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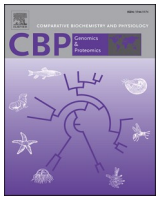

Research article

Transcriptome variation in banded newt (*Ommatotriton vittatus*) during its life cycle and habitat transition

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ABSTRACT

Israel represents the southern limit of the distribution of the banded newt (*Ommatotriton vittatus*). The life cycle of *O. vittatus* includes several distinct phases: eggs, aquatic larvae, a terrestrial phase and an aquatic reproductive phase. We investigated differences in gene expression during the life cycle and transition of banded newts between terrestrial and aquatic habitats using mRNA-seq. We identified ~10 k genes that were differentially expressed (DE) in one of the pairwise comparisons between 3 groups: 1 - terrestrial newts (males and females), 2 - aquatic newts (males and females), 3 - aquatic larvae before metamorphosis. The groups were clearly defined by Principal Components Analysis (PCA). The greatest difference was between aquatic newts (males and females) and aquatic larvae: ~7.4 k DE genes. Of special interest were the ~2.4 k genes DE between the aquatic and terrestrial phenotypes. These included prominent candidates with known roles in kidney function (uromodulin homologs were strongly associated with aquatic lifestyle), tissue structure (keratins), and the thyroid hormone signaling modulator DUOXA1. Additional developmental and metabolic pathways overrepresented among the identified DE genes included "epidermis development", "nervous system development", "nucleotide-sugar biosynthesis". Overall, both metamorphosis and environmental transition of banded newts involve extensive transcriptomic remodeling involving developmental, metabolic, and cellular pathways. Understanding the roles of these pathways and individual genes is instrumental for studies of transition between habitats, especially those affected by climate change. Furthermore, the phenotypic flexibility of the newt and the underlying regulation of gene expression can shed light on the evolution of terrestrial vertebrates.

1. Introduction

The banded newt (*Ommatotriton vittatus,* synonymous with *Triturus vittatus*) is one of three species of genus *Ommatotriton* (*O. nesterovi, O. ophryticus and O. vittatus*) found in Turkey, Syria, and Israel, and are adapted to extremely unstable conditions (Van Riemsdijk et al., 2017; Degani, 2019; Degani and Ahkked, 2021) (Fig. 1). The two species, *O. ophryticus* and *O. vittatus*, differ in trunk vertebra count, genome size and allozyme data (Litvinchuk et al., 2005). The northern taxon, *O. ophryticus*, is subdivided into two geographical fragments: the "western group" populations from western Anatolian Turkey, and the "eastern group" populations distributed in the rest of Turkey and Western Caucasus. *Ommatotriton vittatus* is found in Israel (Degani, 2019), which is the southern limit of the genus's distribution (Fig. 1A), and which is characterized by extreme seasonal changes in the environment. Thus, the habitat described in our study (Nahalit pool in the upper Galilee; full detail in Methods) is aquatic only ~1 month out of the year, and this is typical for newt habitats in Israel; while northern habitats are often at least partially aquatic through most of the year. This fact ostensibly makes the adaptability of the newt to the environmental changes especially significant for its survival, and represents a strong selective pressure for such adaptability, which is likely less pronounced in areas further north, where the climate is less arid.

While amphibians have experienced significant evolutionary changes in reproductive strategies and life cycles, aquatic larvae is an ancestral trait within the three primary amphibian clades (frogs, salamanders, and caecilians) maintained by a substantial number of present-day species, based on a broad study of 4025 species (Liedtke et al., 2022). Additionally, analysis of adaptation to different environmental conditions based on data from multiple species (Sinai et al., 2022)

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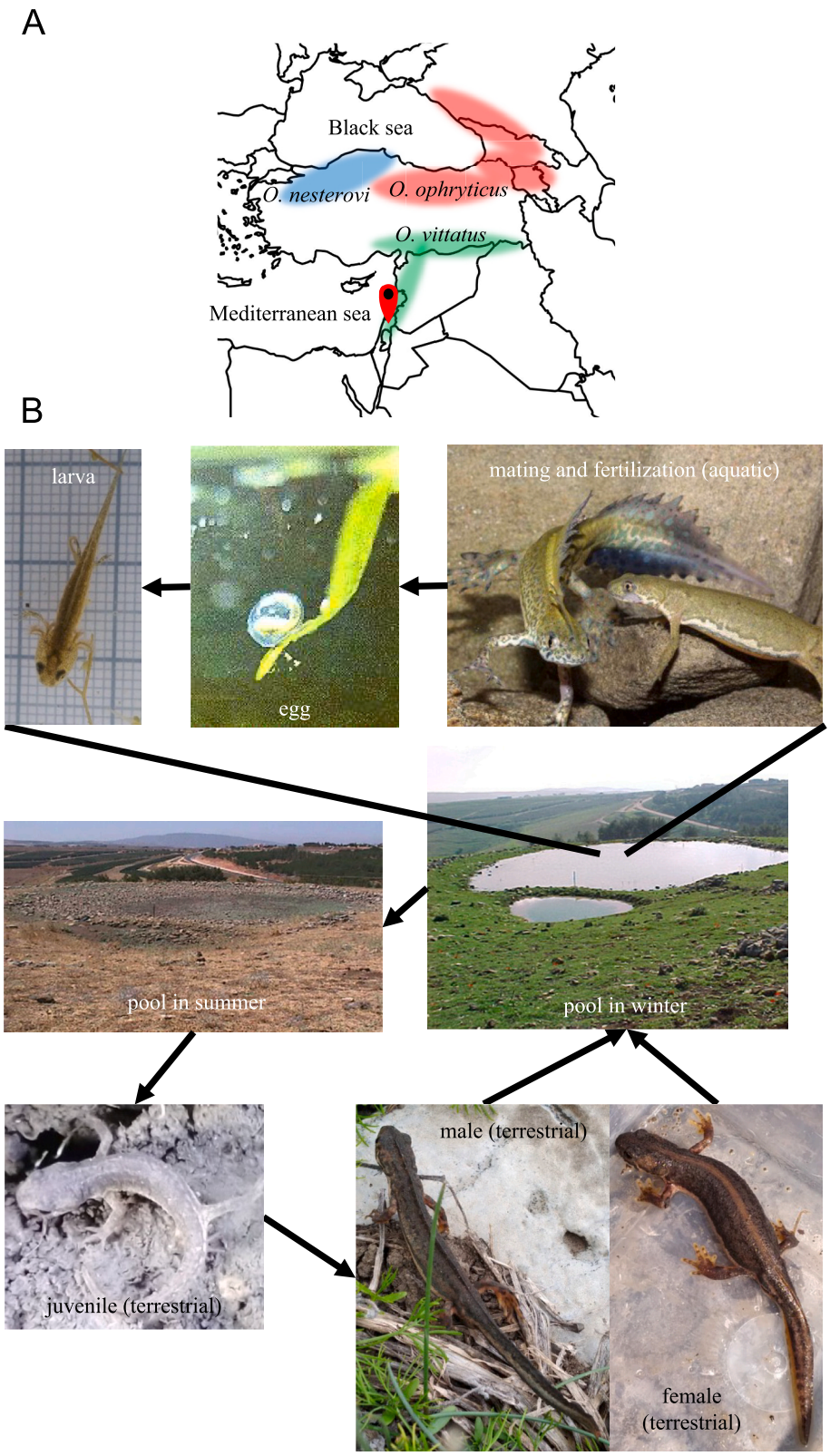

Fig. 1. A. Scheme of the geographical ranges of banded newt species (*O. nesterovi, O. ophryticus and O. vittatus*). Adapted from (Van Riemsdijk et al., 2022b) and World Map by Vemaps.com. The sampling location is marked by a pin. B. Life cycle of *O. vittatus* (Van Riemsdijk et al., 2017; Degani, 2018). (Photographs of the pool and newts in aquatic and terrestrial phases: Gad Degani).

showed that organisms' developmental plasticity in response to temperature changes across latitudes. Crucial traits impacted by semi-arid and arid habitats include a high tadpole count, brief growth and metamorphosis periods, the search for shelter to avoid dehydration, and physiological adjustments for urea accumulation in body fluids (Sinai et al., 2022). Specifically, the adaptation of *O. vittatus* to the southern limit of newt populations in Israel and many aspects of *O. vittatus* biology in Israel have been investigated: its life cycle (Geffen et al., 1987; Pearlson and Degani, 2008), ecological conditions during larval growth (Degani, 1982; Pearlson and Degani, 2007), genetic variation (Degani, 2018) and environmental hiding-place seeking behavior after metamorphosis and genetic differentiation of the larvae in various breeding places (Degani and Ahkked, 2021). The development of the newt *Triturus carnifex*, which is very similar to *O. vittatus*, has been described from egg deposition to hatching and illustrated with the use of photographs of living embryos (D'Amen et al., 2006). The life cycle of *O. vittatus* includes several distinct phases: eggs, aquatic larvae, a terrestrial phase and an aquatic reproductive phase (Degani, 2017) (Fig. 1B). Due to the extreme seasonal variation of its habitat, this species is thus particularly appropriate for a study of transition-related transcriptomic changes.

In the current study, we compared the tail transcriptomes of aquatic larvae, terrestrial adults (females and males), and aquatic adults (females and males), in order to understand how changes in gene expression help this species adapt to aquatic vs. terrestrial lifestyle in the adult phases, as represented by the adult phenotypes, in addition to metamorphosis - related changes in gene expression, referring specifically to the changes from larvae to adults.

2. Materials and methods

2.1. Study area

Nahalit Pool is a winter pool, located on the slopes of an agricultural settlement in the Upper Galilee mountains, among grazing areas for cattle and horses that are rich in annual vegetation (longitude

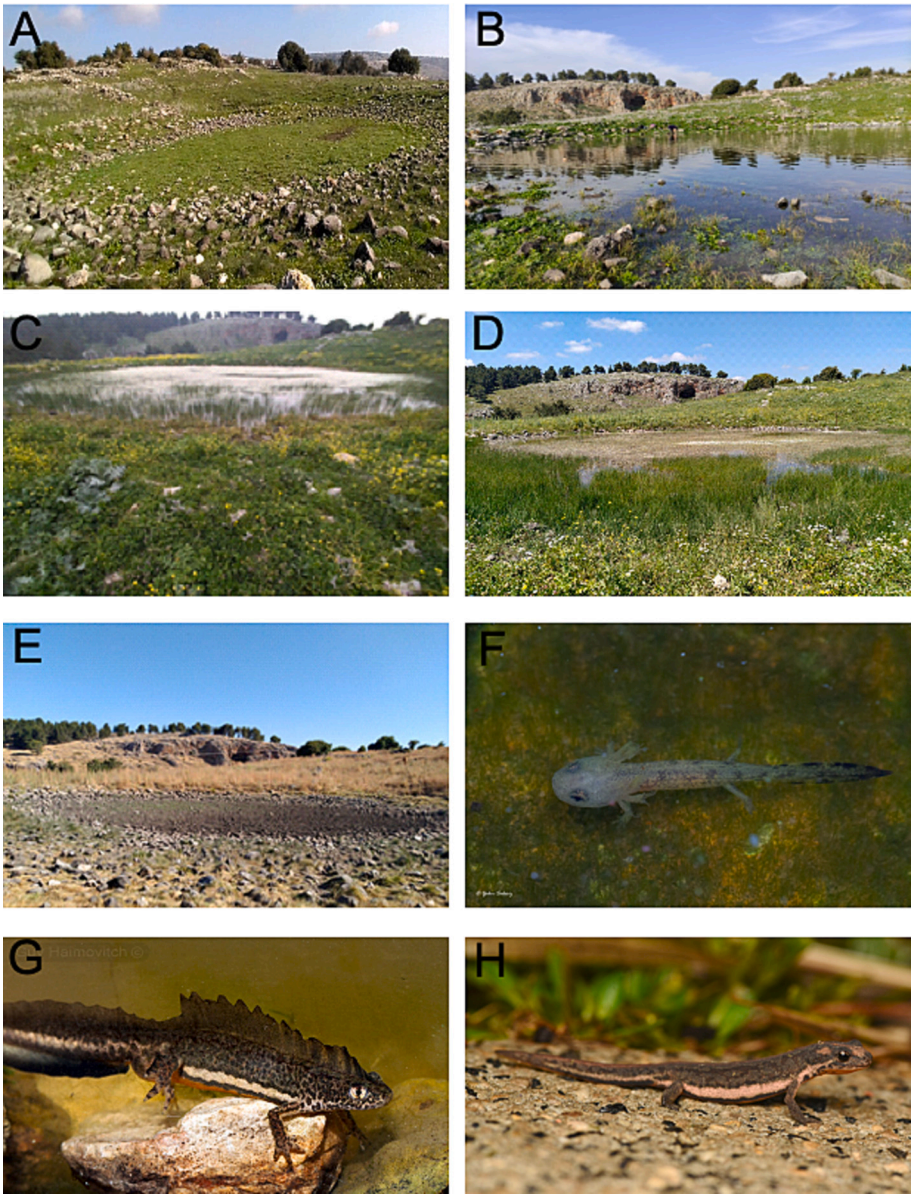

Fig. 2. A–E: Variations in a winter pond (Nahalit Pool—35°27′48″E 33°04′56"N, 665 m ASL) from winter to summer (2021): A – December, B - January 25th, C – February, D – April 27th, and E – May 25th. (Photographs: Gad Degani.) F-H: Phenotypic variation in *O. vittatus*: F – aquatic larva (photograph: Yakov Salaviz), G – aquatic adult male (photograph: Guy Haimovitch), H – terrestrial adult (photograph: Oren Auster).

35°27′48″E, latitude 33°04′56"N, altitude 665 m above sea level) (Degani, 2018). The pools are filled with runoff water; water also seeps in from the settlements' barns and coops. The pool is divided into a deeper part, about 2 m in depth, covering a total area of about 50 m^2 (Fig. 2) which holds water from around January to February (Degani and Ahkked, 2021); and the larger and shallower part, where the depth reaches about 80 cm at the center, the total area covers about 1000 m^2 and holds water from January to June. In both parts of the pool, aquatic vegetation develops in the water: the common water-crowfoot (*Ranunculus peltatus*) and the common spike-rush (*Eleocharis palustris*).

2.2. Sample collection

The study was approved by the Israel Nature and National Park Protection Authority (permit 2020/42661). The pool area and its surroundings were explored from October to May (Degani and Ahkked, 2021) (Fig. 2). While the pool was dry, the entire area was examined once a week for newts in the terrestrial phase hiding under stones (during December 2020). When the pool was filled with water, aquatic adult newts (in January–February 2021) and larvae (in April 2021) were collected from it using a round hand net 40 cm in diameter with a mesh size of 0.1 cm. The net was immersed 40 cm into the water and 3–4 rotational movements of about 1 m from side to side were performed. All terrestrial and aquatic newts and aquatic larvae were released back into their respective habitats after being measured, photographed, and identified to the species level (Degani and Ahkked, 2021). A total of 20 tail samples (clipped tips of tails) were taken from 4 terrestrial females, 6 terrestrial males, 4 aquatic males, 1 aquatic female and 5 aquatic larvae (the phenotypic differences are exemplified in Fig. 2 F—H) and frozen in 1.5-ml tubes with RNALater (Thermo Fisher Scientific) at −20 °C until further analysis.

2.3. RNA extraction

Tissue samples were removed from RNALater and homogenized using a TissueRuptor (Qiagen). Total RNA was extracted from each sample with TRI Reagent (Sigma) using the manufacturer's protocol. The concentration and integrity of RNA were examined using a ThermoFisher Scientific NanoDrop 8000 Spectrophotometer and an Agilent 4150 TapeStation. 13 RNA samples had OD260/280 $\geq$ 1.8 and RNA integrity number (RIN) $\geq$ 7, and these were selected for further analysis.

2.4. Transcriptome analysis

RNA-seq libraries were prepared at the Crown Genomics Institute of the Nancy and Stephen Grand Israel National Center for Personalized Medicine, Weizmann Institute of Science using the in-house polyA-based RNA seq protocol (INCPM mRNA-Seq). Briefly, the polyA fraction (mRNA) was purified from 500 ng of total input RNA followed by fragmentation and generation of double-stranded cDNA. After cleanup with Agencourt AMPure XP beads (Beckman Coulter), end repair, A base addition, adapter ligation and PCR amplification steps were performed. Libraries were quantified by Qubit (Thermo Fisher Scientific) and TapeStation (Agilent). Sequencing libraries were constructed with barcodes to allow multiplexing of 24 samples on an Illumina NovaSeq 6000 machine, using an SP (100 cycles) kit. 100 bp single reads were sequenced on 2 lanes. The output was between 13 and 40 million reads per sample. Fastq files for each sample were generated by bcl2fastq v2.20.0.422. Poly-A/T stretches and Illumina adapters were trimmed from the reads using cutadapt; resulting reads shorter than 30 bp were discarded. Trimmed reads were used for assembly using Trinity (v2.13.2) (Grabherr et al., 2011). After the assembly, read representation was assessed with Bowtie2 (v2.3.4.3) (Langmead and Salzberg, 2012) and completeness was assessed using BUSCO (v5.4.4) (Manni et al., 2021) using the eukaryota_odb10 (default) lineage dataset. Clustering of reads was performed using CD-hit (v4.8.1) (Fu et al., 2012). Transdecoder (v5.7.0) (Haas, BJ. https://github.com/TransDecoder/TransDecoder) was used to predict coding regions from the long transcript of each gene. Eggnog-mapper was used to add functional annotations to the proteins' sequences. The resulting annotation was merged with the DE analysis results (below).

2.5. Differential expression analysis

Abundance estimation was performed by RSEM (alignment-based). Trinity's script was used, the command is included as part of the provided code (Supplementary Information). The resulting counts matrix (Trinity_genes.isoform.counts.matrix) was used for DE analysis. Differentially expressed genes were identified using DESeq2 (Love et al., 2014) with the betaPrior, cooksCutoff and independentFiltering parameters set to False. Default DESeq2 normalization was performed to adjust for library read counts. Raw *P* values were adjusted for multiple testing using the procedure of Benjamini and Hochberg (Benjamini, 2010). Pipeline was run using snakemake (Köster and Rahmann, 2012; Love et al., 2014; Degani and Ahkked, 2021). The 1000 most variable genes from the DESeq2 analysis served for Principal Component Analysis (PCA) and Hierarchical Clustering (HCL) plots using default values. Volcano plots were prepared using the ggplot R package using DESeq2 output.

2.6. GO and pathway analysis

Functional enrichment analysis was performed using g:Profiler (version e109_eg56_p17_1d3191d) with g:SCS multiple testing correction method, applying a significance threshold of 0.05 (Raudvere et al., 2019). *Xenopus tropicalis* was used as a reference species. The gene lists contained DE transcripts, either up- or down-regulated in each comparison, with adjusted *p*-values $<$0.05.

3. Results

The analysis of the transcriptome variation in the tail tissues at the different phases of the newt life cycle shows clear and distinct differences between the gene expression of 3 groups: 1 – terrestrial newts (males and females), 2 - aquatic newts (males and females) and 3 – aquatic larvae before metamorphosis. The clustering of samples according to life cycle phase, but not according to sex, is apparent in PCA (Fig. 3A) and hierarchical tree analysis (Fig. 3B).

De-novo transcriptome assembly from all samples resulted in a total of 143,575 assembled transcripts; 116,464 "genes" were represented. BUSCO completeness analysis searched a total of 255 groups, of which 249 (97.6 %) were complete, 151 (59.2 %) were also single-copy, 98 (38.4 %) were duplicated, 6 (2.4 %) were fragmented, and 0 were missing. BLAST hits were observed for 31,905 genes (min e-value was used to select the best hit). Of them, 15,299 (48 %) were classified as bacterial and were filtered out. 12,117 hits belonged to eukaryotes. Of these, 3388 were similar to known avian genes, 2893 to mammals, 1670 to amphibians, 514 to bony fishes, and 239 to other eukaryotes.

To assess differential expression (DE) of genes between the biological groups, reads from the individual samples were aligned to the assembly. The largest number of DE genes was found between aquatic larvae and aquatic adult newts (7384 DE genes); comparing the aquatic and terrestrial adult groups showed 2399 DE genes (Fig. 4). This was also apparent when looking at the changes in individual genes (volcano plots, Fig. 5). The most prominent DE genes in all comparisons (DE analysis output, Supplementary Information) were those coding for different variants of keratins and homologs of uromodulin. The latter were dramatically downregulated (by 4–5 orders of magnitude in expression) in terrestrial adults vs. aquatic larvae, and likewise dramatically up-regulated in aquatic vs. terrestrial adults. We further screened DE genes for gene names and pathways previously reported to take part in amphibian metamorphosis. Homologs of several known

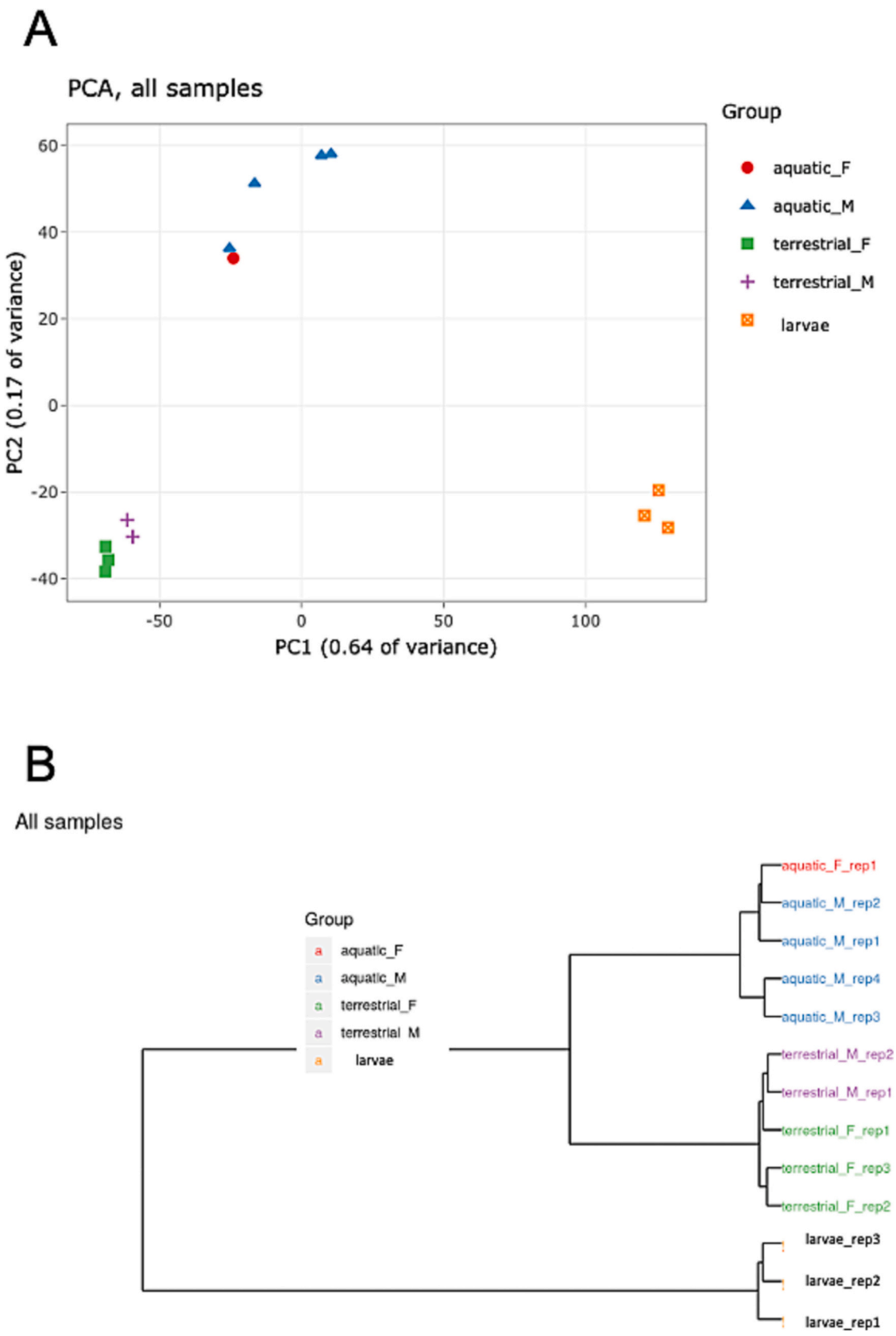

Fig. 3. Triton transcriptomes cluster according to life cycle phase, but not according to sex. A. Principal Component Analysis (PCA) of *O. vittatus* samples: aquatic females and males, terrestrial females and males, and larvae, based on all polyA+ transcripts. B. HCL (hierarchical clustering) of *O. vittatus* samples as in A.

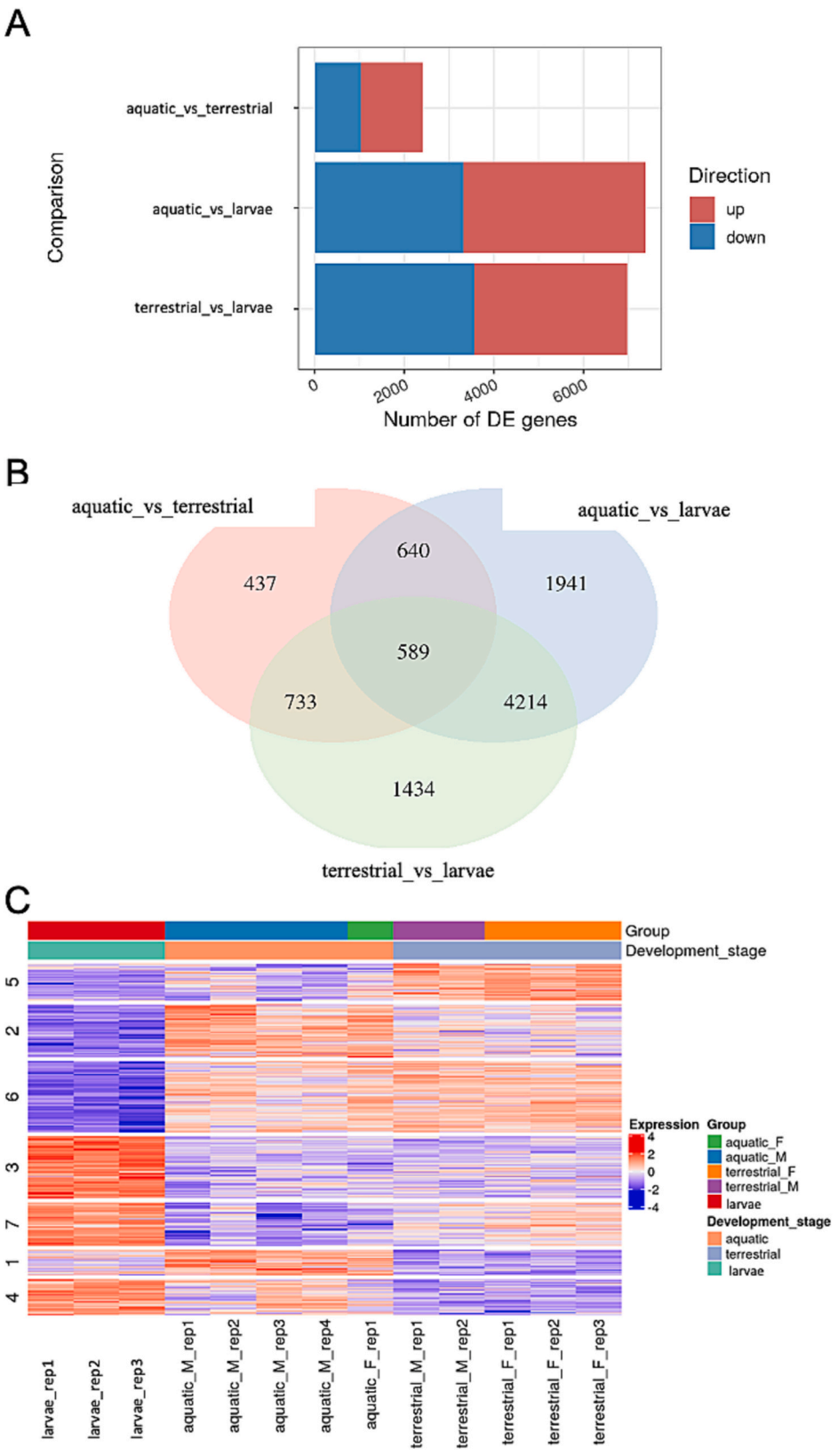

Fig. 4. Statistics of differentially expressed genes between *O. vittatus* sample groups: aquatic females and males, terrestrial females and males, and larvae, based on polyA+ transcripts. A. Numbers of DE genes in each comparison. B. Venn diagram of genes that were DE in one or more comparisons between the groups. C. HCL heatmap of 9988 genes that were DE in at least one pairwise comparison between the groups. Threshold for DE significance: padj $\leq$0.05, $|\log2FoldChange| \geq 1$, min count $\geq$30.

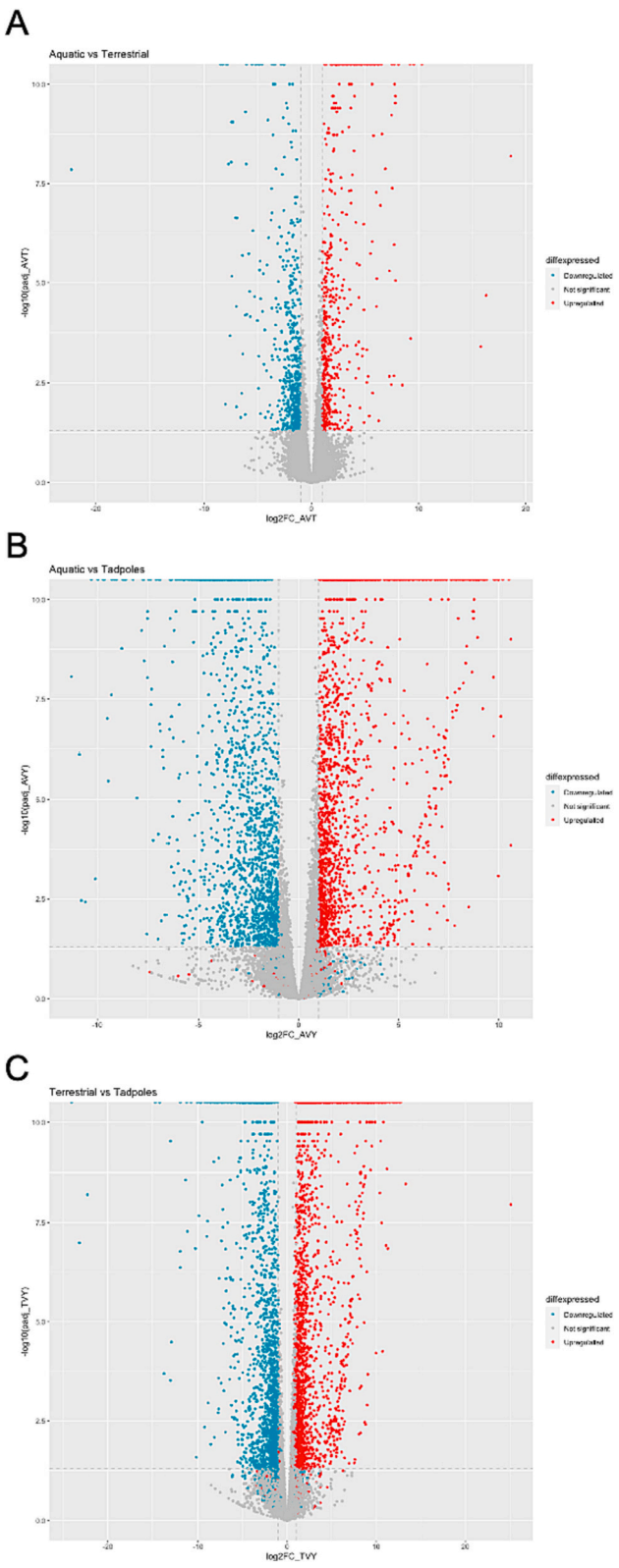

Fig. 5. Volcano plots highlighting differential gene expression between *O. vittatus* sample groups: aquatic females and males, terrestrial females and males, and larvae, based on polyA+ transcripts. Downregulated genes are in cyan, upregulated genes are in red, and genes not showing significant changes in expression are in grey. A. Aquatic adults vs terrestrial adults. B. Aquatic adults vs aquatic larvae. C. Terrestrial adults vs aquatic larvae. Threshold for DE significance: padj ≤ 0.05, $|\log2FoldChange| \geq 1$. All gene expression information can be found in Supplementary Information.

genes involved in thyroid hormone signaling were identified in the newt transcriptome (Supplementary Information); among them, DUOXA1 (Dual Oxidase Maturation Factor 1) and PTH1R (Parathyroid Hormone 1 Receptor) were downregulated ~3-fold (padj = 0.024) and ~2.6 fold (padj = 0.0012), respectively, while DIO3 (Iodothyronine deiodinase) was upregulated ~3-fold (padj = 0.0059) in aquatic vs. terrestrial adults. In agreement with a consistent role in the aquatic-terrestrial switch, DUOXA1 was also upregulated ~8.5-fold (padj = 6.7E-08) in terrestrial adults vs. aquatic larvae; while DIO3 was upregulated 6.6-fold (padj = 0.000002) in aquatic adults vs. aquatic larvae, indicating an association with metamorphosis.

To gain insights into the functions of the DE genes, we performed g_profiler GO and pathway analysis. The top GO terms and pathways for the different comparisons are presented in Table 1 and Supplementary Fig. 1. Overrepresented developmental and metabolic pathways included "epidermis development" (top term in genes upregulated in

Table 1
Top GO terms and pathways overrepresented in each of the different comparisons, based on g_profiler results (Supplementary Fig. 1). All gene expression information can be found in Supplementary Information.

Comparison	Change	Term	p_{adj}
Aquatic adults vs Aquatic larvae	Down	Anatomical structure development	1.05×10^{-15}
		Sarcomere	4.87×10^{-15}
		Nervous system development	8.67×10^{-15}
		Myofibril	1.19×10^{-14}
		Contractile fiber	1.59×10^{-14}
		Cell junction	4.95×10^{-14}
	Up	Epidermis development	1.64×10^{-6}
		Oxidoreductase activity	3.93×10^{-5}
		Protein-glutamine gamma-glutamyltransferase	1.28×10^{-4}
		Aminoacyltransferase activity	4.15×10^{-4}
		Apical junction complex	6.2×10^{-4}
Terrestrial adults vs Aquatic larvae	Down	Sarcomere	8.2×10^{-12}
		Myofibril	2.11×10^{-11}
		Contractile fiber	2.85×10^{-11}
		Nervous system development	1.07×10^{-8}
		Synapse	5.17×10^{-7}
	Up	Oxidoreductase activity	2.46×10^{-5}
		Mitten deformity	2.98×10^{-4}
		Actin binding	4.22×10^{-4}
		Intermediate filament cytoskeleton	1.5×10^{-4}
Aquatic adults vs Terrestrial	Down	Transferase complex	1.87×10^{-2}
		Nucleoside-triphosphatase	3.4×10^{-2}
		GTPase regulator activity	3.4×10^{-2}
	Up	Nucleotide-sugar biosynthetic process	7.52×10^{-5}
		Intermediate filament cytoskeleton	3.19×10^{-3}
		UDP-*N*-acetyl glucosamine biosynthesis	6.16×10^{-3}

aquatic adults vs. larvae), "nervous system development" (downregulated in both aquatic and terrestrial adults vs. larvae), "nucleotide-sugar biosynthesis" (top term in genes upregulated in aquatic vs. terrestrial adults), "oxidoreductase activity" (top term in genes upregulated in terrestrial adults vs. larvae), and "sarcomere" (downregulated in both aquatic and terrestrial adults vs. larvae), potentially indicating the importance of these functions in metamorphosis (from larva to adult) and transition between aquatic and terrestrial lifestyle in the adult phases.

4. Discussion

The life cycle, behavior and genetic variations among *O. vittatus* populations in northern Israel down to the central coastal plains and near the desert, were described previously; see review, (Degani, 2017). Our study found transcriptomic differences not only between aquatic larvae and adult newts, but also between the terrestrial and aquatic adult phases characteristic of the genus *Ommatotriton* (Van Riemsdijk et al., 2017; Van Riemsdijk et al., 2022a; Van Riemsdijk et al., 2022b). Until recently, most "omics" studies of the genus *Ommatotriton* focused on differences between populations (Van Riemsdijk et al., 2017; Van Riemsdijk et al., 2022a; Van Riemsdijk et al., 2022b) and in phylogenetic aspects, unlike our study which for the first time examined the developmental changes in gene expression in an *Ommatotriton* species. We identified DE genes between the different life phases of the newts, namely aquatic larvae and adults in aquatic and terrestrial phases. The overall difference in gene expression between the aquatic larvae and adults is greater than the difference between the terrestrial and aquatic phases, which mirrors the great physiological changes in metamorphosis (see for example Warburg, 1971; Warburg, 1997; Degani, 2018; Degani and Ahkked, 2021) and is in agreement with studies in other amphibians which undergo metamorphosis. Thus, in leopard frogs (*Lithobates sphenocephalus*) 42 % of genes were differentially expressed between aquatic larvae and juveniles (Schott et al., 2022). Similar findings were reported in other newt species, e.g. the ribbed newt (*Pleurodeles waltl*) (Matsunami et al., 2019).

We also identified DE genes between the aquatic and terrestrial phases in adult newts, which to the best of our knowledge has not been described in *O. vittatus* or other newts. Thus, uromodulins being the main protein secreted by the kidneys, the differential expression of uromodulin homologs between aquatic and terrestrial phases of the newt is in agreement with the importance of changes in kidney function between aquatic and terrestrial environments. It is well-known that the pattern of nitrogen excretion changes in the metamorphosis from aquatic larva to terrestrial adult, and also in the switch between terrestrial and aquatic adult phenotypes (Nash and Fankhauser, 1959). Thus, ~75 % of the nitrogen is excreted as ammonia and 25 % as urea in the larval stage; terrestrial adults excrete ~87 % of the nitrogen as urea; and aquatic adults partially reverse this change, doubling ammonia excretion to 26 % (Nash and Fankhauser, 1959). In addition to the known kidney-specific functions of uromodulin in mammals, uromodulin-like genes in amphibians appear to be both more ubiquitously expressed (specifically in the skin), and involved in specific morphological changes (Mori et al., 2009). Expression of uromodulin-like genes in newt fins, and morphologically-related changes in their expression as reported here, is in line with these prior findings.

Additionally, we report the association of elevated DUOXA1 (Dual Oxidase Maturation Factor 1), a positive modulator of thyroid hormone signaling (Szanto et al., 2019), with the terrestrial phenotype. Thyroid hormone signaling is a key trigger of amphibian metamorphosis (Brown and Cai, 2007), and our findings suggest that it may also play a part in the aquatic-terrestrial switch in adult newts (Suggested model, Fig. 6). Notably, the highly conserved DUOX and DUOXA genes which exist in single copies in fishes, underwent tandem duplication in vertebrates before the amphibian divergence (Grasberger and Refetoff, 2006), suggesting a possible functional link to terrestrial adaptation.

The samples examined in the different phases were taken from the newts' tails. Thus, it is not surprising that the GO terms and pathways overrepresented in the different comparisons, are mainly related to structural anatomy and connective tissues, inter-cell connections, muscle and nerve cell generation, etc. Specifically, "nervous system development" was overrepresented among genes highly expressed in larvae; while "epidermis development" was overrepresented among genes upregulated in the aquatic adults, which may be related to the altered physiological roles of the skin (e.g. in gas exchange) in the aquatic vs. terrestrial adult phase.

Our study was limited by the sample size, which was a consequence of the difficulty of capturing newts in the wild. Additionally, ethical considerations dictated that only tail samples be taken. Thus, the observed changes in gene expression may not be representative of those changes that are most functional in the developmental and physiological processes underlying the phenotypes. For example, we would expect the kidneys to produce a clearer picture of the gene expression changes most relevant to the terrestrial-aquatic switch. However, the large number of DE genes identified, and the probable functional role of a subset of them based on prior knowledge, indicate that the use of tail section sampling, although only partially representative, is nevertheless warranted and informative. Future research, to include qRT-PCR validation of changes in the abundance of specific candidate transcripts and in specific tissues, could further elucidate the molecular mechanisms underlying newt

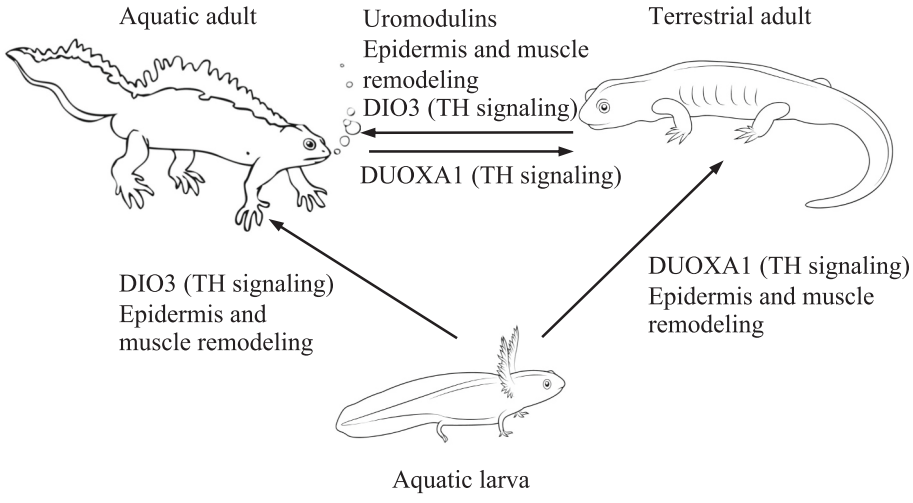

Fig. 6. A proposed qualitative model highlighting the link of selected genes and pathways to different phases in the life cycle of *O. vittatus*.

development and habitat transition. As climate change inevitably impacts the yearly wet-dry cycle of many habitats, the adaptability of the newt to aquatic and terrestrial lifestyles becomes paramount to its survival. Understanding the roles of specific genes and pathways in such transition, and assessing the variability in these traits between and within populations, can help predict population dynamics in vulnerable habitats and direct conservation efforts.

Conclusions: Both metamorphosis and environmental transition of banded newts involve extensive transcriptomic remodeling involving developmental, metabolic, and cellular pathways. Understanding the roles of these pathways and individual genes is instrumental for studies aimed at conservation and preservation, especially in habitats affected by climate change. Furthermore, the phenotypic flexibility of the newt and the underlying regulation of gene expression can shed light on the evolution of terrestrial vertebrates.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2024.101203.

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CRediT authorship contribution statement

Gad Degani: Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Ari Meerson:** Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequence reads are deposited in the SRA (BioProject PRJNA990561). The assembled transcriptome file is available upon request from the authors.

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