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Phenotypic plasticity in the hepatic transcriptome of the European common frog (*Rana temporaria*): the interplay between environmental induction and geographical lineage on developmental response

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Abstract

Phenotypic plasticity might facilitate adaptation to new environmental conditions through the enhancement of initial survival of organisms. Once a population is established, further adaptation and diversification may occur through adaptive trait evolution. While several studies have found evidence for this mechanism using phenotypic traits, much less is known at the level of gene expression. Here, we use an islands system of frog populations that show local adaptation and phenotypic plasticity to pool drying conditions in development time until metamorphoses. We examined gene expression differences in *Rana temporaria* tadpole livers with respect to pool drying at the source population and in response to simulated pool drying in the laboratory. Using a MAGEX cDNA microarray and quantitative real-time polymerase chain reaction (qPCR), we identified an increase in several gene transcripts in response to artificial pool drying including thyroid hormone receptor alpha and beta, carbamoyl phosphate synthetase 1, ornithine transcarbamylase and catalase. In addition, these gene transcripts also showed greater abundance in island populations that developed faster. Hence, the gene transcripts were related to both constitutive response (higher levels in island populations that developed faster) and plastic response (increased abundance under decreasing water levels). This pattern is in accordance with genetic accommodation, which predicts similarities between plastic gene expression and constitutive expression in locally adapted populations.

Keywords: adaptation, amphibians, gene structure and function, life history evolution, molecular evolution, phenotypic plasticity

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Introduction

Organisms can adapt to environmental variation in two different strategies, either by a constitutive trait expression to one environmental state or by expressing phenotypic plasticity. Phenotypic plasticity is defined as the ability of a given genotype to produce different phenotypes in response to different environmental conditions, that is, a slope of a reaction norm (Agrawal 2001). Which of these strategies proves optimal depends on the predictability and variation of the environment and on gene flow between environments (Sultan & Spencer 2002). It has been shown theoretically and empirically that, when the environment is variable, phenotypic plasticity is selected for (Sultan & Spencer 2002; Lind *et al*. 2011). More interestingly, it has been realized that plasticity and constitutive expressions can interact. For example, plasticity might allow a population to survive long enough in a new environment for existing genetic variation, in combination with mutation and/or recombination, to respond to local selection conditions. In such cases, traits that were original plastic might become genetically assimilated or accommodated in the

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new environment (West-Eberhard 2005). Genetic assimilation is the process whereby an environmentally induced phenotype becomes canalized (constitutively expressed) through selection acting upon the developmental system. Genetic accommodation is any type of shift in the elevation of a reaction norm as a result of the new environment the organism is exposed to. While populations or species, locally adapted along an environmental gradient, often express phenotypes that mirror the plastic response in key traits (Gomez-Mestre & Buchholz 2006; Ledon-Rettig *et al.* 2008), much less is known about the genetic mechanisms involved in phenotypic plasticity and constitutive trait expressions. In theory, plasticity may be caused by up- or down-regulation of common developmental pathways, but also by environment-specific gene expression, where genes expressed under different environmental conditions are more or less independent (environmental decoupling) (Snell-Rood *et al.* 2011). Theory suggests that environment-specific gene expression can reduce pleiotropic constraints among genes associated with different environments (Pal *et al.* 2006) and also result in reduced selection (Kawecki 1994), both of which may result in rapid evolutionary diversification of populations (Snell-Rood *et al.* 2011; Snell-Rood 2012). There is some support that traits involved in polyphenisms, which is an extreme form of phenotypic plasticity with discrete morphs, indeed show some degree of environment-specific gene expression, although not strictly decoupling (Snell-Rood *et al.* 2011). Hence, plasticity may directly aid adaptive divergence, because alternative phenotypes can been shaped by selection independent of each other. However, for life history traits, plasticity is seldom expressed as discrete phenotypic classes but instead as a continuous reaction norm (e.g. Newman 1988), and less is known whether environment-specific gene expression (or considerable modulation in gene transcript abundance), which would aid diversification of population, is the norm for such traits.

The simultaneous measurement of mRNA transcript levels representing a large number of genes using DNA microarray analysis allows us to bridge the gap between gene expression and developmental processes, and a greater understanding of phenotypic plasticity and its evolution can be achieved (Moczek *et al.* 2011). By focusing on natural populations, such a multigene approach can be used to investigate whether gene expression differs in individuals expressing phenotypic plasticity-induced traits compared with individuals that express the same trait as a constitutive response. Comparisons of gene transcript abundance profiles are important because they will provide information on developmental pathways that are underlying adaptive responses and the genetic basis of plastic and constitutive expressed traits. One interesting question is whether the constitutive expressed phenotypes show the same gene expression patterns and gene networks as the phenotypic plasticity-expressed phenotypes. This research has just begun, but comparative gene expression data on winged and wingless ant castes and horn morphology in males and between sexes in beetles suggest that a considerable overlap in gene expression between plastic and nonplastic genotypes can be demonstrated within insect orders (Abouheif & Wray 2002; Snell-Rood *et al.* 2011).

Postembryonic development of frogs and their metamorphosis from the larval tadpole stage to juvenile froglet provides an excellent system to study the pattern of gene expression in phenotypes that are either expressed constitutionally (specialized genotypes) or by phenotypic plasticity with regard to development rate. Metamorphosis of the frog tadpole is modulated by a variety of biotic and abiotic variables including hormone action and the presence of steroids and peptides (Rose 2005; Veldhoen *et al.* 2006). For many of these influencing factors, the impact on tissue-specific gene expression patterns at the level of mRNA abundance can be quantified using DNA microarray and qPCR approaches (Veldhoen *et al.* 2002, 2006). The tissue-specific expression profiles occurring during anuran post embryonic development are probably adaptive, that is, they affect development that matches the environmental conditions and occurs as a constitutive developmental as well as a plastic response (Denver 1998; Rose 2005; Gomez-Mestre & Buchholz 2006; Lind *et al.* 2011; Richter-Boix *et al.* 2011). As variation can occur in the status of their aquatic environment, completion of tadpole development must be coordinated with the potentially ephemeral nature of ponds.

Here, we use a well-studied island system in Sweden to investigate differences in hepatic gene mRNA abundance profiles between and within European common frog (*Rana temporaria*) populations that differ in development rate during the tadpole stage. Frogs from islands with temporary pools have a significantly faster development when raised in a common environment compared with frogs from island with permanent pools, and this difference is caused by selection on development rate in response to pool drying on the islands (Lind & Johansson 2007; Lind *et al.* 2011). This response is adaptive because failing to develop before a pool dries out results in death, and metamorphosis at large size has positive effects on fitness components (Smith 1987; Berven 1990; Morey & Reznick 2001; Altwegg & Reyer 2003). In addition, all island populations show an adaptive response to pool drying in that they speed up their development rate under laboratory conditions when subjected to decreasing water levels (Lind & Johansson 2007; Lind *et al.* 2011). Populations with more

variation in drying among pools within the island show a higher degree of plasticity with regard to development rate. Hence, populations show constitutive differences in development rate among islands and they differ in phenotypic plasticity of rate of metamorphosis. Previous studies, using trait expression only, have suggested that plasticity to pool drying is mediated by a decoupling of development and growth rate, but that this plastic decoupling is not mirrored in the fixed difference between locally adapted populations, suggesting a limited role for plasticity in adaptive divergence (Lind & Johansson 2011).

To further understand the genetic basis of plasticity and specialization, additional gene expression data are needed. In the current study, we focus on the frog liver because this organ undergoes a drastic thyroid hormone-mediated reprogramming during tadpole metamorphosis. This includes changes in the expression status of genes important in induction of the metamorphic programme such as the thyroid hormone receptors, *thra* and *thrb*, and the activation of urea cycle genes in preparation for the animal's transition from life in water to land (Helbing *et al.* 1992, 2010; Helbing 2012; Searcy *et al.* 2012). The urea cycle is essential for survival under limited water conditions in order to detoxify nitrogenous waste. In tadpoles, ammonia is rapidly removed by the copious quantities of water bathing the gills. In frogs, however, this mechanism is not suitable due to reduced access to water, and ammonia is fixed in the less toxic form of urea through the actions of five urea cycle enzymes (Atkinson *et al.* 1996).

We raised tadpoles in the laboratory and compared hepatic gene mRNA abundance profiles between island tadpole populations presented with either constant water conditions or a decreasing water environment. More specifically we asked (i) Is there evidence of environment-specific gene expression in plasticity to artificial pool drying? (ii) Are there differences in gene expression status of larvae from islands that differ in pool drying rate? and (iii) How do these two types of responses differ with regard to gene expression?

Materials and methods

Rana temporaria populations from six islands in the Baltic Sea, Northern Sweden, that differ in environmental water conditions as well as in tadpole development rate were chosen based on Lind & Johansson (2007, 2009). Two of the islands have permanent pools (Stora Fjäderägg and Öster Hällskär), two have temporary pools (Ålgrundet and Sävar-Tärnögern) and two have pools that are intermediate with regard to drying (Bredskär and Petlandsskär). For a map and precise location of the populations, see Lind *et al.* 2011. The tadpoles from these populations demonstrate slow, fast and intermediate development rate, respectively. In addition, the populations differ in their degree of phenotypic plasticity with regard to decreasing water levels during tadpole development. Petlandsskär and Bredskär are populations with a high degree of phenotypic plasticity, and the other four have a lower degree of phenotypic plasticity with regard to development rate. In summary, three categories exist with regard to pool drying (permanent, intermediate and temporary) and two categories with regard to phenotypic plasticity in development rate (high and low).

Egg collection and laboratory rearing

We collected egg masses from ten females on each of the six islands in the beginning of May 2010. Because this species usually lays only one egg mass per season (Savage 1961), each egg mass was assumed to have been laid by a separate female *R. temporaria*. Egg masses were brought to the laboratory, and the eggs hatched at a temperature of 22 °C. The tadpoles were allowed to develop to Gosner stage 23 (active swimming, Gosner 1960) at which time 10 offspring per female representing 10–12 families per island were subdivided.

Individual tadpoles were held in plastic containers (9.5 cm × 9.5 cm × 10 cm) filled with 750 mL conditioned nonchlorinated conditioned tap water (Lind & Johansson 2007). The containers were placed in random order on shelves in a walk-in climate chamber. Tadpoles were fed every fourth day with a 1:1 mixture consisting of commercial fish flakes and rabbit food, finely ground using a mortar and pestle. On days 1–8, they were fed 15 mg. During the course of the experiment, the food levels were increased according to the following schedule: 30 mg from days 9–12, 45 mg from days 13–16, 60 mg from days 17–20 and thereafter 75 mg. Every fourth day, prior to feeding, the conditioned water was renewed. To examine the impact of decreasing water levels on development rate, we simulated pool drying for half of the tadpoles by decreasing the water level by 33% every fourth day until day 25, after which the water volume in this treatment was kept constant at 66 mL. Note that all tadpoles analysed for their hepatic gene transcript abundance profiles were collected before this day.

For the analysis of gene expression status, four tadpoles from each family were used, two tadpoles from the constant water treatment and two from the decreasing water treatment. Within each treatment, one tadpole was sampled at experimental day 14 and the other when it reached Gosner stage 37. To study the phenotypic effect of the treatments, we also ran three replicate siblings per condition from three of the

populations (Bredskär, Stora Fjäderägg and Sävar-Tärnögern) to metamorphosis (Gosner stage 42, corresponding to 23–34 days of development) at which time development time and wet weight were measured.

To separate the effects of development time and development stage, one half of the tadpoles were evaluated for hepatic mRNA levels at Gosner stage 37 (Gosner 1960) (corresponding to days 19–21), and to determine the relationship between development rate and transcript abundance, the other half were analysed at day 14 of development (median stage: 34 with a range between Gosner stages 31 and 35). Sampled individuals were euthanized by immersion in Tricaine-S (MS 222; Sigma-Aldrich), and liver tissue was collected and placed in 5 mL RNA*later* preservative solution (Life Technologies Corp., Carlsbad, CA, USA). All preserved tissue samples were initially placed at 4 °C for 24 h prior to storage at −20 °C. At day 14, we noted the development stage (Gosner stage) according to Gosner (1960), and at Gosner stage 37, we noted wet weight and age (development time) of tadpoles. We did not consider maternal effects in the present study but similar past studies have shown that maternal effects are minimal (~5%) in these island populations (Lind & Johansson 2007). The raw data used in the analyses are provided in Tables S5 and S6 (Supporting information).

RNA isolation, MAGEX DNA microarray, quantitative real-time polymerase chain reaction (qPCR) and statistical analyses

Materials and methods are explained in detail in the Appendix S1 along with Figures S1–S6 (Supporting information). The microarray data set has been submitted to the Gene Expression Omnibus (GEO number GSE42274).

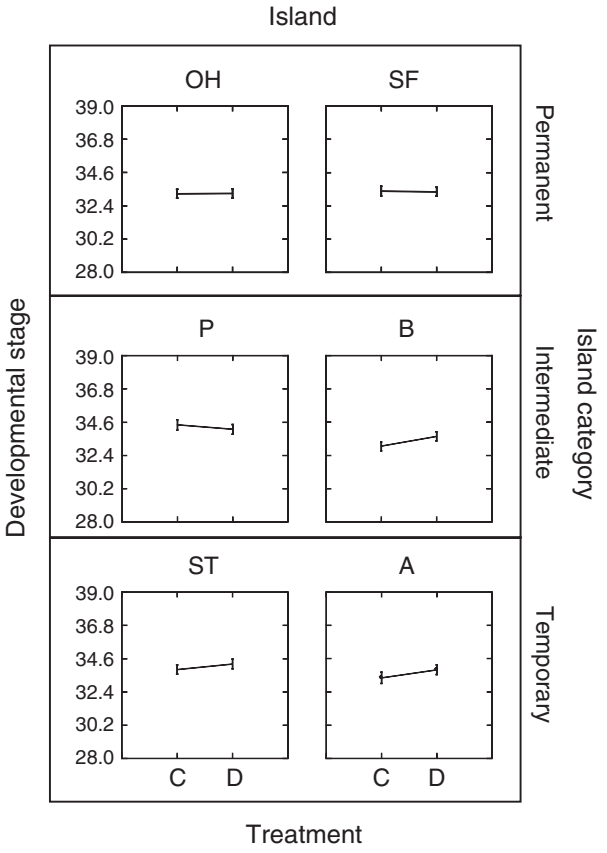

Fig. 1 Relationship between Gosner developmental stage at experimental day 14 and water status for *Rana temporaria* tadpoles originating from six island populations including Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF). Treatments involved maintenance of constant (C) or decreasing (D) water levels, and animal populations were categorized based upon the presence of permanent, temporary or intermediate (mix of permanent and temporary) island pools.

Results

Life history traits

Treatment and island effects on life history traits were measured at three times during development: at day 14, Gosner stage 37 and Gosner stage 42 (metamorphosis). At day 14, larvae had reached a median Gosner stage of 34 (range 31–35). A two-way ANOVA on development stage at day 14 showed a nonsignificant interaction term between treatment and island population, and therefore, this interaction term was removed. The subsequent ANOVA showed a significant island effect ($P < 0.001$), but there was no treatment effect ($P = 0.24$), (Fig. 1). At Gosner stage 37, a two-way ANOVA on development time to stage 37 showed a nonsignificant interaction term ($P = 0.51$) between treatment and population, and when this interaction term was removed from the analysis, treatment and island population had no significant effects on time until development stage 37 ($P = 0.25$ and $P = 0.14$ respectively, Fig. 2). The two-way ANOVA on weight at stage 37 showed a nonsignificant interaction term, and the subsequent test without the interaction term showed a significant treatment effect ($P < 0.001$) with lower tadpole weight in the decreasing water level treatment (Fig. 2). Island populations did not differ significantly ($P = 0.31$). However, at metamorphosis (stage 42), we did find a significant treatment effect in both development time and weight. Tadpoles in the drying water treatment had a faster development time ($\chi^2_1 = 23.70$, $P < 0.001$) at the expense of a lower metamorphic weight ($\chi^2_1 = 144.82$, $P < 0.001$). After removing the highly nonsignificant treatment x population interaction, we found an effect of population on weight

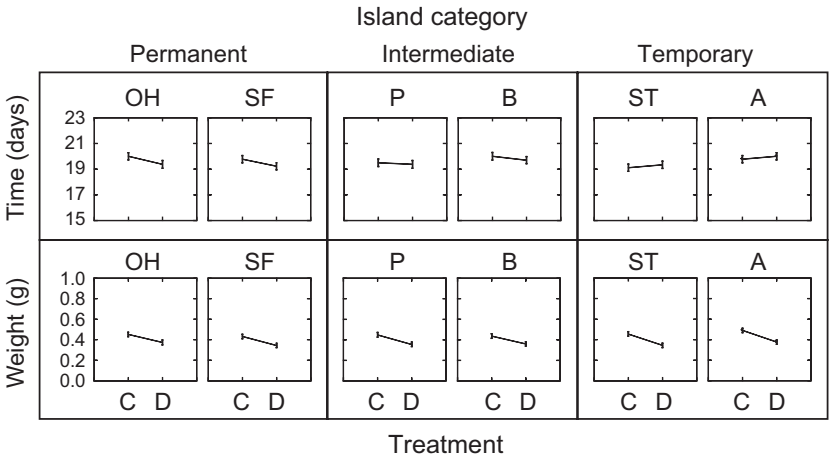

Fig. 2 Relationship between time to reach Gosner stage 37 (days) or weight (g) at Gosner stage 37 with water treatment status for *Rana temporaria* tadpoles originating from six island populations including Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF). Treatments involved maintenance of constant (C) or decreasing (D) water levels, and animal populations were categorized based upon the presence of permanent, temporary or intermediate (mix of permanent and temporary) island pools.

(χ^2_2 = 13.07, P = 0.001), but not on development time (χ^2_2 = 3.44, P = 0.18).

Hepatic gene expression patterns: MAGEX DNA microarray analysis

We initially used the MAGEX DNA microarray on Gosner stage 37 animals from a subset of two temporary and two permanent pool island populations to perform heterologous hybridizations for the evaluation of gene abundance profiles of 497 transcripts. The MAGEX DNA microarray was designed to include a wide range of target gene probes representing a diversity of biological pathways involved in amphibian development and homeostasis. A total of 11 gene transcripts were identified as significantly different across the four islands when animals were in constant water conditions (Table 1, Fig. S2, Supporting information). The majority of these transcripts and/or the proteins they encode have been previously associated with modulation and/or perturbation of thyroid hormone-mediated meta-morphosis (Zhang *et al*. 2006; Helbing *et al*. 2007a, b; Helbing 2012). Of particular note were significant relationships observed in a group of core transcripts encoding proteins that are part of ammonia fixation and urea production pathways. This group comprises four (*cps1*, *otc*, *asl* and *glul*) of a total of six enzymes in the pathway, five of which were present on the MAGEX DNA microarray (Table 1). Spearman rho analysis showed a strong positive correlation (0.67–0.84) between *otc*, *asl* and *glul* transcripts. The remaining transcripts encoded for proteins involved in a variety of cellular processes only two of which grouped together (Table 1). These two transcripts, *psmxc3* and *psmy*, encode for proteasome subunits in the proteasome core complex involved in protein degradation. Additional processes represented were tricarboxylic acid cycle, glucose metabolic process, regulation of growth, apoptotic process and DNA binding (Table 1). When challenged with decreasing water availability, 13 mRNA transcripts were significantly different in abundance between the four selected islands in the liver of Gosner stage 37 tadpoles (Table 2, Fig. S2, Supporting information). Eight of the transcripts that were identified as different between islands under constant water conditions were also significantly different in decreasing water conditions (compare Tables 1 and 2). Again, a number of these transcripts have been previously associated with modulation and/or perturbation of thyroid hormone-mediated metamorphosis. These included *otc*, *asl*, *glul* and protein catabolic process-associated transcripts encoding proteasome subunits (*psmxc3* and *psmy*) and E2 ubiquitin conjugating enzyme (*ube2*). Additional processes represented are glucose metabolic process, apoptotic process, DNA binding, protein complex assembly, actin binding and oxidation–reduction process (Table 2).

We next examined the transcript differences within each island population in response to decreasing water conditions in Gosner stage 37 tadpoles. The greatest number of significantly changed gene transcripts was found in animals from islands having permanent pools (Öster Hällskär and Stora Fjäderägg: 30 and 8 mRNA transcripts, respectively; Fig. S3; Tables S3 and S4, Supporting information). All except for one transcript showed increased abundance upon decreasing water conditions (Fig. S3, Supporting information). There was a notable difference in the number of differentially expressed genes between these populations and those from islands with temporary pools (Ålgrundet and Sävar-Tärnögern: 1 and 0 mRNA transcripts, respectively; Fig. S3, Supporting information). All but one gene showed an increase in transcript abundance in response to decreasing water conditions (Fig. S3, Supporting infor-

Table 1 Gene transcripts at Gosner stage 37 that were significantly different in abundance across island populations maintained under constant water levels following MAGEX DNA microarray analysis*

Gene transcript	Kruskal–Wallis *P*-value	Gene ontology
Carbamoyl phosphate synthetase (*cps1*)†	0.013	GO:0009064 glutamine family amino acid metabolic process/ GO:0000050 urea cycle
Ornithine transcarbamylase (*otc*)†	0.001	GO:0009064 glutamine family amino acid metabolic process/ GO:0000050 urea cycle
Argininosuccinate lyase (*asl*)†	0.004	GO:0009064 glutamine family amino acid metabolic process/ GO:0000050 urea cycle
Glutamine synthetase/glutamate-ammonia ligase (*glul*)	0.011	GO:0009064 glutamine family amino acid metabolic process
Proteasome subunit XC3 (*psmxc3*)	0.019	GO:0005839 proteasome core complex
Proteasome subunit Y (*psmy*)†	0.031	GO:0005839 proteasome core complex
Aconitase (*aco*)†	0.028	GO:0006091 tricarboxylic acid cycle
Aldolase B (*aldob*)	0.042	GO:0006006 glucose metabolic process
Growth associated protein 43 (*gap43*)†	0.030	GO:0040008 regulation of growth
Defender against death (*dad1*)†	0.007	GO:0006915 apoptotic process
High mobility group X (*hmgx*)†	0.016	GO:0003677 DNA binding

*Animals from islands containing permanent pools originated from Stora Fjäderägg and Öster Hällskär, while temporary pool island animals originated from Ålgrundet and Sävar-Tärnögern (n = 6 per island).
†Gene transcript and/or protein has been previously associated with modulation and/or perturbation of thyroid hormone-mediated metamorphosis. See text for references.

Table 2 Gene transcripts at Gosner stage 37 that were significantly different in abundance across island populations maintained under decreasing water conditions following MAGEX DNA microarray analysis*

Gene transcript	Kruskal–Wallis *P*-value	Gene ontology
Ornithine transcarbamylase (*otc*)*,†	0.002	GO:0009064 glutamine family amino acid metabolic process
Argininosuccinate lyase (*asl*)†,‡	0.002	GO:0009064 glutamine family amino acid metabolic process
Glutamine synthetase/glutamate-ammonia ligase (*glul*)‡	0.043	GO:0009064 glutamine family amino acid metabolic process
Proteasome subunit XC3 (*psmxc3*)‡	0.009	GO:0030163 protein catabolic process/GO:0005839 proteasome core complex
Proteasome subunit Y (*psmy*)†,‡	0.002	GO:0030163 protein catabolic process/GO:0005839 proteasome core complex
E2 ubiquitin conjugating enzyme (*ube2*)	0.048	GO:0030163 protein catabolic process/GO:0000278 mitotic cell cycle
Aldolase B (*aldob*)‡	0.0008	GO:0006006 glucose metabolic process
Lactate dehydrogenase B (*ldhb*)†	0.013	GO:0006006 glucose metabolic process
Defender against death (*dad1*)†,‡	0.017	GO:0006915 apoptotic process
High mobility group X (*hmgx*)†,‡	0.045	GO:0003677 DNA binding
Beta tubulin (*tubb*)†	0.021	GO:0006461 protein complex assembly
Nonmuscle tropomyosin (*tpm4*)†	0.030	GO:0003779 actin binding
Alcohol dehydrogenase class 4 (*adh4*)	0.001	GO:0055114 oxidation–reduction process

*Animals from islands containing permanent pools originated from Stora Fjäderägg and Öster Hällskär, while temporary pool island animals originated from Ålgrundet and Sävar-Tärnögern (n = 6 per island).
†Gene transcript and/or protein has been previously associated with modulation and/or perturbation of thyroid hormone-mediated metamorphosis. See text for references.
‡Also significant in constant water conditions. See Table 1.

mation). As permanent pools might dry out under rare instances of extremely dry conditions, it is not surprising that the animals from islands with permanent pools showed a greater modulation in gene transcript profiles compared with those originating from islands with temporary pools, which undergo seasonal drying out at a greater frequency. None of the identified gene transcripts overlapped in identity between islands, but the

majority have been previously associated with thyroid hormone-mediated metamorphosis (Zhang *et al.* 2006; Helbing *et al.* 2007a,b).

Hepatic gene expression patterns: qPCR analysis

The above-mentioned DNA microarray analysis permitted the sampling of a wide range of transcripts over a limited number of island populations (permanent pools: Stora Fjäderägg and Öster Hällskär; temporary pools: Ålgrundet and Sävar-Tärnögern). To complement and extend the microarray data to a greater number of animals, time/developmental points and island populations, we cloned and designed qPCR assays for the following *Rana temporaria* gene transcripts: the thyroid hormone receptors, *thra* and *thrb*, the urea cycle enzymes, *cps1* and *otc*, and catalase (*cat*). All five were chosen because of their central roles in the metamorphic transition of the tadpole to a froglet, three of which were identified as being altered through the MAGEX DNA microarray analysis. A link between *thra* transcript levels and developmental rate has previously been reported in spadefoot toads (Hollar *et al.* 2011). The transcript encoding the catalase enzyme was not present on the MAGEX DNA microarray, but was examined due to its important role in removing H_2O_2 produced through aerobic respiration (Hammond *et al.* 2013). Therefore, *cat* serves as an indicator of oxidative stress due to changing metabolic needs.

For this part of the study, we elected to examine hepatic transcript levels across all six island populations at two collection times: (i) at a common experimental time point (day 14) and (ii) at the same developmental stage attained regardless of how long it took for the tadpole to reach that developmental landmark (Gosner stage 37). At the 14 day time point, the median Gosner stage was 34 (range: 31–35). The abundance levels of the hepatic transcripts were examined with respect to developmental progression by comparing animals that were collected at day 14 with Gosner 37 tadpoles under constant water conditions (Fig. 3). As expected (Helbing *et al.* 1992; Helbing 2012), transcript levels for *thrb* and *cps1* were significantly higher in the Gosner stage 37 tadpoles compared with those in day 14 (Gosner stage 31–35) (Fig. 3). Also expected was a modest to no increase in *thra* mRNAs as development progressed to Gosner stage 37 (Veldhoen *et al.* 2006; Hammond *et al.* 2013). An increase was generally evident with the exception of Bredskär tadpoles where no significant *thra* change was observed (Fig. 3). Bredskär tadpoles also showed a unique profile in *cat* mRNA levels where Gosner stage 37 tadpoles display elevated levels. In contrast, *cat* mRNAs decreased most in the temporary pool islands (Ålgrundet and Sävar-Tärnögern) during development and no difference in the other islands (Fig. 3). *Otc* transcripts did not show a difference between developmental groupings in any island population with the exception of Öster Hällskär where *otc* mRNAs decreased (Fig. 3). The above-mentioned five gene transcripts were evaluated further with respect to island distribution, treatment and the interaction effect by separating the analyses into animals collected on experimental day 14 and animals collected when they reached Gosner stage 37.

Analyses from tadpoles collected on experimental day 14

A two-way ANOVA of *thra* mRNA abundance data with island and treatment as factors showed nonsignificant interaction, and therefore, the interaction term was removed. Treatment showed a significant effect on gene transcript abundance, which was higher in tadpoles under decreasing water levels ($P = 0.005$, Fig. 4), and there was a trend for differences among islands with Stora Fjäderägg and Bredskär showing somewhat higher gene transcript abundance ($P = 0.08$, Fig. 4). The two-way ANOVA on *thrb* mRNA abundance showed a nonsignificant interaction term, and after this term was removed, there was a significant effect of treatment on gene transcript abundance ($P = 0.022$, Fig. 4), and there was no significant difference among islands ($P = 0.11$), but Stora Fjäderägg and Bredskär showed a somewhat higher gene transcript abundance (Fig. 4). The result from the two-way ANOVA on *cps1* showed a significant treatment effect ($P < 0.001$) and island population ($P < 0.001$) effect and a significant interaction term ($P < 0.001$) on gene transcript abundance. While some island population tadpoles showed a higher gene transcript level (Bredskär and Sävar-Tärnögern) when subjected to decreasing water levels, the population of Ålgrundet showed a decreased *cps1* gene expression level under decreasing water levels (Fig. 4). An ANOVA on *otc* showed a significant treatment effect ($P = 0.026$), suggesting that in general, gene transcripts were higher when tadpoles were subjected to decreasing water level (Fig. 4). There was no significant island effect ($P = 0.11$). However, the interaction term was close to significant ($P = 0.074$), suggesting that population differed in their gene transcripts with regard to decreasing water. For example, the Ålgrundet animals showed the opposite pattern with regard to the general trend of a higher abundance transcript. There was a significant interaction term ($P < 0.001$) in the two-way ANOVA of *cat* transcript levels, suggesting that gene transcript abundance in tadpoles differed between treatments among islands. For example, the Bredskär population had higher gene transcript abundance in the drying treatment, while the

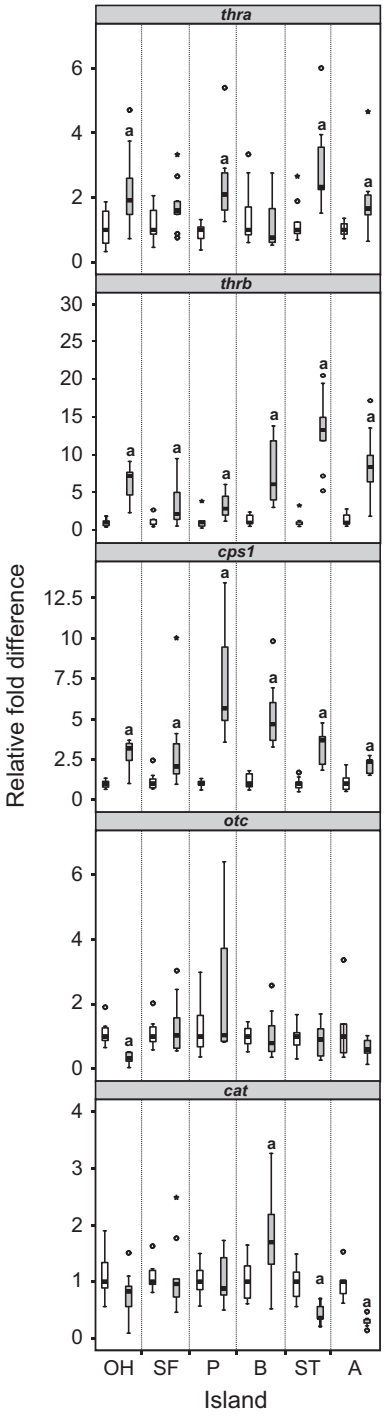

Fig. 3 Comparison of hepatic gene transcript abundance in *Rana temporaria* from Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF) island populations when reared under constant water conditions. The abundance of the indicated gene transcripts: *thra*, thyroid hormone receptor α; *thrb*, thyroid hormone receptor β; *cps1*, carbamoyl phosphate synthetase; *otc*, ornithine transcarbamylase; and *cat*, catalase were evaluated using qPCR analysis. The data from animals collected at experimental day 14 (median Gosner stage 34; range 31–35; white bars) were compared with animals collected at Gosner stage 37 (grey bars) to evaluate developmental progression. Box plots show medians ± first and third quartiles. The whiskers indicate minimum and maximum values excluding outliers and extreme values. Outlier (cases between 1.5 and 3 box lengths from the upper or lower edge of the box) and extreme values (cases >3 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Data are plotted relative to the day 14 transcript abundance levels within each island set with $n = 6$ animals/island/sampling point. Significance relative to the day 14 data ($P < 0.05$) is denoted by 'a'.

Petlandsskär population had lower gene transcript abundance in this treatment compared with those under constant water conditions (Fig. 4). The treatment effect was nonsignificant ($P = 0.19$), while the island effect was significant ($P < 0.001$, Fig. 4).

Within the experimental day 14 animals, a slight increasing relationship between transcript abundance and Gosner stage attained was observed for *thra* and *cat* but not *thrb*, *cps1* or *otc* (Fig. 5). Overall, gene transcript abundance at experimental day 14 showed a strong effect in response to changing water levels. Hierarchical cluster analysis demonstrated that some island category distinction (between fast-developing tadpoles from temporary pool islands and slow/intermediate-developing tadpoles) was achieved with a combination of *thra*, *thrb*, *cps1* and *otc* mRNA transcript levels but only under constant water conditions (Fig. 6). Although *cat* contributed to differences between islands, this transcript did not function to separate by island category and was therefore not included in the hierarchical cluster analysis.

Analyses from tadpoles collected at developmental stage 37

A two-way ANOVA of *thra* mRNA abundance data with island and treatment as factors showed a nonsignificant interaction, and therefore, the interaction term was removed. Treatment showed a nonsignificant effect on tadpole gene transcript abundance ($P = 0.81$, Fig. 7), but island populations differed significantly in their gene transcript abundance ($P = 0.027$) with Öster Hällskär and Bredskär having higher levels of gene transcript abundance in decreasing water conditions (Fig. 7). The two-way ANOVA on *thrb* mRNA abundance data showed a nonsignificant interaction term, and after this term was removed, there was a significant effect of treatment on tadpole gene transcript abundance showing higher levels in the drying treatment ($P = 0.016$, Fig. 7), and there was a trend for a significant island population effect ($P = 0.11$). The result from the two-way ANOVA on

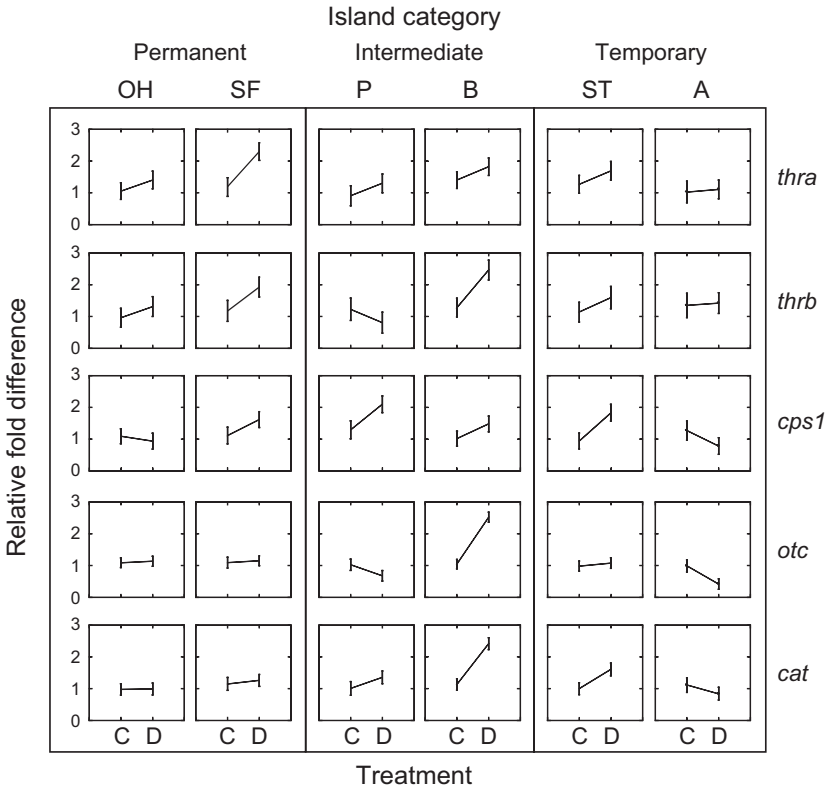

Fig. 4 Relationship between hepatic gene transcript abundance at experimental day 14 and water treatment status for *Rana temporaria* tadpoles originating from six island populations including Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF). Refer to Fig. 3 legend for gene transcript abbreviations. Treatments involved maintenance of constant (C) or decreasing (D) water levels, and animal populations were categorized based upon the presence of permanent, temporary or intermediate (mix of permanent and temporary) island pools.

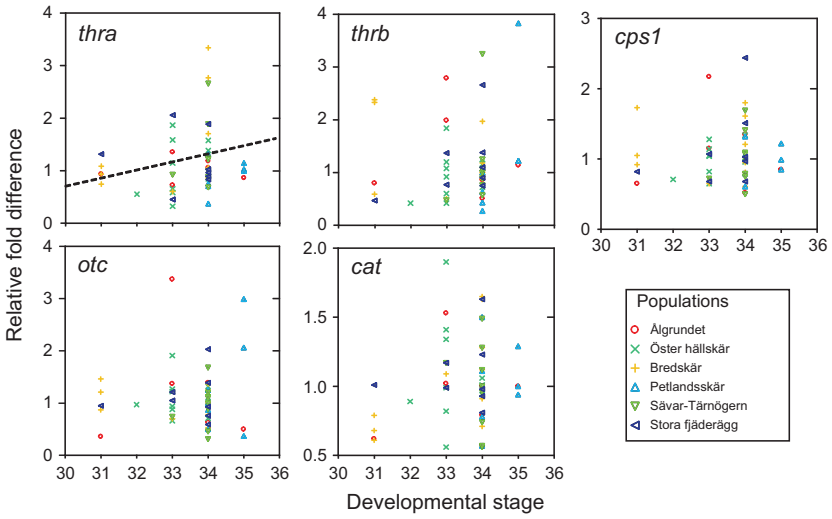

Fig. 5 Relationship between heptic gene transcript abundance and developmental stage (Gosner stage) of *Rana temporaria* tadpoles at experimental day 14 under conditions of constant water level. The line for thra denotes marginally significant relationships between relative fold difference and development stage (see Table 4). Refer to Fig. 3 legend for gene transcript abbreviations.

cps1 showed a nonsignificant interaction term. When this term was removed from the model treatment, island population showed significant effects ($P = 0.05$ and $P < 0.001$, respectively). In general, tadpole *cps1* transcript abundance was higher in the decreasing water level treatment, and tadpoles from the islands Öster Hällskär and Stora Fjäderägg showed low gene transcript abundance at this development stage relative to other islands (Fig. 7). An ANOVA on *otc* transcripts showed a significant interaction term between island populations and treatment ($P = 0.03$). Island populations differed significantly in tadpole gene transcript abundance ($P < 0.001$), but there was no significant main effect on treatment ($P = 0.52$). Hence, *otc* gene transcripts were generally lower in the decreasing water level treatment except for Ålgrundet where the opposite pattern was found (Fig. 7). With respect to *cat* transcript abundance data, the interaction term was nonsignificant

Table 3 Result from modelling the relationship between the differences in development stage and hepatic gene transcript abundance between treatments using populations as factor

	d.f.	F	*P*
thra	1	0.99	0.32
Population	5	1.46	0.22
Error	43		
thrb	1	1.33	0.27
Population	5	0.02	0.88
Error	43		
cps1	1	1.47	0.22
Population	5	0.72	0.40
Error	43		
otc	1	1.19	0.33
Population	5	0.41	0.52
Error	43		
cat	1	1.35	0.26
Population	5	0.37	0.55
Error	43		

and was therefore removed. After removing the interaction term, the two-way ANOVA showed a significant island effect ($P = 0.048$) with tadpoles on Öster Hällskär and Bredskär having lower gene transcript abundance (Fig. 7). The treatment effect was nonsignificant ($P = 0.35$). Hierarchical cluster analysis supported these observations showing moderate clustering according to fast-, intermediate- or slow-developing tadpoles particularly in constant water conditions (Fig. 8).

In summary, gene transcript levels showed differences between islands but there was limited consistency in abundance patterns across islands. Some of the gene transcript profiles also differed with regard to treatment, but this effect was not strong.

Regression models

Comparing plasticity effects. None of the interaction terms between populations and difference in gene transcript abundance were significant (>0.10) and were therefore excluded from the final model. There was no relationship between the difference in gene expression status between treatments and the difference in development stage between treatments (Table 3). This suggests that the five genes do not have a strong effect on the degree of plasticity of development. Instead, they could be important for the induction but not the degree of plasticity.

Comparing constitutive effects. In general, populations differed in development stage but levels of the five gene transcripts showed no strong relationship with developmental stage at experimental day 14 (Table 4). This is in

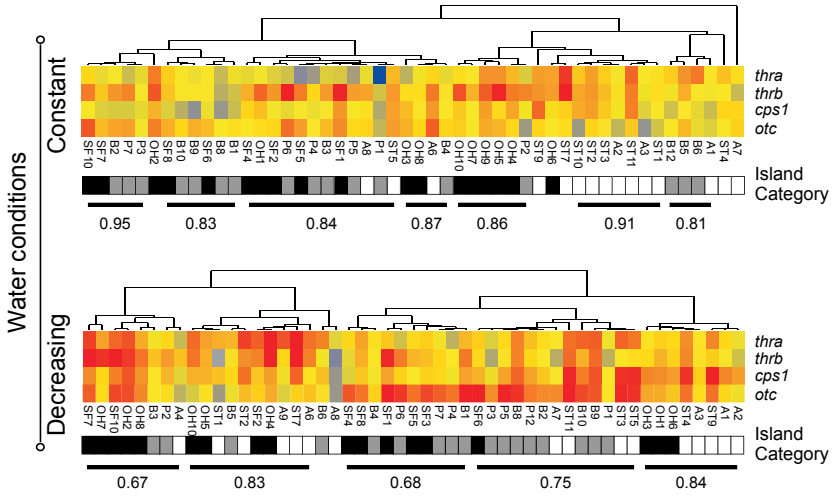

Fig. 6 Interisland hierarchical cluster analysis of hepatic gene transcript abundance profiles derived using qPCR assessment of *Rana temporaria* that were raised in either constant or decreasing water levels and sampled at a fixed date 14 days later. The island and family information for each animal evaluated is depicted below the cluster with the following abbreviations: Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF). Increasing (red), decreasing (blue) and similar (yellow) mRNA abundance using the median of Ålgrundet as the anchor point are shown. Refer to Fig. 3 legend for gene transcript abbreviations. The distribution of individual hepatic mRNA profiles as it pertains to the prevailing water status at each island is noted below each cluster (Island Category) with animals originally sourced from permanent ponds identified with black boxes, those from temporary ponds shown with white boxes and those derived from a mix of permanent and temporary water bodies depicted with grey boxes. The correlation coefficients are shown for the groups indicated by a solid bar selected from the hierarchical clustering. The transcript encoding catalase did not contribute to distinguishing between island categories in this analysis and was therefore omitted.

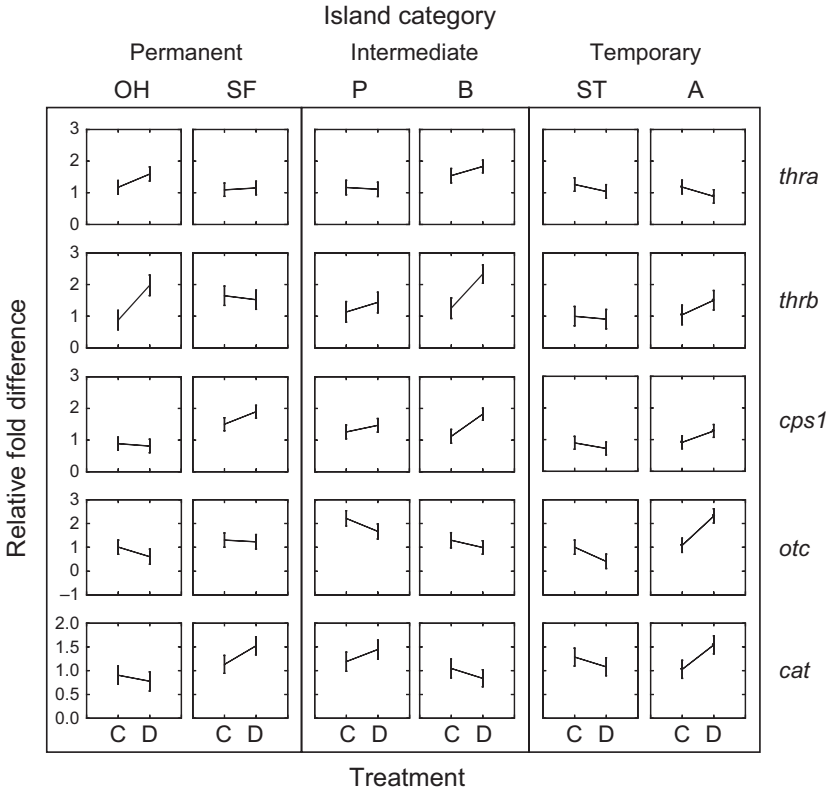

Fig. 7 Relationship between hepatic gene transcript abundance at Gosner stage 37 and water treatment status for *Rana temporaria* tadpoles originating from six island populations including Ålgrundet (A), Sävar-Tärnögern (ST), Bredskär (B), Petlandsskär (P), Öster Hällskär (OH) and Stora Fjäderägg (SF). Refer to Fig. 3 legend for gene transcript abbreviations. Treatments involved maintenance of constant (C) or decreasing (D) water levels, and animal populations were categorized based upon the presence of permanent, temporary or intermediate (mix of permanent and temporary) island pools.

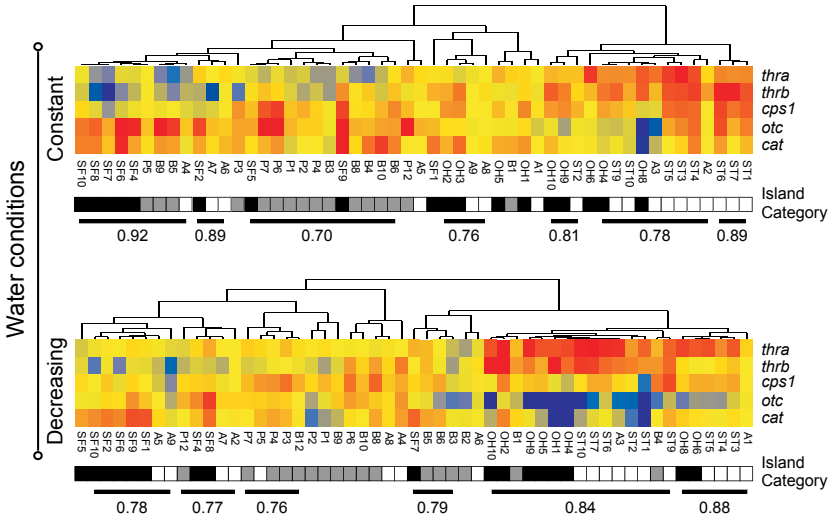

Fig. 8 Interisland hierarchical cluster analysis of hepatic gene transcript abundance profiles derived using qPCR assessment of *Rana temporaria* that were raised in either constant or decreasing water levels and sampled at Gosner stage 37. See the Fig. 6 legend for details.

accordance with the result of treatment effect on development stage at this time point. However, *thra* transcript levels were marginally significant and showed higher abundance at more advanced development stages (Fig. 5), and there was a significant interaction between *thrb* and population, suggesting population-specific effects of *thrb* on development stage at day 14. In conclusion, two of the gene transcripts were associated with development stage under constant water conditions even at this early time of postembryonic development and seem to be important for the constitutive effect of changes in development rate.

Discussion

In the current study, we assessed mRNA transcript abundance differences between island populations of the common frog, locally adapted to different pool dry-

Table 4 The effect of population and gene transcript abundance on development stage at day 14, investigated using ordinal regression. Reported *P*-values, chi-square values and d.f. are for comparison with previous model using likelihood-ratio tests

Model	AIC	LogL	χ^2	d.f.	*P*-value
(A) *thra*					
—	125.78	−58.888			
Population	117.85	−49.926	17.9251	5	0.003*
Population + *thra*	116.57	−48.285	3.2804	1	0.070
Population × *thra*	123.82	−46.910	2.7502	5	0.738
(B) *thrb*					
—	125.78	−58.888			
Population	117.85	−49.926	17.9251	5	0.003*
Population + *thrb*	119.14	−49.570	0.7103	1	0.399
Population × *thrb*	117.79	−43.893	11.3558	5	0.045*
(C) *cat*					
—	125.78	−58.888			
Population	117.85	−49.926	17.9251	5	0.003*
Population + *cat*	118.34	−49.171	1.5092	1	0.219
Population × *cat*	121.64	−45.821	6.7003	5	0.244
(D) *cps*					
—	125.78	−58.888			
Population	117.85	−49.926	17.9251	5	0.003*
Population + *cps*	119.39	−49.697	0.4565	1	0.499
Population × *cps*	127.16	−48.579	2.2370	5	0.815
(E) *otc*					
—	125.78	−58.888			
Population	117.85	−49.926	17.9251	5	0.003*
Population + *otc*	119.84	−49.919	0.0127	1	0.910
Population × otc	125.94	−47.972	3.8941	5	0.565

*denotes significant effects

ing regimes, and also plasticity in gene expression between different water level treatments. We found some differences in gene expression status between islands, such that island tadpoles from fast-developing populations had an increased abundance of hepatic gene transcripts compared with those from slow-developing populations (Fig. 8). This differential gene expression profile was mirrored in the plastic response, where tadpoles subjected to decreasing water level developed faster and demonstrated higher gene transcript levels, in general (Fig. 6). This pattern is predicted by the theory of genetic accommodation, which suggests that the local specialization should mirror the plastic response (West-Eberhard 2005).

While the gene abundance profiles for the plastic response was consistent across our statistical tests, the results for the difference among islands was less consistent among tests. We will discuss the nonconsistence observations below. We found no strong decoupling of expression status between environments in the genes examined. Instead, modulation of gene expression in the same group of genes was responsible for both the constitutive and plastic responses. The lack of decoupling is not surprising because the environmental variation is continuous rather than discrete. Our results are similar to the findings that polyphenisms in horned beetles are caused by environmentally biased, but not independent, gene expression (Snell-Rood *et al.* 2011). Previous studies have shown considerable variation in life history plasticity among the islands, and this variation is caused by heterogeneity in pool drying within islands (Lind & Johansson 2007; Lind *et al.* 2011). As phenotypic plasticity is present in mainland populations (Almfelt 2005), which are the ancestral population that have colonized the island system (Lind *et al.* 2011), it is possible that environmentally based gene expression caused by plasticity has facilitated the variation in constitutive gene expression profiles observed among the island populations.

Phenotypic plasticity in response to pond drying is well studied at the phenotypic level in many amphibian species, and the response to artificial pond drying in the laboratory is to metamorphose at a younger age and lower weight than in constant water level (Newman 1988, 1989; Denver 1997; Richter-Boix *et al.* 2006) and has previously been demonstrated in the present island system (Lind & Johansson 2007; Johansson *et al.* 2010). We did not find a strong response to decreasing water level at day 14 with regard to development stage. However, we found a significant reduction in time to metamorphosis (Gosner stage 42) in response to decreasing water level. We also note that tadpoles had lower mass at Gosner stage 37 in the decreasing water level treatment, but they showed no significant advancement in development, suggesting that the treatment differences in development rate is most pronounced during the later stages of development. Nevertheless, despite the lack of a treatment effect on development at experimental day 14, we found significant treatment effects in gene transcript abundance both at this time and at Gosner stage 37. The phenotypic effects of these changes in gene transcript abundance were, however, not evident until later in the progression of postembryonic development.

Gosner stage 37

To examine treatment-specific effects on gene status, tadpoles must be evaluated at the same development stage so that differences among treatments are not simply the result of stage-dependent effects on mRNA abundance. This was accomplished through analysis of gene transcript profiles in animals reaching Gosner stage 37 across water treatments. Levels of *thrb* and *cps1* mRNA showed increased abundance under decreasing water levels for most of the islands (Fig. 7). Moreover,

we found some island category distinction between fast-, intermediate- and slow-developing island populations. As we observed that decreasing water level resulted in a shorter development time at the cost of a reduced metamorphic weight (results from stage 42), *thrb* and *cps1* seem to be involved in mediating a faster development. Increased abundance of *thrb* mRNA in response to changing aquatic conditions is not an unexpected finding, because thyroid hormones are well known to influence development in tadpoles and *thrb* is a highly responsive gene. When spadefoot toads (*Scaphiopus hammondii*) are exposed to reduced water levels, similar to this experiment, there is an increase in endogenous levels of thyroid hormones (T_3, T_4) (Denver 1998). Both *cps1* and *otc* are also thyroid hormone responsive and are essential for ammonia fixation and removal in the urea cycle pathway (Atkinson *et al.* 1996). In fact, many of the gene transcripts identified using the MAGEX DNA microarray and qPCR analyses in the present study have previously been associated with thyroid hormone-mediated metamorphosis and perturbations thereof (Zhang *et al.* 2006; Helbing *et al.* 2007a,b; Helbing 2012). It is also interesting to note that in addition to urea production/transport (the result of permanent reprogramming of the tadpole liver), several transcripts involved in the regulation of transcription and cell signalling (integral to membrane, cell cycle) were also identified as possible differentiators of island categories.

Although differences in gene expression patterns between closely related populations of amphibians have never been investigated, a study across several species of spadefoot toads have found that the species with highest development rate has a higher levels of both thyroid hormones and *thra* (but not *thrb*) transcript abundance (Hollar *et al.* 2011). Given that we find plastic changes in the induction of *thrb* and population differentiation in *thra* (and a trend in *thrb*), it could be argued that environment-specific gene expression has aided population differentiation. However, although islands differed, we found no consistent significant relationship between transcript level and island drying category, which is the selective force, previously found to be driving adaptive divergence in development rate in this island system (Lind *et al.* 2011). This might seem surprising because past studies have shown that islands from different drying regime categories differ in development rate (Lind & Johansson 2007; Lind *et al.* 2011). However, previous studies have used a regression approach incorporating information from populations derived from more than 10 islands that display a continuous range in the extent of pool drying. Hence, if one or two island populations selected in the present study are demonstrated to express a markedly different developmental status reflective of interisland variation, the reduced number of selected islands could mask the overall pattern previously observed in adaptation to pool drying. Nevertheless, islands were shown to differ in gene expression status, suggesting that, for some of the examined genes, island populations have diverged.

Experimental day 14

A strong effect of water level treatment on tadpole gene transcript abundance at the same time point (experimental day 14) was observed. With a few exceptions, all five transcripts analysed by qPCR showed an increase in abundance with decreasing water levels (Figs 4 and 6). This is not surprising because this represents the combined effect of treatment-specific changes in gene expression status and treatment-specific influence on development rate. Hence, these five gene transcripts are certainly involved in the accelerated development in response to decreasing water levels. While islands differ in abundance of all five gene transcripts at Gosner stage 37, only two (*cps1* and *cat*) showed a significant island effect at day 14. However, tadpoles were at different developmental stages at day 14, which might confound interpretation of the gene transcript profiles. Nevertheless, given that islands differ in development rate, the results suggest that three of the gene transcripts are more important during later stages of the metamorphic programme in affecting progress through development. Hence, we cannot exclude that they are important in determining development early in life, because we did not evaluate mRNA abundance profiles at earlier stages, but we note that small changes in gene transcripts and gene network composition over development time can have a large effect on morphology (Brakefield *et al.* 1996; Abouheif & Wray 2002).

Constitutive and plastic responses

At experimental day 14, we found that tadpoles that had developed to a later Gosner stage had elevated levels of gene transcripts for *thra* and *thrb* (Table 4). Although the pattern could possibly be explained by stage-specific effects on gene expression status (a general increase in transcript during development), it may also reflect a mechanism underlying the constitutive response in development rate in these populations of *Rana temporaria* that includes an inherent alteration in the genetic programming of liver. We did not find a relationship between mRNA levels and development stage for the other three transcripts analysed. This is in accordance with the fact that development stage also did not differ at day 14 between islands. In contrast,

gene expression for the plastic response showed a significant effect at day 14 (Figs 4 and 6). This suggests that although changes in gene expression occur at day 14, there is a certain lag phase until it can be seen phenotypically (i.e. Gosner stage).

An evaluation of the relationship between the difference in gene transcript abundance and development stage between the treatments, which would suggest that an increase in gene transcript levels is directly responsible for the degree of plasticity, demonstrated no strong pattern. This is in contrast with our finding that there are differences in transcripts between the treatments at developmental stage 37 and could have several explanations. First, it may be that there is a transcript change that induces plasticity, but is not involved in the magnitude of plasticity (i.e. plasticity genes), but the finding that these transcripts affect the magnitude of development rate within the control treatment does not support this interpretation. Second, the common time point (day 14) we used for measuring transcript abundance in the present analysis was at an earlier developmental stage (31–35), so plasticity may not be as pronounced as at Gosner stage 37, where we found transcript differences between the treatments (indeed, we did not find any significant treatment effect in development time at stage 37, only at metamorphosis at Gosner stage 42). Hence, more information concerning the status of gene expression during the different stages of development is required to disentangle these effects.

Concluding remarks

We found an increase in abundance of several gene transcripts as a response to artificial pool drying and we also observed elevated transcript levels of select mRNA in tadpoles that had faster development rate. However, as we cannot separate the effect of development rate *per se* and stage-specific gene expression status, we cannot draw any definitive conclusions on whether the plastic increase in gene transcript abundance has aided evolutionary divergence. However, we can conclude that the genes examined show modulation in transcript abundance both in the plasticity and in the constitutive expression of tadpoles. This is not a surprising result, but few studies on natural populations are currently available that show such variation at the level of gene expression (Oleksiak *et al.* 2002; Kvist *et al.* 2013). On the other hand, many more genes than the ones we examined are involved in developmental processes (Abouheif & Wray 2002; Moczek & Nagy 2005), and it remains a challenge to reveal the exact mechanism and contributory factors. A previous study investigating the relationship between development rate and growth rate in this system using a life history approach suggests that different mechanisms are responsible for the plastic and constitutive responses, implying a decoupling of development and growth rate from environment (Lind *et al.* 2011), which we did not find at the molecular level. Hence, whether traits are decoupled or not seems to be trait and stage dependent. Nevertheless, evolutionary divergence in spadefoot toads adapted along a drying gradient has been reported to increase thyroid hormone receptor expression (Hollar *et al.* 2011), which is in agreement with our finding that developmental rate is positively correlated with the levels of *thra*, suggesting that similar mechanisms underlay both the plastic and the constitutive response to drying. More detailed studies to separate the effect of development rate and stage-specific gene expression in combination with nonbiased transcript analysis using RNA-seq methodologies on Ranid species (Helbing 2012) are warranted.

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The study was designed by F.J. and C.C.H. F.J. and M.I.L. collected animals and performed the animal tests. C.C.H. and N.V. performed the microarray and qPCR procedures. All authors contributed to the analyses/interpretation of the data and writing of the manuscript.

Data accessibility

The microarray data set has been submitted to the Gene Expression Omnibus (GEO number GSE42274). The raw data used in the analyses are provided in Tables S5 and S6 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Materials and methods.

Table S1 Spot signal intensities of selected gene transcripts used for microarray normalization.

Table S2 qPCR Primer information.

Table S3 Gene transcripts that were significantly different following MAGEX DNA microarray analysis between constant and decreasing water conditions for the Öster Hällskär population at Gosner stage 37.

Table S4 Gene transcripts that were significantly different following MAGEX DNA microarray analysis between constant and decreasing water conditions for the Stora Fjäderägg population at Gosner stage 37.

Table S5 Gosner stage (stage) and gene transcript (*thra*, *thrb*, *cat*, *cps1* and *otc*) relative fold difference values at day 14.

Table S6 Life history traits (weight in grams and age in days) and gene transcript (*thra*, *thrb*, *cat*, *cps1* and *otc*) relative fold difference values at Gosner stage 37.

Fig. S1 Cycle threshold (Ct) values obtained for the rpL8 normalizer gene transcript for (A) fixed-date and (B) Gosner stage 37 tadpoles analysed in the present study.

Fig. S2 Comparison of hepatic gene transcript abundance between islands using MAGEX DNA microarray analysis of *Rana temporaria* tadpoles reared under different water conditions and evaluated at Gosner stage 37.

Fig. S3 Effects of water conditions on *Rana temporaria* tadpole hepatic gene transcript levels determined using MAGEX DNA microarray analysis.