Oxytocin Signaling Regulates the Homeostatic Response to Cold Stress in Poikilothermic Vertebrates

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Abstract

When exposed to low temperature, homeothermic vertebrates maintain internal body temperature by activating thermogenesis and by altered metabolism, synchronized by neuroendocrine responses. Although such physiological responses also occur in poikilothermic vertebrates, the prevailing notion is that their reactions are passive. Here, we explored molecular hypothalamic and physiological responses to cold stress in the tropical poikilotherm Nile tilapia (*Oreochromis niloticus*). We show that cold exposed tilapia exhibit complex homeostatic responses, including increased hypothalamic oxytocin, plasma glucose and cortisol concomitant with reduced plasma lactate and metabolic rate. Pharmacological or genetic blockage of oxytocin signaling further affected metabolic rate in two cold-exposed poikilothermic models. This indicates that oxytocin, a known thermoregulator in homeotherms, actively regulates temperature-related homeostasis in poikilotherms. Overall, our findings show that the brain of poikilotherms actively responds to cold temperature by regulating metabolic physiology. Moreover, we identify oxytocin signaling as an adaptive and evolutionarily conserved metabolic regulator of temperature-related homeostasis.

Introduction

The notion of physiological homeostasis was conceived more than 140 years ago on the basis of equilibrium thermodynamics. The homeostatic view postulates that while biological systems are unstable by nature, they are regulated to maintain a dynamic equilibrium[1-3]. For example, temperature-related homeostasis is achieved in homeotherms through physiological and central mechanisms, such as metabolic heat generation. By contrast, in poikilotherms, commonly termed "cold-blooded" animals, it is achieved through behavioral modification, such as heat seeking behaviors in responses to cold stress[4]. Nevertheless, poikilotherms exposed to environmental extremes of cold temperatures experience a stressful metabolic challenge, which elicits physiological responses required to maintain cellular homeostasis. Moreover, heat seeking may not resolve the homeostatic needs of tropical poikilotherms under unpredictable extreme cold events, which occur frequently due to global climate change[5,6].

Because poikilotherms cannot generate heat, it is expected that evolutionary pressure would lead their cells and tissues to develop responsive mechanisms to temperature alterations in order to maintain functionality and fitness under suboptimal conditions[7]. Indeed, previous studies have reported various responses to low temperature, including modified glucose or lipid metabolism[6,8-11], altered gene expression and alternative splicing[10,12-14], and endocrine and immune system activity[15,16], which may vary according to the tissue and species. This suggests that the homeostatic response to cold stress in poikilotherms involves multiple physiological pathways and is more complex than currently perceived.

In vertebrates, the homeostatic activity of various organs and tissues is orchestrated by the brain hypothalamic region. The hypothalamus integrates sensory input from the internal and external environments and secretes regulatory neuropeptides and monoamines, which continually fine-tune physiological functions and maintain body homeostasis[17,18]. Several studies have demonstrated the thermoregulatory roles of hypothalamus in homeotherms[19,20].

Bony fish (Class *Osteichthyes*), which comprise the largest group of vertebrates[21], are poikilothermic[6,13]. As such, they have developed various strategies to survive in extreme cold waters, like hibernation and plasma antifreeze proteins. Yet, these

strategies are utilized mainly by species inhabiting low-temperature environments, which encounter these conditions routinely throughout their life history[22]. In the last century, tropical fish species are increasingly exposed to extreme cold events due to several factors. First, climate change leads to increased weather events of extreme heat but also of extreme cold[5]. Second, the continuous expansion of the aquaculture industry leads to culture of tropical species in subtropical climates[23,24]. Third, aquaculture escapees, fish introductions for recreational angling and migration from saturated ecosystems result in species invasion into new ecosystems and ecological niches[23,25,26]. Exposure of a tropical fish to cold conditions would challenge its homeostasis and generate physiological stress. Yet, some species manage to survive exposure to cold temperatures and even thrive in climates that include cold seasons, which differ from their ecological life history. Understanding the central regulation of these homeostatic processes is important for ecological conservation and aquaculture, and may shed much needed light on the evolutionary origins of physiological thermoregulation in poikilothermic organisms.

Nile tilapia (*Oreochromis niloticus*) is one of the most important cultured fish worldwide. Originating from Africa, it is now cultured in more than 85 countries. With high reproductive rate, aggressive behavior, and a wide range of feeding sources, tilapia escapees have successfully invaded many ecosystems, including in sub-optimal climates[27,28]. Although some physiological and molecular homeostatic responses of tilapias to cold stress have been demonstrated[6,9,11,12,15], the general metabolic relevance and hypothalamic regulation of these processes in poikilothermic fish remain mainly unexplored. In the present study, we examined the influence of cold exposure on Nile tilapia metabolism. As previously demonstrated in other piscine species[29], we found a direct correlation between temperature and resting (standard) metabolic rate (RMR), as well as alterations in stress-related metabolic parameters upon exposure to extreme cold. In search of a central regulatory pathway that controls these physiological responses, we performed transcriptome analysis of Nile tilapia hypothalamus. Results showed that oxytocin (Oxt), a key regulator of core temperature in homeothermic mammals[30], is markedly elevated upon exposure to extreme cold. Indeed, analysis of metabolic responses using pharmacological Oxt-receptor antagonist (ORA)[31,32] in tilapia or genetic perturbation of Oxt signaling in zebrafish (*Danio rerio*) showed an Oxt-dependent decline in RMR during extreme

cold exposure. These findings indicate that Oxt signaling is involved in the central thermoregulation of the physiological response to cold in poikilotherms, a function that was leveraged by homeotherms later in evolution. More broadly, our findings suggest that neuroendocrine pathways can modulate poikilothermic adaptiveness to climate change within physiological boundaries.

Results

Physiological response to cold stress

Survival temperatures of poikilothermic species are strongly correlated with their geographical distribution[33], as reflected by the limited geographical expansion of Nile tilapia to tropical and subtropical areas[23,34]. Therefore, we utilized this fish as our model for studying regulation of homeostatic response to cold stress in poikilothermic vertebrates. To characterize the effects of cold temperature exposure on RMR and physiology of Nile tilapia, we analyzed the fish metabolic rate while reducing water temperature from 25^{o}C to 14^{o}C, at a rate of -1^{o}C/h, followed by plasma analysis for major stress indicators and metabolic parameters (**Fig. 1**). This analysis showed direct correlation between temperature and RMR in Nile tilapia (**Fig. 1a-c**). In agreement with previous findings[11,15,35], plasma cortisol and glucose levels significantly increased upon cold exposure, supporting a physiological stress response (**Fig. 1d-e**). Plasma lactate levels significantly decreased, which was also expected considering the reduction in RMR (**Fig. 1f**). These results are in line with cold-induced gluconeogenesis, which has previously been demonstrated by us and others[6,9]. No significant changes were found in plasma levels of total protein, triglycerides or growth hormone (**Fig. 1g-i**).

Central pathways involved in the response to cold stress

In vertebrates, homeostatic functions are orchestrated by the hypothalamus, which serves as the central sensory and regulatory hub of peripheral body systems[17,36,37]. During an adaptive response, the hypothalamus generates a quiescent reaction to a strong metabolic challenge, while maintaining low physiological noise from other affected systems[3]. Hence, a reduction in the environmental temperature should induce a hypothalamic response that would change physiological and endocrine parameters,

153 such as plasma levels of lactate, glucose and cortisol. To determine whether such
154 homeostatic regulation occurs in fish hypothalamus, we dissected midbrains of Nile
155 tilapia to include the diencephalon and optic tectum, which encompass all
156 hypothalamic nuclei[38,39] (**Fig. 2a**). Isolated midbrains from cold-exposed and
157 normothermic fish were subsequently assessed for changes in gene expression levels
158 by transcriptome analysis. First, the accuracy of midbrain dissections was confirmed
159 by the expression of known hypothalamic neuroendocrine markers of the various
160 hypothalamic regions, including *avp* (LOC100708704), *tac1*, *gal* (*galn*), *pomc*
161 (*pomca*), *agrp* (LOC100691312), *trh*, *crh* (*crhb*) and others. Results showed that the
162 expression of these genes was not significantly altered in the hypothalamus of cold-
163 exposed Nile tilapia, compared to the controls (**Supp. Fig. 1**), confirming the integrity
164 and uniformity of the hypothalamic dissections in both groups.

165 Next, we interrogated gene expression changes in the hypothalamus of cold-exposed
166 Nile tilapia, using IsoDE2[40]. We found that 927 genes were significantly upregulated
167 upon cold exposure, whereas 1971 genes were significantly downregulated (>2-fold
168 change, P<0.01) (**Fig. 2b; Supp. Table 1**). To understand the biological processes
169 that were affected by the differentially expressed genes, we next performed functional
170 annotation analysis. Submission of the 911 downregulated genes that were expressed
171 above 1 TPM (transcripts per million) in the control to g:Profiler[41] yielded seven
172 significant GO terms, which included broad functions such as “receptor signaling
173 activity” and “peptide receptor activity” (**Table 1**). This suggested a generalized
174 suppression of signaling pathways in Nile tilapia hypothalamus upon cold exposure.
175 The 485 upregulated genes that were expressed above 1 TPM in the cold-exposed fish
176 were significantly enriched for 21 GO terms (**Table 2**). These included the cellular
177 component hemoglobin complex, apoptotic processes and cell death, as well as
178 circadian rhythm pathways such as “circadian regulation of gene expression” and
179 “rhythmic process”, suggesting that cold exposure affects the circadian rhythm of Nile
180 tilapia (**Table 2**).

181 As expected by the altered metabolic rate, analysis of the most highly expressed
182 upregulated genes revealed the presence of several genes encoding hemoglobin
183 subunits, suggesting that the adaptive response of cold-exposed Nile tilapia may
184 require increased brain oxygenation (**Fig. 2c**). While the general suppression of genes
185 related to signaling pathways seems to support the concept of reduced metabolic

responsiveness in cold-exposed poikilotherms[22], we discovered that *oxt*, the gene encoding OXT neuropeptide, was significantly upregulated in the hypothalamus of Nile tilapia upon cold exposure (**Fig. 2c**). Similar analysis of the most highly expressed downregulated genes revealed suppression of several factors involved in the regulation of mRNA expression and processing (**Fig. 2d**). Our transcriptomic analysis was further validated by real-time PCR quantification of cold-induced (**Supp. Fig. 2**) and cold-suppressed (**Supp. Fig. 3**) mRNAs. These results support differential gene activation or suppression according to their involvement in specific cellular and physiological functions. Furthermore, the exceptional responsiveness of *oxt* expression to cold exposure suggests that OXT signaling is involved in the central regulation of the homeostatic response to cold stress in poikilotherms.

OXT signaling reduces metabolism in poikilotherms under extreme cold conditions

The homeostatic response of homeothermic mammals to cold stress involves hypothalamic activation of OXT-neurons, which in turn elicit energy expenditure and thermogenesis to maintain core temperature[42,43]. However, in poikilothermic fishes low temperatures usually suppress feeding[44] and, therefore, increased energetic expenditure may exhaust the energy storage of the animal and thereby risk its survival. Thus, we next aimed to determine whether the observed increase in *oxt* expression is related to poikilotherm thermoregulation through OXT signaling, or merely a result of globally altered regulation of gene expression. For this purpose, we used the OXT receptor-specific antagonist (ORA) L-368,899, which was shown to block OXT pathway from fish to mammals[31,32]. Nile tilapia were injected intraperitoneally with 1 mg/kg BW ORA and analyzed for their metabolic rate. Results showed that ORA significantly suppressed the cold-driven reduction in metabolic rate, an effect that was not detected in ORA-injected fish maintained in normothermy (**Fig. 3a-b**). Importantly, the effectiveness of ORA was seen only down to ~19^{o}C, suggesting that oxytocinergic regulation is only effective within the life history-shaped metabolic constrains of the species. This finding was accompanied by a significant reduction of plasma cortisol in ORA-treated fish exposed to cold stress (**Fig. 3c**). ORA did not affect plasma levels of glucose or lactate, supporting temperature-related metabolic rate regulation by OXT through modulation of physiological stress response.

The timeframe of the experiment was dictated by the previously reported pharmacokinetics of L-368,899[32]. Therefore, although ORA did not affect glucose and lactate levels (**Fig. 3d** and **Fig. 3e**, respectively), we could not exclude the involvement of OXT in gluconeogenesis and lactate metabolism, as these effects may require prolonged activation. ORA probably led to increased *oxt* expression due to activation of a feedback loop aimed to regain OXT receptor (OXTR) activity. Interestingly, this effect was more robust under normothermic conditions (**Fig. 3f**). Real-time PCR analysis of mRNAs which were also used for transcriptome validation showed that their expression was either unchanged or affected mainly by the change in temperature, rather than by ORA treatment (**Fig. 3g** and **Supp. Fig. 4**). The second most downregulated gene in response to cold, *anserinase* (*ansn*; **Fig. 2d**), is an orthologue of the homeothermic carnosinase enzyme unique to poikilothermic vertebrates[45,46]. Interestingly, ORA significantly induced *ansn* mRNA expression (**Fig. 3h**). These catabolic enzymes and their anserine/carnosine substrates have been associated with cognitive functioning, neurovascular activity and physiological homeostasis of histidine-containing dipeptides[45-47]. This further supports the specific activity of OXT in homeostatic regulation of tilapia's metabolic response to cold stress.

Evolutionary conservation of OXT signaling in temperature-related homeostasis

Our findings in Nile tilapia suggested that OXT signaling is a central regulatory pathway for temperature-related metabolic homeostasis in poikilotherms. To expand the evolutionary relevance of our findings, we used zebrafish (*Danio rerio*) as a complementary species. Although both species belong to the class of ray-finned fish (*Actinopterygii*), they are separated by over 300 million years of evolution[48]. Furthermore, while Nile tilapia originate from Africa, zebrafish originate from the Indian subcontinent and naturally experience a wider temperature range, making it more resilient to temperature extremes[9]. Thus, we used *oxt* and *oxtr* knockout ($oxt^{-/-}$ and $oxtr^{-/-}$, respectively) zebrafish germlines[49,50] to analyze the involvement of OXT signaling in the central regulation of temperature-related metabolism in a distant poikilothermic species (**Fig. 4a).** Our analysis demonstrated that under normothermic but not under extreme cold conditions, $oxtr^{-/-}$ and, to a lesser extent, $oxt^{-/-}$ mutant zebrafish display significantly reduced RMR (**Fig. 4b**). This finding suggests that OXT signaling is involved in maintaining basal metabolic functions in zebrafish. In

view of the suppressed baseline RMR in *oxt*$^{-/-}$ and *oxtr*$^{-/-}$, the possible link between OXT signaling and zebrafish RMR during cold exposure was analyzed by subtracting the baseline RMR from the average RMR in each temperature (**Fig. 4c**). A direct correlation was found between reduced water temperature and zebrafish RMR; yet, this RMR suppression was faster in wild-type (WT) than in *oxt*$^{-/-}$ and *oxtr*$^{-/-}$ mutants (**Fig. 4c**). These findings support OXT signaling as a key pathway in the regulation of zebrafish baseline metabolic maintenance and cold-induced homeostatic adaptation.

Discussion

Homeostasis is a fundamental dogma in physiology. It states that environmental perturbations elicit physiological responses in the organism, which strive to regain stability and maintain fitness[1,3]. While the central and physiological responses of homeotherms to cold stress have been extensively studied, research of low temperature-related homeostasis in poikilothermic vertebrates has been narrowed to heat seeking behaviors, antifreeze protein production or hibernation[4,22]. Nevertheless, several studies demonstrated that active physiological and central modifications occur in poikilothermic fish exposed to cold stress[9,10,12,13]. Because the vertebrate hypothalamus serves as the homeostatic regulator of many physiological processes, its active response to extreme cold should support a thermoregulatory function. However, central pathways orchestrating such homeostatic responses have yet been identified[7]. Our current findings provide pioneering evidence for a central neuroendocrine regulation of metabolic rate in a poikilothermic vertebrate under cold stress conditions. We show that extreme cold exposure elicits a physiological stress response, which is accompanied by transcriptional upregulation of *oxt* in the midbrain-hypothalamic compartment. Next, we used an OXTR-specific antagonist and genetic KO models to demonstrate that OXT signaling regulates both metabolic rate and homeostatic physiology in cold-exposed poikilothermic vertebrates. Importantly, these data can also expand our understanding of the ecological impacts of globally increased incidences of extreme whether events and of invasive fish species, inadvertently introduced by the constantly expanding global aquaculture.

Acute cold exposure was shown to induce stress parameters including increased levels of plasma cortisol and catecholamines from fish to human[11,15,51]. However, while

283 mammals exposed to extreme cold exhibit induction of energetic expenditure[4,51], data
284 by us and others[22] show that rapid cold exposure in fish leads to reduced metabolic
285 rate. As cold exposure suppresses feeding and activity in poikilothermic fish[44,52],
286 reduction in energetic expenditure is clearly beneficial for its fitness and survival
287 under these conditions. Therefore, although these endocrine responses are widely
288 conserved throughout evolution, their physiological manifestations should still differ
289 according to the physiological constrains of the species and its life history.

290 The responses of poikilothermic fish to sharp declines in environmental temperatures
291 were generalized to behavioral thermoregulation or cessation of physiological
292 activity, whereas adaptive physiological mechanisms were considered only in polar or
293 endothermic fish species[22]. Nevertheless, despite the expected RMR reduction in
294 cold-exposed Nile tilapia, our data support an active homeostatic adaptation to the
295 new conditions within the physiological boundaries of a tropical poikilotherm. This is
296 well reflected by the increased levels of plasma cortisol, which is a known regulator
297 of stress-related adaptation affecting multiple metabolic pathways[53]. Furthermore,
298 cortisol was previously shown to significantly increase gluconeogenesis from lactate
299 in the liver of several fish species[53], which can explain why lactate levels remained
300 low although glucose levels increased in cold-exposed fish. These data support a more
301 active and complex homeostatic response to extreme cold than previously assumed.

302 Carbohydrate homeostasis is mainly performed in the liver[54], corticosteroids are
303 regulated by interrenal chromaffin cells of fish or adrenal cortex in mammals[17], and
304 catecholamine homeostasis involves synthesis in the autonomic nervous system and
305 adrenal medulla and may also affect glucose homeostasis[55,56]. If these physiological
306 changes are to increase the animal's fitness, they must be synchronized with signals
307 from the internal and external environments. While previous works have analyzed
308 whole-brain transcriptomes[9,12,13], our analysis focused in the vertebrates central
309 homeostatic center, the hypothalamus[36]. Hence, in our search for a central regulator of
310 the homeostatic response to cold stress in the poikilothermic Nile tilapia, we
311 performed transcriptome analysis of the midbrain-hypothalamic compartment. The
312 results suggested increased expression of genes involved in oxygen transport, cellular
313 apoptosis and circadian rhythm pathways and suppressed expression of genes
314 involved in pathways of peptide-related receptor signaling. This generally supports
315 our initial hypothesis that poikilotherms exhibit active regulation of cold-driven

316 homeostatic responses. Furthermore, the general suppression of peptide-related
317 signaling is in line with our finding that cold exposure strongly affected midbrain *oxt*
318 expression, suggesting that OXT is involved in the central regulation of the
319 homeostatic response to cold stress. OXT is a known homeostasis-controlling
320 neuropeptide involved in the regulation of metabolic physiology, behavioral and
321 neuroendocrine stress responses and was recently suggested as a mediator of
322 interactions between these homeostatic functions[30,57]. Additionally, OXT peptide
323 sequence is evolutionarily conserved from worms to humans and so are some of its
324 functions[58]. OXT and its cognate receptor are known regulators of mammalian core
325 temperature by activation of physiological heat generation pathways[30,42]. OXT and
326 OXTR mutant mice displayed impaired thermoregulation[42,59] and their central
327 recovery was sufficient to restore this function[43,60], further supporting a direct
328 involvement of OXT in temperature-related homeostasis. Nonetheless, to our
329 knowledge, the role of OXT in temperature-related metabolic homeostasis of
330 poikilothermic vertebrates has not yet been elucidated.

331 Strikingly, administration of ORA suppressed the temperature-dependent reduction in
332 standard metabolic rate of cold-exposed tilapia. In addition, ORA affected plasma
333 cortisol and central expression of specific mRNAs, supporting OXT signaling as an
334 active and specific modulatory pathway of cold-related metabolic rate and physiology
335 in a poikilothermic vertebrate model. These findings suggest that extreme cold
336 exposure induce oxytocinergic signaling in the hypothalamus in order to suppress
337 general energetic expenditure. Aiming to gain an evolutionary perspective of our
338 findings, we analyzed our recently generated *oxt*$^{-/-}$[49] and *oxtr*$^{-/-}$[50] zebrafish lines under
339 similar temperature challenge. Both mutant lines exhibited lower RMR under
340 normothermy, suggesting that OXT signaling has an important role in maintaining
341 general metabolism in the animal. This is in agreement with previous findings that
342 mouse *Oxt*$^{-/-}$ and *Oxtr*$^{-/-}$ models demonstrate imbalanced energetic consumption
343 versus expenditure[42]. Nonetheless, as seen in tilapia, mutant zebrafish exposed to
344 extreme cold displayed lower RMR reduction rate compared to WT zebrafish.

345 In light of our current findings, we suggest that OXT signaling is a key regulator of
346 low temperature-related metabolism in poikilothermic vertebrates. Moreover, we
347 propose that instead of passive metabolism in poikilothermic vertebrates exposed to
348 low temperatures[22], there is an adaptive regulation of metabolic homeostasis, within

physiological constrains. Therefore, while oxytocinergic signaling in homeotherms provoke internal heat production to maintain activity in cold environments, it actively suppresses energetic expenditure in cold exposed poikilotherms aiming to preserve energetic storage under low activity conditions. This notion should be incorporated into predictive modeling for aquaculture and invasive potential when considering introduction of non-native poikilotherms[23,25,28], taking into account their homeostatic range in addition to their life history ecosystem. The relatively high amenability of aquaculture species to genome editing and rapid industry growth[61] suggest that these considerations should also be applied to genome-edited lines intended for aquaculture in their native ecosystems. It was recently suggested that global temperature extremes may risk more than 60% of piscine species, mainly by affecting embryonal and reproductive life stages[62]. The high importance of OXT signaling to the embryonal development of hypothalamo-neurohypophyseal system[63] and regulation of reproductive functions and behaviors were previously demonstrated from invertebrates to humans[58]. Thus, we suggest OXT as an evolutionarily conserved key neuroendocrine regulator of thermo-metabolic homeostasis in animals.

Materials and Methods

Animals

The experiments were approved by the Agricultural Research Organization Committee for Ethics in Using Experimental Animals (approval number: 806/18 IL). Nile tilapia males (body weight, 80±15 g) were raised in cylindrical 250-liter tanks (n = 8 fish/tank). Temperature was maintained at 24–26ºC. Ammonia and nitrite levels were monitored. Fish were fed twice daily ad libitum with commercial tilapia feeds (Zemach Feed Mills^{TM}, Israel). Zebrafish (body weight, 0.49±0.12 g) were maintained under standard procedures as previously described[64]. Briefly, all genotypes were bred and reared at 28.5°C under 14 h/10 h light/dark cycle. Embryos were raised at 28.5°C in 30% Danieau's medium supplemented with 0.01 mg/L methylene blue. Fish were deprived of food for 24 h prior to metabolic measurements to avoid digestion-related oxygen consumption.

Metabolic rate analysis

The effect of gradual temperature decline on RMR was measured using an intermittent-flow respirometry system (Loligo Systems, Viborg, Denmark). Tilapia system included eight 1L acrylic cylindrical chambers that were equally allocated to control or cold exposure groups. Chambers were placed in a thermally regulated water tank with separated compartments of 77 L each. Temperature of control group was maintained at 26°C (±0.5°C) using a standard heating element, while temperature of treatment group was regulated using a refrigerated/heated bath circulator (Arctic Series A25, ThermoFisher Scientific, USA) linked to a submerged heat exchanger coil. Oxygen levels in tanks were maintained at 90%-100% O_2 saturation using multiple air stones and water were recirculated through a UV lamp apparatus to avoid bacterial growth. Each respirometric chamber was connected to two separate water pumps (Eheim, Germany). One was used for flushing between subsequent measurements, whereas the other was used for recirculating water to allow for dissolved oxygen measurement via a flow-through oxygen cell and a mini spot sensor connected through an optical fiber to a Witrox oxygen meter. Temperature was continuously monitored using a software-integrated thermometer; system operation as well as data monitoring were performed using AutoResp software (ver. 2.2.2, Loligo

Systems). The respirometric system was drained and cleaned between runs to prevent the development of biofilm which may cause background respiration.

The experiment included 24 fish (n=12 fish/treatment) and was divided into 3 subsequent and identical runs of 8 fish per run (n=4 fish/treatment) over the course of one week. In each run, fish were weighed and randomly placed in either treatment or control chambers for a 4-hour acclimation. Overnight measurements began at 5 pm and lasted for 15 h, during which temperature for treatment group was reduced from 26°C to 14°C (±0.5°C) at an average rate of 0.75°C/h. Mass-specific O_2 consumption ($\dot{M}O_2$; mg O_2/kg/h) was continuously measured in 8.5 minute cycles, each composed of a "flush" (3 min), "wait" (0.5 min) and "measure" (5 min) periods. Duration of measurement cycles was empirically tested to avoid reaching below 80% O_2 saturation and minimize additional effect of respiratory stress. For each animal, RMR was analyzed in 1°C (±0.5) bins.

Zebrafish system included eight 11.5 mL glass cylindrical chambers. Chambers were placed in one of two thermally regulated 10 L water tanks connected through a 70 L reservoir. Temperature was maintained at 27°C (±0.5°C) using a standard heating element followed by gradual decrease averaged at ~1°C/h using a refrigerated/heated bath circulator (Arctic Series A25, ThermoFisher Scientific, USA) linked to a heat exchanger coil submerged in the main reservoir. Oxygen levels in tanks were maintained at 90%-100% O_2 saturation. Each respirometric chamber was connected to two separate miniature impeller pumps (PU10700, Loligo Systems). Flow scheme and regulation were as described for tilapia. $\dot{M}O_2$ was continuously measured using 300 seconds cycles, each composed of a "flush" (90 seconds), "wait" (30 seconds) and "measure" (180 seconds) periods. To avoid inter-measurements bias, every trial contained 1-2 fish from each genotype, which were randomly assigned to the respirometric chambers. For each animal, RMR was analyzed in 1°C (±0.5) bins.

Pharmacological treatment

To assess the involvement of OXT signaling in the metabolic rate at rest of cold-exposed Nile tilapia, fish were intraperitoneally injected with L-368,899 (ChemCruz, Dallas, TX, USA), which is a known ORA[31,32]. Control fish were intraperitoneally injected with saline. Metabolic analysis of pharmacologically treated fish was

performed as described above, with slight modifications. Analysis was performed in four replicates, each included 4 control (2 normothermy and 2 cold- exposed) and 4 ORA-treated (2 normothermy and 2 cold-exposed) fish. In view of the previously described pharmacokinetics of L-368,899[29,30], temperature reduction in the cold-exposed group was modified to 1.25-1.5 °C/h and started immediately following ORA administration.

Tissue collection and biochemical analysis

At the end of each run, blood was collected via the caudal vein using a 23-gauge hypodermic needle rinsed with heparin (200 IU/ml). Blood glucose levels were measured using a FreeStyle Optimum glucometer (Abbott Diabetes Care, Witney, UK). Plasmas were separated from blood cells and platelets by centrifugation at 4°C/3.2 g for 20 min, transferred to 1.5 mL tubes and stored at -80°C until further analysis. Following blood collection, weight and total length were measured for each fish and the diencephalon, including the preoptic area, were micro-dissected and snap-frozen in liquid nitrogen. Subsequently, plasma lactate, triglycerides and total protein content, as well as cortisol and growth hormone (GH) levels, were measured. Lactate, triglycerides and total protein were quantified using the Cobas c111 analyzer (Roche diagnostics GmbH, Mannheim Germany) as previously described by Segev-Hadar et al.[38]. Cortisol and GH were quantified according to previously published protocols by Yeh et al.[65] and Mizrahi et al.[66].

RNA extraction and transcriptome sequencing

Total RNA was extracted using TRIzol® reagent (Life Technologies Corporation, Carlsbad, USA) according to manufacturer protocol and purified to remove remaining DNA contamination using the TURBO DNA-free™ kit (Invitrogen, USA). RNA concentration and purity were determined using an Epoch Microplate Spectrophotometer (BioTek, USA). RNA samples from treatment and control groups (n=4/treatment) were sequenced at the Technion Genome Center (Technion Institute of Technology, Haifa, Israel). RNA integrity was tested using an Agilent 2200 TapeStation (Agilent Technologies, USA). Subsequently, poly (A) mRNA was isolated from the total RNA with poly (dT) oligo-attached magnetic beads, and cDNA libraries were prepared using the TruSeq RNA Sample Preparation Kit (Illumina,

USA) following the manufacturer protocol. Eight cDNA libraries were sequenced on a single lane by the HiSeq2500 sequencing platform (Illumina, USA) at 2 × 100 bp paired-end (PE) reads. The data have been deposited in the GEO database (accession number GSE159019).

Gene expression analysis. The *O. niloticus* genome was downloaded from NCBI on April 2019 (v.1.0). Reads were aligned to the genome using Hisat2[67], and gene expression was determined using IsoEM2. Differential gene expression was determined using IsoDE2[40], which reports a confident fold-change (P<0.01). Genes with a confident fold-change >2 were considered differentially expressed. Upregulated and downregulated genes expressed above 1 TPM in at least one condition were submitted to g:Profiler[41] for functional annotation analysis using *O. niloticus* as background. Real-Time PCR validation of selected genes was performed as previously described[38]. Briefly, possible genomic DNA contamination was eliminated by treatment with Invitrogen TURBO DNA-freeTM kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. DNase-free total RNA (0.5 µg) was reverse-transcribed using Verso cDNA kit (Thermo Fisher Scientific; naïve fish) or High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific; ORA experiment) according to the manufacturer's protocol. cDNA was stored at −20°C until quantitation by real-time PCR. Hypothalamic gene expression levels were analyzed by quantitative PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems, Inc. Foster City, CA, USA). *elongation factor 1 alpha* (*ef1α*) and *β-actin* served as reference genes (**Supp. Table 2**). Each reaction consisted of 5 µL SYBRR green dye (Thermo Fisher Scientific, Vilnius, Lithuania), 0.5 µL of 2 µM forward and reverse primers, 2.5 µL of ultra-pure water (UPW) and 1.5 µL of cDNA template (diluted 1:16 in UPW). Analysis was performed in duplicates. Controls without the cDNA were used to test for non-specific amplification. Specificity of the primers was validated by Sanger sequencing and melt curve analysis was used to confirm amplification of a single product. Amplification was performed under the following conditions; 95.0°C for 20 sec, 40 cycles at 95.0°C for 3 sec, and 60.0°C for 30 sec, followed by one cycle at 95.0°C for 15 sec and 60.0°C for 1 min, 95.0°C for 15 sec for the generation of the melting curve. Fluorescence signals of the target and reference genes in the control and treatment

groups were analyzed using StepOne software Version 2.3. Relative quantification of within-tissue expression was determined using the $2^{-\Delta\Delta}$CT method[68].

Statistical analysis

Values are presented as mean ± standard deviation (SD). The level of significance was set to $p < 0.05$ in all performed analyses. A two-way ANOVA (Tukey's multiple comparisons) was used to test for RMR differences between normothermic and cold-exposed fish. Unpaired Student's t-test test was used for analyzing the physiological parameters measured and for analyzing Real-Time PCR data of transcriptome validation. Analysis of RMR differences of the pharmacological analysis demonstrated that the data significantly diverged from linearity. Therefore, the data were analyzed using non-linear regression followed by an extra sum-of-squares F test which demonstrated that the data sets could not be represented by a single curve ($p=0.0035$). Analysis of physiological parameters measured and Real-Time PCR data were performed using a two-way ANOVA (Tukey's multiple comparisons). A two-way ANOVA (Tukey's multiple comparisons) was used to test for RMR differences between different zebrafish genotypes at specific temperatures. Due to baseline RMR differences of the zebrafish mutants, data were plotted as delta RMR (RMR[27°C]-RMR[X°C]). Data sets were further analyzed by linear regression which demonstrated that slopes are significantly different ($p=0.0013$). Statistical analyses performed using GraphPad Prism 7.03.

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Author contributions

J.B., A.C. and R.N.K. conceived and designed the project. A.S.H., S.K., L.H., A.B. and T.N. performed *in vivo* metabolic analyses, physiological and molecular analyses. A.M.O, K.C.H and R.N.K performed the bioinformatics analysis. G.L. designed the metabolic analysis of zebrafish mutants and contributed *oxt*$^{-/-}$ and *oxtr*$^{-/-}$ germlines. J.B. prepared the figures. J.B., A.C., R.N.K. and A.M.O. wrote the manuscript. All authors reviewed the manuscript.

Declaration of interests

The authors declare that no competing interests.

Figures and Tables

Figure 1

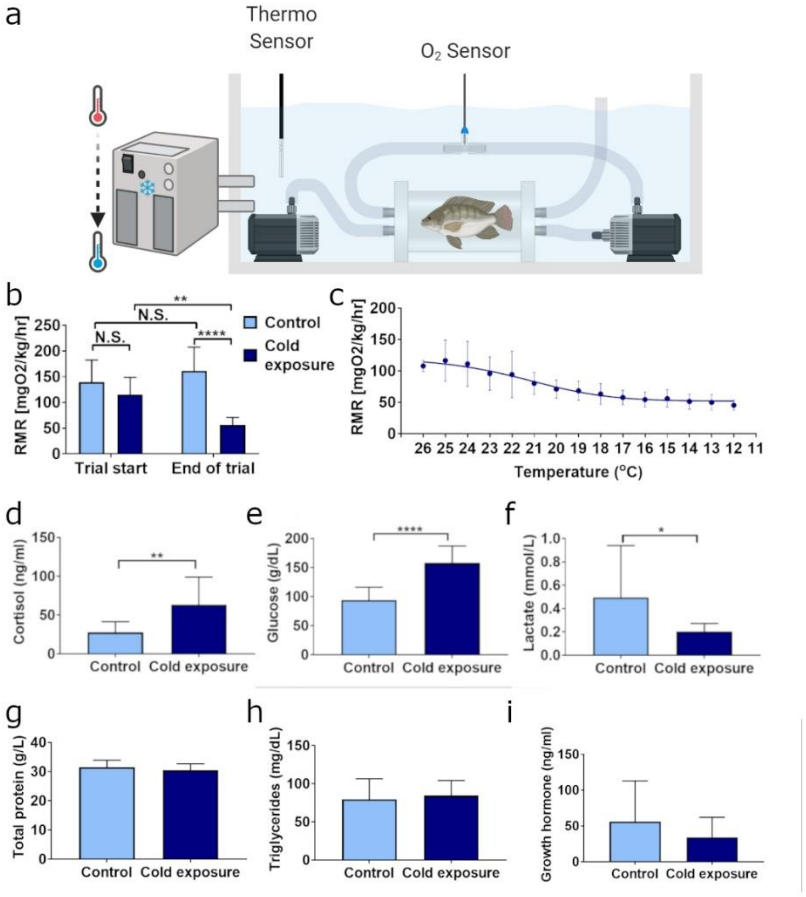
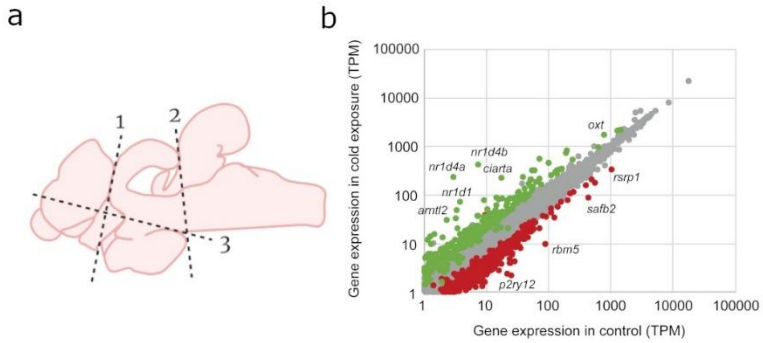


Figure 1. Cold exposure suppresses metabolic rate and elicits physiological stress response in Nile tilapia. (**a**) Schematic representation of the experiment. Fish were individually placed into 1L acrylic chambers connected to an oxygen measurement cell and submerged into temperature regulated water reservoir. (**b**) Analysis of the average metabolic rate at rest (RMR) of Nile tilapia prior to cold exposure (26°C and 25°C for control and cold-exposed fish, respectively) and following it (26°C and 14°C for control and cold-exposed fish, respectively) indicate a significant reduction in the RMR of exposed tilapia. (**c**) RMR analysis of cold-exposed fish in 1°C bins revealed a direct but nonlinear correlation of the fish RMR with the environmental temperature. (**d** and **e**) Plasma cortisol and blood glucose significantly increased following cold exposure; however, paradoxically, plasma lactate levels were decreased (**f**). (**g-i**) No significant changes were detected in plasma protein, triglyceride (TG) or growth hormone (GH) levels. The data are presented as mean ± SD.*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. n=12 fish/treatment.

Figure 2



c

Gene	Full name	control	cold exposure
LOC109201896	hemoglobin subunit alpha-A	1432	2226
LOC109201893	hemoglobin subunit beta-A-like	1256	2130
oxt	oxytocin/neurophysin I prepropeptide	767	1728
LOC109201892	hemoglobin subunit alpha-A	628	976
cbx7a	chromo domain-containing protein cec-1	196	829
LOC100699085	histone H1.0-B	192	733
LOC100707730	histone H1.10	71	551
fkbp5	peptidyl-prolyl cis-trans isomerase FKBP5	245	543
tef	TEF transcription factor	101	455
nr1d4b	nuclear receptor subfamily 1 group D member 1	7.5	430

high TPM
low TPM

d

Gene	Full name	control	cold exposure
rsrp1	arginine and serine rich protein 1	1013	337
ansn	anserinase	549	181
clk4a	dual specificity protein kinase CLK4	488	208
safb2	scaffold attachment factor B2	439	90
LOC100695290	cysteine-rich protein 2	393	161
cbln3	cerebellin-1	248	118
eif5	eukaryotic translation initiation factor 5	220	106
fam107b	family with sequence similarity 107 member B	200	80
srsf2a	serine/arginine-rich splicing factor 2	156	72
crebzf	CREB/ATF bZIP transcription factor	138	59

high TPM
low TPM

Figure 2. Cold exposure induces a transcriptional hypothalamic response of major neuroendocrine and metabolism related pathways. (**a**) Nile tilapia brains were micro-dissected to include all the major hypothalamic nuclei, including the preoptic area. (**b**) Transcriptome analysis of hypothalami revealed an increased expression of over 900 genes and suppressed expression of about 2,000 genes in response to cold exposure. (**c**) Analysis of the most highly expressed upregulated genes identified *oxt* as the most cold-responsive neuroendocrine factor, suggesting its involvement in the adaptive response of Nile tilapia to cold exposure. (**d**) Analysis of the most highly expressed downregulated genes yielded mainly genes involved in mRNA expression and processing. TPM, transcripts per million.

Figure 3

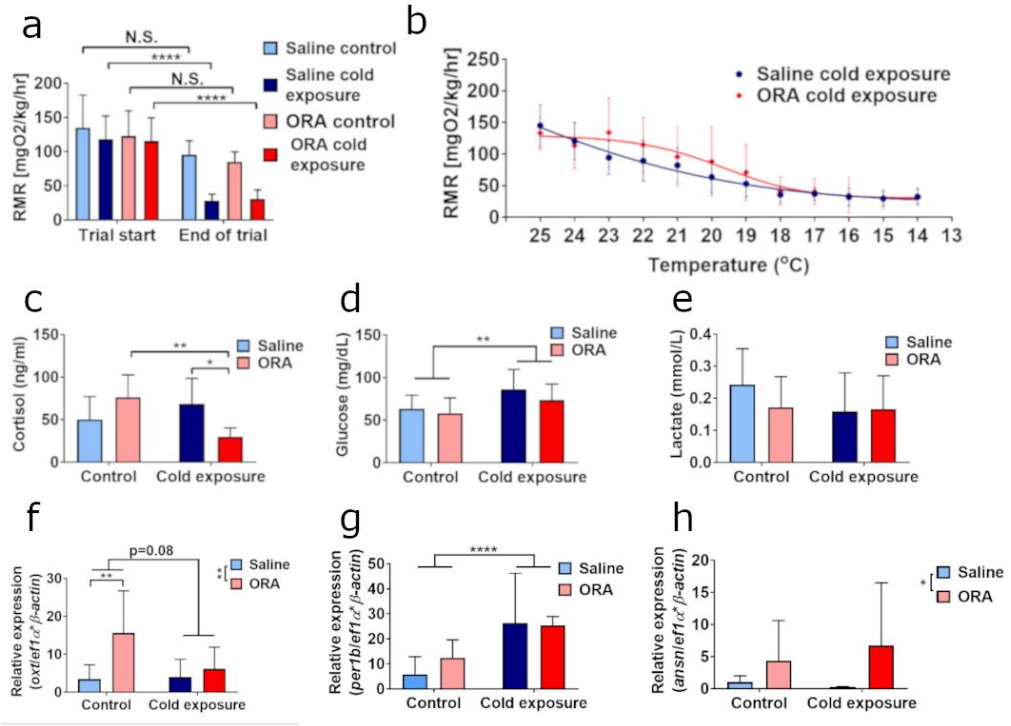


Figure 3. Oxt-receptor antagonist inhibits the cold-induced decline in metabolic rate and the physiological stress response in Nile tilapia. (**a**) Analysis of the average metabolic rate at rest (RMR) of Nile tilapia prior to cold exposure (24-24.5°C for all groups) and following it (24°C and 14°C for control and cold-exposed fish, respectively) indicate a significant reduction in the RMR of cold-exposed tilapia, regardless of the ORA treatment. (**b**) Nonetheless, regression analysis of normothermic and cold-exposed fish RMR in 1°C bins showed a significant perturbation of the fish RMR by ORA administration (p=0.0035). The cold-induced increase of plasma cortisol (**c**) but not of blood glucose (**d**) or lactate (**e**) was significantly blunted following ORA treatment. As expected by the blockage of OXT signaling, OXT expression was significantly increased by ORA (**f**). (**g,h**) While most analyzed genes were responsive to the cold exposure, the expression of the enzyme-coding *anserinase* responded significantly to ORA treatment. The data are presented as mean ± SD.*p < 0.05; **p < 0.01; ****p < 0.0001. n=12 fish/treatment.

Figure 4

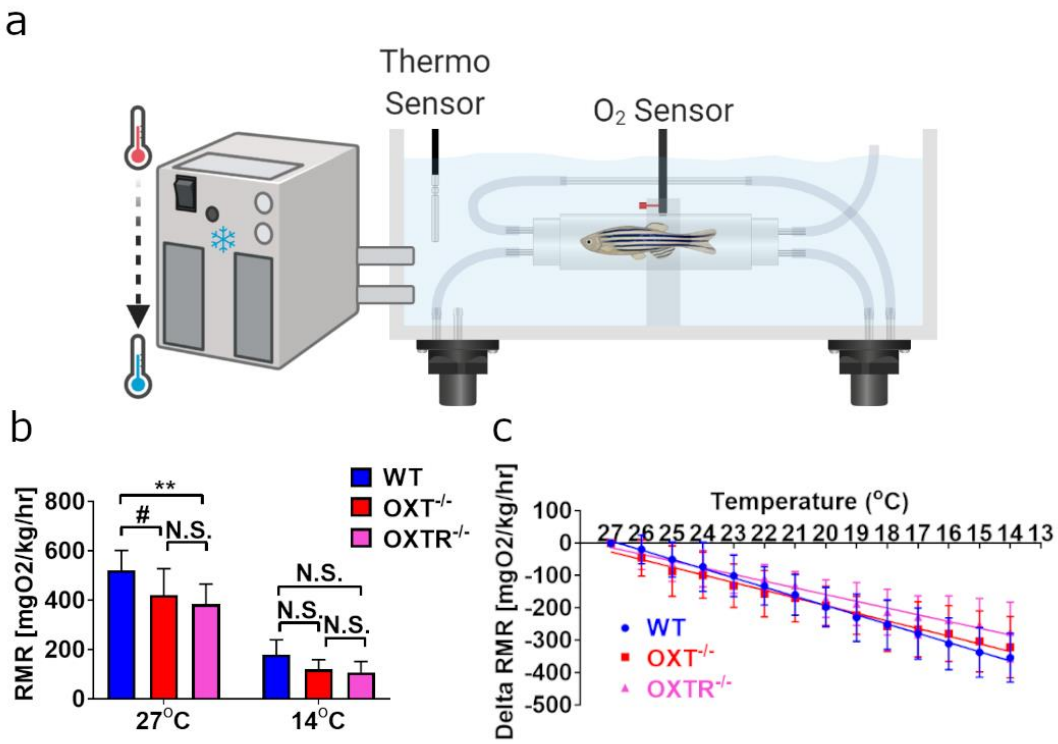


Figure 4. Genetic perturbation of OXT signaling alters basal and cold-adaptive metabolic rate in zebrafish. (**a**) Fish were individually placed into a glass chamber connected to an optical fiber for oxygen measurement, positioned in a temperature-regulated water reservoir. (**b**) As compared to wild-type (WT) controls, *oxt*$^{-/-}$ and *oxtr*$^{-/-}$ zebrafish displayed significant reduction in RMR under normothermic temperature (27°C), but not during cold exposure (14°C). (**c**) Therefore, delta RMR ($RMR_{temperature}$-RMR_{27^oC}) was used to identify the adaptive cold-related effects of OXT perturbation, within the genetic background of each line. Similarly to the oxytocinergic effects detected in Nile tilapia, regression analysis of RMR data demonstrated that genetic ablation of *oxtr* or *oxt* significantly delayed RMR suppression caused by reduced water temperature (p=0.0013). The data are presented as mean ± SD. **p < 0.01; #p=0.0792; N.S., not significant. n=7-9 fish/treatment.

820 **Table 1. Functional annotation of genes downregulated upon cold exposure.**

GO term	Term name	Adjusted P-value	Number of genes
GO:0004930	G protein-coupled receptor activity	0.00339	43
GO:0008523	G protein-coupled peptide receptor activity	0.007352	13
GO:0001653	Peptide receptor activity	0.007909	13
GO:0038023	Signaling receptor activity	0.030744	54
GO:0060089	Molecular transducer activity	0.030744	54
GO:0004888	Transmembrane signaling receptor activity	0.033708	50
GO:0007166	Cell surface receptor signaling pathway	0.047196	40

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823 **Table 2. Functional annotation of genes upregulated upon cold exposure.**

GO term	Term name	Adjusted *P*-value	Number of genes
GO:0032922	Circadian regulation of gene expression	1.24E-6	6
GO:0005634	Nucleus	3.50E-5	45
GO:0003677	DNA binding	0.00024	37
GO:0007623	Circadian rhythm	0.00031	7
GO:0140110	Transcription regulator activity	0.00044	24
GO:0048511	Rhythmic process	0.00046	7
GO:0003700	DNA-binding transcription factor activity	0.00049	22
GO:0046983	Protein dimerization activity	0.00493	16
GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	0.00618	7
GO:0006270	DNA replication initiation	0.01200	4
GO:1901363	Heterocyclic compound binding	0.01401	90
GO:0097159	Organic cyclic compound binding	0.01504	90
GO:0097659	Nucleic-acid templated transcription	0.01522	36
GO:0032774	RNA biosynthetic process	0.01596	36
GO:0005833	Hemoglobin complex	0.01640	4
GO:0098531	Ligand-activated transcription factor activity	0.01833	5
GO:0004879	Nuclear receptor activity	0.01833	5
GO:0043231	Intracellular membrane-bounded organelle	0.03371	49
GO:0006915	Apoptotic process	0.03556	14
GO:0012501	Programmed cell death	0.04078	14
GO:0048523	Negative regulation of cellular process	0.04088	21
GO:0003676	Nucleic acid binding	0.04799	58
GO:0065007	Biological replication	0.04885	100

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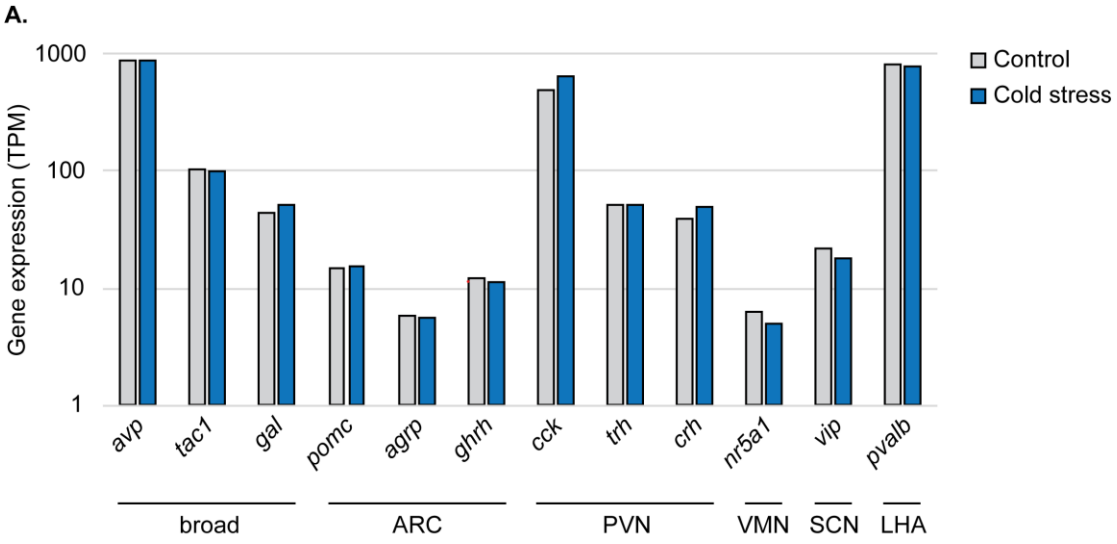
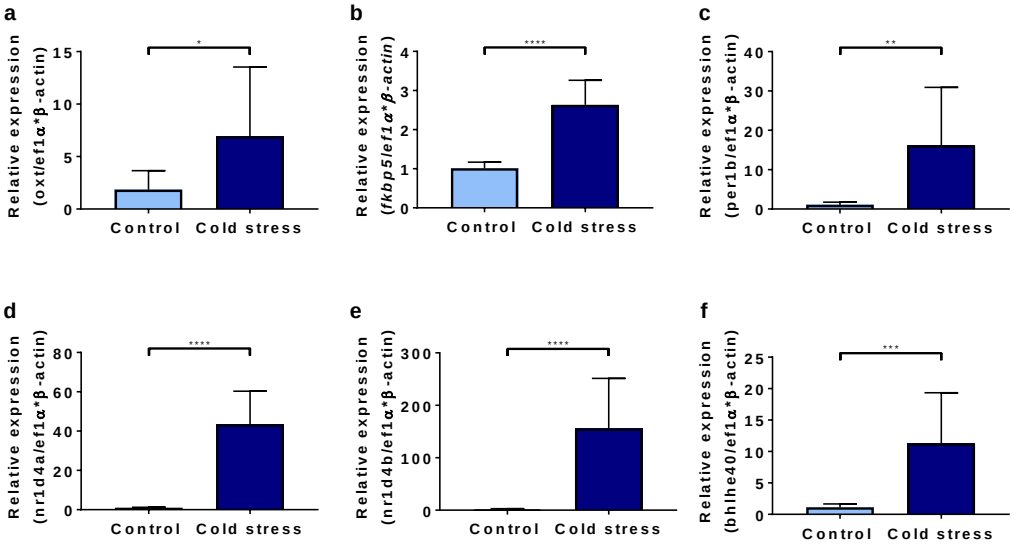
Supplemental Information

Supplemental Figure 1



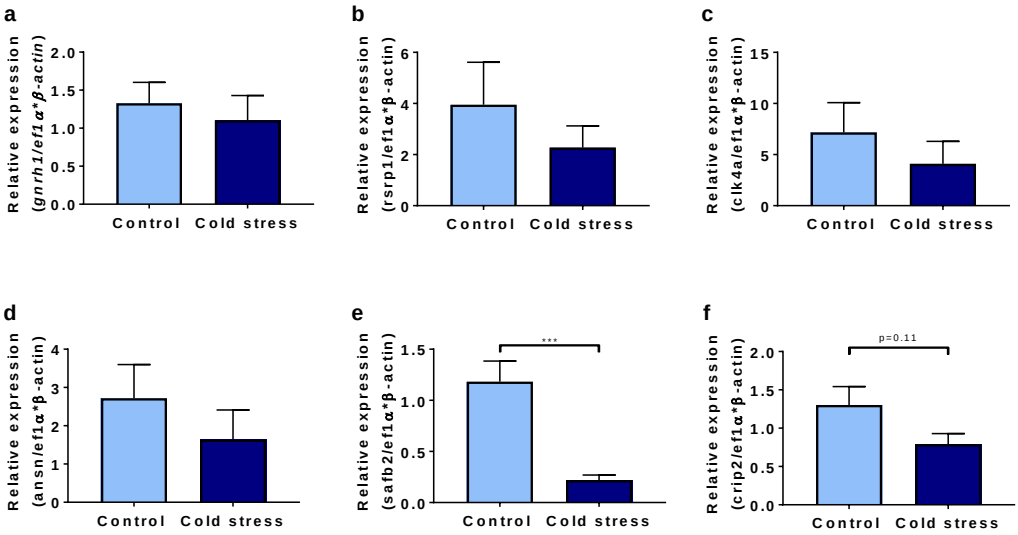
Supplemental Figure 1. Validation of hypothalamic microdissection by RNAseq analysis for expression of hypothalamic markers. No significant differences were observed for *LOC10070874* (*avp*), *tac1*, *gal*, *pomc* (*pomca*), *LOC100691312* (*agrp*), *ghrh*, *cck*, *trh*, *crh* (*crhb*), *nr5a1*, *LOC100705021* (*vip*) or *LOV100710987* (*pvalb*). Genes are grouped by the hypothalamic nucleus they mark in the mammalian brain. ARC, arcuate nucleus; PVN, paraventricular nucleus; VMN, ventromedial nucleus; SCN, suprachiasmatic nucleus; LHA, lateral hypothalamic area.

Supplemental Figure 2



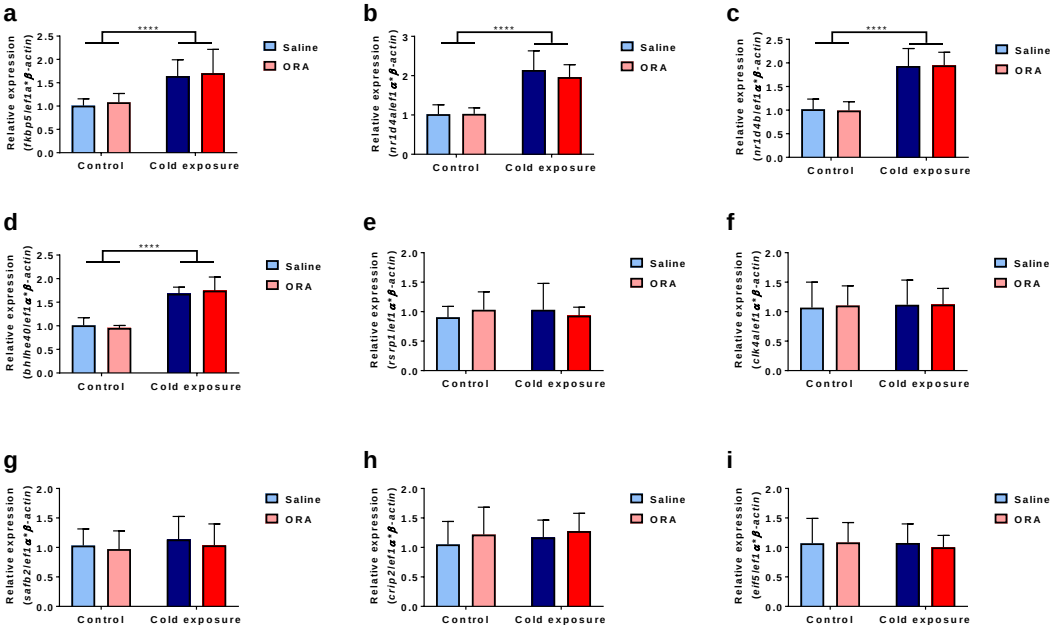
Supplemental Figure 2. Validation of transcriptomic analysis results for identified cold-induced genes. Total RNA was extracted from midbrains of control (n=11) and cold-exposed (n=9) tilapia and was analyzed by real-time PCR for cold-induced expression of various genes. Similar to the transcriptome data, *oxytocin* (**a**), *fkbp5*(**b**), *per1b* (**c**), *nr1d4a* (**d**), *nr1d4b* (**e**) and *bhlhe40* (**f**) displayed significantly increased expression in response to cold exposure. The data are presented as mean ± SD.*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Supplemental Figure 3



Supplemental Figure 3. Validation of transcriptomic analysis results for identified cold-suppressed genes. Total RNA was extracted from midbrains of control (n=11) and cold-exposed (n=9) tilapia and was analyzed by real-time PCR for cold-suppressed expression of various genes. Similar to the transcriptome data, *gnrh1* (**a**), *rsrp1* (**b**), *clk4a* (**c**), *ansn* (**d**), *safb2* (**e**) and *crip2* (**f**) displayed clear trends for decreased expression in response to cold exposure. The data are presented as mean ± SD. ***$p < 0.001$.

Supplemental Figure 4



Supplemental Figure 4. Expression analysis of cold-responsive genes upon administration of Oxt-receptor antagonist. Total RNA was extracted from midbrains of control and cold-exposed tilapia that were intraperitoneally injected with either saline or 1 mg/kg body weight ORA and was analyzed using real-time PCR. Most genes displayed similar trends of responsiveness as we identified in non-injected tilapia. Analyzed genes included *fkbp5* (**a**), *nr1d4a* (**b**), *nr1d4b* (**c**), *bhlhe40* (**d**), *rsrp1* (**e**), *clk4a* (**f**), *safb2* (**g**), *crip2* (**h**) and *eif5* (**i**). The data are presented as mean ± SD. ****p < 0.0001. n=8 fish/treatment.

877 **Supplemental Table 1. Gene expression of cold-exposed Nile tilapia**

878 See attached Excel file

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880 **Supplemental Table 2. Oligos used in the current study**

Primer	Position	Sequence : (5' to 3')	Efficiency (%)	R^2	Product size
OXT_F	110	AGCTAACAAAAATGACCGGAGC	107.774	0.998	187
OXT_R	279	CAGCAGATACTTGGCCCGAA			
FKBP5_F	684	TCCTCCCAGCTCTTCAGTAGT	123.46	0.989	150
FKBP5_R	834	AGGTGCACGTTAACAACTGATCC			
nr1d4_a_F	2143	TCAGGCACCTTCCAGGTTCT	99.02	0.998	104
nr1d4_a_R	2247	AAAGTGGGCAGCGGGTAAG			
nr1d4_b_F	1014	GCGCAAATTACGACGGTGTC	76.797	0.792	100
nr1d4_b_R	1114	CCATACCTCCGGTTTTGGTGA			
per1_b_F	3097	GACATGACCCCGACTTCTCC	147.298	0.988	121
per1_b_R	3218	TTCTCCGGCTGTCCCTATCA			
bhlhe40_F	381	GCTGACATGCAAGGAATGGAC	109.375	0.97	108
bhlhe40_R	489	GATAAGTCGGTGGGGCAACT			
RSRP1_ F	167	ATTTGCCACAGCGTTTGCT	92.346	0.985	129
RSRP1_R	296	CTGTTCCCTTGGCCATTGTC			
CLK4a_F	389	CTTGGGCTCAGCACATACGA	89.018	0.993	132
CLK4a_R	521	CTGTGTGCGTCAGCTTGTTC			
ANSN_F	246	ACTGTGGCTCAGAAACTCCG	90.858	0.996	120
ANSN_R	366	TACCAAACTGAGCCGTCACC			
SAFB2_F	1483	CTGTGGAGCGGGCTAAAAATG	96.168	0.996	199
SAFB2_R	1682	AACAGGCTCTCCCTTGGACT			
CRIP2_F	284	CACGATGGAAGGCCCTACTG	94.957	0.968	190
CRIP2_R	474	GCTTTTGATGGTGCCTTGGG			
EIF5_F	939	AATTTGTGCTGTGTGCCGAG	89.005	0.975	166
EIF5_R	1105	ATCATTGCTCTCAGGTGGGT			
EF1a_F	640	GGAGACCAGTGACAAGATGAG	103.96	0.999	158
EF1a_R	798	GTTCCGATACCGCCAATCT			
β-actin_F	140	CCACCCAAAGTTCAGCCATG	90.379	0.956	121
β-actin_R	261	ACGATGGAGGGGAAGACAG			

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