Cold temperature blocks thyroid hormone-induced changes in lipid and energy metabolism in the liver of *Lithobates catesbeianus* tadpoles

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**Abstract**

**Background:** Exposure of the American bullfrog *Lithobates catesbeianus* tadpoles to low temperature affects many biological processes including lipid metabolism and the thyroid hormone (TH) signaling pathway, resulting in arrest of TH-induced metamorphosis. To clarify what molecular events occur in this phenomenon, we investigated the glycerophospholipid and fatty acid (FA) compositions, the activities of mitochondrial enzymes and the transcript levels of related genes in the liver of control (26 °C) and cold-treated (4 °C) tadpoles with or without 5 nM 3,3',5-triiodothyronine (T3).

**Results:** Exposure to T3 decreased the tail height and polyunsaturation of FAs in the glycerophospholipids, and increased plasma glucose levels and transcript levels of primary TH-response genes including TH receptor, and some energy metabolic (cox4, srebp1 and fas) and FA chain elongase genes (elovl3 and elovl5). However, these T3-induced responses were abolished at 4 °C. Exposure to cold temperature enhanced plasma glucose, triglyceride and free FA levels, monounsaturation of FAs, mitochondrial enzymes activities (cytochrome c oxidase and carnitine palmitoyltransferase; U/g liver), with the upregulation of the genes involved in glycogenolysis (pygl), gluconeogenesis (pck1 and g6pc2), FA β-oxidation (acadl), and cholesterol uptake and synthesis (hmgcr, srebp2 and idlr1), glycerophospholipids synthesis (pcyt1, pcyt2, pemt, and pparg), and FA monounsaturation (scd1) and chain elongation (elovl1 and elovl2). T3 had little effect on the cold-induced changes.

**Conclusions:** Our study demonstrated that exposures to T3 and cold temperature exert different effects on lipid metabolism, resulting in changes in the FA composition in glycerophospholipids, and suggests that a cold-induced signal may block TH-signaling pathway around primary TH-response genes.

**Keywords:** Tadpole, Cold exposure, Thyroid hormone, Glycerophospholipid, Fatty acid, Desaturase, *Lithobates catesbeianus*
(TRs) [4], which up- or down-regulate primary and secondary TH-response genes. Tissue-specific activation or suppression of these TH-response genes may affect gene regulation cascades responsible for tissue-specific transformation during metamorphosis. Findings from recent in vivo and in vitro studies revealed that transcription of the TH-response genes TRβ (a primary TH-response gene) and ornithine transcarbamylase (a secondary TH-response gene) remains at basal levels when L. catesbeianus tadpoles and cells are exposed to cold temperature (4 °C) in the presence of T3 for 3–6 days [3, 5]. However, in zebrafish, TH signal may still be active at low temperature. Findings from a recent report demonstrated that T3 and its metabolite 3,5-diiodothyronine affected swimming performance, metabolic rate, and tissue-specific regulatory enzyme activities, depending on the actual temperature and thermal history of the zebrafish [6]. Whether TH signaling is completely blocked when tadpole metamorphosis is arrested by exposure to cold temperature is not known.

Temperature affects the integrity and fluidity of biological membranes, which are determined by the glycerophospholipid composition of membranes and the fatty acid (FA) composition of the membrane glycerophospholipids [7]. Ectothermic organisms are able to adapt to cold temperature by changes in lipid metabolism. The most well-known and consistent response to cold temperature is an increase in the unsaturation of FAs in glycerophospholipids. Acyl-CoA Δ9 desaturase (stearoyl-CoA desaturase), which introduces a double bond in the Δ9 position of acyl-CoA, is the enzyme that is responsible for this response to cold temperatures and has been studied in detail in yeast [8] and in fish [9–11]. In addition, significant changes in the glycerophospholipid composition and the FA composition of glycerophospholipids have been reported in several species of fish with exposure to cold temperature for 2–7 days [9, 11, 12]. These changes in lipid composition at cold temperatures may optimize the fluidity of the membranes and influence the activity of membrane proteins.

This study was conducted to clarify what effect(s) TH has on the composition of membrane glycerophospholipids and FAs and transcript levels of genes involved with energy and lipid metabolism in L. catesbeianus tadpoles, what effect(s) cold temperature (4 °C) has on any changes induced by TH, and what effect(s) cold temperature has independent of TH. In addition, we assessed whether TH counters or enhances the response to cold temperature. Animals were reared in the presence or absence of T3 at 4 or 26 °C (control temperature). The glycerophospholipid composition of hepatic membranes and the FA composition of glycerophospholipids were analyzed by thin-layer chromatography (TLC) followed by gas chromatography (GC), and transcription levels of genes involved with energy and lipid metabolism were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). As possible effective sites, activities of the enzymes in mitochondrial membrane were also assayed.

Results

Morphological and plasma biochemical parameters

Tail height was the most sensitive of the morphological parameters tested to T3 (Additional file 1: Table S1). Exposure to T3 at 26 °C significantly reduced the tail height at day 3, and body weight and length, and tail length and height at day 7. However, the reductions in morphological parameters observed at 26 °C following exposure to T3 were absent at 4 °C. In tadpoles that were not exposed to T3, exposure to cold temperature did not affect these morphological parameters at day 3 or 7 (Additional file 1: Table S1).

Exposure to T3 and/or cold temperature had variable effects on plasma biochemical parameters in tadpoles (Fig. 1). By day 7 at 26 °C, exposure to T3 increased the plasma concentration of glucose (p < 0.05). This effect of T3 on the glucose concentration disappeared at 4 °C on day 7. Glucose and triglyceride (TG) concentrations were 2.0-fold to 2.5-fold higher at 4 °C than at 26 °C on day 3 (p < 0.05); however, these increases in concentrations were not statistically significant on day 7, and were not affected by T3. On both days 3 and 7, the plasma concentration of cholesterol did not differ significantly among the four groups. Exposure to cold temperature, but not to T3, greatly affected the ratio of TG/cholesterol (3-fold) in plasma. Neither exposure to T3 nor cold temperature (4 °C) altered the concentration of free FAs in plasma, except in the cold-treated tadpoles on day 7.

Hepatic glycerophospholipid composition and the FA composition of glycerophospholipids

Of the glycerophospholipids investigated in tadpole liver, more than 50 % were phosphatidylcholine (PC) and approximately 20 % were phosphatidylethanolamine (PE). Both phosphatidylserine (PS), phosphatidylinositol (PI) comprised about 10 % of the glycerophospholipids, whereas phosphatidylglycerol (PG) comprised 3 % and cardiolipin (CL) less than 1 %. The glycerophospholipid composition did not differ significantly among the four groups on both days 3 (Fig. 2A) and 7 (Fig. 2B).

For total glycerophospholipids (Table 1), exposure to T3 decreased the relative abundance of 20:5n-3 at day 7, however, this T3-induced decrease was not observed at 4 °C. Exposure to cold temperature decreased the relative abundance of 20:4n-6 at day 7. T3 did not affect this cold-induced reduction. For each glycerophospholipid
Exposures to T3 and cold temperature had only a modest effect on the FA composition at both days 3 and 7, except for PG (Additional file 2: Table S2-5), in which exposure to cold temperature enhanced the proportion of 18:1n-9 by 10-fold at day 3 and 4-fold at day 7. T3 did not affect this cold-induced reduction.

Exposure to T3 decreased the unsaturation index (UI, the average number of double bonds per 100 FA molecules) in total glycerophospholipids on days 3 and 7 (Fig. 3A). The T3-induced decrease was not detected at 4 °C. The UI value of FAs did not significantly differ between the control and cold-treated tadpoles in the absence of T3.

The ratio of monounsaturated FA (MUFA)/saturated FA (SFA), which partially reflects Δ⁹ desaturase activity, was not changed in total glycerophospholipids with exposure to T3 (Fig. 3B), whereas it increased significantly on day 3 with exposure to cold temperature. The cold-induced response was not observed on day 7, and was not affected by T3.

The ratio of 20:4n-6/18:2n-6 and/or the ratio of 20:5n-3/18:3n-3, both of which partially reflect the sequential reactions consisting of the Δ⁶ desaturase and very long chain FA elongase activity of the ω-6 and ω-3 polyunsaturated FAs (PUFAs), respectively (see Additional file 3: Figure S1B), decreased in total glycerophospholipids with exposures to T3 and cold temperature on day 3 or 7 (Fig. 3C, D). These T3-induced or cold-induced responses were not enhanced additively or synergistically at 4 °C and with T3.

The ratio of Σω-3 FA/Σω-6 FA, which indicates a preference of the ω-3 and ω-6 FA utilization in glycerophospholipids, increased in total glycerophospholipids (Fig. 3D) at day 7 with exposure to cold temperature but not with exposure to T3. Exposure to T3 did not affect this cold-induced response.

Hepatic mitochondrial membrane enzymes activity
Cytochrome c oxidase (COX) and carnitine palmitoyltransferase (CPT) activities, when estimated as U/mg membrane protein, did not differ significantly among the four treatment groups (Fig. 4). However, when estimated as U/g wet weight of the liver used, although COX and CPT activities did not significantly increase with exposure to T3, these activities increased 2.8-fold to 3.4-fold with exposure to cold temperature at day 3 (Fig. 4A, B), indicating that the amount of mitochondrial protein per liver wet weight was increased by exposure to cold temperature. This increase in enzyme activities was significantly greater than that in the ratio of membrane protein (mg) to liver (g), 1.9, at least on day 3. Exposure to T3 did not affect the cold-induced response.

Transcript amounts of hepatic genes
Primary TH-response gene transcripts
Five TH-response genes that are known to respond directly to TH through TH response elements in X. laevis...
or *L. catesbeianus* (thrb, nfc, thibz, dio3 and mmp11) [3, 5, 13], except for the dio3 on day 7, were upregulated with exposure to T3. However, the T3-induced responses were not observed at 4 °C (Fig. 5).

**Energy metabolism gene transcripts**

The effect of exposure to T3 or cold temperature was variable on transcript amounts of energy metabolism genes. We detected 6 TH-response genes (cox4, srebpl, fas, pparα, acaa2 and sc4mol) and 11 cold-response genes (pygl, g6pc2, mdh, pck1, ppara, acadl, hmgcr, sreb2, ldlr1, cyp7a1 and soat1) at day 3 or 7 in the tadpole liver (Additional file 4: Figure S2). The T3-induced responses were not observed at 4 °C, whereas most of the cold-induced responses were not affected by exposure to T3 (Fig. 6).
There was no change in the transcript amount of the glycogen phosphorylase gene (pygl) with exposure to T3, however, it increased with exposure to cold on days 3 and 7, suggesting the enhancement of glycogenolysis in the liver by cold stimuli. No effects of cold and T3 exposures on days 3 and 7 were detected on the transcript amounts of four genes in the glycolysis pathway (hk, aldb, pgk1 and ldhb). Exposure to T3 at 26 °C but not at 4 °C increased the amounts of the cox4 transcript on days 3 and 7 without significant effects on the pgc1a expression, whereas cold exposure increased the transcript amounts of three genes involved in gluconeogenesis (g6pc2, mdh and pck1) on at least day 3 regardless of whether tadpoles were exposed to T3 or not.

Neither exposure to T3 nor cold temperature affected the transcript amount of the gene that is involved in TG synthesis (dgat1). Of the three genes that are involved in FA synthesis (acb, srebpl and fas), the srebpl and fas transcript levels were high on at least day 3 of exposure to T3 at 26 °C but not at 4 °C, although the acb transcript levels were affected by neither exposure to T3 nor cold temperature.

There was a reduction in the transcript amounts of the transcription factor ppara gene, which plays an important role in β-oxidation, and the acaa2 gene at 26 °C on day 3 with exposure to T3. However, there was no change in the transcript amounts of the other β-oxidation genes (acadl and cpt1a) and ceramide synthase gene (cers). Exposure of cold temperature reduced significantly the transcript amount of ppara and enhanced the transcript amount of acadl on day 3. The cold-induced responses were not affected by exposure to T3.

Of the genes involved in cholesterol synthesis and uptake (hmgrc, sc4mol, srebpl2 and ldlr1), the amount of the sc4mol transcript decreased with exposure to T3 on day 3 at 26 °C, but this T3 effect was not detected at 4 °C, whereas the amounts of the hmgrc, screbpl2 and ldlr1 gene transcripts increased with exposure to cold temperature on at least day 3. Exposure to T3 did not affect most of the cold-induced increases.

At 26 °C, exposure to T3 did not have a significant effect on the transcript levels of three genes involved in bile synthesis (cyp7a1, cyp8b1 and hsd3b7), two genes involved in the synthesis of cholesterol ester to accumulate it as cytoplasmic lipid droplets (soat1) or to transport it via blood lipoproteins into the liver (lcat), or one gene involved in estradiol and arachidonic acid synthesis (hsd17b12) [14]. The cyp7a1 transcript amount decreased on days 3 and 7 with exposure to cold temperature. In contrast, the cyp8b1, hsd3b7, lcat and hsd17b12 transcript amounts were hardly affected by cold exposures on days 3 and 7. The soat1 transcript amount increased on day 7 of cold exposure. These cold-induced changes were not affected by exposure to T3.
Glycerophospholipid metabolism gene transcripts

The effect of exposure to T3 was not observed, however the effect of exposure to cold temperature was variable on transcript amounts of glycerophospholipid metabolism genes (Additional file 3: Figure S1A). We detected four cold-induced or -suppressed genes (pcyt1, pcyt2, psd1 and pemt) at day 3 and one cold-induced gene (pparg) at day 7. Exposure to T3 did not alter these cold-induced responses (Fig. 7A).

In the de novo glycerophospholipids synthesis pathway, the pcyt1, pcyt2 and pemt transcript amounts were significantly up-regulated whereas the psd1 transcript amount was down-regulated on day 3 of cold exposure. However, these effects were lost on day 7. Cold exposure on day 7 enhanced the amount of the pparg transcript that encodes a receptor for 20:4n-6 metabolites. There were no effects of exposure to cold temperature on the expression of the other genes tested: those involved in phosphatidic acid synthesis (gk and lpaata), the de novo glycerophospholipid synthesis (pld1, cds1 and cds2), the remodeling pathway (reacylation of membrane glycerophospholipids from glycerolysospholipids) (cpla2 and agpat3).

Very long chain FA desaturation and elongation gene transcripts

The effect of exposure to T3 or cold temperature was variable on transcript amounts of very long chain FA desaturase and elongase genes (Additional file 3: Figure S1B), in which 2 TH-response genes (elovl3 and elovl5) and 3 cold-response genes (scd1, elovl1 and elovl2) were detected in the tadpole liver. The T3-induced responses at 26 °C were not observed at 4 °C whereas the cold-induced responses were not affected by exposure to T3 (Fig. 7B).

Among the three desaturase genes tested, the effect of exposure to T3 was not detected in these transcript amounts. The amounts of Δ⁹ desaturase (scd1) transcript, but not the Δ⁵ (fads2) and Δ⁷ (fads1) desaturase transcripts, increased on at least day 3 of cold exposure. Among the five elovl genes tested, the elovl3 and elovl5 transcript amounts increased on at least day 3 with
exposure to T3 at 26 °C, whereas the elovl1 and elovl2 transcript amounts increased on day 3 and day 7, respectively, with exposure to cold temperature. Neither exposure to T3 nor cold temperature affected the amount of the elovl6 transcript.

Discussion
The present study demonstrates that exposures to T3 and cold temperature for up to 7 days have different effects on components of membrane lipids with dynamic changes in transcript levels for energy/carbohydrate/lipid metabolism (Table 2). Exposure of tadpoles to T3 at 26 °C induced primary and secondary TH-response genes, resulting in tail regression. In the liver, remodeling of membrane lipids, FA synthesis and mitochondrial activity were activated with elevated plasma glucose level, while lipolysis and sterol synthesis were suppressed. Exposure to cold temperature and exposures to both cold temperature and T3 induced similar changes in the liver: remodeling of membrane lipids, lipogenesis (sterol and/or ceramide synthesis), lipolysis, mitochondrial activity, glycogenolysis, probably glyceroneogenesis/TG/FA cycle, with transient elevation of plasma glucose and TG. We detected 13 TH-response genes including at least 5 primary TH-response genes and 19 cold-response genes. Under our experimental conditions, morphological, biochemical and transcriptional changes induced by T3 in the tadpoles reared at 26 °C were not observed at 4 °C, although the majority of the changes induced by cold temperature were not affected by exposure to T3.

Effects of exposures to T3 and cold temperature on the FA composition of glycerophospholipids
In contrast to the glycerophospholipid composition which was hardly affected by exposure to T3 or cold temperature, the FA compositions of the total glycerophospholipids and specific classes of the glycerophospholipids were variously changed in response to these stimuli. At least three different steps may contribute to these changes: (1) the monounsaturation of FAs, which is deduced from the ratio of MUFA/SFA, predominantly depending on the Δ9 desaturase activity, (2) the polyunsaturation of FAs, which are deduced from the ratios of 20:4n-6/18:2n-6 and 20:5n-3/18:3n-3, probably reflecting sequential reactions consisting of Δ6 desaturase, very long chain FA elongase ELOVL5 and Δ5 desaturase (Additional file 3: Figure S1B), and (3) the acylation and deacylation that determine the preference for FA classes in the glycerophospholipids, which the ratio of Σω-3 FA/Σω-6 FA presumably reflected, in the de novo
synthesis pathway and the cycle of acylation and deacylation (Additional file 3: Figure S1A). Exposure to T3 suppressed the step (2) alone, leading to the decline in the UI value; whereas exposure to cold temperature enhanced the steps (1) and (3) but suppressed the step (2), leading to no change in the UI value.

Changes in the UI values and FA ratios described above are also reported in T3-treated mice [15] and cold-tolerant fishes [12, 15]. However, there are marked differences in these responses among the species. The UI value and the MUFA/SFA, 20:4n-6/18:2n-6, 20:5n-3/18:3n-3 FA ratios were elevated in mouse liver in response to T3 [15], unlike tadpole liver. After cold-exposure, the MUFA/SFA ratios increased in carp liver [16] but not in rainbow trout liver [12, 17]. Unlike in the tadpole liver, in carp and rainbow trout livers [12, 16, 17] the 20:4 n-6/18:2n-6 or 20:5 n-3/18:3n-3 ratio increased. The ratio of Σω-3 FA/Σω-6 FA dramatically decreased in carp but increased in rainbow trout liver [12, 16]. These observations suggest that the pathway of very long chain FA unsaturation, elongation or acylation/deacylation may be differently regulated in a species-specific manner to maintain lipid homeostasis or membrane fluidity in response to T3 and cold stimuli. The variety of the species-specific responses may result in the different changes in FA compositions among species.

In this study, we detected glycerophospholipid-specific changes in the FA compositions, i.e., 18:1n-9 in PG, in the cold-treated tadpoles (Additional file 2: Table S2-5). Although it is unclear at present why the elevation in the proportion of 18:1n-9 is notable in PG, a higher turnover rate of PG is likely to provide a possible explanation. Alternatively, glycerophospholipid class-specific acylation and deacylation pathway may contribute to the PG-specific elevation of C18:1n-9 proportion [18]. As PG is a precursor of CL that is the constituent lipid of the mitochondrial inner membrane, the increase in 18:1n-9 composition of PG is likely to affect mitochondrial functions with exposure to cold temperature.

**Effects of exposures to T3 and cold temperature on mitochondrial enzyme activities**

Although THs have important roles in mitochondrial function and biogenesis through direct actions of TRs in mitochondria and indirect actions of TRs by which regulatory genes such as pgc1a/b are activated in nucleus and the translational products act as transcription factors in mitochondria in mammals [19], the COX and CPT activities and the pgc1a transcript levels hardly responded to exposure to T3 in the tadpole liver, despite the strong upregulation of the cox4 transcript by T3. Such a discordance between the enzyme activity and its transcript abundance was also reported for fish COX [6, 20], suggesting the presence of non-transcriptional regulation.

Exposure to cold temperature may alter mitochondrial functions probably by increasing the amount of mitochondrial proteins and another unknown mechanism under our experimental conditions. The COX and CPT activities (U/g tissue but not U/mg protein) increased in the liver mitochondria membranes of cold-treated tadpoles, in agreement with a previous report in rainbow trout muscle mitochondria membranes [21]. This increase occurred without the activation of the pgc1a gene, which encodes a master regulator of mitochondrial biogenesis and functions. Therefore, tadpole liver may increase the mitochondrial protein content rather than the copy number of the mitochondrial genome in response to cold stimulus, as proposed previously [20]. Our data also suggest that the cold-induced increase in COX and CPT activities may also be explained by an unknown additional mechanism. It is unclear whether the cold-induced increase in COX and CPT activities is because of changes in the glycerophospholipid milieu in mitochondria.
mitochondrial membranes as shown previously in the carp red muscle mitochondria [22] or increases in the amounts of COX and CPT per mitochondrial membrane proteins.

Effects of exposures to T3 and cold temperature on transcript amounts of TH-response genes

T3-induced transcriptional activation or suppression at 26 °C, including that in 5 primary TH-response genes,
Table 2 Effects of thyroid hormone and cold exposures on biochemical and transcriptional changes in the liver of *L. catesbeianus* tadpoles

| Treatment | Possible events | Biochemical and transcriptional changes |
|-----------|----------------|----------------------------------------|
| T3(+) at 26 °C |
| (Enhancement) Tail regression |
| Remodeling of membrane lipids |
| Lipogenesis (FA synthesis) |
| Glycogenolysis? |
| Mitochondrial activity |
| Lipolysis (β-oxidation) |
| Lipogenesis (sterol synthesis) |
| T3(−) at 4 °C |
| (Suppression) Lipolysis (β-oxidation) |
| Lipogenesis (sterol synthesis) |
| (Unclear) FA/GT synthesis |

No parenthesis of the column “Biochemical and transcriptional changes” indicates significant changes on both day 3 and day 7. Gene names are shown in Additional file 5: Table S3.

Chol: cholesterol, COX: cytochrome c oxidase, CPT: carnitine palmitoyltransferase, FA: fatty acid, FFA: free fatty acid, MUFA: monounsaturated fatty acid, PG: phosphatidylglycerol, SFA: saturated fatty acid, TG: triglyceride, UI: unsaturation index. Parentheses indicate the day showing significant changes: 3d on day 3, 7d on day 7.

was lost in the liver with exposure to cold temperature. In contrast, almost all of cold-induced gene activation or suppression was not affected by exposure to T3. As the primary TH-response genes may have TH response elements in their regulatory regions [13], cold stimuli may affect the T3-dependent activation of these genes through a common molecular mechanism. Our previous studies indicated that exposure to cold temperature abolished the T3-induced increase in the amount of acetylated histone H3 at lysine 9 in the thrb gene [5], suggesting that cold-induced signal may affect a transcriptional step between TR transactivation and the histone modification in at least the thrb gene. It is unclear whether the signal derived from cold stimuli interferes with the transactivation of the liganded TRs or with the interaction of TRs with other transcriptional regulatory proteins such as co-activators, nucleosome remodellers and histone modifiers on TH response elements in the primary TH-response genes. It was recently reported that T3 signaling persists even at 5 °C in at least some primary TH-response genes of the bullfrog tadpoles. These phenomena were tissue-specific and clearly detected in tadpole tail fin [23]. In fish, hypothyroidism impaired swimming performance and metabolic rates in cold-acclimated but not warm-acclimated fish whereas T3- or diiodothyronine-treatment restored these performances [6], indicating that cold acclimation sensitizes TH actions. In the transcriptome analysis of zebrafish larvae exposed to cold temperature [10], rev-erb subfamily of nuclear receptors was proposed as candidate master genes of cold response. However, the bullfrog rev-erb gene transcript was not respond to exposure to cold temperature (data not shown). Further studies about the T3 effects on T3-response genes in the liver of cold-acclimated hypothyroid tadpoles are required.
**Effects of exposures to T3 and cold temperature on energy metabolic pathways**

Exposures to T3 and cold temperature dramatically and differently altered the energy metabolisms in the tadpole liver. Exposure to T3 at 26 °C increased plasma glucose level, which may be induced by enhanced glycolysis and activation of glucose 6-phosphatase that occur during spontaneous or TH-induced metamorphosis [24–26]. However, our transcriptional data (pgyl, g6pdh, mdh and pck1) did not support T3-dependent glycolysis and gluconeogenesis. It is likely that mechanisms other than transcriptional regulation, e.g., the modulation of enzyme activity by phosphorylation, may be involved in the activity of rate-limiting enzymes for glycolysis such as glycogen phosphorylase if plasma glucose is mainly derived from the liver. There has been no solid evidence that elevated plasma glucose is derived from the larval tissues such as tail and intestine that are hydrolyzed during metamorphosis. On the other hand, exposure of tadpoles to cold temperature increased plasma glucose level with elevated amounts of the transcripts for pgyl, g6pdc2, mdh and pck1 on day 3 but not on day 7, suggesting a transient activation of glycolysis and gluconeogenesis. This hyperglycemia may have an advantage to acclimate to cold temperature. The bullfrog *L. catesbeianus* [27], as well as other northern frogs: *Rana sylvatica* [28] and *Pseudacris triseriata* [29], resists to freezing for survival at sub-zero temperature, by increasing plasma glucose level as a cryoprotectant. *R. sylvatica* and *P. triseriata* mobilize glucose from liver glycogen.

Increases in plasma TG (on day 3) and free FA (on day 7) levels and in transcript abundance of the hepatic pck1 (on day 7) with exposure to cold temperature suggest that TG/FA cycle may be activated between the liver and the other tissues such as fat bodies. In mammals, this cycle is usually activated under starvation with activation of phosphoenolpyruvate carboxylase in the liver and with its suppression in the adipose tissue [30]. A possible scenario in the tadpoles is that lipids are hydrolysed into free FAs and glycerol in the fat bodies or other tissues [31–34] and then free FAs are re-esterified back to TG either in the fat bodies or in the liver, where glyceroneogenesis is activated to make glycerol 3-phosphate for TG synthesis. Increases in hepatic phosphoenolpyruvate carboxylase activity and plasma glycerol level were reported in the rainbow smelt in response to cold temperature [31]. However, it is unclear why there is a difference in timing of the increases in plasma free FA and TG levels in the tadpoles reared at 4 °C.

Exposure to cold temperature enhanced the transcript abundances of genes for cholesterol, cholesterol esters and/or ceramide syntheses, and suppressed those for cholesterol catabolism into bile acids, with a trend toward a decrease in plasma cholesterol level. This strongly suggests that free sterol level is low in the liver cells. The liver have to provide lipid and cholesterol building blocks to every tissue for extensive reconstruction of membranes. Remodeling of the membrane lipids may be a prerequisite for coping with cold-induced changes in the fluidity of lipid membranes and activity or conformation of membrane proteins [35]. Changes in cholesterol content of lipids may be most critical for short-term cold acclimation [36, 37].

Among the transcripts for transcription regulatory proteins, striking changes were found in the amounts of the srebpl and srebpt2 transcripts, which encode important transcription factors involved in lipogenesis and cholesterol homeostasis, respectively [38]. The srebpl gene was up-regulated by exposure to T3 whereas the srebpt2 gene was up-regulated by exposure to cold temperature. The increase in the ratio of TG/cholesterol in plasma detected with exposure to cold temperature may reflect the increase in cellular uptake of cholesterol from plasma via the ldlr activation by the srebpt2. The ppara gene was suppressed or not affected by exposures to T3 and cold temperature with relatively high TG levels in plasma, suggesting that the liver may not be under conditions of energy deprivation [39]. Nevertheless, FA β-oxidation, which is usually activated by the transcription factor PPARα that the ppara gene encodes, was activated with exposure to cold temperature. The underlying molecular mechanism is unclear.

Approximately half of the biochemical and transcriptional changes in response to cold temperature, are detected on day 3 only (Table 2), and returned to normal on day 7. These may be the transient stress responses to cold stimuli, as seen in mammalian and fish species [40, 41].

**Effects of exposures to T3 and cold temperature on membrane lipid metabolic pathways**

Very long chain FAs with >C22 are produced from long chain FAs with >C18, are essential for components of membrane lipids (e.g., sphingolipids and glycerophospholipids) and precursors of lipid mediators. Fatty acyl desaturases and ELOVLs are rate-limiting enzymes for FA chain desaturation and elongation with characteristic substrate-specificity (Additional file 3: Figure S1B). Dysfunction of these mammalian enzymes exert abnormality of lipid synthesis, storage, metabolism in liver or adipose tissues, immune cell homeostasis, inflammatory reactions, and sterility, in an enzyme-specific manner [42]. Although we cannot clearly state the functional meaning of the changes in the transcript amounts of elongases and desaturase genes, the nature of lipid membranes may be specifically altered to optimize the membrane fluidity, structure or activity of membrane proteins, in response to exposure to T3 or cold temperature.
In spite of various changes in the transcript amounts of the metabolic enzymes for glycerophospholipids and very long chain FAs in response to exposures to T3 and cold temperature, there were weak correlations between the transcript amounts and the glycerophospholipid or FA amounts. Only the upregulation of the scd1 gene by cold exposure was correlated with the increase in the MUFA/SFA ratio. In mammals, SREBP1c plays a pivotal role in the activation of the scd1 gene [43]. As the srebp1 gene was not up-regulated by exposure to cold temperature in the tadpole liver, post-translational regulation as found in mammals [42] may be important to activate SREBP1 protein as a transcription factor. The ratios of 20:4n-6/18:2n-6 and 20:5n-3/18:3n-3 declined with exposures to T3 and/or cold temperature, nevertheless the transcript amounts of the genes fads1 and fads2 for Δ5 and Δ6 desaturases, respectively, which are rate-limiting enzymes for PUFA conversion (Additional file 3: Figure S1A), were not affected by these exposures. In the present study, we have mainly investigated the de novo glycerophospholipid synthesis pathway (Kennedy pathway) (Additional file 3: Figure S1A). However, the remodeling pathway by cycle of deacylation-reacylation of membrane glycerophospholipids (Lands’ cycle) (Additional file 3: Figure S1A), in which a number of enzymes are involved [44], may also profoundly affect the compositions of the FAs in the glycerophospholipids [18].

Conclusions
We demonstrated that pre-metamorphic tadpoles induced a variety of metabolic changes by short-term exposures to T3 and cold temperature, which include changes in biochemical parameters in plasma, and FA compositions of the glycerophospholipids, mitochondrial activities and transcript levels of the genes for energy and lipid metabolisms in the liver. In particular, the pathway of the FA desaturation and chain elongation was differently affected at several steps. The morphological, biochemical and transcriptional changes induced by T3 in the tadpoles reared at 26 °C were almost completely abolished by exposure to cold temperature. Cold-induced arrest of amphibian metamorphosis may be due to the block of T3-signaling pathway at around the activation of primary TH-response genes. In nature, the bullfrog tadpoles overwinter with an increase in body mass during the coldest months of the year at high latitudes. This life stages may have a significant benefit in survival and fecundity.

Methods
Animals and experimental design
Tadpoles of the American bullfrog *L. catesbeianus* were collected from Saitama or Ibaraki, Japan, and commercially obtained. They were maintained in aerated and dechlorinated tap water at 22–26 °C and were ad libitum fed boiled spinach three times a week. Their developmental stages were estimated according to the criteria of Taylor and Kollros [45]. After they were acclimated to laboratory conditions for 1–2 weeks, tadpoles at stages X–XIII were divided into four groups (6–8 individuals/group) and placed in 6 L-aquaria with 4–5.3 L of dechlorinated tap water. For the two cold-treated groups, two aquaria were moved into a cold room and the water temperature was gradually reduced to 4 °C at a rate of less than 1 °C/h, on the evening before starting experiments. For the two other groups, the water temperature in the remaining two aquaria was maintained at 26 °C as a control. The next morning, the experiments were started by adding T3 (final concentration 5 nM) into one aquarium at 4 and 26 °C and dimethyl sulfoxide (final 0.00025 %) into the other aquarium at 4 and 26 °C, and were continued for 3 or 7 days. Aquaria water was changed every day. Tadpoles were not fed during the experiment. Tadpoles were anesthetized by immersion in 0.2 % 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO, USA), after which their body weight, body length, and tail length and height were measured, their blood was collected from the heart using heparinized capillaries, and their liver was dissected. The plasma was separated from the blood cells by centrifugation at 3000×g for 15 min at 4 °C and was stored at −35 °C for later use.

All housing and experimental procedures were conducted in accordance with the code of ethics of the Animal Welfare Committee of Shizuoka University.

Measurements of plasma parameters
The concentration of glucose in plasma was determined by the mutarotase-glucose oxidase method [46] using a kit (Glucose C II-testWako), TGs by the glycerol-3-phosphate oxidase method [47] using a kit (Triglyceride E-testWako), cholesterol by the cholesterol oxidase method [48] using a kit (Cholesterol E-testWako), and free FAs by the acyl-CoA synthetase and acyl-CoA oxidase method [49] using a kit (NEFA C-testWako). All kits were used according to the manufacturer’s directions. Plasma parameters were quantified by scanning absorbance at 490 nm for glucose with a microplate reader (Emax, Molecular Devices, CA, USA), and absorbance at 600 nm for TGs and cholesterol and at 550 nm for free FAs with a spectrophotometer (U-3210, Hitachi Koki, Tokyo, Japan).

Membrane lipid analysis
Lipids were extracted from tadpole liver according to the method described elsewhere [50]. In brief, liver from two tadpoles were pooled (0.2–0.4 g), added to methanol:chloroform (2:1, v/v; 0.3 mL) solvent, and
crushed by a glass rod. After leaving the mixture at room temperature for 30 min, the supernatant was recovered by centrifugation at 1400×g for 10 min at 4 °C. The extract was washed with 0.9 % NaCl (1 mL) by agitating with a vortex mixer and centrifuging at 1400×g for 10 min at 4 °C. This process was repeated twice. The lower organic layer containing the extracted lipids was stored at −30 °C for analysis.

The extracted lipids (50 μL) were separated by TLC on a silica gel plate (KN3315756, Merck Millipore, Darmstadt, Germany) using a solvent system of chloroform:methanol:petroleum ether:acetone:acetic acid:water (20:15:10:5:1:3:1, v/v). The lipid spots were visualized under ultraviolet light after spraying with primuline (0.01 % in 80 % acetone). Each lipid spot was identified by co-chromatography with the standards: PC, PE, PS, PI, PG and CL. Each lipid spot was scraped off the silica gel plate and the silica powder was transferred to a screw-capped glass test tube. Pentadecanoic acid (100 μL of 50 μg/mL) was added to each tube as an internal standard, then methanolysis was carried out with 1 N methanolic HCl (1 mL; Sigma) for 30 min at 80 °C. Next, hexane (1 mL) and 0.9 % NaCl (1 mL) was added to the acidic solution and the solution was centrifuged at 1400×g for 10 min at 4 °C, resulting in the formation of two phases. The hexane layer was collected and evaporated by an evaporator (TC-8, TAITEC, Saitama, Japan). The residual FA methyl esters were re-suspended with hexane to 30 μL and analyzed by GC (QP2010SE, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector on a capillary column (BPX5, 30 m × 0.25 mm; SGE Analytical Science, Melbourne, Australia), according to the manufacturer's instructions. All assays were started by mixing the mitochondria-rich fraction (50 μL; 12.5 μg protein), enzyme assay buffer (425 μL), and reduced cytochrome c (25 μL), and were done at 25 °C in triplicate using a spectrophotometer. Activity was calculated by the decrease in absorbance at 550 nm using an extinction coefficient of 21.84 mmol/L/cm (manufacturer’s protocol) between ferrocytochrome c and ferricytochrome c and is expressed as μmoles/min (U) cytochrome c transformed/mg protein or U/g liver (first order reaction).

CPT activity was measured according to the method described elsewhere [51, 53]. Enzyme assay solution [250 μL of 116 mM Tris–HCl, pH 8.0, 2.5 mM ethylenediaminetetraacetic acid, 0.2 % Triton X-100, 0.5 mM 5,5′-dithiobis (2-nitrobenzoic acid)] was mixed with the mitochondria-rich fraction (20 μL; 20 μg protein) and distilled water (210 μL) in a cuvette. Next, 2 mM palmitoyl-CoA (10 μL) and 62.5 mM l-carnitine (10 μL; or enzyme assay solution for blank assay) were added to the mixture. Absorbance at 412 nm was monitored immediately at 0.2 min intervals with a spectrophotometer and the production of 5-thio-2-nitrobenzoic acid was calculated using an extinction coefficient of 13.6 mmol/L/cm [51]. CPT activity was determined as an l-carnitine-dependent rate that was obtained from the difference between the rates with and without l-carnitine, and is expressed as mU produced 5-thio-2-nitrobenzoic acid/mg protein or mU/g liver.

The protein content of the mitochondria-rich fractions was estimated by the micro-Lowry method [54] with bovine serum albumin as the standard using a microplate reader.

**Preparation of mitochondria-rich fractions and enzyme assays**

Mitochondria-rich fractions were prepared from the liver as described previously [51, 52], with some modifications. In brief, livers from two tadpoles were pooled (0.2–0.4 g), homogenized immediately in ice cold buffer (1 mL; 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid, 3 mM Tris–HCl, pH 7.5, and 1 mM reduced glutathione) in a Potter–Elvehjem homogenizer, and centrifuged at 5000×g for 10 min at 4 °C. The superficial lipid layer was removed, and the supernatant was centrifuged at 9000×g for 10 min at 4 °C twice. The pellet containing mitochondria was re-suspended in homogenization buffer (0.3 mL) and stored at −80 °C until used.

COX activity was measured by monitoring the oxidation of reduced cytochrome c at 550 nm using a kit (Cytochrome C Oxidase Activity Assay Kit, BioChain, Newark, CA, USA), according to the manufacturer’s directions. All assays were started by mixing the mitochondria-rich fraction (50 μL; 12.5 μg protein), enzyme assay buffer (425 μL), and reduced cytochrome c (25 μL), and were done at 25 °C in triplicate using a spectrophotometer. Activity was calculated by the decrease in absorbance at 550 nm using an extinction coefficient of 21.84 mmol/L/cm (manufacturer’s protocol) between ferrocytochrome c and ferricytochrome c and is expressed as μmoles/min (U) cytochrome c transformed/mg protein or U/g liver (first order reaction).

**RNA extraction and RT-qPCR analysis**

Tadpole liver (−0.1 g) was lyzed with guanidinium thiocyanate solution (1000 μL) [55]. The total RNA was isolated with phenol and chloroform. To confirm its integrity, RNA (1 μg per lane) was electrophoresed in a 1 % agarose gel containing 2.0 M formaldehyde, and 28S and 18S rRNAs were visualized by ethidium bromide staining. Complementary DNAs were synthesized from the total RNA (200 ng) in 10 μL of 1× Taqman RT buffer using Taqman RT reagents kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The expression of individual genes was estimated in triplicate using Power SYBR Green Master Mix and ABI Prism 7000 sequence detection System (Applied Biosystems) with a specific primer set (each 200 nM), as shown previously [5]. Detailed information of primer sets and RT-qPCR conditions is shown in Additional files 5 and 6: Tables S3 (Additional file 5) and S4 (Additional file 6). We included controls lacking cDNA template to determine the specificity of target cDNA amplification.
All assays produced unique dissociation curves. The endpoint used in real-time PCR quantification, \( C_q \) was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold and is a function of the amount of target DNA present in the starting material. Quantification was determined by applying the \( 2^{-\Delta\Delta C_q} \) formula and calculating the average of the values obtained for each sample. Eligibility of this formula was verified by qPCR using RT-qPCR or RT product of total RNA as a template at different concentrations that covered 3–5 orders of magnitude. To standardize each experiment, the amounts of the test transcripts were divided by those for the ribosomal protein L8 (rpl8) and lactate dehydrogenase B (ldhb) transcripts, we used the actb as a reference gene.

### Statistical analysis

All assay data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by one-way or two-way analysis of variance using the Fisher’s test for multiple comparisons to show significant differences. \( p < 0.05 \) was considered statistically significant.

### Additional files

**Additional file 1: Table S1.** Morphological changes of the tadpoles exposed to cold and 3,3′,5-triiodothyronine (T3).

**Additional file 2: Tables S2-1 to S2-6.** Changes in compositions of fatty acids in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and cardiolipin from the tadpole liver.

**Additional file 3: Figure S1.** Pathways of glycerophospholipid synthesis (A) and very long chain fatty acid desaturation and elongation (B) related to the transcription data. Transcription data and full names of genes are shown in Fig. 7 and its legend. Transcription regulatory protein is boxed in green, hepatic lipids and fatty acids analyzed (see Table 1 and Fig. 2) are boxed. 3,3′,5-Triiodothyronine-response and cold-response genes on day 3 or day 7 are marked in red and blue, respectively. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; Cho-p, choline phosphate; CDP-Cho, cytidine diphosphate choline; CDP-Etn, cytidine diphosphate ethanolamine; PA, palmitic acid; POA, palmitoleic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

**Additional file 4: Table S2.** Energy metabolic pathways related to the transcriptional data. Transcription data and full names of genes are shown in Fig. 6 and its legend. Transcription regulatory proteins are boxed in green, plasma biochemical components analyzed (see Fig. 1) are boxed. Enzyme activities examined are underlined 3,3′,5-Triiodothyronine-response and cold-response genes on day 3 or day 7 are marked in red and blue, respectively.

**Additional file 5: Table S3.** The list of primers used in quantitative polymerase chain reaction.

**Additional file 6: Table S4.** Detailed quantitative polymerase chain reaction information.

### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CL | cardiolin | | COX | cytochrome c oxidase |
| CPT | carnitine palmitoyltransferase | | FA | fatty acid |
| GC | gas chromatography | | MUFa | monounsaturated fatty acid |
| PC | phosphatidylcholine | | PG | phosphatidyglycerol | |
| PI | phosphatidylinositol | | PS | phosphatidylserine | |
| PI | phosphatidylglycerol | | GLA | γ-linolenic acid | |
| PE | phosphatidylethanolamine | | AA | arachidonic acid | |
| PS | phosphatidylserine | | OA | oleic acid | |
| PI | phosphatidylglycerol | | LA | linoleic acid | |
| CL | cardiolipin | | GLA | γ-linolenic acid | |
| Cho-p | choline phosphate | | EPA | eicosapentaenoic acid | |
| CDP-Cho | cytidine diphosphate choline | | DPA | docosapentaenoic acid | |
| CDP-Etn | cytidine diphosphate ethanolamine | | DHA | docosahexaenoic acid | |

### References

1. Frieden E, Wahlborg A, Howard E. Temperature control of the response of tadpoles to triiodothyronine. Nature. 1965;205:1173–6.
2. Viparina S, Just JJ. The life period, growth and differentiation of Rana catesbeiana larvae occurring in nature. Copeia. 1975;1975:103–9.
3. Murata T, Yamauchi K. Low-temperature arrest of the triiodothyronine-dependent transcription in Rana catesbeiana red blood cells. Endocrinology. 2005;146(1):256–64. doi:10.1210/en.2004-1090.
4. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocrin Rev. 2010;31(2):139–70. doi:10.1210/er.2009-0007.
5. Mochizuki K, Goda T, Yamauchi K. Gene expression profile in the liver of Rana catesbeiana tadpoles exposed to low temperature in the presence of thyroid hormone. Biochem Biophys Res Commun. 2012;420(4):845–50. doi:10.1016/j.bbrc.2012.03.085.
6. Little AG, Kunise T, Kannan K, Seebacher F. Thyroid hormone actions are temperature-specific and regulate thermal acclimation in zebrafish (Danio rerio). BMC Biol. 2013;11:26. doi:10.1186/1741-7007-11-26.
7. Hazel JR. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? Annu Rev Physiol. 1995;57:19–42. doi:10.1146/annurev.ph.57.030195.000315.
8. Nakagawa Y, Sakamoto N, Kaneko Y, Harashima S. Mg2+ is a putative sensor for low temperature and oxygen to induce OLE1 transcription in Saccharomyces cerevisiae. Biochem Biophys Res Commun. 2002;291(3):707–13. doi:10.1006/bbrc.2002.6507.
9. Hiseh SL, Kuo CM. Stearoyl-CoA desaturase expression and fatty acid composition in milkfish (Chanos chanos) and grass carp (Ctenopharyngodon idella) during cold acclimation. Comp Biochem Physiol B Biochem Mol Biol. 2005;141(1):95–101. doi:10.1016/j.cbb.2005.02.001.
10. Long Y, Li L, Li Q, He X, Cui Z. Transcriptomic characterization of temperature stress responses in larval zebrafish. PLoS One. 2012;7(5):e37209. doi:10.1371/journal.pone.0037209.
11. Tiku PE, Gracey AY, Macarney AJ, Beynon RJ, Cossins AR. Cold-induced expression of Δ5-desaturase in carp by transcriptional and posttranslational mechanisms. Science. 1996;271(5250):815–8.

12. Kräfte E, Marty Y, Guderley H. Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout Oncorhynchus mykiss: roles of membrane proteins, phospholipids and their fatty acid compositions. J Exp Biol. 2007;210(Pt 1):149–65. doi:10.1242/jeb.02628.

13. Shi YB. Amphibian metamorphosis, from morphology to molecular biology. New York: Wiley-Liss; 1999. p. 102–22.

14. Saloniemi T, Jokela H, Strauss L, Pakarinen P, Poutanen M. The diversity of Δ9-desaturase in carp by transcriptional and posttranslational mechanisms. 200900528.

15. Borras J. Gilthead sea bream liver proteome altered at low temperature on proximate composition and fatty acid profiles in Sparus aurata. J Exp Biol. 2005;249(1):477–86. doi:10.1242/jeb.046854.

16. Wodtke E. Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp (Cyprinus carpio L.). Biochim Biophys Acta. 1981;640(3):698–709.

17. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. J Physiol. 1979;236(1):91–101.

18. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.

19. Weitzel JM, Iven KA. Coordination of mitochondrial biogenesis by thyroid hormone. Mol Cell Endocrinol. 2011;342(1–2):1–7. doi:10.1016/j.mce.2011.05.009.

20. O’Brien KM. Mitochondrial biogenesis in cold-acclimated fishes. J Exp Biol. 2011;214(Pt 22):4757–65. doi:10.1242/jeb.064854.

21. St-Pierre J, Charest P-M, Guderley H. Relative contribution of quantitative and qualitative changes in mitochondria to metabolic compensation during seasonal acclimatization of rainbow trout Oncorhynchus mykiss. J Exp Biol. 1998;211(26):2961–70.

22. Wodtke E. Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp (Cyprinus carpio L.). Biochim Biophys Acta. 1981;640(3):698–709.

23. Hammond SA, Veldhoen N, Helbing CC. Influence of temperature on thyroid hormone signaling and endocrine disruptor action in Rana (Lithobates) catesbeiana tadpoles. Gen Comp Endocrinol. 2015;196–9. doi:10.1016/j.ygcen.2016.04.001.

24. Frye BE. Metabolic changes in the blood sugar and the pancreatic islets of the frog, Rana clamitans. J Exp Zool. 1964;155(2):215–23.

25. Farrar ES, Frye BE. Comparison of blood glucose and liver glycogen of larval and adult frogs (Rana pipiens). Gen Comp Endocrinol. 1973;21(3):513–6.

26. Frieden E, Mathews H. Biochemistry of amphibian metamorphosis. Ill. Liver and rana pipiens. Am J Physiol. 1958;193(1):107–19.

27. Steiner AA, Petenisco SQ, Bentegani LG, Branco LG. The importance of glucose for the freezing tolerance/tolerance of the anuran amphibians Rana catesbeiana and Bufo paracnemis. Rev Bras Biol. 2004;60(2):321–8.

28. Storey KB, Storey JM. Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze–thaw cycles. Can J Zool. 2006;84(1):49–56.

29. Swanison DL, Graves BM, Koster KL. Freezing tolerance/intolerance and lipid storage by thyroid hormone in mouse liver. Cell Biosci. 2014;4:38. doi:10.1186/2045-3701-4-38.

30. Wodtke E. Lipid adaptation in liver mitochondrial membranes of carp acclimated to different environmental temperatures: phospholipid composition, fatty acid pattern, and cholesterol content. Biochim Biophys Acta. 1978;529(2):280–91.

31. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol. 1979;236(1):91–101.

32. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.

33. Shi YB. Amphibian metamorphosis, from morphology to molecular biology. New York: Wiley-Liss; 1999. p. 102–22.

34. Gracey AY, Fraser EJ, Li W, Fang Y, Taylor RR, Rogers J, Brass A, Cossins AR. Coping with cold: an integrative, multi-tissue analysis of the transcriptome of a poikilothermic vertebrate. Proc Natl Acad Sci USA. 2004;101(48):16970–5.

35. Weber LW, Boll M, Stampfl A. Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. World J Gastroenterol. 2010;16(21):3081–7.

36. Sokolovic M, Sokolovic A, Wehkamp D, Loren Ver, THEMART A, DE Waart DR, Gilhuys-Pederson LA, et al. The transcriptomic signature of fasting murine liver. BMC Genom. 2008;9:528. doi:10.1186/1471-2164-9-528.

37. Davis KB. Temperature affects physiological stress responses to acute confinement in sunshine bass (Morone chrysops x Morone saxatilis). Comp Biochem Physiol A Mol Integr Physiol. 2004;139(4):433–40. doi:10.1016/j.cbpa.2004.09.012.

38. Jain S, Brout BC, Stevenson JR. Cold stress leads to increased splenocyte responsiveness to concanavalin A, decreased serum testosterone, and increased serum corticosterone, glucose, and protein. Life Sci. 1996;59(3):209–18.

39. Shi YB. Amphibian metamorphosis, from morphology to molecular biology. New York: Wiley-Liss; 1999. p. 102–22.

40. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol. 1979;236(1):91–101.

41. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.

42. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol. 1979;236(1):91–101.

43. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.

44. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol. 1979;236(1):91–101.

45. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.

46. Hazel JR. Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol. 1979;236(1):91–101.

47. Hazel JR. The incorporation of unsaturated fatty acids of the n-9, n-6, and n-3 families into individual phospholipids by isolated hepatocytes of thermally-acclimated rainbow trout, Salmo gairdneri. J Exp Zool. 1983;227(2):167–76.