A Photoreceptor Contributes to the Natural Variation of Diapause Induction in *Daphnia magna*

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Abstract

Diapause is an adaptation that allows organisms to survive harsh environmental conditions. In species occurring over broad habitat ranges, both the timing and the intensity of diapause induction can vary across populations, revealing patterns of local adaptation. Understanding the genetic architecture of this fitness-related trait would help clarify how populations adapt to their local environments. In the cyclical parthenogenetic crustacean *Daphnia magna*, diapause induction is a phenotypic plastic life history trait linked to sexual reproduction, as asexual females have the ability to switch to sexual reproduction and produce resting stages, their sole strategy for surviving habitat deterioration. We have previously shown that the induction of resting stage production correlates with changes in photoperiod that indicate the imminence of habitat deterioration and have identified a Quantitative Trait Locus (QTL) responsible for some of the variation in the induction of resting stages. Here, new data allows us to anchor the QTL to a large scaffold and then, using a combination of a new mapping panel, targeted association mapping and selection analysis in natural populations, to identify candidate genes within the QTL. Our results show that variation in a rhodopsin photoreceptor gene plays a significant role in the variation observed in resting stage induction. This finding provides a mechanistic explanation for the link between diapause and day-length perception that has been suggested in diverse arthropod taxa.

Key words: **diapause, resting-stage, QTL mapping, association mapping, *Daphnia magna*, Rhodopsin.**

Article

Introduction

Diapause is a widespread adaptation that allows organisms to survive seasonal habitat deterioration by producing an arrested development form. In diapausing organisms, therefore, the induction of a resting phase represents a crucial fitness-related life history trait, as failure to enter diapause at the appropriate time would lead to the extinction of the individual's genotype (Deng 1996). Whereas often understood as an overwintering strategy (Schmidt et al. 2005; Tyukmaeva et al. 2011; Paolucci et al. 2013; Lehmann et al. 2014; Sim et al. 2015), diapause is actually induced by a number of environmental cues that signal various types of local habitat deterioration, such as desiccation, overcrowding or decreased food supply (Gilbert and Schreiber 1998; Koch et al. 2009; Hahn and Denlinger 2011; Varela-lasheras and van Dooren 2014). In species occurring across a broad range of habitats, these cues may vary locally, so that diapause induction or its intensity can show geographic variation (Schmidt et al. 2005; Winterhalter and Mousseau 2007; Tyukmaeva et al. 2011; Chen et al. 2013) and local adaptation (Meuti and Denlinger 2013; Roulin et al. 2013).

Across animal taxa, diapause occurs at different developmental stages, from eggs to adults, but is often triggered by changes in photoperiod (Denlinger 2002). Studies of mutants have demonstrated the role of circadian clock genes (e.g. *period*, *Clock*), as well as genes that encode photoreceptors, on the photoperiodic and seasonal induction of resting stages (Syrová et al. 2003; Stehlik et al. 2008; Meuti and Denlinger 2013). However, with the exception of the well-documented case of the dauer larvae in *C. elegans* (for review see Fielenbach and Antebi 2008) and *Drosophila sp* (Williams et al. 2006; Schmidt et al. 2008; Tauber 2008; Salminen et al. 2015; Parker et al. 2016), genes and pathways that contribute to the natural variation of diapause induction have been poorly explored. As such genes are thought to play an important role in local adaptation, they constitute a good model for studying genes under locally divergent selection. Here, we investigate the genes that induce diapause in *Daphnia magna*, a freshwater planktonic crustacean that occurs across a wide range of habitats.

In *Daphnia magna*, diapause induction is a phenotypic plastic trait that depends on the environmental conditions (Stross and Hill 1965). *D. magna* reproduces by cyclical parthenogenesis, i.e., females can repeatedly switch from asexual reproduction, producing clonal offspring, to sexual reproduction, in which they produce embryonic resting stages (also called diapausing eggs or ephippia). In *D. magna*, resting stage production is tightly linked to sexual reproduction, and sexually produced resting stages are the sole means

of surviving harsh conditions. We have previously shown that, on a European scale, resting stage production correlates with a change in photoperiod that signals the imminence of habitat deterioration (freezing or desiccation), and is therefore locally adapted (Roulin et al. 2013). Using a F2 genetic cross, we have also identified a Quantitative trait locus (QTL) in the genome of *D. magna* that influences this trait (Roulin et al. 2013). However, due to the fragmentation of the genome assembly at the time of that study and a strongly deleterious mutation close to the region of interest that distorted transmission ratios and confounded fine mapping, the QTL could not be well mapped, impeding our ability to identify the candidate genes.

As a next step, we undertake in this study to pinpoint candidate genes responsible for inducing resting stage production. To do so, we first assemble our genomic region of interest using PacBio reads. We then produce a new mapping panel, using a genetic cross without the deleterious mutation, and narrow the region of interest down to 75 kb, which includes 14 predicted genes. Using association mapping of natural isolates of *D. magna*, we are able to locate one of these candidate genes—a rhodopsin photoreceptor gene—as a main contributor to the variation of resting stage production. Finally, we test for evidence of selection in this region, confirming that the rhodopsin photoreceptor gene shows a signature of selection consistent with local adaptation.

Results

QTL Analysis and Identification the Genomic Region of Interest

In the current study, we used resting stage (also referred as diapausing egg or ephippia) production as a measure of diapause induction. The QTL analysis for resting stage production presented here is an update of the QTL analysis presented in the introduction and published in 2013 (Roulin et al. 2013). In this earlier analysis, we used a preliminary version of the *Daphnia magna* genetic map published since then by Routtu et al. (2014). We used here the published version. Based on resting stage production data collected on 180 F2-lines (Roulin et al. 2013), the updated QTL analysis gave very similar results compared with the previously published analysis (Roulin et al. 2013). Only one peak, located on the linkage group 8 (former linkage group 10, fig. 1A) was observed ($LOD_{<LG8@56.5cM>} = 10.4$, $LOD_{<threshold>} = 3.8$). The QTL explained 22% of the variance between the F2 lines.

Due to the fragmentation of the current *D. magna* draft genome (version 2.4), the Single Nucleotide Polymorphisms (SNPs) present in the QTL confidence interval mapped on small scaffolds or unassembled contigs. To anchor the QTL to a larger scaffold, we used long PacBio reads and successfully extended the sequence of the contig carrying the SNPs with the highest LOD score (contig 28655, LG8 at 56.5 cM) from 791 nucleotides (nt) to 16.5 kilobases (kb) (fig. 2A). Using a blast search, we found that the termini of this extended sequence unambiguously flanked a 15 kb gap (stretch of N) in region 772–787 kb of a 1.2 megabase (Mb) scaffold, i.e., scaffold 1036 (fig. 2B).

To verify that we had correctly anchored the QTL, four additional SNPs located on scaffold 1036 at position 648, 710, 740, and 840 kb were sequenced in the 180 F2 lines used for the original QTL mapping and incorporated into the genetic map and the analysis. All four of them located in the QTL region on LG8 from ~53 to 57.5 cM. We found that the four SNPs showed an association with resting stage production ($LOD_{648kb} = 9.9$, $LOD_{710kb} = 11$, $LOD_{740kb} = 11$, $LOD_{840kb} = 9.5$) and thus supported the anchoring of the QTL for resting stages production on scaffold 1036. The 95% confidence interval of the refined QTL included 11 SNPs located on LG8 from 54 to 61.6 cM (fig. 1A).

Narrowing Down the Region of Interest

A previously identified locus of sterility (Routtu et al. 2010, 2014) located within the confidence interval of the QTL led to strong segregation distortion in our region of interest. If homozygous AA at this sterility locus, F2 females (known as Red-Dwarf or RD) do not reproduce. Because such genotypes cannot be maintained as clonal cultures, there are no AA genotypes at the locus of sterility (between 57 cM and 57.5 cM on LG8, about 785–840 kb on scaffold 1036, fig. 1B) and a deficit of AA genotypes in the surrounding genomic region. This result prompted us to test (i) whether the RD and resting stage production loci might be the same, or (ii) if they were two distinct loci, whether the distortion of genotype frequencies caused by the RD locus produced a bias in the QTL analysis.

To test these questions, we developed a new panel of lines without the RD allele. We selected an F2-line (F2-1371) that putatively did not carry the sterility allele, i.e., BB from 57cM to 81 cM on LG8 (from 785 kb on scaffold 1036 and upstream), but heterozygous AB between 45.8 cM and 56.5 cM on LG8 (downstream 782 kb on scaffold 1036 and beyond the lower confidence interval border of the QTL, fig. 1C). This F2-line was self-crossed and resulted in an F3 panel with a total of 82 recombinant lines. During the hatching of the F3 lines, no Red Dwarf phenotypes were observed, confirming that the cross was free of the Red Dwarf allele and that the RD locus is located upstream of 785 kb on scaffold 1036.

We genotyped the 82 F3-lines for four SNPs located on scaffold 1036 at positions 710, 740, 782, and 785 kb and phenotyped them for resting stage production. As expected, all lines were homozygous BB for the SNP at 785 kb. For the three other SNPs, the ratio of the AA, AB, and BB genotypes did not deviate from the expected 1:2:1 ratio ($X^2 = 3$, $P = 0.2$ at each marker, fig. 1D). BB genotypes produced significantly fewer resting stages than did AA and AB genotypes, whereas no difference was observed between AA and AB genotypes (fig. 1D), which coincides with our earlier results from the F2 panel (Roulin et al. 2013). This finding confirms that the RD and resting stage production loci are distinct, and that the RD locus did not bias the former QTL analysis. As all F3 lines are homozygous BB from 785 kb on scaffold 1036 and upstream the QTL higher confidence interval border, it also demonstrates that the QTL for resting stage production must be downstream 785 kb (57 cM on LG8). This reduces the QTL confidence interval from 7.6 cM (54–61.6 cM) to

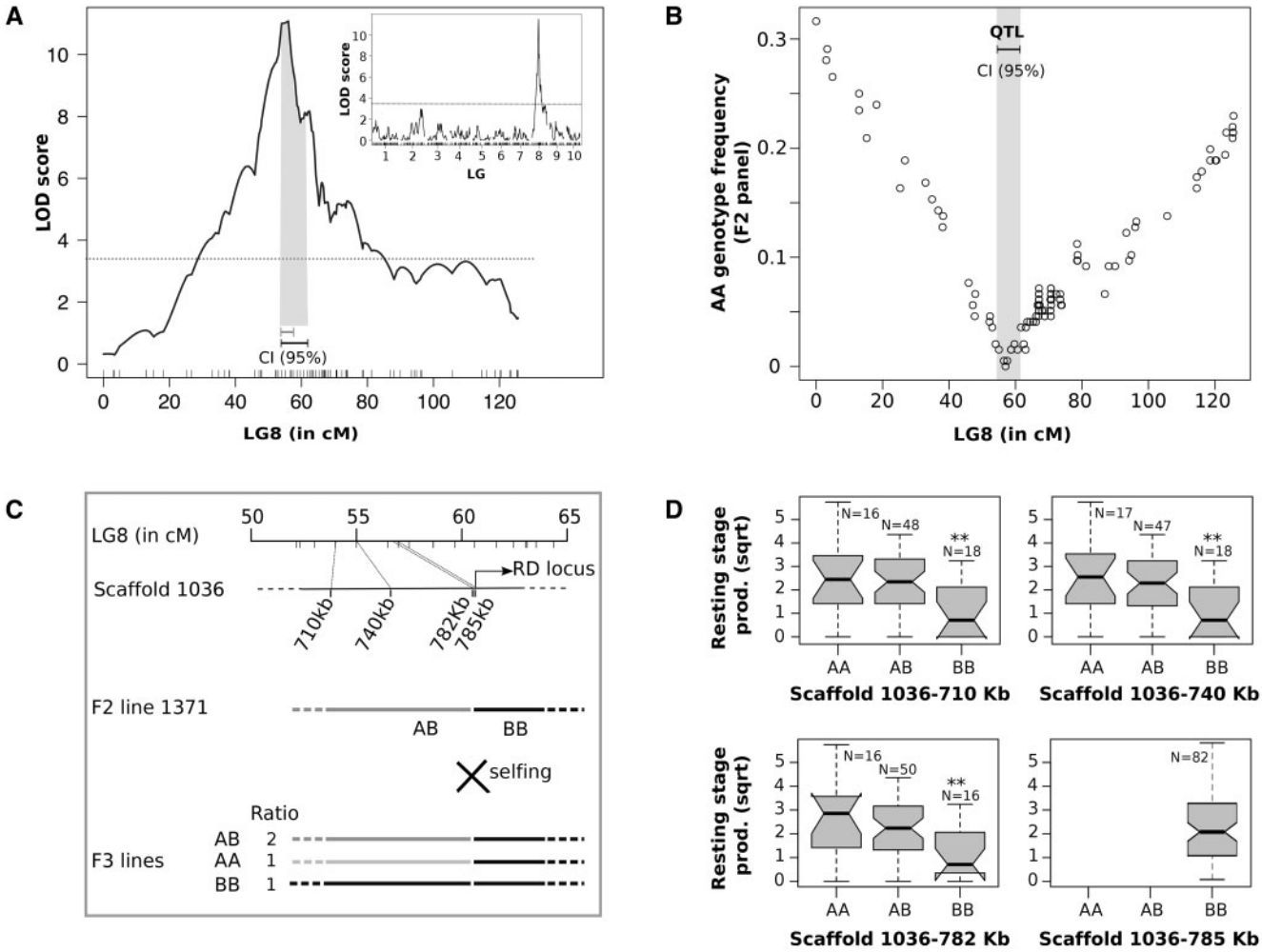

Fig. 1. QTL analysis. (*A*) QTL analysis for LG8. The gray zone and the black bar indicate the initial 95% Confidence Interval (CI) of the F2 QTL study. The gray bar represents the reduced confidence interval after analysis of the F3 panel. The dashed horizontal line corresponds to the LOD threshold for significance. The inset figure shows the QTL analysis performed with the entire *D. magna* genetic map. (*B*) Frequency of AA genotypes on LG8 in 180 F2-lines. The gray zone delimits the QTL confidence interval (CI) ranging from 54 cM to 61.6 cM. (*C*) Strategy used to develop the F3 panel to exclude the RD allele. F2 line 1371 had a recombination breakpoint between the RD locus (homozygote BB) and the QTL peak (heterozygote AB). Selfing of F2 line 1371 resulted in 82 F3 lines. (*D*) Resting stage production (square-root transformed) of the 82 F3 lines in relation to the genotypes at each of four loci in the region of interest. The number of F3 lines per genotype (N) is displayed above each box plot. Notches display the confidence interval around the median. ** indicate $P < 0.01$ from a linear model analysis.

3 cM (54–57 cM) (fig. 1A), equivalent to a 75 kb region spanning from 710 kb to 785 kb on scaffold 1036. Using the annotation available for the *D. magna* genome (version 2.4) and a blast search against the NCBI and flybase databases, we identified a transposable element and 14 genes in this region of interest: a G protein-coupled receptor rhodopsin, two cytochrome P450, a glucose dehydrogenase, an ankyrin repeat domain-containing protein, a GTPase-activating protein, a cGMP-protein kinase, and seven expressed or conserved proteins (supplementary table S1, Supplementary Material online). Note that a second G protein-coupled receptor rhodopsin is flanking the QTL confidence interval. This gene has been therefore included for further analyses.

Association Mapping in Natural Populations

Because both the F2 and F3 lines showed too few recombinants in the 75 kb region of interest, all 14 genes present in this region were equally good candidates. To further refine our analysis, we therefore performed a targeted association mapping between SNPs located in our 75 kb of interest and resting stage production in natural populations. Similar to QTL mapping, association mapping also depends on recombination rate; however, because recombination events in natural populations accumulate over many generations (vs. few generations in laboratory crosses), they allow us to map the trait of interest on a finer-scale (Stinchcombe and Hoekstra 2008).

To assess resting stage production, we chose 62 *D. magna* clones originating from eleven subpopulations of a larger Finnish metapopulation (from which the *D. magna* genome reference clone Xinb3 is derived). Although separated by only a few kilometres, these subpopulations showed clear differences in resting stage production (fig. 3A). The inheritability of resting stage production was relatively high ($h^2 = 0.5$), suggesting that the variation of the trait is largely due to genetic effects.

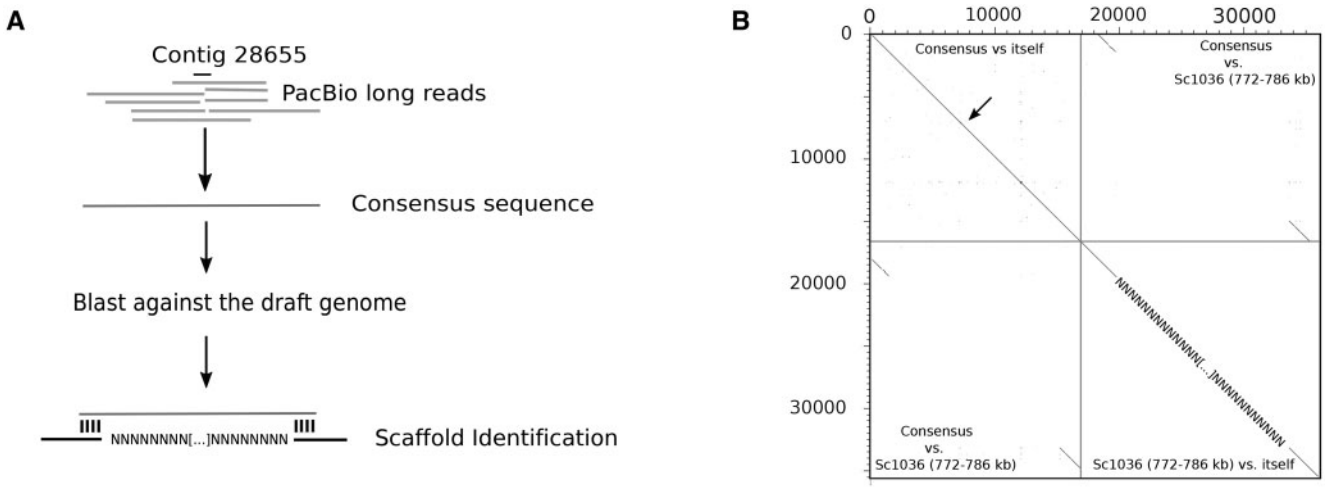

Fig. 2. QTL anchoring. (*A*) Methodology applied to extend the sequence of contig 28655 (the short contig containing the SNP with the best LOD score) with long PacBio reads and to anchor the QTL on a larger scaffold. In the first step, a consensus sequence is built, which is used in the dot plot of the B panel (*B*) Pairwise comparisons between the consensus sequence obtained with the PacBio reads and scaffold 1036 (subset region ~772–786 kb). In regions where two sequences are similar, a row of high scores runs diagonally across the dot matrix, allowing for a direct comparison of sequences homology. A dot plot between two identical sequences thus results in a perfect diagonal. The top right and bottom left corners show that the extremities of the extended consensus sequence are flanking a stretch of N (unassembled region) in the region 772–786 kb of scaffold 1036. The arrow indicates the position of contig 28655 on the consensus sequence.

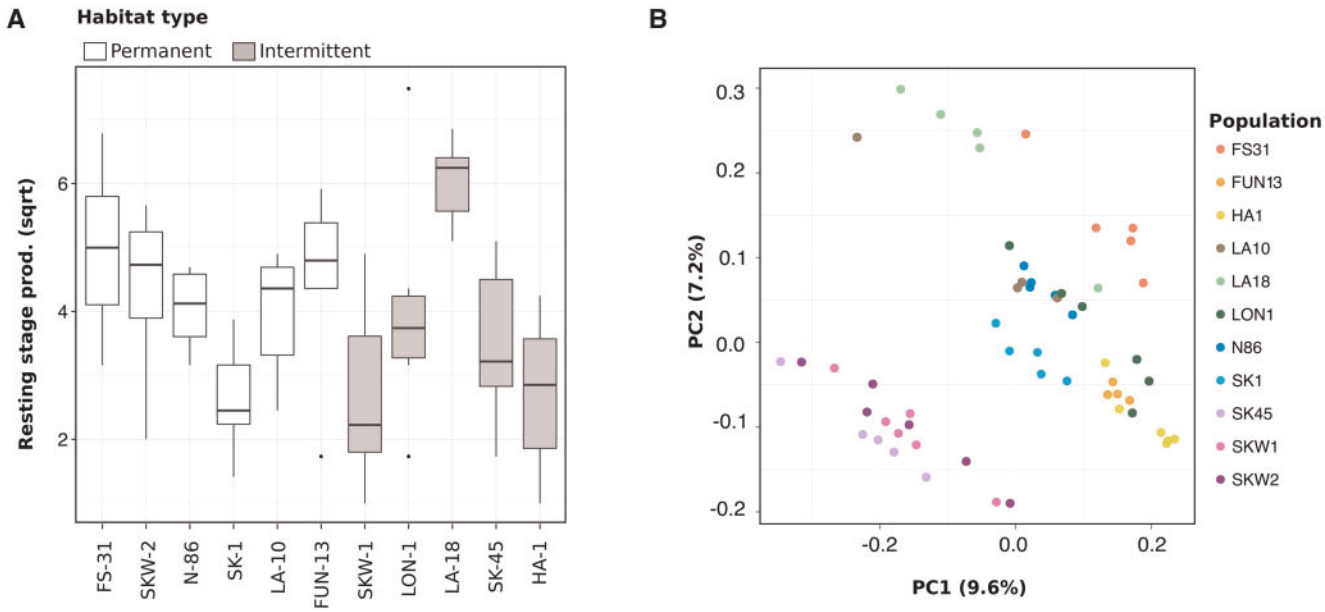

Fig. 3. Resting stage production and population structure. (*A*) Box plots of resting stage production (square-root transformed) per population. Box plots show the median, upper, and lower quartiles. Black dots represent outliers. Populations from permanent and intermittent habitats are highlighted in white and gray respectively. (*B*) Principal Component Analysis (PCA) performed on the 57 clones using 2,990 SNPs.

We genotyped the 62 clones using a Genotyping by Sequencing (GBS) approach (Elshire et al. 2011). Five clones did not pass the quality control either because of the bad quality of their illumina reads or because their coverage was too low. Of the 57 remaining clones, ~50,000 SNPs were generated. After stringent filtering, 2,990 SNPs were kept and used to compute a PCA and a kinship matrix. The PCA showed an intermediate level of population structure, with less than 20% of the variance explained by the first two axes (fig. 3B). Nevertheless, two clades (SKW-1, SKW-2, SK-45 vs. FS-31, FUN-13, HA-1, LA-10, LA-18, LON-1, N-86, SK-1) could be distinguished. The kinship matrix revealed that clones within populations are to some degree genetically related to each other, with a within-population kinship coefficient spanning from 0.008 to 0.3. We therefore corrected the association mapping with the Eigenstrat method, which uses the principal components of the genomic kinship matrix to adjust for possible (sub)stratification (Price et al. 2006).

Only four GBS markers were located in our 75 kb of interest. We therefore Sanger sequenced nine additional loci on

scaffold 1036 at positions 703, 712, 715, 718, 730, 740, 750, 775, and 785 kb. Each amplified locus showed multiple mutations, resulting in 39 SNPs and indels (small insertions or deletions) that were used to perform the association mapping with resting stage production. The analysis revealed that two SNPs (located at 711,932 nt and 712,064 nt) were significantly associated with resting stage production, even when we controlled for population structure (P_{corr}= 0.028 and 0.032, respectively; fig. 4A). These SNPs were in strong linkage disequilibrium and are located in the intron of a 4.3 kb long G protein-coupled receptor rhodopsin.

To confirm and locate the peak of association more precisely, we further Sanger sequenced the entire G protein-coupled receptor rhodopsin and identified 36 additional SNPs and indels that we incorporated into our dataset. By performing a new association analysis, we found that the SNP at 711,632 nt showed the strongest sign of association (P_{corr}= 0.014, fig. 4A). This SNP explained 11% of the variance in resting stage production and is located in the middle of the gene intron (fig. 4A). In total, nine SNPs located between 711,632 and 712,578 nt showed a significant association with resting stage production (P_{corr} between 0.014 and 0.035, supplementary table S2, Supplementary Material online). Of those nine sites, three were homozygous in the F1 line used to generate the QTL mapping F2 panel, indicating that those sites are monomorphic in the F2 line panel (supplementary table S2, Supplementary Material online). The signal of association decreased for SNPs located toward the 3′ and 5′ ends of GPCR rhodopsin (fig. 4A). The signal was not significant for SNPs located in the adjacent gene (at 715,494 nt, P_{corr} = 0.52, fig. 4A, supplementary table S2, Supplementary Material online), confirming that the association we observed is restricted to the GPCR rhodopsin. The only nonsynonymous mutation found in an exon of the GPCR rhodopsin gene (710,413 nt) was not associated with resting stage production (P_{corr}= 0.9).

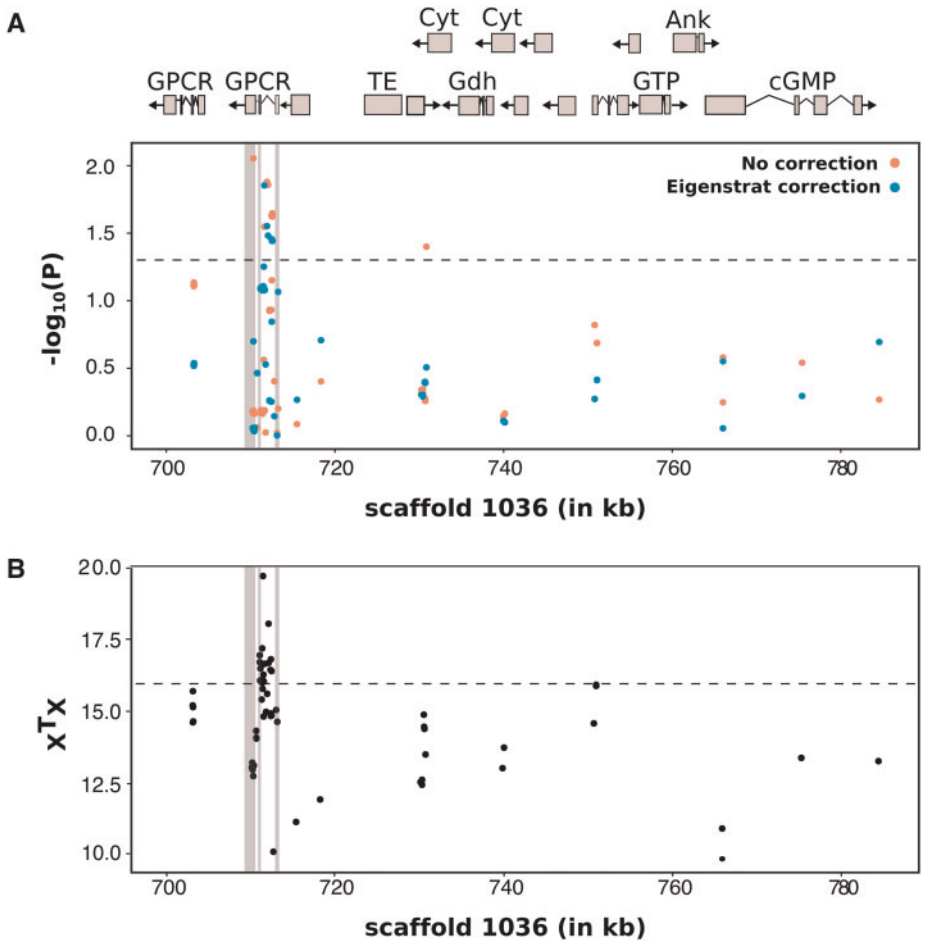

FIG. 4. Association mapping and selection analysis in natural populations. (*A*) Association mapping between resting stage production and the SNPs located on scaffold 1036 between 700 and 785 kb, without (orange) and with control for genetic structuration (blue, Eigenstrat correction). The dashed line corresponds to the threshold of significance (P= 0.05). *P*-values (P) are -$\log_{10}$ transformed for clarity. The boxes on top of the figure display the schematic position of each gene present in the region. Only genes with known functions have their name displayed; GPCR: GPCR rhodopsin; TE: transposable element; Cyt: Cytochrom P450; Gdh: Glucose dehydrogenase; GTP: GTPase-activating protein; Ank: Ankrin repeat domain-containing protein; cGMP: cGMP-protein kinase. The exon/intron regions of the GPCR rhodopsin gene are highlighted in dark gray and white, respectively. (*B*) Selection analysis for SNPs located on scaffold 1036 between 700 and 785 kb. The dashed line corresponds to the threshold of significance at 1% (X^TX >16).

Selection Analysis

The natural populations studied here occur in highly variable habitats, with habitat permanency being one of the more overt differences: in summer, large rock-pools are less likely to desiccate ("permanent" pools FS-31, SKW-2, N-86, SK-1, FUN-13, and LA-10) than smaller pools ("intermittent" pools SKW-1, LON-1, LA-18, SK-45, and HA-1). We hypothesized that this difference may produce locally divergent selection for the induction of resting stages, but in testing the effect of the habitat permanency using a linear mixed-effects model as described in Roulin et al. (2015), we found that this factor did not contribute to variation in resting stage production among populations ($P = 0.45$, fig. 3A). Thus, no evidence exists for local adaptation of resting stage production with regard to rock-pool permanency. However, we found highly significant differences in resting stage production among populations ($P < 0.001$, fig. 3A), with the population effect explaining 28% of the variance. We therefore asked whether SNPs located in our candidate region displayed evidence of divergent selection. To do so, we computed the X^TX statistics, a differentiation measure estimate corrected for population structure and demography. Briefly, we simulated 100,000 SNPs under the demographic model inferred from the actual dataset (GBS dataset). X^TX statistics were then computed for the pseudo-observed dataset (POD) to determine their distribution under a neutral model. We then compared the observed values of these distributions to determine the probability of neutrality for each SNP.

The only SNPs or indels that displayed signs of divergent selection in our region of interest were located in the GPCR rhodopsin (between 711,182 nt and 711,578 nt); some of these SNPs were also significantly associated with resting stage production (fig. 4B, supplementary table S2, Supplementary Material online). Seventeen of the 39 SNPs found in the GPCR rhodopsin indeed showed X^TX values significant at the 1% threshold ($X^TX > 16$) when compared with the POD, suggesting that they were more differentiated than expected under neutral assumptions. The strongest signature of divergent selection was observed at the SNP located at 711,565 nt ($X^TX > 19$), very close to position 711,632 nt, where the peak of the association mapping was observed.

Discussion

Identifying the genes involved in fitness related traits and quantifying their effects on phenotypic variation constitutes an important step in understanding how populations maintain genetic diversity and adapt to their local environment (Stinchcombe and Hoekstra 2008; Fuller et al. 2015). In *Daphnia magna*, the induction of sexual reproduction and diapause is directly linked to fitness, as resting stages are the sole means for populations to survive periods when planktonic life is not possible. The complementary approaches employed in this study found consistent evidence that a G protein-coupled receptor (GPCR) rhodopsin gene plays a role in the variation of resting stage production. As this gene is a photoreceptor, our results are congruent with the observation that diapause induction is linked to day-length perception. As we found signs of divergent selection in the GPCR rhodopsin, this study constitutes a compelling example of the fine scale mapping of a gene contributing to adaptive phenotypic plasticity.

Rhodopsin Photoreceptors and Light Transduction

In arthropods, it is well established that photoreceptors are used to detect day-length variation and to initiate photoperiodic diapause (Denlinger 2002; Saunders 2012; Meuti and Denlinger 2013). In *Daphnia*, nothing is known about the potential genes involved in such mechanisms, yet, the induction of resting stage production is known to be influenced by changes in day-length as well (Stross and Hill 1965; Stross 1971; Deng 1996; Roulin et al. 2013). Thus, we initially predicted that photoreceptors or genes involved in light transduction, rather than genes involved in the oogenesis process itself (Takahashi and Ohnuma 2016), might play a role in the natural variation of this trait.

Our targeted mapping analysis showed that the GPCR rhodopsin, located on scaffold1036 between 708,969 and 713,276 nt, is the only gene significantly associated with resting stage production in our region of interest. Rhodopsins are molecules composed of an opsin, a membrane protein belonging to the large GPCR gene family, which, coupled with a vitamin-derived chromophore, allows light transduction (Terakita 2005; Palczewski 2006). Because GPCR rhodopsins universally serve as light sensors in animals (Terakita 2005; Montell 2013; Xiong and Bellen 2013) and are suspected to be a photoreceptor responsible for the circadian clock entrainment in some arthropods (Veerman 2001), we believe that we have identified here a prime candidate for the natural variation of diapause induction.

Consistent with our results, other studies have suggested that GPCR rhodopsins are involved in the induction of resting stage production in the eyeless mite *Amblyseius potentillae* (Veerman et al. 1983), and, more recently, in the North American butterfly *Limenitis arthemis* (Frentiu et al. 2015) as well as in the cyclical parthenogentic aphid *Acyrthosiphon pisum* (Le Trionnaire et al. 2008; Jaquiéry et al. 2014). This last example is especially striking, as, similar to *D. magna*, *A. pisum* produces resting stages as part of a switch from asexual to sexual reproduction. Even though additional potential candidate genes were pointed out in these studies (Le Trionnaire et al. 2008; Jaquiéry et al. 2014), they may jointly with our results, underlie a conserved role of GPCR rhodopsins and light perception/transduction in the switch from asexual to sexual reproduction in cyclical parthenogenetic organisms.

Interestingly, we observed a discrepancy between the size-effects of the QTL and of the GPCR rhodopsin on resting stage production (22% vs. 11%, respectively). This difference might simply reflect that QTL analyses have a tendency to overestimate size effects (Allison et al. 2002; Vales et al. 2005; Chenoweth and Mcguigan 2010). In addition, the QTL mapping used parental lines from far distant populations (Finland vs. Germany), whereas the association mapping was performed within the subpopulations of the Finnish metapopulation. Nevertheless, taking into account that the total

genetic variation of resting stage production is only about 50% (heritability $h^2 = 0.5$) and that quantitative fitness-related traits tend to be explained by multiple loci of small effects (Houle 1992; Roff 2002), we can consider the GPCR rhodopsin to contribute significantly to this trait.

Selection on the Rhodopsin Receptor Gene

Interestingly, we found evidence for divergent selection at SNPs that cover the GPCR rhodopsin receptor, some of them also significantly associated with resting stage production (fig. 4). Within the Nordic *D. magna* metapopulation studied here, one of the most variable environmental factors is habitat permanency. Whereas small rock pools may completely desiccate in summer, larger rock pools are less likely to do so. Thus, we initially hypothesized that *D. magna* from populations in small rock pools produce resting stages at a higher rate during the late-spring photoperiod than populations from larger pools. However, our results did not support this hypothesis. What they did find, however, correlates with a previous study we conducted with the same *D. magna* genotypes, but a different dataset (Roulin et al. 2015): large genetic effect for resting stages production among populations (fig. 3A). This finding suggests some other kind of selection that acts locally.

Many processes associated with the metapopulation dynamic could blur the expected pattern of local adaptation (for discussion, see Roulin et al. 2015). Among them, seasonal selection might shape allele frequencies across ponds (Bergland et al 2014). In seasonal habitats with winter freezing and summer droughts, Daphnia populations alternate between phases of rapid asexual population growth and resting phases. During asexual growth, selection against resting stage production is strong, as females that produce resting stages instead of asexual offspring will reduce their clonal representation in the population. However, towards the end of the planktonic phase of the population cycle, females need to produce resting stages to outlast the oncoming drought or frost. This process is a form of fluctuating directional selection. The ideal strategy would be to produce asexually until habitat deterioration is imminent, and then switch completely to resting stage production, although this would require that females could anticipate the best moment for switching, which seems not to be the case (Altermatt and Ebert 2008, Oecologia). Rather, since asexual growth can be terminated by both summer droughts and winter freezing in Nordic rock pool populations, these populations have evolved a strategy of high resting egg production throughout the summer (Altermatt and Ebert, 2008; Roulin et al 2013), even though selection will act against females that produce resting eggs during the asexual growth phase. How long this selection acts and how effective it is likely depends primarily on the length of the asexual growth phase, which differs strongly among rock pools, with smaller pools being more likely to dry up during summer. Pools with southern exposures also dry up faster, but have a potentially longer planktonic phase, as the higher temperatures encourage hatching from resting stages to start earlier in the spring, and good conditions last longer into the fall. Wind (predominately from the southwest) and the pool's wind exposure may also affect the drought process. Furthermore, the length of the asexual period varies widely from year to year, as local climate fluctuates with wind, sun, rain and temperature differences. Thus, rock pool populations can be expected to diverge, even on the smallest local scale, in their selection for or against resting stage production, leading to strong allelic frequency fluctuations across generations and to an increased variance of resting stage production within the metapopulation (Bell 2010). The signal of selection observed in our study may be a read-out of this variation. Seasonal selection at the photoreceptor genes identified here could be tested by collecting samples early and late during the season.

Functional Impact

All SNPs that show a significant or marginal association with variation in resting stage production are located in the intron of the GPCR rhodopsin gene (fig. 4A). A growing number of studies have shown that intronic polymorphisms have functional effects (Visser et al. 2012, for review see Cooper 2010). Indeed, such polymorphisms can alter existing splicing donor or receptor sites (Khan et al. 2002; Laere et al. 2003; Yuan et al. 2006; Cooper 2010; Seo et al. 2013; Shepherd et al. 2015), activate cryptic splice sites (Coulombe-huntington et al. 2009; van Kuilenburg et al. 2010), or serve as complex regulatory elements by modulating chromosome folding (Visser et al. 2012). Thus, our study may constitute a new case of a functional intronic polymorphism (Cooper 2010), and variation in resting stage production, may be linked, in part, to the regulation of the GPCR rhodopsin expression rather than to a change in its amino acids composition.

The SNP that showed the highest signal of association in our relatively small sample size might not be actually be the SNP responsible for resting stage variation. Indeed, the SNP that showed the strongest association with resting stage production in the natural populations is monomorphic in the F2 lines used for the QTL mapping and is, thus, unlikely to be the causal mutation. The peaks of our association mapping and selection analyses are also slightly shifted (<80 nt), again suggesting that we may not have, as yet, identified the SNP responsible for resting stage variation. To identify the causal mutation(s) with certainty, a larger sample size would be necessary. Overall, we have identified a 1 kb region associated with resting stage production that also displayed signs of divergent selection. Given the quantitative effect of the GPCR rhodopsin, pinpointing the causal mutation(s) with an association mapping-based approach may not be the most fruitful approach (Mccarthy and Hirschhorn 2008). We believe that combining a CRISPR/Cas-mediated targeted mutagenesis approach, an approach still challenging in *D. magna* but recently developed by Nakanishi et al. (2014), with an expression analysis would provide a more efficient way to validate the functional impact of the intronic mutations identified in the GPCR rhodopsin.

Conclusion

QTL mapping, association mapping, and molecular evolution analysis are powerful methods for discovering genomic

regions associated with ecologically relevant traits. Even though each of these methods comes with a statistical uncertainty, combining them, as we do in this study, powerfully increases resolution and lends confidence to the process of discovering the candidate gene. Using such a combined approach, we have identified a gene with a significant effect on resting stage production—one that also shows a signature of population divergence. We were unable to elucidate functional aspects of the link between genotype and phenotype; however, the finding of a photoreceptor gene influencing a trait induced by a photoperiod cue provides a functional hint and fits our prediction. Furthermore, this result corresponds with suggestions of a mechanistic link in other arthropods. Genetic manipulation would be necessary to functionally characterize the GPCR rhodopsin. In addition, genome-wide association studies and genome scan analyses could investigate the trait complexity at the scale of the whole genome and in a broader spectrum of natural populations. Here, we explain only part of the total genetic variance observed among natural clones, indicating that other genomic regions or epistatic effects play a role in shaping this trait.

Materials and Methods

Assessment of Resting Stage Production

We used resting stage production as a measure of diapause induction. For all experiments, we followed the same protocol as in an earlier QTL analysis (Roulin et al. 2013) where we assessed resting stage production under standardized conditions, i.e., 20 °C with 16 h of light per day. Briefly, to avoid similarities between replicates due to maternal effects, we started independent replicates using 1- to 3-day-old females that were grown separately in 400-mL jars for three generations. Individual females of the third generation were then placed in 400-mL jars. As resting stages are produced only in crowded conditions (Roulin et al. 2013), females were allowed to grow populations for 4 weeks. The number of resting stages produced was then counted in each jar. Two replicates per clones were used.

Updated QTL Analysis in F2 Recombinant Lines

The genetic map used for the QTL analysis is described in Routtu et al. (2014). Briefly, two inbred parental lines, one originating from Germany (Iinb1, genotype AA) and one originating from Finland (sequenced reference clone, Xinb3, genotype BB), were crossed. These two parental lines showed contrasting phenotypes for resting stage production (Roulin et al. 2013). The resulting F1 clone was self-crossed to produce 353 F2 lines. These F2 lines were genotyped for 1,324 Single Nucleotide Polymorphisms (SNPs) and used to generate a SNP-based genetic map (Routtu et al. 2014).

Resting stage production was previously assessed in a subset of 180 F2 lines (Roulin et al. 2013). Since the data were not normally distributed, we performed a single-QTL genome scan using a nonparametric model. The QTL analysis was performed in R v.3.1.3 (R development core team 2013) using the package R/qtl v.1.36-6 (Broman et al. 2003), and the 95% confidence interval was estimated using the R/qtl module scanboot with nonparametric bootstrapping and 1,000 replicates. The frequency of AA genotypes in the QTL region was assessed in the same 180 F2 lines.

QTL Anchoring

As long PacBio reads were generated for the genome of the reference clone Xinb3 (~60X coverage, unpublished data), we used those to anchor the QTL region to a large scaffold. We performed a Blastn search (Altschul et al. 1990) using the PacBio reads as queries against the contig that showed the highest association with resting stage production (contig 28655, length = 791 nt, $LOD_{<\text{contig }28655>} = 10.4$). Taking into account the higher error rate of the PacBio technology, we kept reads that showed significant hits (identity >85%) with the entire contig 28655 and corrected them with LSC (Au et al. 2012) using short reads (illumina) available for the same clone. The corrected PacBio reads were then properly oriented and aligned with ClustalW (Larkin et al. 2007) to produce a consensus sequence. Repeating the same procedure with the consensus sequence as a seed, we extended the length of the sequence successively until both of its extremities showed a significant Blast hit with another scaffold of the genome draft. Dot plots were performed with the graphical dot-matrix program Dotter (Sonnhammer and Durbin 1995).

Four additional SNPs flanking the anchoring region, namely on scaffold 1036 at positions ~648, 710, 740, and 840 kb, were sequenced in the 180 F2 lines described above. These four SNPs were then placed in the genetic map using the R package Rqtl (Broman et al. 2003). The QTL analysis and the confidence interval calculation were then run a second time with those SNPs integrated into the genetic map.

F3 Lines Development

A large number of females from the F2 line F2-1371 were raised in 400-mL jars in crowded conditions to ensure both sexual egg and male production (necessary for egg fertilization). The resulting resting stages were collected, dried and manually opened to optimize the hatching success. The subsequent F3 lines were phenotyped for resting stage production and genotyped for four SNPs located on scaffold 1036 at position 710, 740, 782, and 785 kb. The association between resting stage production and the different genotypes at each of the four SNPs was tested using linear models, with resting stage production (square-root transformed) as a dependent variable and genotypes as explanatory variables. The observed ratios of AA, AB, and BB genotypes were compared with the expected 1:2:1 ratio using a Chi^2 test. All statistical analyses and figures were conducted in R version 3.1.3.

Association Mapping in Natural Populations

We chose 62 clones from 11 different populations that were part of a large rock pool metapopulation (pool codes: FS-31, N-86, SK-1, FUN-13, LA-10, LA-18, SKW-1, SKW-2, LON-1, SK-45, and HA-1) in southwest Finland. These subpopulations are separated by only few kilometres and were the subject of a previous study (Roulin et al. 2015). We genotyped the populations by extracting DNA from the 62 clones using the QIAGEN DNeasy Blood & Tissue Kit. Primers were designed

to amplify nine loci on scaffold 1036 at positions 703, 712, 715, 718, 730, 740, 750, 775, and 785 kb. Later, primers were designed to fully amplify the GPCR rhodopsin located on scaffold1036 between 708,969 nt and 713,276 nt. These loci were sequenced in the 62 clones by Sanger sequencing. To assess the specificity of these primers, we performed a Blast search using the generated sequences as queries against the *D. magna* genome. For each locus, the generated sequences were then aligned with ClustalX (Larkin et al. 2007) and used to genotype the 62 clones. SNPs with a minor allele frequency <0.05 were removed from the analysis.

To adjust the association mapping for potential genetic structure, the DNA of the same 62 clones was also sent to the GBS platform at Cornell University (http://www.biotech.cornell.edu, Elshire et al. 2011). DNA samples were digested with restriction enzyme PstI. We used the SNP file provided by the platform. The generated SNPs were sorted with vcftools (Danecek et al. 2011) according to the following criteria: maximum of 10% of missing individuals, minimum allele frequency (maf) of 0.05 and a minimum and maximum coverage of 20 and 250, respectively. Using the R package GenAbel (Aulchenko et al. 2007), the resulting SNPs were used to compute a Principal Component Analysis (PCA) and to calculate the kinship matrix between clones. SNPs falling on scaffold 1036 between 700 kb and 785 kb were added to the SNPs we had previously identified with Sanger sequencing.

We performed an association mapping between the SNPs in our region of interest on scaffold 1036 and resting stage production using the R package GenAbel. The association was first tested without correction for potential genetic structure and then adjusted for potential population structure and clone relationship using the Eigenstrat method implemented in GenAbel. Heritability of resting stage production was also calculated with the library GenAbel with $h^2 = {\sigma_G}^{2/}({\sigma_G}^2 + {\sigma_e}^2)$, where ${\sigma_G}^2$ is the variance explained by the genetic effects and ${\sigma_e}^2$ is the variance explained by the residual effects. All figures were produced with the R package ggplot2 (Wickham 2009).

Analysis of Selection

"Permanent pools" were defined as rock pools that were less likely to desiccate in summer (pools FS-31, SKW-2, N-86, SK-1, FUN-13, and LA-10), whereas "intermittent pools" were defined as rock pools with a high chance of desiccating (pools SKW-1, LON-1, LA-18, SK-45, and HA-1). Intermittent pools are, on average, smaller than permanent pools (Roulin et al. 2015). The effect of the habitat type on resting stage production (square-rooted to fit normal distribution of the residuals) among populations was tested using linear mixed-effects models as described in Roulin et al. (2015). Briefly, we first built a full model that included the habitat type (permanent vs. intermittent) as a fixed effect and the populations nested in habitat type as random effects. We then tested the significance of the habitat type by comparing the Akaike Information Criterion (AIC) of this full model with the AIC of a model from which the habitat type was removed. Standard deviations of population and residual effects were extracted from the full model and used to calculate variance components as described in (Crawley 2013). Models were run using the package nlme in R (Pinheiro and Bates 2014).

To assess whether SNPs located in the candidate region displayed evidence of selection and local adaptation, we performed an outlier analysis using the software BAYPASS (Gautier 2015). This method elaborates on the BAYENV model (Coop et al. 2010; Günther and Coop 2013) that estimates and explicitly accounts for the hierarchical structure and demographic history of populations to detect signatures of local adaptation from variation in allele frequencies. We computed the X^TX statistics, a differentiation measure estimate corrected for population structure. To assess whether SNPs displayed significant departure from neutrality, we first considered the extent to which a given SNP differed from the other SNPs by determining the centile in which its X^TX value lay in the distribution of the whole actual dataset (sorted GBS dataset). We further confirmed the outlier status by calibrating the X^TX statistics as suggested by Gautier (2015). This required simulating 100,000 SNPs under the demographic model inferred from the actual dataset by BAYPASS using the R function simulate.baypass() provided with the software. BAYPASS default parameters for the core model were used: MCMC chains were run for 25,000 iterations after a 5,000 iterations burn-in period. Samples were taken from the chain every 25 iterations. The X^TX was then computed for this pseudo-observed dataset (POD) to determine its distribution under a neutral model. Finally, we compared the observed values to this distribution to determine the probability of each SNP being neutral.

Gene Functions

Genes located in the defined region of interest were identified using the annotation file (gff) available for the current assembly of the genome (unpublished data). Gene sequences were retrieved using the function extractseq from EMBOSS (Rice et al. 2000). To validate the genes' functions, a Blastx search using the gene sequences as queries was then performed against the NCBI (nonredundant) and flybase (http://flybase.org) databases.

Supplementary Material

Supplementary tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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