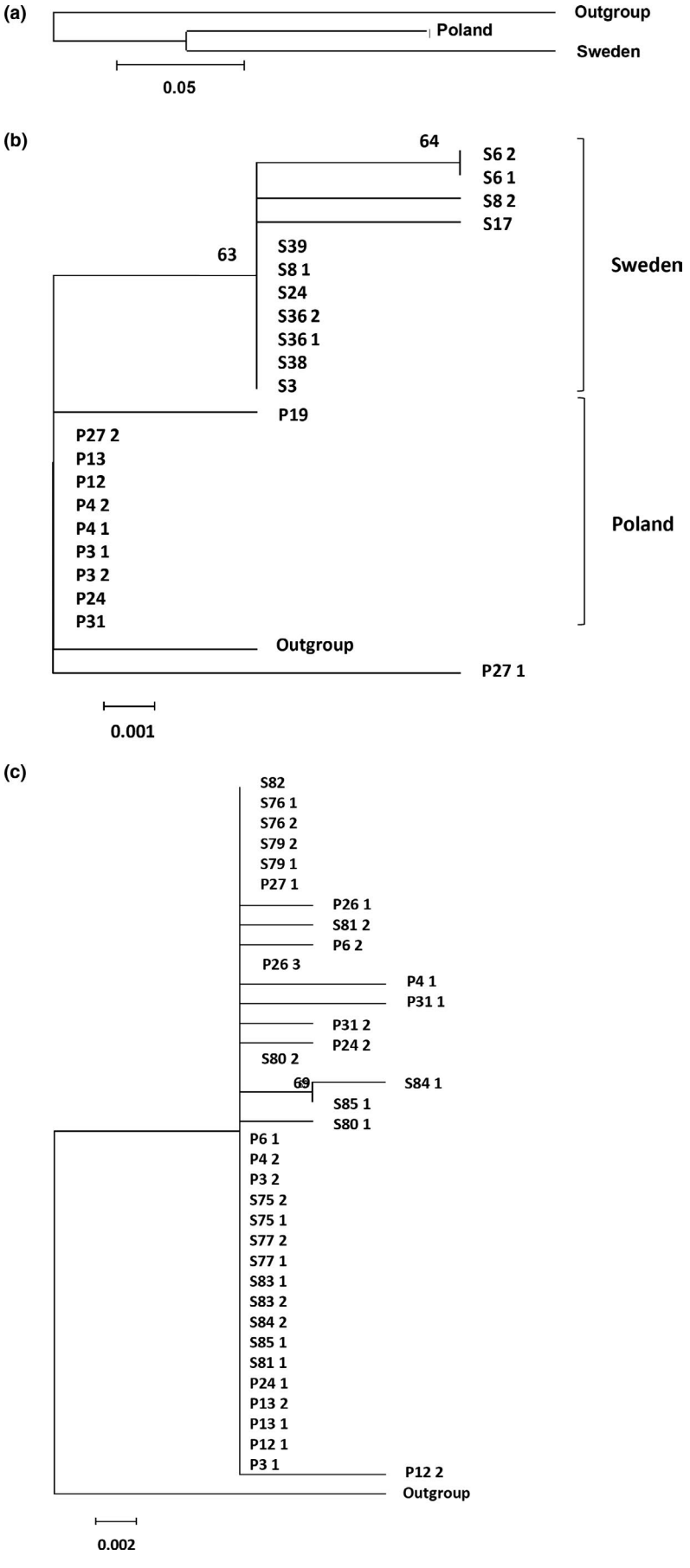

FIGURE 1 (a) UPMGA microsatellite tree with bootstrap test of phylogeny of the two populations. A geographically distinct population from Belgium served as outgroup. (b, c) Maximum likelihood phylogenetic trees with bootstrap test of phylogeny based on haplotype sequences of the Swedish and the Polish *D. magna* populations for the protease CT448 (b) and the single-copy gene *ATP synthase gamma chain* (c). The haplotypes from the Polish population all start with a "P" followed by a number, whereas the Swedish haplotypes are assigned with "S" followed by a number. Brackets indicate the clustering of the clones of a population within a tree

bottleneck, which often results in an excess of singletons, Tajima's *D* was calculated for the intron sequences of all genes. Tajima's *D* was not different from zero for eight of nine intron sequences (Appendix S4), indicating that the site frequency spectrum did not deviate from that predicted by neutral theory. The same was true for two other neutrality tests (Fu & Li's D^*, Fu's F_s). Only the intron sequence set of one single-copy gene (smad anchor for receptor activation) showed significantly negative values of Tajima's *D*, Fu & Li's D^* and Fu's F_s (Appendix S4). Consequently, we cannot rule out the possibility that this gene experienced any non-neutral evolution, which led us to exclude it for the subsequent analyses. However, haplotype data for eight intron sequences, including all protease introns, remained for further analyses. Also, the mismatch distribution, i.e., the distribution of the number of differences between sequence pairs, was not different from zero for seven of eight intron sequence sets, except for vitamin k-dependent γ-carboxylase. Six intron sequence data sets, including the proteases, suggested no evidence for changes in population size (i.e., no ongoing population expansion) and no signs of selection affecting the evolution of these non-coding sequences.

In contrast to the six neutral intron sequences, Tajima's *D*, Fu & Li's *D* and Fu's F_s were significantly negative for *CT448* (Table 1) but not for *CT802* and *CT383* (except for a significant Fu's F_s), which hints at positive selection due to a selective sweep or a recent population expansion in *CT448*.

In order to investigate if selection was working on the two other chymotrypsin genes, the K_a/K_s ratio at each site of all genes, i.e., protease and single-copy loci, were calculated with Selecton. The exon sequences of five single-copy genes showed no selected sites (data not shown). Only one single-copy locus, i.e., ATP synthase gamma chain, showed significantly positively selected sites with a significance level of 0.05 of the likelihood ratio test between the M8 model and the null model M8a. *CT383* showed no selected sites (Figure 2a). In *CT802*, positively selected sites were found (Figure 2b), ($p < .001$) when comparing the M8 model (positive selection) against the null model M8a (which does not allow for positive selection) by means of a likelihood ratio test (ln = −1144.78 vs. ln = −151.33). Interestingly, positively selected sites of *CT802* were found along the entire gene sequence with five sites being close to disulphide bridges of the enzyme and one concerning the active centre of the protease (Figure 2b).

4 | DISCUSSION

Digestive proteases in the gut of *Daphnia* are inhibited by cyanobacterial protease inhibitors. By feeding protease inhibitor encapsulated in liposomes, Von Elert, Zitt, and Schwarzenberger (2012) demonstrated that growth rate reduction was an effect of the inhibitors only and not due to other cyanobacterial products. Nevertheless, it has been shown that *Daphnia* are able to respond to such inhibitors by an increase in protease gene expression (Schwarzenberger et al., 2010). This increased protease gene expression was demonstrated to be maternally transferred to the offspring of pre-exposed mothers, which causes higher somatic growth rates of their offspring in comparison to offspring from naïve mothers (Schwarzenberger & Von Elert, 2013).

Several findings strongly suggest that differences between populations in tolerance to cyanobacterial protease inhibitors result from differences in the molecular structure of digestive proteases of *Daphnia*. (a) Schwarzenberger et al. (2012) demonstrated that clones of *D. magna* that showed differences in tolerance to dietary protease inhibitors differed in the nucleotide sequences of their digestive proteases. (b) Von Elert et al. (2012) demonstrated that tolerance to cyanobacterial protease inhibitors was acquired by remodelling the affected digestive protease type. (c) Recently, we demonstrated that higher tolerance of a *Daphnia* population to protease inhibitors is consistent with a reduced copy number of a particular chymotrypsin gene (Schwarzenberger et al., 2017), which hints at selection of few tolerant proteases together with the elimination of sensitive and therefore redundant isoforms. (d) In the study, the Swedish and the Polish population differ in tolerance to dietary chymotrypsin inhibitors (*cf.* Schwarzenberger et al., 2017) which goes along with a different chymotrypsin band pattern (i.e., different chymotrypsin isoforms; Appendix S5). These findings strongly suggest that the presence of tolerant digestive protease isoforms is a result of selection.

As a first approach to identifying potential differences in the molecular structure of the three chymotrypsins between the tolerant (Swedish) and sensitive (Polish) population (Schwarzenberger et al., 2017), we compared the genetic distance of three protease genes with those of single copy genes, assuming that the latter would represent neutral loci. The F_{ST} values for the neutral loci were well in the range known from *Daphnia* literature (Hebert & Finston, 1996; Kuster, Schwarzenberger, & Von Elert, 2012; Mort & Wolf, 1986). However, the genetic distance between populations was much higher for the proteases than for the neutral loci. Since F_{ST} values are regarded as a useful first step for the identification of candidate genes that might have been under selection (Beaumont, 2005), we concluded that adaptive selection has likely been driving the evolution of these protease genes.

In *Daphnia* studies, the relevance of dispersal and strong founder effects have led to the need to compare numerous populations to differentiate founder effects from selection (Bohonak & Jenkins, 2003). Bohonak and Jenkins (2003) discussed molecular genetic population differentiation. In contrast to this, functional molecular approaches offer the opportunity to test for positive selection even within single genomes (Hansen, Olivieri, Waller, & Nielsen, 2012), and do not necessarily require more than a single population. Therefore, we focused on the chymotrypsin genes of the tolerant population to investigate if the digestive chymotrypsins might have been under positive selection. Non-coding sequence regions should not be targets of any direct selection factor. Therefore, in order to calculate selection of chymotrypsin genes, we compared coding regions of the three chymotrypsin genes with their non-coding regions, and with intron sequences of single-copy genes that proved to be neutral markers.

Gene	Tajima's D	*p*-value	Fu & Li's D	*p*-value	Fu's Fs	*p*-value	*N*
CT383	−0.644	.29	−0.866	.24	−4.561	.024*	5
CT448	−1.845	.01*	−2.735	<.01*	−2.653	.03*	11
CT802	−1.148	.11	−0.985	.19	−0.999	.28	13

N, Number of haplotypes per protease locus.
*Indicates significance.

TABLE 1 Results and their respective *p*-values for the population genetic tests of the exon sequences of the haplotypes of the three proteases (*CT383*, *CT448* and *CT802*) from the Swedish *Daphnia magna* population

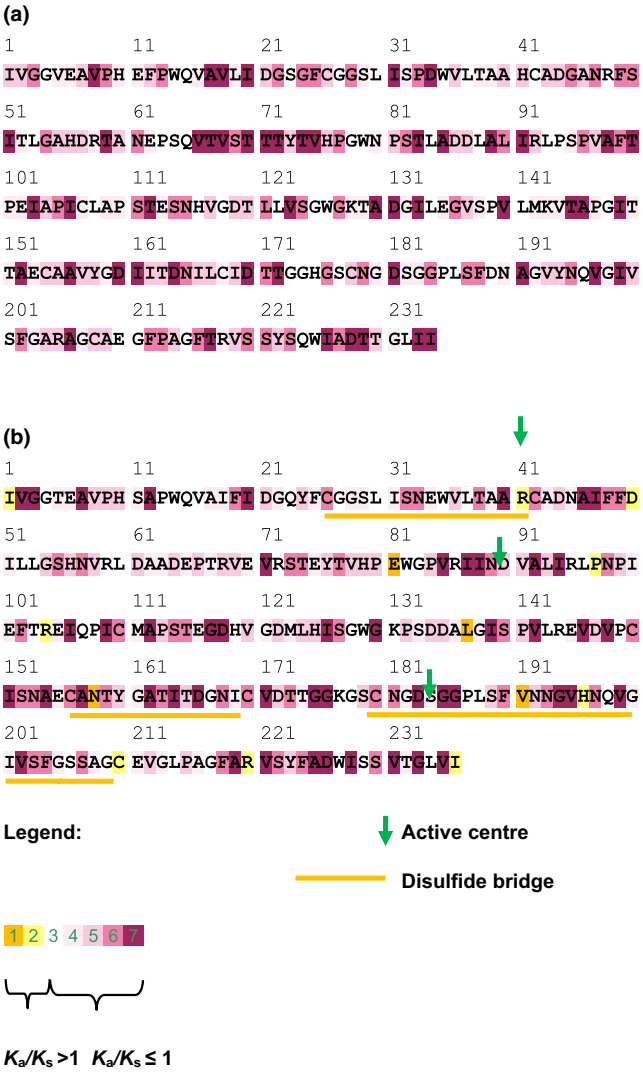

FIGURE 2 Placement of site-specific selection on the protein sequences of (a) *CT383* and (b) *CT802*. The K_a/K_s score for each codon was calculated with Selecton (see legend; a site highlighted in yellow represents evidence of strong positive selection, whereas a site highlighted in purple represents purifying selection). For *CT802* the structural information is also delineated

One chymotrypsin gene, i.e., *CT383*, was not found to be selected. Several causes are imaginable: (a) The difficulties in sequencing due to long, repetitive elements resulted in a low number of sequenced haplotypes; this might have led to nonsignificant population genetic tests for this locus. (b) *CT383* has a minor role in the tolerance to dietary chymotrypsin inhibitors and was therefore not a target of selection. (c) Not the protease itself, but rather its promotor region has been under selection. The latter is a possibility because the relative increase in gene expression of *CT383* was much higher than that of *CT448* and *CT802* in a *D. magna* clone feeding on dietary chymotrypsin inhibitors (Schwarzenberger et al., 2010).

In contrast, we found evidence for adaptive evolution in the two other chymotrypsin genes of the Swedish population. In *CT802* we detected positively selected sites which was based on the analysis of K_a/K_s ratios. However, since this test was developed for species comparisons and not for intraspecific comparison, we cannot completely rule out that the detection of selected sites in *CT802* might simply reflect polymorphism rather than true divergence. Nevertheless, one site that was positively selected is part of the active centre of the encoded enzyme. This finding suggests that this site might have undergone selection and putatively codes for a more stable *CT802* isoform.

In *CT448*, the analysis of polymorphism frequencies supported the hypothesis of positive selection acting on the *CT448* gene, putatively due to a recent selective sweep. Selective sweeps decrease the variability of a selected gene within a population. Therefore, a population undergoing a selective sweep should differ from a non-selected population in this target gene. Here, such a probable scenario might have resulted in the clear clustering of the Swedish population in the genealogy of *CT448*. This selective sweep might have been caused by the presence of a certain type of protease inhibitors in the Swedish lake. If another type of protease inhibitor becomes dominant, another allele of *CT448* might be more successful. The putative selective sweep of *CT448* is also supported by the finding that a 100% amino acid substitution between the Swedish (Gln) and the Polish *D. magna* population (Glu) has occurred at position 34, which is close to the active centre. This amino acid substitution might have resulted in a more stable isoform of *CT448* in the Swedish population.

Both findings, i.e., the putative positively selected sites in *CT802* and the probable selective sweep in *CT448* (eventually leading to the 100% amino acid substitution), might influence the tertiary structure of the chymotrypsins. Such a change in tertiary structure might have caused the higher resistance of the tolerant population to inhibition by protease inhibitors in comparison to the Polish population (Schwarzenberger et al., 2017). In future studies, it will be interesting to investigate whether different isoforms differ in stability to protease inhibition, e.g., via recombinant expression and subsequent kinetic analysis of different isoforms of *CT448* and *CT802*.

Here, we have demonstrated in a population genetic approach that protease genes from a tolerant Swedish *D. magna* population have most probably been targets of selection, and we

assume that these selected proteases cause the higher tolerance of the population's digestive proteases to chymotrypsin inhibition (Schwarzenberger et al., 2017). Therefore, we conclude that the selection of digestive proteases by dietary protease inhibitors, in addition to reduction of gene copy numbers, is an important mechanism underlying local adaptation of tolerant *Daphnia* populations to protease inhibitors.

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AUTHOR CONTRIBUTIONS

A.S., and E.v.E. designed all experiments that were performed by A.S. A.S., and M.H. calculated the population genetic tests. A.S. wrote the manuscript. All authors interpreted the results and read and approved the manuscript.

DATA AVAILABILITY STATEMENT

Gene sequence data were uploaded to GenBank and can be found under the accession numbers MN556344–MN556548. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Agrawal, M. K., Zitt, A., Bagchi, D., Weckesser, J., Bagchi, S. N., & Von Elert, E. (2005). Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, *20*, 314–322. https://doi.org/10.1002/tox.20123

Beaumont, M. A. (2005). Adaptation and speciation: What can F-st tell us? *Trends in Ecology & Evolution*, *20*, 435–440. https://doi.org/10.1016/j.tree.2005.05.017

Blom, J. F., Baumann, H., Codd, G. A., & Jüttner, F. (2006). Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp3,(E)-Dhb7]microcystin-RR. *Archiv Fuer Hydrobiologie*, *167*, 547–559. https://doi.org/10.1127/0003-9136/2006/0167-0547

Blom, J. F., Bister, B., Bischoff, D., Nicholson, G., Jung, G., Süssmuth, R. D., & Jüttner, F. (2003). Oscillapeptin J, a new grazer toxin of the freshwater cyanobacterium *Planktothrix rubescens*. *Journal of Natural Products*, *66*, 431–434. https://doi.org/10.1021/np020397f

Bohonak, A. J., & Jenkins, D. G. (2003). Ecological and evolutionary significance of dispersal by freshwater invertebrates. *Ecology Letters*, *6*, 783–796. https://doi.org/10.1046/j.1461-0248.2003.00486.x

Brown, E. A., Pilkington, J. G., Nussey, D. H., Watt, K. A., Hayward, A. D., Tucker, R., … Slate, J. (2013). Detecting genes for variation in parasite burden and immunological traits in a wild population: Testing the candidate gene approach. *Molecular Ecology*, *22*, 757–773. https://doi.org/10.1111/j.1365-294X.2012.05757.x

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., … Boore, J. L. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science*, *331*, 555–561. https://doi.org/10.1126/science.1197761

Cousyn, C., De Meester, L., Colbourne, J. K., Brendonck, L., Verschuren, D., & Volckaert, F. (2001). Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 6256–6260. https://doi.org/10.1073/pnas.111606798

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, *147*, 915–925.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*(3), 693–709.

Ger, K. A., Hansson, L. A., & Lürling, M. (2014). Understanding cyanobacteria-zooplankton interactions in a more eutrophic world. *Freshwater Biology*, *59*(9), 1783–1798. https://doi.org/10.1111/fwb.12393

Ghadouani, A., Pinel-Alloul, B., & Prepas, E. E. (2003). Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. *Freshwater Biology*, *48*, 363–381. https://doi.org/10.1046/j.1365-2427.2003.01010.x

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hansen, M. M., Olivieri, I., Waller, D. M., & Nielsen, E. E. (2012). Monitoring adaptive genetic responses to environmental change. *Molecular Ecology*, *21*, 1311–1329. https://doi.org/10.1111/j.1365-294X.2011.05463.x

Hebert, P. D. N., & Finston, T. L. (1996). Genetic differentiation in *Daphnia obtusa*: A continental perspective. *Freshwater Biology*, *35*, 311–321. https://doi.org/10.1046/j.1365-2427.1996.00501.x

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, *7*, 1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

Kuster, C. J., Schwarzenberger, A., & Von Elert, E. (2012). Seasonal dynamics of sestonic protease inhibition: Impact on *Daphnia* populations. *Hydrobiologia*, *715*(1), 37–50. https://doi.org/10.1007/s10750-012-1303-x

Lewontin, R. C., & Krakauer, J. (1973). Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics*, *74*(1), 175–195.

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Mort, M. A., & Wolf, H. G. (1986). The genetic structure of large lake *Daphnia* populations. *Evolution*, *40*, 756–766. https://doi.org/10.1111/j.1558-5646.1986.tb00535.x

Ramos-Onsins, S. E., & Rozas, J. (2002). Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, *19*(12), 2092–2100. https://doi.org/10.1093/oxfordjournals.molbev.a004034

Sarnelle, O., & Wilson, A. E. (2005). Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, *50*, 1565–1570. https://doi.org/10.4319/lo.2005.50.5.1565

Schneider, S., Roessli, D., & Excoffier, L. (2000). *Arlequin ver. 2.000: A software for population genetics data analysis*. Geneva, Switzerland: Genetics and Biometry Laboratory, University of Geneva. http://anthro.unige.ch/software/arlequin.

Schwarzenberger, A., D'Hondt, S., Vyverman, W., & Von Elert, E. (2013). Seasonal succession of cyanobacterial protease inhibitors and *Daphnia magna* genotypes in a eutrophic Swedish lake. *Aquatic Sciences*, *75*, 433–445. https://doi.org/10.1007/s00027-013-0290-y

Schwarzenberger, A., Keith, N. R., Jackson, C. E., & Von Elert, E. (2017). Copy number variation of a protease gene of *Daphnia*: Its role in population tolerance. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *327*, 119–126. https://doi.org/10.1002/jez.2077

Schwarzenberger, A., Kuster, C. J., & Von Elert, E. (2012). Molecular mechanisms of tolerance to cyanobacterial protease inhibitors revealed by clonal differences in *Daphnia magna*. *Molecular Ecology*, *12*, 4898–4911. https://doi.org/10.1111/j.1365-294X.2012.05753.x

Schwarzenberger, A., Sadler, T., Motameny, S., Ben-Khalifa, K., Frommolt, P., Altmüller, J., … von Elert, E. (2014). Deciphering the genetic basis of microcystin tolerance. *BMC Genomics*, *15*, 776. https://doi.org/10.1186/1471-2164-15-776

Schwarzenberger, A., Sadler, T., & Von Elert, E. (2013). Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of *Daphnia magna*. *Journal of Experimental Biology*, *216*, 3649–3655. https://doi.org/10.1242/jeb.088849

Schwarzenberger, A., & Von Elert, E. (2013). Cyanobacterial protease inhibitors lead to maternal transfer of increased protease gene expression in *Daphnia*. *Oecologia*, *172*, 11–20. https://doi.org/10.1007/s00442-012-2479-5

Schwarzenberger, A., Zitt, A., Kroth, P., Mueller, S., & Von Elert, E. (2010). Gene expression and activity of digestive proteases in *Daphnia*: Effects of cyanobacterial protease inhibitors. *BMC Physiology*, *10*, 6. https://doi.org/10.1186/1472-6793-10-6

Schwerin, S., Zeis, B., Lamkemeyer, T., Paul, R. J., Koch, M., Madlung, J., … Pirow, R. (2009). Acclimatory responses of the *Daphnia pulex* proteome to environmental changes. II. Chronic exposure to different temperatures (10 and 20 degrees C) mainly affects protein metabolism. *BMC Physiology*, *9*, 8.

Scoville, A. G., & Pfrender, M. E. (2010). Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *PNAS*, *107*, 4260–4263. https://doi.org/10.1073/pnas.0912748107

Smith, M. J., & Haigh, J. (1974). The hitch-hiking effect of a favorable gene. *Genetics Research*, *23*(1), 23–35. https://doi.org/10.1017/S0016672300014634

Smith, V. H., & Schindler, D. E. (2009). Eutrophication science: Where do we go from here? *Trends in Ecology & Evolution*, *24*, 201–207. https://doi.org/10.1016/j.tree.2008.11.009

Stern, A., Doron-Faigenboim, A., Erez, E., Martz, E., Bacharach, E., & Pupko, T. (2007). Selecton 2007: Advanced models for detecting positive and purifying selection using a Bayesian inference approach. *Nucleic Acids Research*, *35*, W506–W511. https://doi.org/10.1093/nar/gkm382

Storz, J. F., Sabatino, S. J., Hoffmann, F. G., Gering, E. J., Moriyama, H., Ferrand, N., … Nachman, M. W. (2007). The molecular basis of high-altitude adaptation in deer mice. *PLOS Genetics*, *3*, 448–459. https://doi.org/10.1371/journal.pgen.0030045

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, *123*(3), 585–595.

Takezaki, N., Nei, M., & Tamura, K. (2014). POPTREEW: Web version of POPTREE for constructing population trees from allele frequency data and computing some other quantities. *Molecular Biology and Evolution*, *31*, 1622–1624. https://doi.org/10.1093/molbev/msu093

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729. https://doi.org/10.1093/molbev/mst197

Von Elert, E., Agrawal, M. K., Gebauer, C., Jaensch, H., Bauer, U., & Zitt, A. (2004). Protease activity in guts of *Daphnia magna*: Evidence for trypsin and chymotrypsin enzymes. *Comparative Biochemistry & Physiology Part B*, *137*, 287–296. https://doi.org/10.1016/j.cbpc.2003.11.008

Von Elert, E., & Jüttner, F. (1997). Phosphorus limitation not light controls the exudation of allelopathic compounds by *Trichormus doliolum*. *Limnology and Oceanography*, *42*, 1796–1802. https://doi.org/10.4319/lo.1997.42.8.1796

Von Elert, E., Zitt, A., & Schwarzenberger, A. (2012). Inducible tolerance in *Daphnia magna* to dietary protease inhibitors. *Journal of Experimental Biology*, *215*, 2051–2059. https://doi.org/10.1242/jeb.068742 https://doi:

Xia, H., Camus-Kulandaivelu, L., Stephan, W., Tellier, A., & Zhang, Z. (2010). Nucleotide diversity patterns of local adaptation at drought-related candidate genes in wild tomatoes. *Molecular Ecology*, *19*, 4144–4154. https://doi.org/10.1111/j.1365-294X.2010.04762.x

Yampolsky, L. Y., Schaer, T. M. M., & Ebert, D. (2013). Adaptive phenotypic plasticity and local adaptation for temperature tolerance in freshwater zooplankton. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1776), 20132744. https://doi.org/10.1098/rspb.2013.2744

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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