1 At least two expressed genes for transcription factors Pitx2 and Rpx are present in 2 common carp and are upregulated during winter acclimatization.

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25 ABSTRACT

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The mechanisms of seasonal acclimatization in eurythermal fish such as common 27 carp are not fully understood. Here, we concentrate on the regulation of pituitary 28 29 factors, as this organ was shown to be highly affected by seasonal changes. We 30 cloned and sequenced two different cDNAs for each of the transcription factors Pitx2 and Rpx, known to play a role in pituitary development. We show that these genes 31 are conserved throughout evolution, to different degrees depending on the specific 32 domain considered. Finally, we show that the cDNAs for both factors are clearly up-33 regulated during the winter season, in sharp contrast to other regulators such as Pit1 34 or pituitary hormone genes such as prolactin (prl) and growth hormone (gh). Our 35 results suggest that increased expression of Pitx2 and Rpx contributes to seasonal 36 37 adaptation of common carp to winter conditions.

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40 Key words: Pitx2; Rpx; gene expression; carp fish; seasonal control; acclimatization;

42 INTRODUCTION

Eurythermal fish rearrange their molecular and cellular functions to compensate for 43 the circannual environmental seasonal changes of their habitat. This acclimatization 44 process occurs through a cyclical reprogramming of molecular processes, involving 45 in common carp (Cyprinus carpio) the modulation of transcriptional and translational 46 events in various tissues (Krauskopf et al., 1988; Figueroa et al., 1994; Goldspink et 47 al., 1995; Kausel et al., 1999; Molina et al., 2002). Likewise, although much less 48 complex than the process of acclimatization, long-term adaptation to different 49 50 temperatures also involves reprogramming of gene expression in different tissues 51 (Gracey et al., 2004), including carp pituitary gland (Figueroa et al., 1997; Arends et al., 1998). In fish, the pituitary seems to function as a central node controlling the 52 adaptive compensatory response to stressors or environmental changes. Increased 53 expression of prolactin (Prl) mRNA, the most versatile pituitary hormone, occurs in 54 55 the rostral pars distalis (RPD) of summer-acclimatized carp, as compared to the negligible level of transcription detected in winter-acclimatized fish (Figueroa et al., 56 57 1994). Photoperiod appears to be a particularly relevant factor for modulation of prl transcription in carp (Figueroa et al., 1997). Another pituitary hormone of the Prl 58 59 family that is up-regulated during the summer season is growth hormone (Gh) 60 (Figueroa et al., 2005). In good correlation with these observations, expression of the main regulator of these genes, the pituitary-specific transcription factor Pit1 is 61 62 strongly increased during acclimatization of the common carp (Cyprinus carpio) from winter to summer (Kausel et al., 1999). 63

During embryogenesis, various other transcription factors were shown to be involved in morphogenesis of the primordial pituitary and in specification and differentiation of the specific cell types in mammals (Zhu *et al.*, 2007). In zebrafish similar functions

are played by pit1 (Herzog et al., 2003; Nica et al., 2004), eya1 (Lopez et al., 2006; 67 Nica et al., 2006) or asc1 (Pogoda et al., 2006), suggesting a good conservation of 68 regulation of pituitary ontogenesis from fish to man (Pogoda and 69 the 70 Hammerschmidt, 2009). Rpx1 and Pitx2 are two members of the "paired-like" 71 homeobox domain gene family that are expressed in mouse at early stages in development (Olson et al., 2003; Chou et al., 2006). Pitx2 is required for formation of 72 various organs, including palate, heart, lung, muscle and tooth (Amendt et al., 1998). 73 74 It is mutated in the human Rieger syndrome and has been well studied for its role in 75 determination of left-right asymmetry during embryogenesis (Essner et al., 2000). In the pituitary, Pitx2 deficiency leads to defects in cell proliferation (hypoplasia) as well 76 as to expansion of the Pit1 lineage and differentiation of gonadotrope cells. It binds 77 to and activates the promoters of the prl, gh, gsua, fshb, lhb, pomc genes in 78 combination with other, neighboring factors (Quentien et al., 2002). Different 79 80 mutations affecting human PITX2 DNA-binding or trans-activation have been described in Rieger patients (Quentien et al., 2006). Besides its N-terminal, 81 82 conserved DNA binding homeodomain, the Pitx2 factor contains a C-terminal domain (OAR, otp-, aristaless-, rax-domain) that is involved in regulation of DNA-binding and 83 84 transcriptional activation (Amendt et al., 1999).

Rpx1 (Hesx1, Anf1) is a transcriptional repressor transiently expressed in the mouse pituitary primordium and playing a role in early determination and differentiation (Hermesz *et al.*, 2003). It is among the first factors expressed specifically in the pituitary primordium and is maintained until e13.5. Mouse embryos lacking Rpx1 display pituitary displasia, reduced proencephalon, anophtalmia or microphtalmia, defects in the olfactory tract and hypothalamus (Dattani *et al.*, 1998). Maintenance of Rpx1 expression in the pituitary beyond e13.5 in transgenic mouse embryos results in loss of lineages depending on the related factor Prop1 indicating that a tight
control of the opposing actions of these two factors is required for normal
development in mouse. Prop1 is required for onset of Pit1 expression and thus for
differentiation of the entire Pit1 lineage (Zhu *et al.*, 2007).

The two factors Pitx2 and Rpx are thus involved at different levels of pituitary development. Here, we obtained the cDNAs for their homologs in common carp (*Cyprinus carpio*) and we investigated their expression during seasonal acclimatization. We show that two different homologs are present for each gene, that all are expressed in adult carp pituitary and that their expression is clearly upregulated during winter season, in contrast to other regulatory genes such as *pit1*.

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105 MATERIALS AND METHODS

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107 Animals and tissue preparation

Adult male carp (*Cyprinus carpio*) weighing about 1000 - 1500g were caught during winter and summer and maintained in a fixed 3 x 4 m cage submerged 2 m in an affluent of the same river. The water temperatures in winter and in summer were 8 -10°C and 18 - 20°C, respectively.

Pituitary glands from winter- and summer-acclimatized carp were dissected and either fixed immediately for *in situ* hybridization in cold 4% paraformaldehyde or frozen in liquid nitrogen and stored at -80°C for RNA extraction.

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116 **RT-PCR amplification of Pitx2 and Rpx cDNAs**

117 Total RNA was isolated according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). RNA was treated with RNase-free DNase I (Invitrogen) and reverse 118 119 transcribed using SuperScript II (Invitrogen) and oligo dT₁₅ (Invitrogen). PCR was 120 carried out with 2.5 units of Tag DNA Polymerase (Invitrogen) on 0.5 µg cDNA in a solution containing 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM 121 122 dNTPs, and 0.5 mM forward and reverse primer. Amplification was performed for 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation 123 for 1 min at 72°C. Amplification products were subcloned into pGEM-T-Easy 124 125 (Stratagene) and sequenced from both sides.

126Two different carp *pitx2* cDNAs were cloned from a single individual, clone pcPitx2₁₁₀127(Pitx2-10s: 5'GAAGAGACAAAGGCGGCAACGAAC-3' / zPitx2-4a: 5'-128TCTTACACCGGTCTATCCAC-3') corresponds to gene-I deposited in GenBank

5'-129 accession number EF051103, and pcPitx27 (Pitx2-s: TGGTTCAAGAATCGACGGGCAAAATGG-3' 5'-130 Ptx3a: 1 GTTACAAGTGTCCCGGTAGAC-3') to gene-II accession number EF051104 131 (subscript numbers refer to laboratory clone numbers). The carp rpx 5'-cDNA 132 sequence was amplified by 5'-RACE (RML-RACE Kit, Ambion) and antisense 133 cRpx3a 5'-GCAAGTTCTTCACGTATATC-3' yielding pcRpx46. From the same 134 135 individual, two different cDNAs, pcRpx₆₄ and pcRpx₆₀, were obtained with anf1s 5'-5'-TGAACTGGTACATCGGGCGCAGGCC-3' / 136 zRpx4a CTCTCAGTGTTCTTCTCTGC-3'. Clearly overlapping sequences from pcRpx49 and 137 138 pcRpx64 were combined and deposited as cRpx under accession number 139 EF051105.

140 For quantitative real-time RT-PCR experiments, amplification was performed by denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 30 s at 141 72°C. The primers used were: carp *B-actin* derived from sequence M24113: cbeta-142 143 acts 5'-GGACCTGTATGCCAACACTG-3' and cbeta-acta 5'-GTCGGCGTGAAGTGGTAACA-3' (amplicon size in cDNA 281bp); carp *pitx2*: 144 5'-GAGAGGAGATCGCTGTTTGG-3' 5'-145 cPitx2-11s and cPitx2-12a CAGCCCAGTTGTTGTACGTG-3' (amplicon size 198bp); carp rpx: cRpx-11s 5'-146 CTGGATCTCCAGATGGCTTC-3' and cRpx-12a 5'-TCCACTGAAGCACCACTGTC-147 148 3' (206bp). The obtained Ct values were first normalized relative to ß-actin and the 149 log-fold ratio between winter and summer samples was obtained by using summer values as calibrator. 150

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152 *In situ* hybridization

153 Carp pituitary sections were obtained using a cryostat, placed on gelatinized slides 154 and stored at -80°C. *In situ* hybridization was performed on sections of different 155 specimens (summer- and winter-acclimatized carp) in parallel as previously 156 described (Figueroa *et al.*, 1994), except that the washing step was carried out at 157 42°C.

As probe, the carp pitx2 specific antisense oligonucleotide cPitx2-2a 5'-158 CCGAATTTAGAGAGGGGTTGC-3' was used as described (Kausel et al., 1999). 159 Control hybridization was performed with sense oligonucleotide Pitx2-1s 5'-160 161 AGCCCTACGATGACATGTATC-3'. A zebrafish specific *rpx* riboprobe was utilized 162 as described previously (Kausel et al., 1999). Sections from four individual winter carp and four individual summer carp were processed in parallel. Quantification of 163 the label in the tissue sections was performed using an automated image digitizing 164 system Image-Pro-Plus 3.0 as described earlier (Kausel et al., 1999). Three to five 165 166 sections from each individual were analyzed in this way and mean optical densities were calculated. Differences were assessed using the Student's t-test. P<0.08 for 167 168 *pitx2* and P<0.09 for *rpx* were considered significant.

170 **RESULTS**

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172 Characterization of two distinct *pitx*2 cDNAs in carp pituitary

173 We obtained total RNA from adult carp pituitary and synthesized cDNA by reverse 174 transcription using an oligo(dT) oligonucleotide as primer. This cDNA was used to perform PCR reactions using primer pairs designed against various parts of the 175 176 zebrafish *pitx2* sequence. Several amplified fragments were obtained, corresponding 177 to overlapping regions of the carp *pitx2* cDNA. Sequence analysis and assembly of overlapping clones with identical sequences allowed us to obtain two different partial 178 179 cDNA sequences coding for Pitx2 in carp. Both sequences start in exon III, the longer one at position 471 (cPitx2-I, GenBank Accession EF051103) and the shorter 180 181 one at position 646 (cPitx2-II, GenBank Accession EF051104) relative to the coding sequence of the aligned zebrafish sequence (Fig. 1). Only the cPitx2-I cDNA covers 182 183 the homeo-domain at the N-terminus. The carp sequences are highly conserved and 184 also present a high degree of similarity to the zebrafish sequence. Alignment of the amino acid sequences deduced from the cDNA clones reveals the same high 185 186 conservation between the two carp sequences, the zebrafish sequence and even to 187 the human and mouse sequences (Fig. 2). Only four positions differ between the two carp sequences and only three between each carp sequence and zebrafish. A very 188 189 high degree of similarity is also observed relative to mammalian sequences, human 190 or mouse.

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192 Characterization of two distinct *rpx* cDNAs in carp pituitary

The same cDNA from adult carp pituitary was used to perform PCR reactions using 194 195 primers designed against the zebrafish rpx sequence (GenBank accession 196 NM131349). Several overlapping fragments were obtained, corresponding to various 197 regions of the carp rpx cDNA. Assembly of overlapping clones with identical 198 sequences allowed us to obtain two different cDNA sequences covering the entire coding region for carp rpx (Fig. 3). These two cDNAs differ by several single base 199 pair changes, but importantly the shorter sequence (cRpx-II) presents a deletion of 200 201 21 nucleotides relative to the longer sequence (cRpx-I, GenBank Accession 202 EF051105). In the rest of the sequence, the two carp cDNAs display a similarity of 203 95% at the nucleotide level. Relative to the zebrafish sequence, cRpx-I presents 15 silent substitutions in the C-terminal region, coding for the homeo-domain, while 204 205 several non-synonymous substitutions and 2 insertions are present in the N-terminal region (Fig. 3). Alignment of the amino acid sequences deduced from the cDNA 206 207 clones reveals a very high similarity between the two carp sequences, while 208 conservation is very high to other species such as zebrafish or human only in the homeo-domain (Fig. 4). In the N-terminal region, similarity to the zebrafish sequence 209 210 is still high while the conservation to the human and mouse sequences is low (Fig. 4). A striking observation is the putative extension of the N-terminal region in 211 the carp Rpx sequences, due to the presence of an additional, more upstream ATG 212 213 translation initiation codon giving rise to an open reading frame in frame with the rest 214 of the coding region. This extension would be a unique feature for carp Rpx. Although supported by the perfect codon usage in carp, we cannot at this stage 215 decide whether the upstream ATG is really used. The carp sequences present three 216 deletions relative to mammalian Rpx genes in the N-terminal region consistent with 217 218 the ones in the zebrafish.

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220 Pitx2 and Rpx expression during acclimatization

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To evaluate modulation of *pitx2* and *rpx* genes expression during seasonal 222 acclimatization and to map the cellular distribution of their mRNAs, in situ 223 hybridization was performed on successive sections of pituitaries from male adult 224 225 winter and summer carp. Using antisense riboprobes corresponding to the 5'-region 226 of carp rpx cDNA or antisense oligonucleotides for pitx2, specific transcripts were 227 detected in carp pituitary sections (Fig. 5). No signal was obtained after incubation of the samples with digoxigenin labeled sense probes (Fig. 5). In striking contrast to 228 229 increased *prl* and *gh* signals in summer carp (Figeroa et al., 1994; 2005), the signals observed with *pitx2* and *rpx* probes were much stronger in pituitaries from winter 230 231 carp, especially in the proximal pars distalis (Fig. 5, A and B). This became evident 232 with the semi-quantitative analyses obtained upon digitalization of the hybridization 233 signals (Fig. 5). In addition, when *pitx2* and *rpx* mRNA levels were compared in total RNA from 3 different winter and summer carp pituitaries by quantitative real-time RT-234 235 PCR, a clear induction was observed in winter (log-fold = 4.4 for *pitx2* and 3.5 for 236 *rpx*) (Fig. 5C).

238 DISCUSSION

239 We have cloned and sequenced the cDNA for the carp *pitx2* and *rpx* genes. In both cases, we obtained at least two different cDNAs. The fact that most of the 240 241 sequences derive from overlapping identical clones argues against reverse transcription or sequencing errors, moreover, for rpx, one cDNA displays a 21 242 nucleotide (7 codon) deletion. The presence of multiple differences, especially in 243 244 view of the outstanding conservation of *pitx2*, strongly suggests that these cDNAs do 245 not represent different alleles of the same gene, but rather duplicated gene copies. 246 In teleosts, the presence of duplicated genes is relatively common (Ferris and Whitt, 247 1977). Particularly in tetraploids such as carp, many occurrences of duplicated genes have been described (Kausel et al., 2006). In some of these duplicate loci, only one 248 copy is effectively transcribed (Ferris and Whitt, 1977). The duplicate rpx and pitx2 249 250 cDNA sequences identified here from one individual pituitary show that both genes 251 are expressed in the same organ.

252 Analysis of the Pitx2 amino acid sequences revealed an outstanding conservation, between the two carp sequences, between carp and zebrafish and between fish and 253 mammalian. No substitution occurred in the homeo-domain or in the C-terminal OAR 254 255 domain, only minor differences are found in the central part of the sequence 256 (Furukata et al., 1997). These observations confirm the importance of these two 257 conserved regions, which are involved in DNA-binding and/or interaction with other 258 transcriptional cofactors (Olson et al., 2003; Amendt et al., 1998, 1999). One 259 interesting feature is the doublet substitution SA in cPitx2-I to PT in cPitx2-II, compared to the zebrafish PA sequence at position 1013 (Fig. 2). This appears as 260 an example of divergent evolution of two duplicated gene copies, where possibly an 261 "ancestral", zebrafish sequence PA diverged to SA in one copy and to PT in the 262

other. The functional significance of these changes remains to date unclear. None of
the mutations in the carp *pitx2* sequences corresponds to a mutation described in a
Rieger patient at the corresponding position in the human sequence, suggesting that
the two encoded proteins in carp are likely to be functional (Tümer and Bach-Holm,
2009).

The Rpx amino acid sequence appears to be less conserved, only the homeo-268 269 domain displays an important similarity between fish and mammals (Fig. 4). 270 Interestingly, the deletion in cRpx-II results in deletion of the two C-terminal amino acids of the generally recognized 60 amino acid long homeo-domain. Similarly, the R 271 272 at position 446 is mutated to C in the cRpx-I sequence, although this was not the 273 case in all the other clones that we obtained. Although these amino-acids are part of 274 the extensive homology domain, these observations might indicate that these 275 particular residues are not crucial for Rpx function. The two carp Rpx factors share 276 with the zebrafish factor the deletions in the central region relative to mammals. These regions might be specifically involved in the action opposing Prop-1 during 277 278 pituitary development in mammals, as there seems to be no homolog for this factor in teleosts (Olson et al., 2003; Mantovani et al., 2006). In contrast, both carp Rpx 279 sequences retain the two N-terminal regions deleted in zebrafish and present an 280 281 additional putative N-terminal extension. The importance of these regions is not clear 282 to date.

We obtained the cDNAs for Pitx2 and Rpx from RNA extracted from one adult carp pituitary, indicating that both genes are expressed in this gland in adults. This observation is confirmed by the *in situ* hybridization experiments, where we detect both mRNAs mainly in the PPD of adult pituitaries. Expression of these transcription factors was mainly studied during embryogenesis, thus it is interesting that we 288 observed their expression in adults (Chou et al., 2006; Pogoda and Hammerschmidt, 2009). In rats, the three Pitx2 variants were found to be expressed in adult brain and 289 pituitary in overlapping patterns (Smidt et al., 2000). Pitx2 expression was also 290 291 detected in pituitaries of adult rat and human, as well as in pituitary cell lines and in 292 certain prolactinomas, where it was shown to be required for full expression of *Prl* (Quentien et al., 2002). Rpx is not detected in adult mice (Tümer and Bach-Hohn, 293 2009), however its expression was found in adult human normal pituitaries and in all 294 types of pituitary adenomas (Mantovani *et al.*, 2006). Thus, our observation in carp is 295 consistent with the notion that in most vertebrates, these two factors play a role in 296 297 adult pituitary function.

During the seasonal acclimatization of carp, photoperiod appears to act as a relevant 298 299 modulator of pituitary gene expression (Figueroa et al., 1997). In particular, pituitary hormones Prl and Gh are highly expressed in summer, as well as one of their 300 301 important regulators, the transcription factor Pit1 (Figueroa et al., 1994; 2005; Kausel et al., 1999). When we investigated the expression levels of *pitx2* and *rpx* by *in situ* 302 303 hybridization and quantitative RT-PCR, it clearly appeared that both cDNAs are 304 significantly induced in winter relative to summer carp, in sharp contrast to all other genes that were previously studied. At present, we do not know whether both genes 305 coding for each factor are induced in winter, but the steady-state level of the cDNAs 306 307 for each factor were clearly increased in winter. Only two genes up-regulated in winter have been described before, the gene for nucleolin, which is concomitant to a 308 severe reorganization of nucleoli, nucleolar segregation and inhibition of ribosome 309 synthesis (Alvarez et al., 2003) and the gene for macroH2A-1 (Pinto et al., 2005), 310 which was proposed to be involved in DNA methylation and chromatin remodeling 311 312 (Buschbeck et al., 2009). While these genes' up-regulation represents an attractive 313 model for down-regulation of general transcription and protein synthesis in winter, 314 our observation of an up-regulation of the Pitx2 and Rpx transcription factors hints at a more specific action on defined genes. Although expression of both genes is not 315 restricted to pituitary during development and in the adult, the observed modulation 316 in this central regulatory gland might be part of a general endocrine mechanism to 317 down-regulate the metabolism of the entire organism in winter (Alvarez et al., 2003). 318 319 Rpx is known as a transcriptional repressor interacting with several cofactors such as the Groucho factor Tle1 (Olson et al., 2003) and the DNA methylase DNMT1 (Sajedi 320 321 et al., 2008). During mammalian development, its down-regulation is required for 322 formation of the Prop-1 and Pit1 dependent lineages (Olson et al., 2003). Prolonged 323 Rpx expression in mouse leads to pituitary hypoplasia (Olson et al., 2003). Similarly, in the adult, it is conceivable that Rpx up-regulation could lead to the observed 324 325 repression of Pit1 expression and down-regulation of its target genes. In contrast, 326 Pitx2 is considered to be a transcriptional activator, controlling the expression of its 327 target genes in combination with other factors such as Pit1 (Quentien et al., 2002). It is interesting to note in this context that Pitx2 was recently shown to interact with 328 329 nucleolin, one of the two factors up-regulated in winter (Huang et al., 2009). The authors describe a DNA microarray study where 868 genes were up-regulated and 330 331 191 were down-regulated by Pitx2 in human cells (Huang et al., 2009). Moreover, its transcriptional activity is controlled by phosphorylation (Espinoza et al., 2005). It will 332 certainly be interesting in the future to determine the target genes of these two 333 regulators and to investigate their effects during seasonal acclimatization. Candidate 334 335 target genes are the Pit1 dependent pituitary hormones, such a prl, gh, sl or tshb, all 336 of which are potentially involved in the control of general metabolism (Pogoda and 337 Hammerschmidt, 2009).

In conclusion, we cloned two cDNAs coding for each carp Pitx2 and Rpx and we show that both of these factors are clearly up-regulated in winter. Our results suggest that these two transcription factors play a role in adaptation of common carp to seasonal environmental changes.

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344 ACKNOWLEDGMENTS

- This work was supported by grants 1070724 from FONDECYT to G.K., the "Fonds"
- de la Recherche Fondamentale Collective" 2.4555.99, the SSTC PAI P5/35 and the
- 347 University of Liège GAME project. M.M. is a "Chercheur Qualifié du F.N.R.S."

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Fig.1: Alignment of *pitx2* cDNAs sequences from carp and zebrafish. Z: *Danio rerio* transcription factor *pitx2a* (pitx2a) mRNA, complete cds, 1820bp AF156906; I: *Cyprinus carpio pitx2* gene I EF051103; II: *Cyprinus carpio pitx2* gene-II EF051104. Grey shading highlights the homeodomain; in bold divergent amino acids and corresponding codons, slash indicates an amino acid change between the two carp sequences; the sequence of the degenerated oligonucleotides is underlined; arrows indicate exon-intron sites.

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Fig.2: Very high conservation of Pitx2 protein domains in vertebrates. An alignment
of amino acid sequences derived from mammalian and fish Pitx2 cDNAs is shown.
Asterix indicates identical amino acids; colon indicates conserved amino acid
substitutions and single point semiconserved amino acid substitutions; carp
sequences are in bold; grey shading highlights the homeobox DNA binding domain;
OAR domain in italics.

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490 Fig.3: Sequence alignment of *Rpx* nucleotide sequences.

491 Z: Danio rerio rpx (GenBank accession NM131349ZF); I: Cyprinus carpio rpx I cDNA 492 (GenBank accession EF051105); II: Cyprinus carpio rpx II cDNA (GenBank 493 accession GU585761). The derived carp amino acid sequence is shown above the 494 carp rpx II cDNA sequence, in bold the amino acids different relative to the zebrafish 495 sequence are shown; slash indicates amino acid changes between the two carp 496 sequences; grey shading highlights the homeodomain; the sequence of the 497 oligonucleotides derived from zebrafish is underlined. Fig.4: Alignment of amino acid sequences derived from mammalian and fish Rpx cDNAs. Asterix applies to identical amino acids; colon to conserved amino acid substitutions, single point to semiconserved amino acid substitutions, in bold carp sequences, grey shading highlights homeobox DNA binding domain.

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504 Fig.5: Increased *pitx2* and *rpx* expression in somatotrophs of winter- compared to 505 summer-acclimatized carp. (A) pitx2 and (B) rpx specific transcripts by in situ hybridization in rostral pars distalis, region of somatotrophs, of summer- and winter-506 507 acclimatized carp pituitary sagittal sections (anterior to the left; dorsal to the top). The 508 inset presents control hybridizations with the respective sense probes. Graphs represent differences of signals quantified in digitalized pictures of four individuals 509 (n=4) from each season. Columns represent mean integrated optical density (IOD) 510 with standard deviation indicated by bars. Student's t-test (A) P<0.08; (B) P<0.09 511 512 was considered significant. (C) Real-time RT-PCR quantification of pitx2 and rpx mRNA from individual carp pituitaries. The columns represent the log-fold ratio 513 between the means of three winter-acclimatized relative to three summer-514 acclimatized individuals +/- standard deviation. 515

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Figure 2

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  ACCATCORCAGCATCOTOGOGACTOGACAGACCOGGACCOCCAGAACCGTCCTGTCAGCACCT
17
I ACCATOGACAGCATCCTGGGACTGGATCGACCGGGGGAGCAGAGAAC----ATGTC-----CT
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   Y R F W T D V K F A G Q H R G V V A D S
I TACCONCECTGORCAGACOTORAACCAGCOGGACAGAACCOCOGCGTGOTOGCAGACAGT
I TACAGGCCCTGGACAGACGTGAAGCCAGCATGTCAGAATCGTCGAGAGTGGTGACAGAAAGT
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   G A S V D V H V N E D S K E Y S K F F A
1 GOTGCTTCAGTGGATGTGAGGGTGAATGAAGACAGTAAATCTTACAGTAAAOCCCCCAGCA
I GATGCTCCAGTGGATGTGAGAGAAAATGAAGATGGTAAATCTTTCAGTAAATCACCAACT
                                                         242
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II
                                         OCOGACADCTTTCTCT
  GACTCTTACAGGAGAACACTGAACTGOTACATCGGCCGCAGACGGGGGACAGCTTTCTCT
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11 AGTGTTCAGATCAAGATATTGGAGAGTGTGTGTTTCAAGTGAACTCACACCCGGGCATTGAT
I AGTGTTCAGATCAAGATATTGGAGAGTGTGTTTCAAGTGAACTCATACCCAGGCATTGAT
2 AGTGTTCAGATCAAGATATTAGAGAGTGTTTTTCCAAGTGAACTCATACCCAGGTATTGAT
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   I H E E C A E K L H L D E D H I Q I/V H F
11 ATACGTGAAGAACTTGCTAAGAACTGCATCTAGATGAGGACAGAATCCAGATTTGGTTC
I ATACGTGAAGAACTTGCTAAGAAACTGCATCTAGATGAAGACAGGATTCAGGTTTGGTTC
2 ATACOTGANGARCTTGCARAGAGCTTCARTTAGATGAGGACAGAATCCAGATTTGGTTC
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   UNRRAKLER/CEHRESOFLHVR
II CAGAACAGAAGAGCGAAGCTGAAGCGT-----CTCATGGTGAAG
  CAGAACAGAAGAGCGAAGCTGAAGTOTTCACACAGAGAATCTCAGTGTGAAG
2 CAGAACAGAAGAGCAAAGCTGAAGCOTTCGCACAGAGAGCCCCAGTTCCTCATGGTGAAA 482
   HVLSDFQS/TSHEEH*
II AATGTCCTCAGTGATITACAAACCA
  AACUTCCTCAGTGATTTACAGTCCA
2 AACGTCCTCAACGATTTACAAATCGOCAGAGAAGAACACTGGGGGGTAGAACTACATTCCT
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1 TATIATTATTATTATTATTATTATCATCATTATTATTCTCTCTCTCTACCAAATTGTAA
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Figure 3

Η.	sapiens	MSPSLOEGAQLGENKPSTCSFSIERILGLDQKKDCVPLMK	4.0
N.	musculus	MSPSLREGAQLRESKPAPCSFSIESILGLDQKKDCTTSVR	40
D.	rerio	WASLANSPSVFTIDSILGLDRPEORTC	27
c.	carpio	MKTRRRGLDLQMASLTVSTHQNRSMPRQCAFTIDSILGLDRPDPRTVLSA	50
Ħ,	sapiens	PHBFWADTCSSSGKDGNLCLHVPNPPSGIBFPSVVDHPMPEERASKYENY	90
М.	musculus	PHRPWTDTCGN5EKDGNFPLHAPDLPSETSFPCPVDHPRPEERAPKYENY	90
D.	rerio	PYRPWTDVEPACGNRRVVTESDAPVDVRENEDOKS	62
C.	carpio	PYRPWTDVKPAGQNRGVVADSGASVDVRVNEDSKS	85
		*1***1** * 1 · · * * *1 1	
Η.	sapiens	FSASERLSLKRELSWYRURRPRTAFTQNQIEVLENVFRVNCYFGIDIRED	140
м.	musculus	FSASETRELERVYRORRFRTAFTQNQVEVLENVFRVNCYPGIDIRED	140
D.	rerio	FSKSPIDSYRRTLNWYIGRRFRIAFSDVQIKILESVFQVNBYFGIDIREE	112
C.	carpio	YSKPPADSYRRTLNWYIGRRPRTAFSSVQIKILESVFQVNSYPGIDIREE	135
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Ħ.	sapiens	LAOKLNLEHDRIQIWFONRRAKLERSHRESOFLMAKKNPNTNLLE	185
м.	musculus	LAOKLNLEEDRIQIWFONRRANMERSBRESCFLMAKKFFNPDLLK	185
D.	rerio	LAKKLOLDEDRIGIWFONRBAKLERSHRESOFLMVKNVLN-DLOIGBEEH	161
c.	carpio	LAKKLHLDEDRIGVWFQNRRAKLKCSHRESQFLMVKNVLS-DLQS	179

Figure 4

