Changes in corticotropin-releasing hormone (CRH) in the larval *Euphlyctis cyanophlyctis* exposed to predation risk

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by

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Certificate

This is to certify that this dissertation entitled “Changes in corticotropin-releasing hormone (CRH) in the larval *Euphlyctis cyanophlyctis* exposed to predation risk” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents study/work carried out by Ms. Nila P B at the Department of Zoology, Savitribai Phule Pune University, Ganeshkhind, Pune under the supervision of Prof. Narahari P. Gramapurohit, during the academic year 2022-2023.

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To amma, achan and achu

Declaration

I hereby declare that the contents embodied in the report entitled “Changes in corticotropin-releasing hormone (CRH) in the larval *Euphlyctis cyanophlyctis* exposed to predation risk” are the results of the experimental work carried out by me at the Department of Zoology, Savitribhai Phule Pune University, Pune, under the supervision of Prof. Narahari P. Gramapurohit and it has not been submitted elsewhere for any other degree

Nila P B

Date:10/04/2023

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Abstract

Predation is a principal selection pressure acting on prey, often leading to changes in physiology, morphology, behaviour, and life-history traits of prey. Vertebrates respond to predation by activating their neuroendocrine stress axis (hypothalamus-pituitary-adrenal axis). Activation of the neuroendocrine stress axis results in the release of corticotropin-releasing factor (CRF) from the hypothalamus that acts on corticotropes of the anterior pituitary to release adrenocorticotrophic hormone (ACTH). ACTH reaches the adrenal cortex, through peripheral blood and stimulates the cortical cells to secrete glucocorticoids (GCs) which mediate the antipredator responses of prey animals by integrating many physiological processes. CRF being the key regulator of HPA axis, holds a key role in predator induced stress responses of animals. The present study intends to determine the changes in CRF distribution and expression after a chronic predation stress on larval *Euphlyctis cyanophlyctis.* After rearing the tadpoles under chronic predator stress from Gosner stages 25-44, changes in the distribution of CRF in the brain of tadpoles was checked using Immunohistochemistry and the CRF gene expression was assessed using qRT-PCR. The immunohistochemical results show that robust CRF expression was observed in the hypothalamus of tadpoles which were exposed to predator. These results were corroborated by RT-PCR studies which showed the increased levels of mRNA in the brain of tadpoles experiencing predation stress. The results of our study are discussed in the context of previous studies in other vertebrates.

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Contributions

Contributor name	Contributor role
N.P Gramapurohit	Conceptualization Ideas
N.P Gramapurohit	Methodology
	Software
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Nila P B	Formal analysis
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1 Introduction

1.1 Stress

Biotic and abiotic factors play a crucial role in shaping the life-histories of organisms in diverse environments. Abiotic factors such as environmental temperature play a pivotal role in development and survival of the organisms. Similarly, predation acts as a major selection pressure on prey animals, often determining their spatial and temporal distribution and abundance in an ecosystem (Chivers & Brown, 1996; Mathis & Smith, 1993) . Due to the ubiquitous nature of predators, prey species living in various ecosystems have developed novel strategies to escape, evade or deter predators (Scherer & Smee, 2016). These responses to predation risk can range from changes in behaviour, morphology, life-history traits, or a combination of these(Kats & Dill, 1998). Since the antipredator responses are potentially costly to develop and maintain for prey, these responses vary depending on the type of predator and the level of threat posed by predators (Scherer et al., 2017). Predation is therefore an important biotic stressor for prey species that alters behaviour, morphology, physiology, and life history traits in the prey population (Ferrari et al., 2010; Lima & Dill, 1990).

Predator recognition is the first step in eliciting an appropriate antipredator response. Prey organisms can recognize predators with the help of visual, chemical, auditory and vibration from predators, as well as from other members of the same or different species (McCoy et al., 2012; Munoz & Blumstein, 2012). In aquatic environments, chemical cues from predators (kairomones, dietary cues) or prey when attacked, captured, or, ingested is more reliable in accessing the predator presence (Ferrari et al., 2010; Wisenden, 2000).

According to Selye (1936), animals react to physical or mental challenges in three distinct stages, which he dubbed the general adaptation syndrome (GAS): the alarm response, the resistance stage and following prolonged exposure, the exhaustion stage. Selye coined the term "stress" to characterize the physiological condition resulting from the GAS and labelled the causal factors "stressors" (Selye, 1956). A critical notion that underpins the GAS theory is the link between stress and adaptation; that is, stress is a reaction that facilitates the adaptation of an organism to the presence of a stressor. A stress response is elicited in an individual when a stressor disrupts physiological homeostasis. It can range from behavioural to

physiological responses that help to maintain homeostasis. The endocrine system involving epinephrine and glucocorticoids released from the adrenal medulla and cortex respectively constitute a major stress response. Immediately after perceiving the stressor, the sympathetic nervous system secretes norepinephrine and the adrenal medulla secretes epinephrine. Subsequently, glucocorticoids are secreted from the adrenal cortex. Glucocorticoids mediate the behavioural effects of stress as these steroid hormones can easily diffuse through the blood-brain barrier, unlike epinephrine (Weil-Malherbe et al., 1959).The secretion of glucocorticoids is regulated along the hypothalamic-pituitary-adrenal axis (H-P-A axis)

1.1.1 Vertebrate stress response

The hypothalamic-pituitary-adrenal (HPA) axis (Figure 1) is the major neuroendocrine system that mediates an individual's response to stress (McEwen, 2007). It involves three main components: the hypothalamus, the pituitary gland, and the adrenal glands (McEwen, 2007). The hypothalamus is responsible for activating the HPA axis by releasing two neuropeptides, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) in response to stress. CRF and AVP synergistically act on the anterior pituitary to release adrenocorticotropic hormone (ACTH; Vale et al., 1981). ACTH circulates through the bloodstream to the adrenal glands, located on top of the kidneys. In the adrenal tissue, ACTH binds to its receptors on zona fasciculata of cortex and not only stimulates the conversion of cholesterol esters to free cholesterol but also activates the steroidogenic pathway. The adrenal cortex, then releases glucocorticoids (cortisol in humans, corticosterone in rodents) that help the body to respond to stress (Nicolaides et al., 2015). Glucocorticoids are released into the systemic circulation in addition to storing a small quantity in the adrenal gland. Glucocorticoids have a variety of effects on the body that includes increasing blood sugar levels, suppressing the immune system, and aiding in the metabolism of fats, proteins, and carbohydrates, all of which inherently try to allocate energy resources efficiently at the time of crisis (Ulrich-Lai & Ryan, 2014). The secretion of glucocorticoids via the HPA axis promotes the homeostatic adaptation to stress.

The HPA axis is tightly regulated by a negative feedback loop, which helps to maintain the body's homeostasis. When glucocorticoids' levels in the blood exceed beyond a certain threshold, they inhibit the release of CRF and ACTH, which then reduces the production of glucocorticoids by the adrenal glands (Jp et al., 1995).

Chronic activation of the HPA axis due to prolonged stress can lead to dysregulation of glucocorticoids production, which can have negative effects on the body. High levels of cortisol have been associated with an increased risk for a variety of health problems, including depression, anxiety, obesity, and cardiovascular disease (McEwen, 2007; Ulrich-Lai & Ryan, 2014)

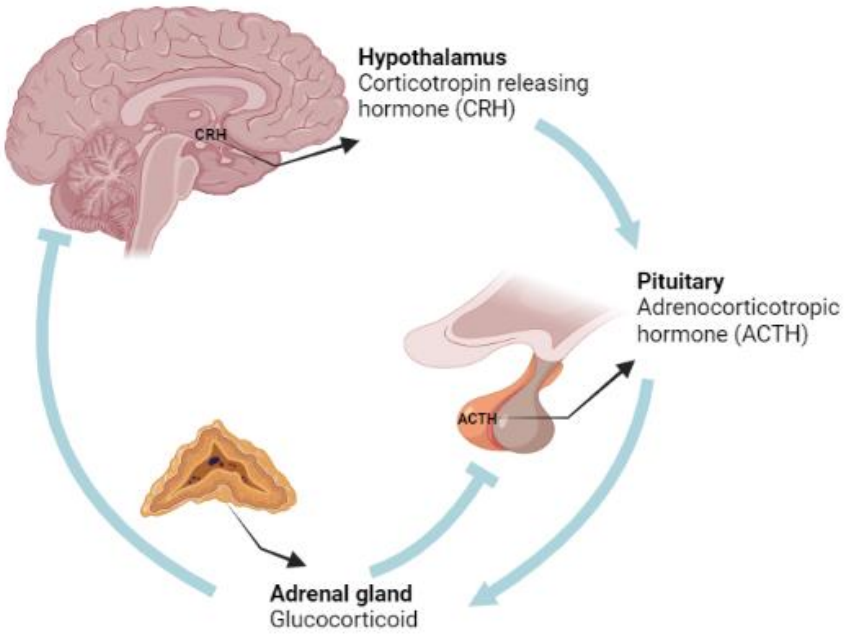


Figure 1 : Hypothalamic pituitary adrenal axis:(Figure created using the Biorender software) Hypothalamic pituitary adrenal axis is the major neuro endocrine system that gets activated in response to stress. The Corticotropin releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus, which reaches the anterior pituitary and stimulates the adrenocorticotropic hormone (ACTH) secretion. ACTH then acts on the adrenal gland to induce the secretion of glucocorticoids. Glucocorticoids mediates the behavioural and physiological responses to maintain homeostasis.

1.1.2 Amphibian stress response

Hypothalamic-pituitary-interrenal (HPI) axis, is the major system that evokes the physiological stress response in amphibians. As HPA axis of the higher vertebrates, hypothalamus, pituitary gland, and interrenal tissue, work together to regulate the production and release of stress hormones such as corticosterone (Denver, 1997). The HPI axis is activated in response to a wide range of stressors, including physical, environmental, and social stress (Romero, 2004). For instance, exposure to a predator or a sudden change in temperature can activate the HPI axis and stimulate the release of corticosterone (Narayan et al., 2013). Hypothalamus

being the central regulator of the HPI axis releases corticotropin-releasing factor (CRF) and arginine vasotocin (AVT) in response to stress. CRF and AVT synergistically stimulate the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH), which binds to the melanocortin 2 receptor (MC2R) in the interrenal tissue to stimulate the production of glucocorticoids such as corticosterone (CORT; (Sapolsky et al., 2000). CORT plays an essential role in the stress response of amphibians by increasing glucose availability, suppressing the immune system, and promoting survival in stressful situations (Sapolsky et al., 2000). However, chronic activation of the HPI axis can lead to negative effects on the health and survival of amphibians. Overall, the HPI axis is a critical component of the stress response in amphibians and plays a vital role in their ability to adapt and survive in their environment.

1.2 Corticotropin-releasing factor

Corticotropin-releasing factor (CRF) or the corticotropin-releasing hormone (CRH) is a 41-amino acid peptide first characterized neuropeptide that is primarily involved in the stress response of vertebrates (Vale et al., 1981). CRF is produced by the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus in response to stress situations. It is released at the median eminence and drains into the portal system (hypothalamus-hypophyseal portal system). It is then transported to the anterior pituitary where it acts on the corticotropes to secrete adrenocorticotropic hormone (ACTH). CRF works synergistically with arginine vasopressin (AVP; arginine vasotocin- AVT is the amphibian hormone) to regulate ACTH secretion (Vale et al., 1981). ACTH stimulates adrenal cortex (Interrenal tissue in amphibians) to secrete glucocorticoids in response to stress. In addition to acting as a releasing factor for ACTH, CRF is also one of the major TSH-secreting factors in amphibians (Denver, 1988).

1.2.1 Other peptides in the CRF family and receptors

Urotensin-I of fish, Sauvagine of frog and Urocortins 1-3 are peptides belong to the same family of vertebrate hormones that includes CRF (Boorse et al., 2005; Boorse & Denver, 2006; Dautzenberg & Hauger, 2002; Deussing & Chen, 2018)

CRF and the related peptides act via two G-protein coupled receptors; corticotropin-releasing hormone receptor 1 (CRF_1) and corticotropin-releasing hormone receptor 2 (CRF_2). The G protein-coupled receptors on the binding of the ligand, lead to the activation of adenylyl cyclase and the resulting increase in

intracellular cAMP. Their actions are modulated by a secreted CRF binding protein (CRF-BP), which regulates the bioavailability of CRF to its receptors (Dautzenberg & Hauger, 2002; Deussing & Chen, 2018; A. F. Seasholtz et al., 2001) The binding affinity of CRF to CRF-BP is similar or more than that of the CRF receptors (A. Seasholtz et al., 2002). CRF-BP is a phylogenetically ancient molecule, which has conserved its structural and functional properties (A. Seasholtz et al., 2002). CRF-BP genes have been isolated in rat, sheep, frog and honeybee (Behan et al., 1996; Brown et al., 1996; Cortright et al., 1997; Huising & Flik, 2005; Potter et al., 1992).

CRF is known to have a higher affinity for CRF_1. Urocortin 1 binds to both CRF_1 and CRF_2. Urocortin 1, 2 and 3 sauvagine, and urotensin have a 100-fold higher affinity to CRF_2 compared to the species CRF homolog. Urocortin 2 and urocortin 3 show no binding affinity to CRF_1 (Dautzenberg & Hauger, 2002; Hsu & Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). CRF_1 receptors are seen in the cerebral cortex, cerebellum, olfactory bulb, medial septum, hippocampus, amygdala, and pituitary in mammals (Potter et al., 1994) whereas CRF_2 receptors are limited to raphe nuclei, lateral septum (LS), cortical and medial amygdalar nuclei, and paraventricular (PVN) and ventromedial hypothalamic nuclei (Bale & Vale, 2004; Dautzenberg & Hauger, 2002; Hauger et al., 2003)

1.2.2 CRF-stress models

Studies in the rainbow trout (*Oncorhynchus mykiss*) have shown that in a hierarchical social system, the subordinate trout has significantly elevated CRF1 mRNA (Doyon et al., 2003). Similarly, CRF mRNA levels were increased in the subordinate rat when exposed to chronic social stress (Albeck et al., 1997). However, this effect is kept in check by reduced CRF receptors in the anterior pituitary (Hauger et al., 1988). The expression pattern of CRF in the neurons of the preoptic area (POA) and caudal neurosecretory system (CNSS) of the trout is stressor-specific (Bernier et al., 2008). For instance, exposure to hypoxia could increase CRF expression in the non-preganglionic nucleus of the vagus nerve (NPO) in the fish (Bernier & Craig, 2005). CRF-related peptides contribute to the regulation of the hypothalamic-pituitary-interrenal (HPI) axis and mediate the reduction in food consumption under hypoxic conditions in the rainbow trout (Bernier & Craig, 2005). Stress can elevate CRF expression, which is an initial step in the cascade that leads to the synthesis and release of glucocorticoids (Rivier & Vale, 1983). The expression of CRF and CRF-R2

mRNA in the forebrain was higher in high-responder (HR) trout than low-responder (LR) trout after confinement, which may be related to the divergence in stress coping displayed by these rainbow trout strains (Backström et al., 2011).

1.2.3 Other roles of CRF

CRF is the primary neurohormone that regulates the hypothalamic-pituitary axis in the vertebrate stress response. Apart from its role in mediating ACTH secretion, CRF also has other physiological roles. In non-mammalian vertebrates, CRF also works as a thyrotropin (TSH) releasing factor (Denver, 1988). Thyroid hormones playing a primary role in amphibian metamorphosis, make CRF a key player in facilitating metamorphosis (Tata, 1993). CRF induces TSH secretion in the pituitary of amphibians (Denver, 1988). CRF_1 mRNA expression was seen during pre-metamorphosis, which increased during pro-metamorphosis and reached a peak during the metamorphic climax (Manzon & Denver, 2004). While CRF_2 mRNA was low during pre-metamorphosis and early pro-metamorphosis, it increased tremendously during late pro-metamorphosis and metamorphic climax (Manzon & Denver, 2004).This indicates the action of CRF via CRF_2 in inducing the TSH release from the pituitary during the metamorphic climax. The action of CRF via CRF_1 is primarily involved in eliciting the stress response that is the increased CORT production throughout the larval stage (Glennemeier & Denver, 2002).

CRF also has a cytoprotective role in cells (Radulovic et al., 2003). CRF is expressed in the tadpole tail muscles, which helps in their survival until metamorphosis. It also slows down tail regression thereby directing the metamorphic timing. Tadpole tail being an important locomotory organ aiding in feeding and escaping from predators in aquatic ecosystem, the cytoprotective role of CRF helps in the survival of individuals delaying the time of transition till the conditions are favourable. CRF-BP regulates the cytoprotective action of CRF in the tail. The CRF-BP mRNA expression increases in the tadpole tail during spontaneous metamorphosis. The forced CRF-BP expression in the tadpole tail accelerated the loss of tail muscle cells *in-vivo* (Boorse et al., 2006). Environmental stressors like hypoxia upregulated CRF and urocortin 1 mRNA and strongly downregulated CRF-BP mRNA (Boorse et al, 2006). During the metamorphic climax, the tail regression is directed by the upregulation of CRF-BP by thyroid hormones thereby reducing the bioavailability

of CRF. The effect of environmental stressors on the tail regression and timing of metamorphosis has a significant role in the quality of life of an adult individual

2 Aim of the study

Previous studies have shown that *Euphlyctis cyanophlyctis* tadpoles lack an innate predator recognition mechanism against gape-limited predators such as dragonfly nymph that cannot eat a larger prey (Supekar & Gramapurohit, 2017). However, they can learn to recognize a dragonfly nymph as predator by associating the conspecific alarm cues with the predator kairomones. This learned recognition of dragonfly nymph has been well described in the previous research work in our laboratory. Larval *E. cyanophlyctis* exposed to a combination of conspecific alarm cues and predatory odours throughout Gosner stages 1-33 responded by reducing their activity (Supekar & Gramapurohit, 2017). Similarly, these tadpoles are capable of assessing the intensity of predation and respond to predation stress accordingly (Supekar & Gramapurohit, 2020). Under predator-induced stress situations the whole-body CORT levels were elevated in animals facing moderate and high predation risk (Supekar & Gramapurohit, 2020). The increasing corticosterone levels with increasing intensity of predation encouraged us to study the effect of predation on the expression pattern of CRF at the level of central nervous system as CRF is a key player in the activation of HPI axis. Therefore, we aim to determine the effect of predation on the expression pattern of CRF protein and its gene (mRNA) in the brain. Studies done so far on the changes in expression pattern of CRF in anuran brain have focused on assessing their baseline expression pattern or their expression change after exposing to an acute stress. In this study we are rearing the *E. cyanophlyctis* tadpoles from developmental stage 25-44 under chronic predation stress and assessing their effect on brain upon reaching the metamorphic climax.

3 Materials and Methods

3.1 Collection and rearing of experimental animals:

Six pairs of adult *E. cyanophlyctis* were collected from a pond located in the M.I.T. (ARAI Tekdi) campus, Pune, Maharashtra, India on 29 July 2022, and quickly transported to the laboratory where each pair was kept separately in the buckets filled with aged tap water for spawning. Spawned eggs were collected the following day and housed in a glass aquarium (45cm x 30cm x 10cm) with aged tap water until they were

hatched. The adults were returned to nature. Hatchlings from all the clutches were thoroughly mixed and 90 tadpoles were randomly chosen for experimentation. Remaining tadpoles were reared separately and were used for feeding dragonfly nymphs. Developmental stages were identified as per Gosner (1960) standard table for staging anuran tadpoles. The tadpoles (n = 90) were divided into two groups: control group reared without any predator and treatment group reared with predators feeding on conspecific tadpoles. Each experimental unit consisted of a glass aquarium (45cm x 30cm x 10cm) with two predator cages (with or without predators). Tadpoles were reared at a density of 3/L of water and each treatment was replicated thrice. Six experimental units, three with predator treatment and three without any predator were set up. The experiment was initiated when the tadpoles reached stage 25.

Since nymphs of the dragonfly are known to feed voraciously on different stages of anuran larvae and are cosmopolitan in their distribution, they (fourth instar) were used as predators (Figure 2). The predators housed in transparent, perforated plastic jars (8 cm diameter x 11cm height) and fed 2 stage and size matched conspecific tadpoles once in three days (Figure 3). Each aquarium was filled with 5L of aged tap water. The water was changed every third day followed by the addition of partially-boiled spinach. The tadpoles were exposed to predators from stages 25 – 44. On reaching stages 43-44, the tadpoles were transferred to separate containers with little quantity of water to facilitate metamorphosis. At the completion of metamorphosis, the froglets were anesthetized using diethyl ether and the brain was dissected and processed for immunohistochemical or molecular studies.

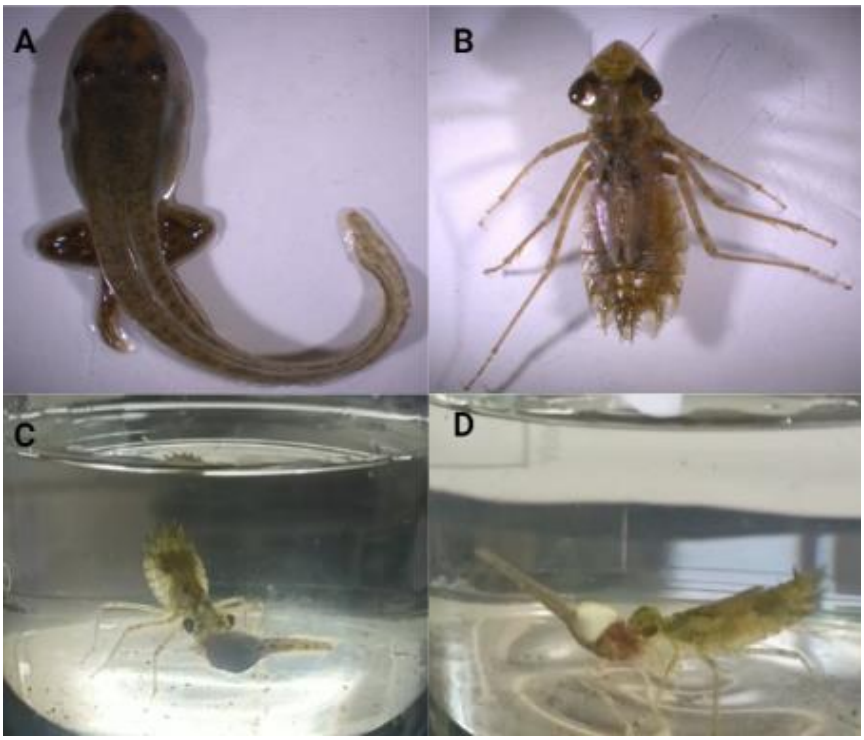


Figure 2: Interaction between tadpole and dragonfly nymph A) *Euphlyctis cyanophlyctis* tadpole of developmental stage 40 B) Dragonfly nymph of fourth instar C) Dragonfly nymph attacking the tadpole D) Dragonfly nymph feeding on the tadpole

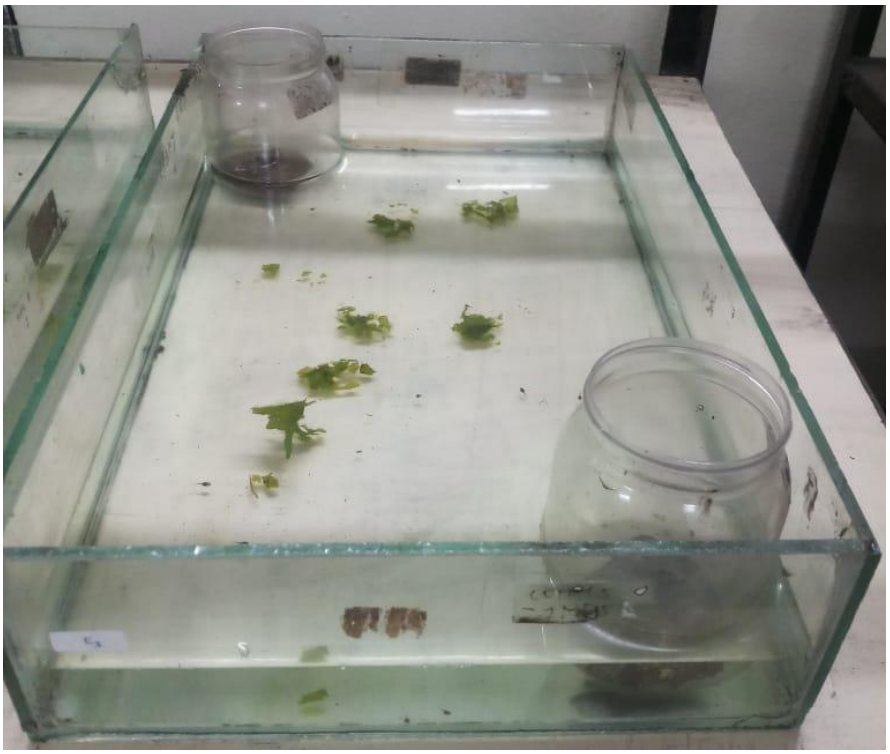


Figure 3: Experimental setup Tadpoles were reared in a glass aquarium at a density of 3/ litre of water. Each aquarium had two perforated jars placed diagonally opposite to each other. The jars were used to house dragonfly nymphs along with conspecifics in the treatment group and are left empty in the control group.

3.2 Tissue processing for histological studies

The dissected brains were fixed in Bouin's fluid for 24 h. Subsequently, the fixed brain tissues were dehydrated using increasing grades of ethanol (30% - 100%), cleared in xylene and embedded in paraffin (58°C - 60°C). Tissues were sectioned in sagittal and transverse planes (7 µm) using a rotary microtome (RM2125 RTS, Leica). The tissue sections were spread and heat fixed onto clean slides coated with Mayer's albumin and processed for further staining procedures.

3.3 Hematoxylin-Eosin staining

The tissue sections were deparaffinized in xylene and subsequently hydrated in alcohol grades (100%-30%) and distilled water. Subsequently, the tissue sections were stained with Haematoxylin for 3 min and Eosin (0.2%) solution in 95% ethanol. The sections were cleared in xylene, and mounted in distyrene plasticizer xylene (DPX) and photographed using a Bright field microscope (Axioscope A-1, Zeiss).

3.4 Tissue processing and sectioning for Immunohistochemical studies

The brain was fixed in Bouin's solution for 24 h at 4°C and subsequently cryopreserved in 10% and 20% sucrose (2 h each), and 30% sucrose (overnight) for immunohistochemistry. The tissue was then embedded in a freezing medium (Shandon cryomatrix) at -20°C and processed for cryosectioning. For paraffin sections, the brain tissues following their fixation in Bouin's were dehydrated in different grades of ethanol (30%-100%), cleared in xylene, and embedded in paraffin wax (58°C- 60°C). The paraffin blocks were stored at room temperature until sectioning. Paraffin sections of 16µm were cut using the rotary microtome (Leica) and cryosections of 20µm were taken using the cryotome (CM 1960, Leica). Tissue sections were fixed on poly-L-Lysine precoated slides.

3.5 Immunohistochemistry

Tissue sections were deparaffinized and hydrated in alcohol grades before processing for immunohistochemical protocol (Gaupale et al., 2013). The tissue sections were washed in distilled water followed by three washes in phosphate buffered saline (PBS) and kept in hydrogen peroxide in methanol (0.3%) for 1 h to quench endogenous peroxidase activity. The tissue sections were then washed thrice in PBS and treated with blocking agent 0.5% BSA and 0.5% gelatin in PBS for one hour. After washing thrice, the sections were incubated with normal goat serum (1:40 dilution, Vectastain). After one hour the excess normal goat serum was removed and the sections were

incubated with polyclonal rabbit CRF antibody (1:75 dilution, Santacruz SC-21000) containing 0.5% BSA and gelatin for 24 h at 4°C. The sections were washed and incubated with biotinylated goat anti-rabbit IgG antibody (1:200 dilution, Vectastain) for 1 h at room temperature. Tissue sections were then washed thrice and incubated with ABC reagent (1:100 dilution, Vectastain ABC kit) for 1 h at room temperature. After three washes, sections were incubated with 3,3 diaminobenzidine tetrahydrochloride (DAB) in tris buffer (0.05M, pH 7.2) containing 0.02% H_2O_2 for 8 min. The tissue sections were then washed in distilled water, dehydrated in alcohol grades (50%, 70%, 90%, 100%), cleared in xylene, and mounted in DPX. Tissue sections were photographed using Bright field microscope (Axioscope A-1, Zeiss)

3.6 Details of the antibody

The CRF antibody used in the present study was commercially available and were procured from Santa-Cruz Biotechnologies USA. The CRF SC-21000 is a rabbit polyclonal antibody of amino acids 1-196 representing the full-length CRF of human origin.

3.7 RNA Isolation

Brain tissues cryopreserved at -80° C were homogenised in 500 µl of TRIzol reagent. The homogenate was then incubated for 30 min on ice. Chloroform (100 µl) was added and the contents were thoroughly mixed. The tube was kept on ice for 15 min followed by centrifugation at 12000 rpm for 15 min at 4° C. The aqueous layer was transferred to another centrifuge tube and equal quantity of isopropanol was added to it. The tube was incubated on ice for 30 min and then stored at -20° C overnight to precipitate RNA. Next day, the tube was centrifuged at 13000 rpm for 25 min at 4° C. The pellet obtained was washed with 70% ice chilled ethanol. It was again incubated on ice for 5 min and centrifuged at 13000 rpm for 25 min at 4° C. Ethanol was removed and the pellet was allowed to dry at room temperature. After removing the traces of alcohol, the pellet was dissolved in 20 µl DEPC treated water. The concentration of RNA was determined by Nanodrop and the RNA was diluted accordingly to perform cDNA synthesis.

3.8 cDNA preparation

cDNA (20 µl) was prepared using Verso cDNA synthesis kit (Thermo Fischer). The components were added as mentioned in Table 1

Components	Volume
5X cDNA synthesis buffer	4 µl
dNTP Mix	2 µl
RNA primer oligo dT	1 µl
RT enhancer	1 µl
Verso enzyme mix	1 µl
Template (RNA)-150 ng	5 µl
NFW	6 µl
Total volume	20 µl

Table 1: Composition of the reaction mixture used for cDNA synthesis.

Conditions of thermal cycler used for cDNA synthesis are shown in Figure 4.

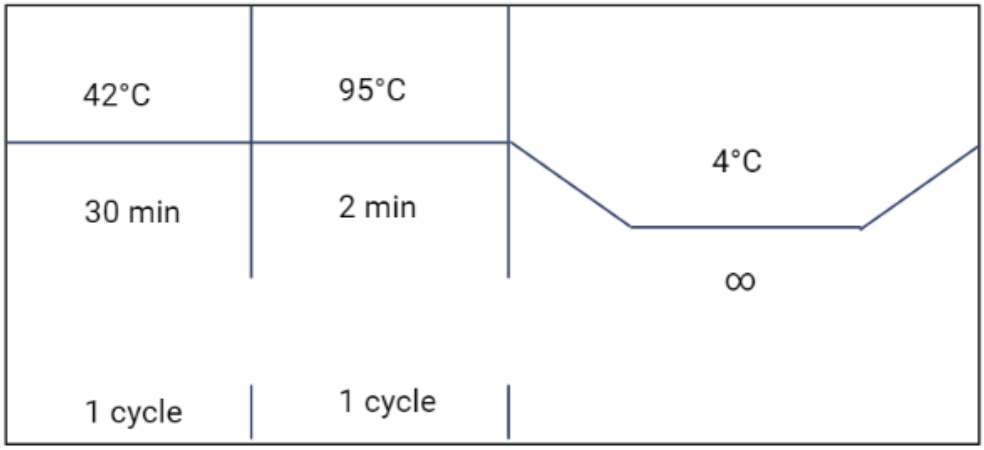


Figure 4: Reaction conditions for thermal cycler for cDNA synthesis

3.9 Primer designing

Primers for CRF and β-actin (house-keeping gene) were designed for *E. cyanophlyctis* from the homology analysis and multiple sequence alignment of 15 closely related species of frogs. Homology alignment was carried out using Clustal Omega tool. NCBI PrimerBlast was used for designing the primers. The details of primers are shown in Table 2.

CRF gene primers

	Sequence (5' ⟶ 3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TAAATCCCCAGCATCTTCTC	20	51.80	45.00	2.00	0.00
Reverse primer	CACGCTTTGCTTTTTCTGT	19	51.90	42.11	2.00	0.00

Beta-actin gene primers

	Sequence (5' ⟶ 3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TACTGAAGCTCCACTAAACCCAAAAG	26	56.9	42.31	1.00	0.00
Reverse primer	GTCCAGCACGATACCAGTTGT	21	57.2	52.4	0.00	0.00

Table 2: Primer details: CRF and Beta-actin

3.10 Real-time Polymerase chain reaction

Quantitative real-time PCR was performed on Real-Time PCR detection system (Applied Biosystems Bio-Rad, USA) in a total of 10µl reaction volume. Following reaction composition was used for RT-PCR reaction set up (Table 3). The reaction was carried out in duplicates with β-actin as the endogenous (house-keeping) control. The thermal cycling conditions were as follows – 1 cycle of 95° C for 10 min, 40 cycles of 95° C for 15 sec and 49° C for 1 min, followed by melt curve analysis (95° C for 15 sec, 49° C for 1 min, and 95° C for 15 sec)

Sr.No	Chemicals	Volume (µl)
1	5X SYBR Green	5.0
2	Forward & Reverse Primer mix	1.0
3	Nuclease free water	2.0
4	cDNA	2.0
Total volume		10

Table 3: Reaction composition for RT-PCR

3.11 Statistical analysis

The data on duration of larval period and the number of CRF-ir cells between control and treated groups were analysed using student t test while the data on mRNA analysis between the groups were analysed using Mann-Whitney test.

4 Results and Discussion

4.1 Growth and metamorphosis

Tadpoles were reared with adequate food and periodic water changes to ensure ideal conditions. The period of exposure to predation risk ranged from August-November, 2022 for the majority of tadpoles. All the tadpoles reached metamorphic climax (stage 42). However, mortality was observed during their transition from metamorphic climax to metamorphosis (n =19). Growth was very slow in a few individuals, which did not develop even the hindlimbs after seven months of rearing [(control (n=2) and treated (n=2)] and hence were not considered for the analysis. Tadpoles exposed to the predation risk by dragonfly nymphs took significantly more time (t = 2.864, p=0.0059) to metamorphose in comparison to control groups tadpoles (Figure:5).

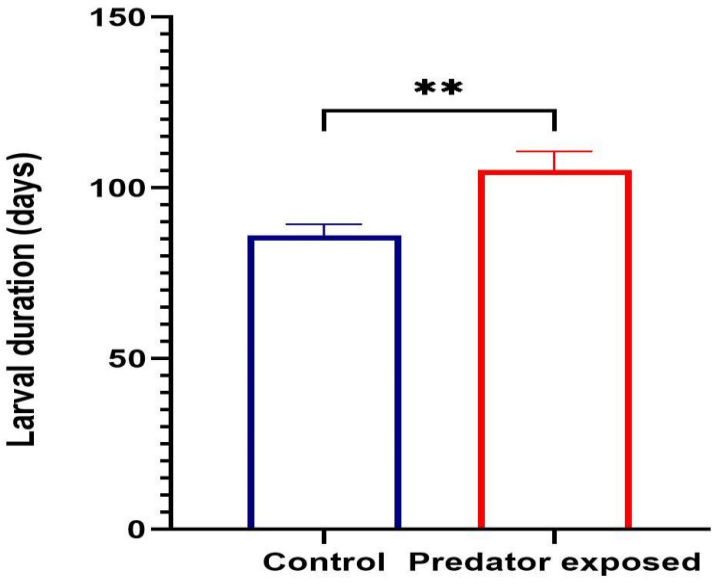


Figure 5: Larval duration (Mean ±SEM) **of tadpoles from control and predator exposed groups.**

[n=27(control), n=31(treated)],

** indicate significant difference at α=0.01 [student's t test]

4.2 Gross morphology of the amphibian brain

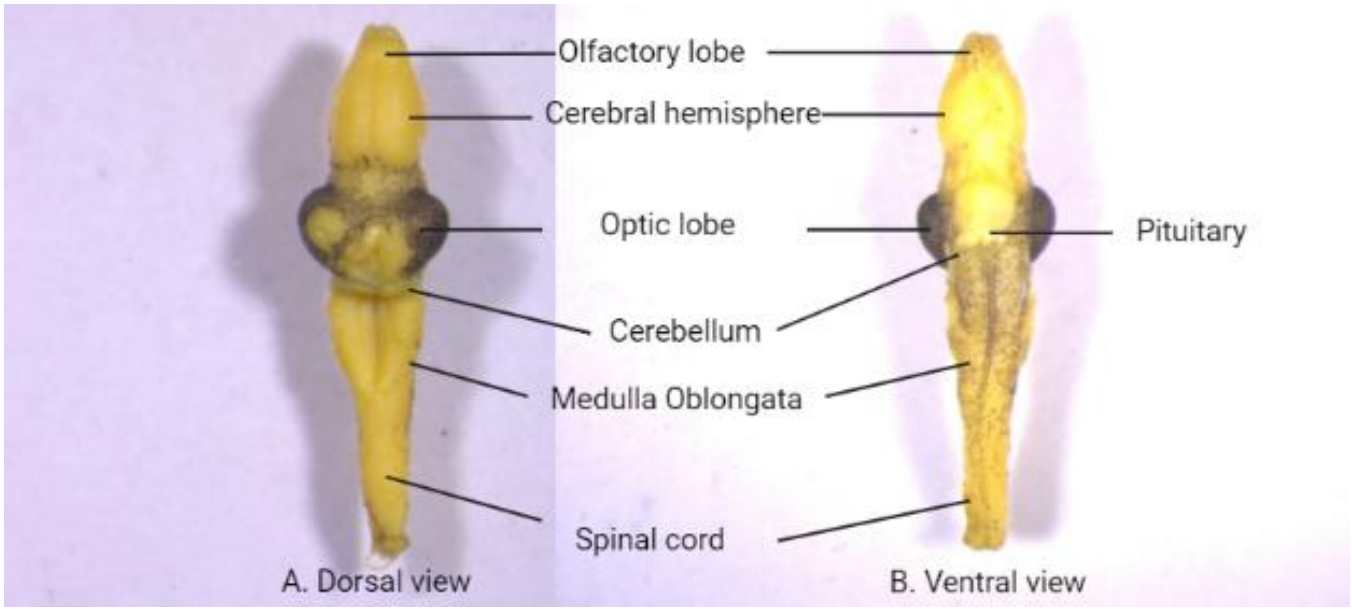


Figure 6: Frog Brain- A) Dorsal view B) Ventral view

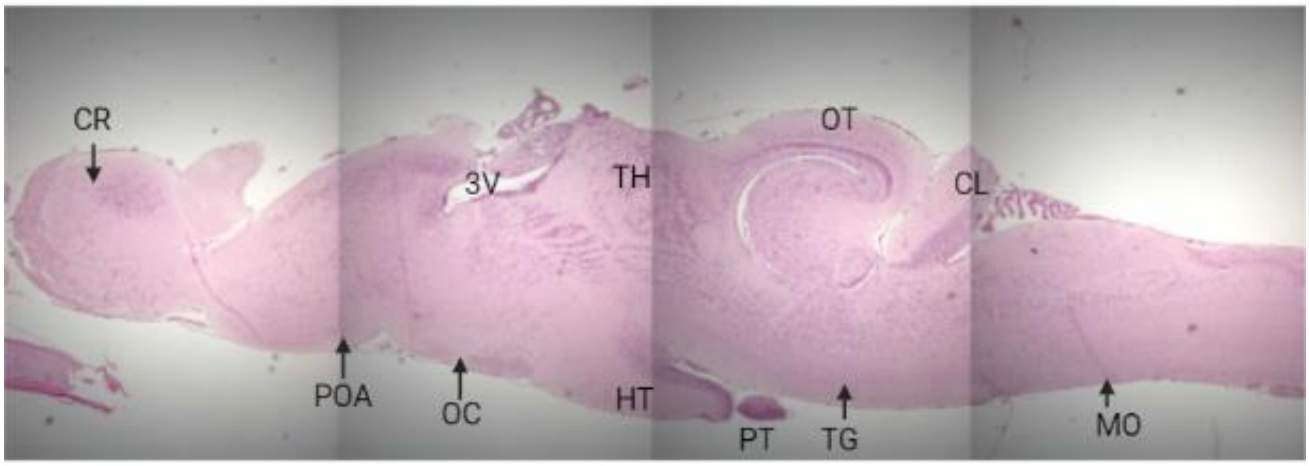


Figure 7: Midsagittal section of adult *Euphlyctis cyanophlyctis* brain-Histology (CR-Cerebrum, POA- Preoptic area, OC-Optic chiasma, 3V- Third ventricle, TH-Thalamus, HT-Hypothalamus, PT-Pituitary, OT-Optic tectum, TG-Tegmentum, CL-Cerebellum, MO-Medulla Oblongata)

The brain of an adult frog is divided into three parts: forebrain, midbrain and hindbrain. Forebrain consists of olfactory lobe, cerebral hemispheres and diencephalon. Olfactory lobe is almost merged with the cerebral hemispheres and each cerebral hemisphere encloses a large cavity called lateral ventricle. The diencephalon is

situated just behind the cerebrum and has a small cavity, the third ventricle. The thin roof of diencephalon is called the epithalamus and the floor is called the hypothalamus. Pituitary is situated below the hypothalamus, and acts as an important endocrine gland. The two optic nerves cross each other in front of the pituitary making the optic chiasma. Midbrain consists of optic lobes and the hindbrain consists of cerebellum and medulla oblongata. Medulla oblongata continues further as the spinal cord.

4.4 Immunohistochemical localization of CRF in the brain of adult *Euphlyctis cyanophlyctis*

Adults of *E. cyanophlyctis* collected from nature were used to study the distribution pattern of CRF immunoreactivity. Mid-sagittal sections of the frog brain passing through the pituitary are shown in the figure 8. In the adult brain, a strong CRF positive immunoreaction was observed in the hypothalamic area (Figure 8C). Immunoreaction was also localized to neuronal cells situated towards the thalamic region of the brain. However, there was no immunoreaction in any other regions of the brain (Figure 8B). Similarly, there was no immunoreaction in the pituitary gland (Figure 8B). Brain sections which were incubated without primary antibody did not show any reaction (Figure 8A).

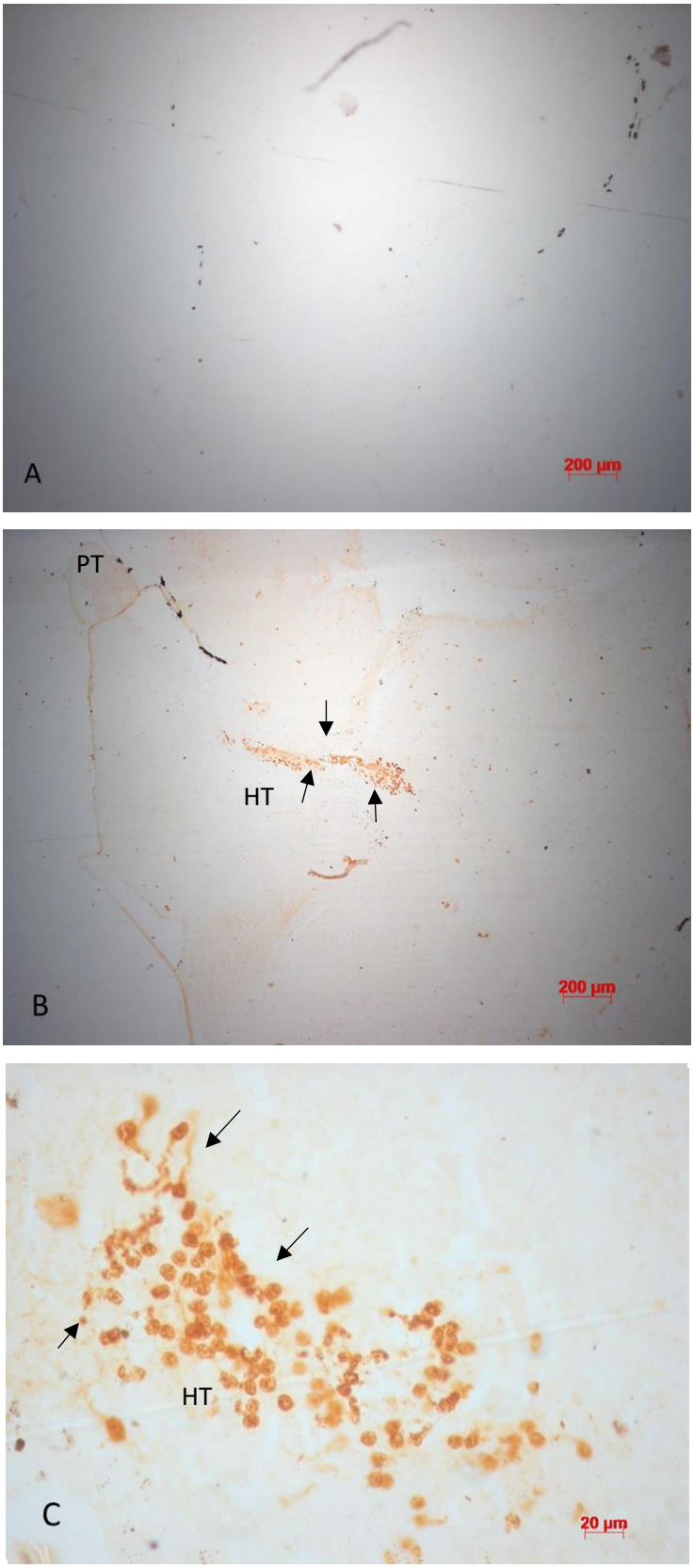
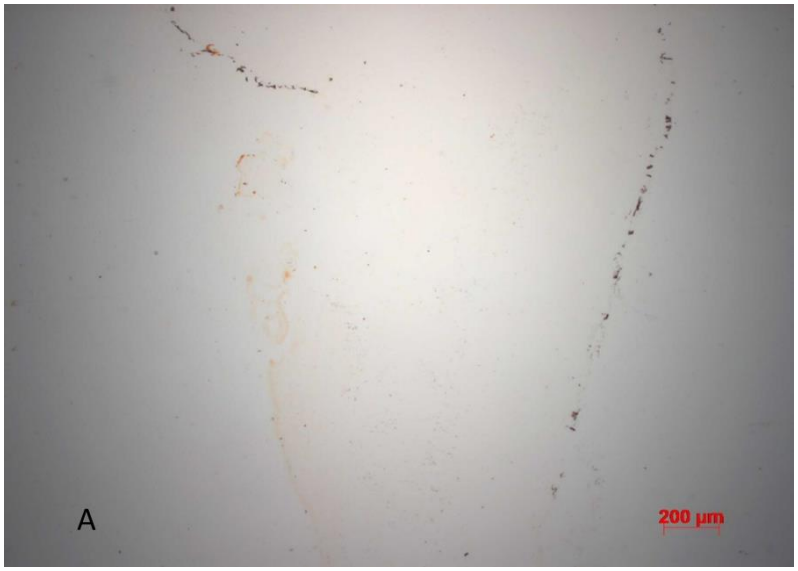
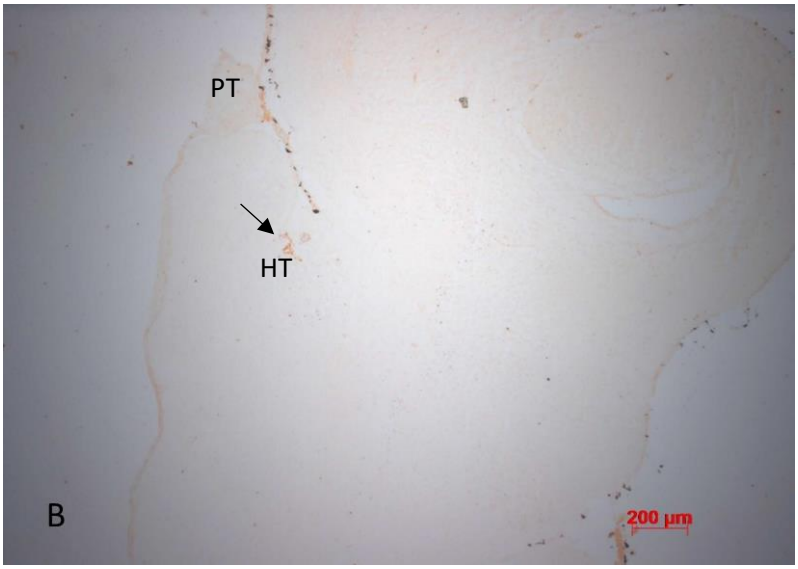


Figure 8: Midsagittal section of the adult brain of *E. cyanophlyctis* showing immunohistochemical localization of CRF: A) Negative control section without primary antibody incubation (B & C) Depicting strong CRF positive cells present in the hypothalamic region. CRF-ir cells are shown by the arrows)

4.5 Localization of CRF in the brain of *E. cyanophlyctis* reared as controls.

In the brain of control froglets, a weak immunoreaction for CRF was observed in the hypothalamic region (Figure 9 B). Only a few cells were positive for CRF-ir (Figure 9C). Surprisingly, other regions of the brain were negative for CRF immunoreaction (Figure 9 B). Brain sections which were incubated without primary antibody did not show any reaction (Figure 9A).





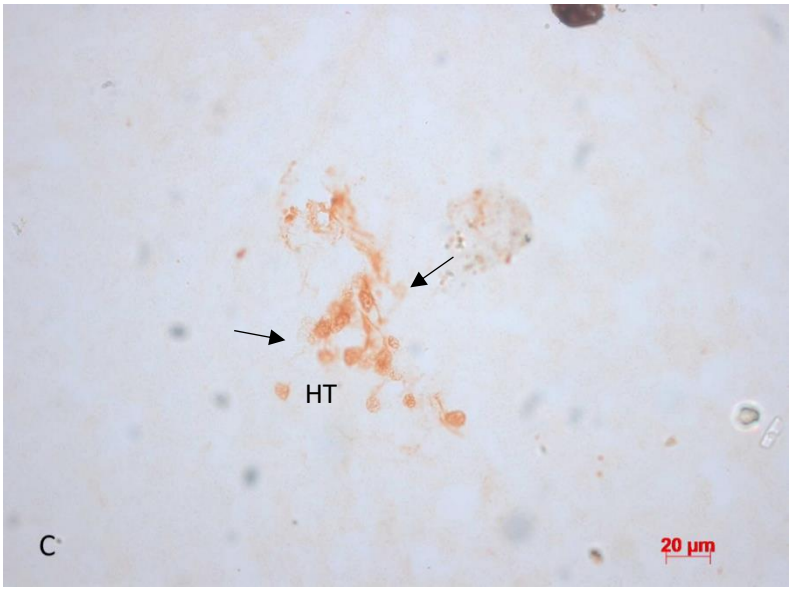
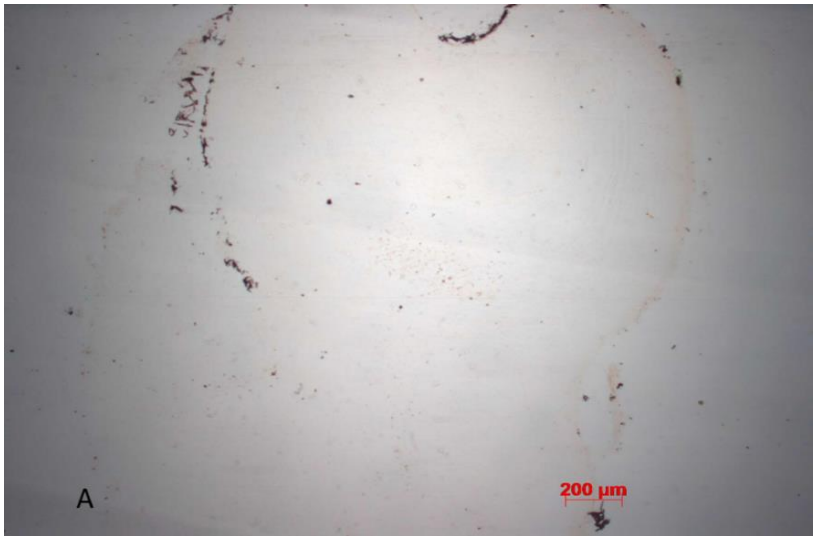
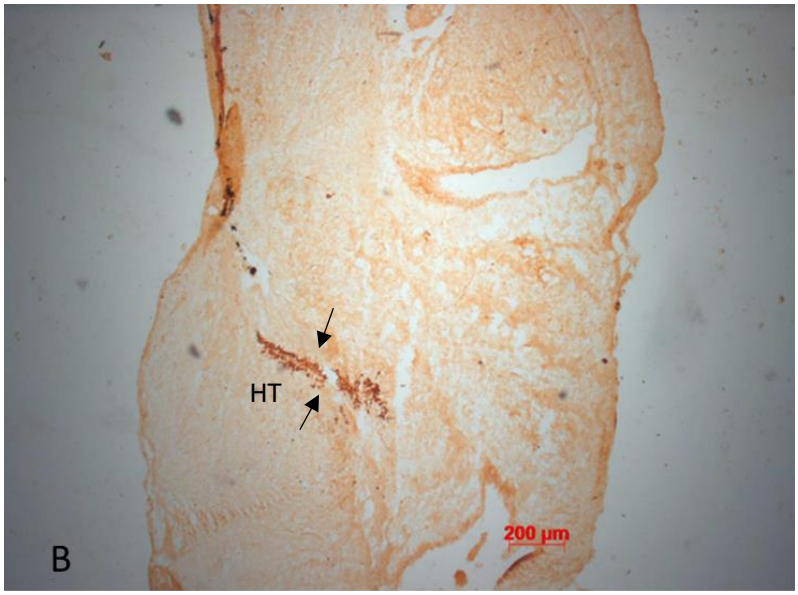


Figure 9: Midsagittal section showing immunohistochemical localization of CRF in the brain of control group froglets A) Negative control section without primary antibody incubation (B & C) Weak CRF positive immunoreaction present in the hypothalamic region (CRF-ir cells are shown by the arrows)

4.6 Localization of CRF in the brain of *E. cyanophlyctis* exposed to predator

The brain of *E. cyanophlyctis* exposed to dragonfly nymph as tadpoles exhibited a strong CRF immunoreaction in the hypothalamus. More number of immunoreactive cells were seen in the hypothalamic region of the brain with secretory granules passing through the nerve fibres (Figure 10 B & C). Brain sections which were incubated without primary antibody did not show any reaction (Figure 10A). Surprisingly, no other brain region showed CRF immunostaining (Figure10 B). In comparison with the control group of animals, CRF-ir cells in the predator-exposed group was more (Figure11). Further, quantification of the immunoreactive cells in the brain of control and predator-exposed groups revealed that significantly ($t=4.814$, $p=0.0003$) greater number of cells were positive for immunoreaction of CRF in the individuals exposed to predation stress in comparison to the control group (Figure 12).


A
200 µm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B
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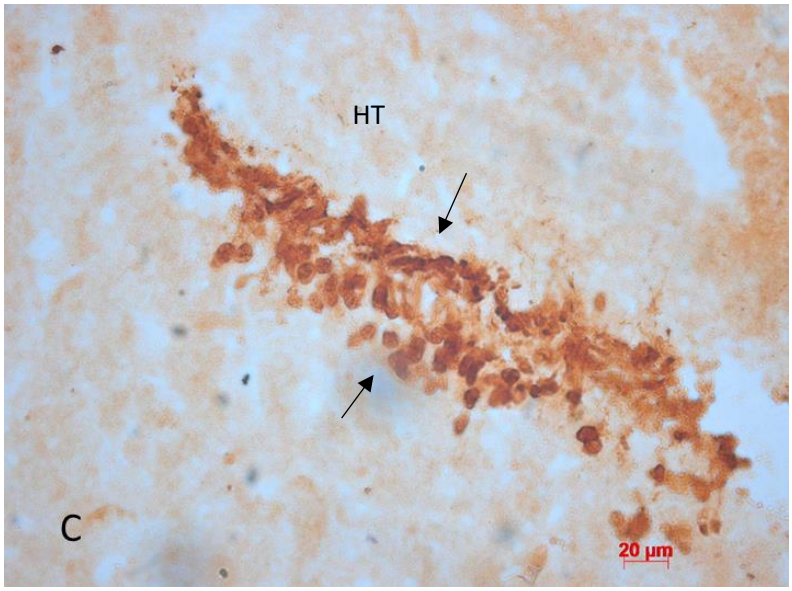

Figure 10: Midsagittal section showing immunohistochemical localization of CRF in the brain of *E. cyanophlyctis* exposed to predator: A) Negative control section without primary antibody; B & C) Strong CRF positive immunoreaction present in the hypothalamic region (CRF-ir cells are shown by the arrows)

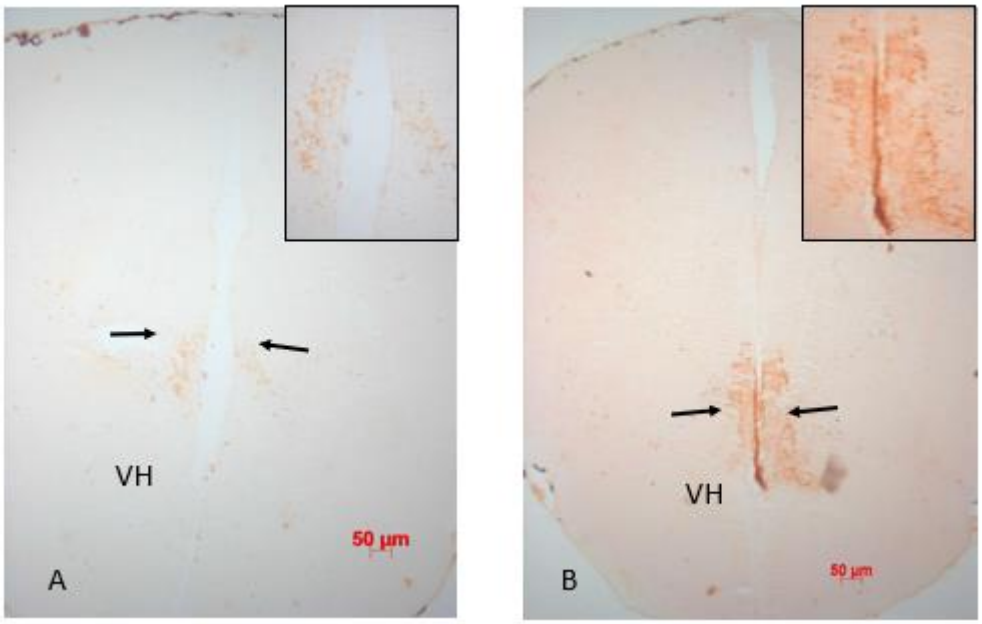

Figure 11: Transverse section showing immunohistochemical localization of CRF in the hypothalamus: A) control group B) predator-exposed group. Enlarged view of the CRH-ir area is shown in the inset (CRF-ir cells are shown by the arrows)

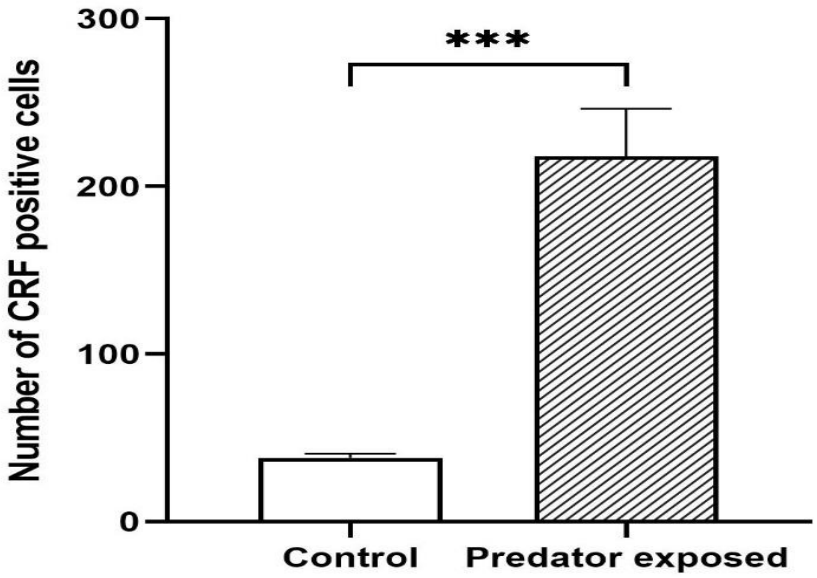

Figure 12: Number of CRF positive cells (Mean ± SEM) in the control and predator exposed *E. cyanophlyctis*. Note that increase in the total number of CRF positive cells in predator exposed group than that of the control group.

*** indicates significant difference ($p<0.001$) between control and predator-exposed groups (unpaired t test)

4.7 CRF gene expression

Quantitative real-time PCR results showed that the expression of CRF gene was elevated in the predator exposed group (n=5). The RT-PCR results were analysed by the $2^{-\Delta\Delta Ct}$ method with β-actin as the endogenous control. Fold change of CRF was 3.35 for the predator exposed group (Figure 13). The relative gene expression of CRF with the control group has a significant difference ($p=0.0025$); Mann-Whitney test).

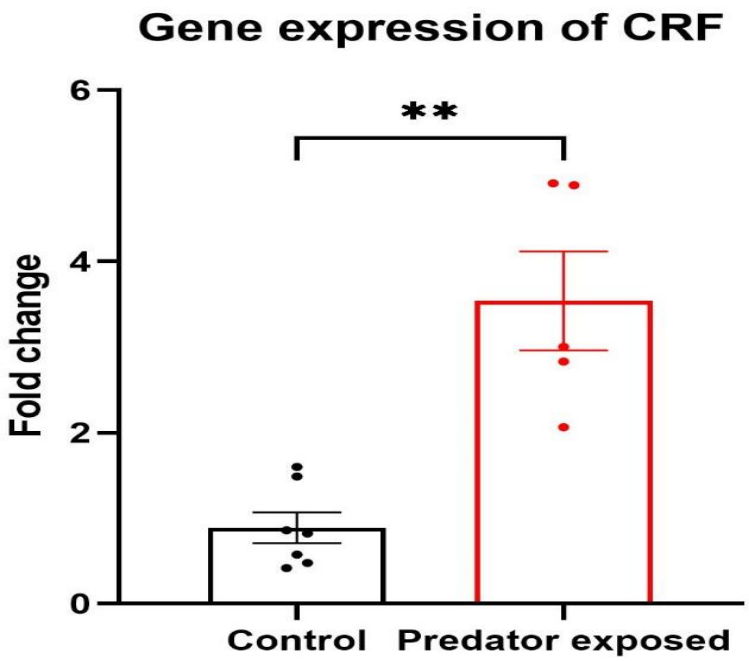

Figure 13: Fold change of CRF (Mean ±SEM) **in control and predator exposed group:** Fold change determined by the quantitative real-time PCR (qRT-PCR) analysis of CRF in control and predator exposed groups of brain tissues. All data were normalized with β-actin expression and given as relative to control. [n=7 for control, n=5 for predator exposed)],

** indicate significant difference at α = 0.01

5 Discussion

Different environmental and ecological factors act as short-term or long-term stressors thus influencing different aspects of life-history of organisms. A biotic stressor such as predation often acts as a major determinant of spatial and temporal distribution of prey animals in different environments. Hence, prey species have evolved novel strategies such as changes in behaviour, morphology and life-history traits to minimize predation risk (Scherer et al., 2017). To respond to predation risk appropriately, prey species are expected to recognise their potential predators. For this purpose, prey species have either innate or acquired predator recognition mechanisms. A combination of both mechanisms may exist depending on the species and the complexity of habitat. Learning to recognise potential predators in prey animals is accomplished by chemical, visual and auditory cues (McCoy et al., 2012; Munoz & Blumstein, 2012)

Vertebrate prey respond to different stressors including predation by activating the HPA axis (Romero, 2004). In amphibian prey this axis is represented by HPI. The stress response is initiated by the release of corticotropin releasing factor (CRF) from

the hypothalamus, which acts on the corticotropes of anterior pituitary to secrete corticotropin (ACTH). Corticotropin stimulates the interrenal tissue to secrete glucocorticoids (Denver, 1997; Sapolsky et al., 2000). Glucocorticoids mediate the downstream changes to exhibit stress responses. Corticotropin releasing factor thus plays a key player in eliciting antipredator response of vertebrates.

CRF has been a well-studied peptide since its identification in 1981 (Vale et al., 1981). For instance, Immunohistochemical studies have shown that CRF-immunoreactive cell bodies and fibres are widely distributed in the CNS of *Xenopus laevis* (Yao et al., 2004). Further, a strong expression of CRF was found in the magnocellular and parvocellular divisions of the preoptic area (POA), locus coeruleus (LC), cerebellum, and rostral spinal cord. CRF-ir cells were also found in the amygdala and bed nucleus of stria terminalis (BNST). Similarly, strong CRF-ir fibres were seen in the median eminence (Yao et al., 2004). The first study that presented the immunological evidence for localization of CRF-like peptide in the amphibian brain indicated the presence of CRF-like peptides in the preoptic area, retro chiasmatic, and dorsal preoptic regions. Radioimmunoassay studies using the pieces of brain of *Rana ridibunda* revealed that a substantial amount of CRF is present in the hypothalamus and infundibular recess (Tonon et al., 1985). Yao et al. (2004) were the first to show the effect of a stressor on the CRF distribution in the CNS of a non-mammalian species. When exposed to an acute shaking stress the CRF expression in *Xenopus laevis* was shown to have a difference compared to the non-stressed control group. Further, an increase in CRF staining and the number of CRF-ir positive neurons was observed in the anterior preoptic area of stressed animals. Increased CRF-ir cells was also observed in BNST and medial amygdala (Yao et al., 2004). However, there are no studies on the effect of chronic stress on the distribution of CRF in the central nervous system of Indian amphibians. In the present study, tadpoles of *E. cyanophlyctis* were subjected to chronic stress by exposing them to predation risk throughout metamorphosis (stage 25–44) to assess the distribution of CRF in the CNS. Immunohistochemical studies revealed an intense immunoreactivity against CRF antibodies in the hypothalamus of *E. cyanophlyctis* brain. Greater number of immunoreactive cells were observed in the brain of froglets experiencing predation stress. This suggests a potential role of CRF in eliciting a stress response against a chronic stressor such as predation risk. Though a robust change in the distribution of CRF could be observed in two groups of animals, CRF expression was seen only in

the hypothalamus. No other regions of the brain were positive for CRF immunoreactivity. Similarly, CRF immunoreaction was seen only in the hypothalamic region of the adult *E. cyanophlyctis* brain. In contrast, previous studies have shown the CRF-immunoreaction in the other regions of the brain to hypothalamus (Yao et al., 2004). Interestingly, CRF-ir cells were also observed in the pituitary gland in addition to hypothalamus of *Indosylvirana indica* exposed to predation stress (personal observations). Therefore, absence of CRF-immunoreactivity in the other regions of the brain could be due to either be a species-specific variation in the distribution of CRF or less specificity of the antibody used. Since the antibody used in our study was raised against the human sequence. In contrast, the antibody used by Yao et al. (2004) antibody was raised against *Xenopus* specific sequence. The specificity of our antibody needs to be confirmed with western blot analysis.

The effect of social stress in rainbow trout (*Oncorhynchus mykiss*) has shown a persistent increase in CRF mRNA expression in the subordinate animals (Doyon et al., 2003). CRF mRNA expression increased in the preoptic area of both the dominant and subordinate animals at the time of establishment of social hierarchy, which returns to basal levels in the dominant fish after the establishment of social dominance. However, the levels remain high in the subordinate fish (Doyon et al., 2003). Consequently, the cortisol (the principal glucocorticoid in fish) level was also high in the subordinate fish indicating a continuously activated HPI axis in socially stressed animals (Doyon et al., 2003). In our study we used the whole brain tissue to assess the expression pattern of CRF gene using quantitative RT-PCR. Our results show an upregulation of CRF gene in the predator exposed animals. However, among individual variation in the pattern of expression was higher. A potential limitation of our study was the limited sample size. Unfortunately, we had fewer number of individuals for molecular analysis due to unprecedented death of experimental animals during the transition from metamorphic climax to completion of metamorphosis. A larger sample size could have minimized the among individual variation in the expression of CRF mRNA. Moreover, western blot analysis could have provided us with the further evidence of increased levels of CRF protein.

In summary, with available data we can say that the CRF expression was more in the predator treated (chronically stressed) animals indicating a continuously activated HPI axis. However, we do not have any data on how the CRF-BP and the CRF receptors are distributed in such a scenario. Also, how other players in this axis are

regulated to counteract a chronic predatory stress needs to be investigated. Additionally, species-specific variations in response to predation stress needs to be investigated by studying a greater number of species.

6 References

Albeck, D. S., McKittrick, C. R., Blanchard, D. C., Blanchard, R. J., Nikulina, J., McEwen, B. S., & Sakai, R. R. (1997). Chronic Social Stress Alters Levels of Corticotropin-Releasing Factor and Arginine Vasopressin mRNA in Rat Brain. *The Journal of Neuroscience*, *17*(12), 4895–4903. https://doi.org/10.1523/JNEUROSCI.17-12-04895.1997

Backström, T., Schjolden, J., Øverli, Ø., Thörnqvist, P.-O., & Winberg, S. (2011). Stress effects on AVT and CRF systems in two strains of rainbow trout (Oncorhynchus mykiss) divergent in stress responsiveness. *Hormones and Behavior*, *59*(1), 180–186. https://doi.org/10.1016/j.yhbeh.2010.11.008

Bale, T. L., & Vale, W. W. (2004). CRF and CRF Receptors: Role in Stress Responsivity and Other Behaviors. *Annual Review of Pharmacology and Toxicology*, *44*(1), 525–557. https://doi.org/10.1146/annurev.pharmtox.44.101802.121410

Behan, D. P., Grigoriadis, D. E., Lovenberg, T., Chalmers, D., Heinrichs, S., Liaw, C., & De Souza, E. B. (1996). Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: Implications for the treatment of CNS disorders. *Molecular Psychiatry*, *1*(4), 265–277.

Bernier, N. J., Alderman, S. L., & Bristow, E. N. (2008). Heads or tails? Stressor-specific expression of corticotropin-releasing factor and urotensin I in the preoptic area and caudal neurosecretory system of rainbow trout. *Journal of Endocrinology*, *196*(3), 637–648. https://doi.org/10.1677/JOE-07-0568

Bernier, N. J., & Craig, P. M. (2005). CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *American Journal of Physiology-*

Regulatory, Integrative and Comparative Physiology, *289*(4), R982–R990. https://doi.org/10.1152/ajpregu.00668.2004

Boorse, G. C., Crespi, E. J., Dautzenberg, F. M., & Denver, R. J. (2005). Urocortins of the South African Clawed Frog, Xenopus laevis: Conservation of Structure and Function in Tetrapod Evolution. *Endocrinology*, *146*(11), 4851–4860. https://doi.org/10.1210/en.2005-0497

Boorse, G. C., & Denver, R. J. (2006). Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. *General and Comparative Endocrinology*, *146*(1), 9–18. https://doi.org/10.1016/j.ygcen.2005.11.014

Boorse, G. C., Kholdani, C. A., Seasholtz, A. F., & Denver, R. J. (2006). Corticotropin-Releasing Factor Is Cytoprotective in Xenopus Tadpole Tail: Coordination of Ligand, Receptor, and Binding Protein in Tail Muscle Cell Survival. *Endocrinology*, *147*(3), 1498–1507. https://doi.org/10.1210/en.2005-1273

Brown, D. D., Wang, Z., Furlow, J. D., Kanamori, A., Schwartzman, R. A., Remo, B. F., & Pinder, A. (1996). The thyroid hormone-induced tail resorption program during Xenopus laevis metamorphosis. *Proceedings of the National Academy of Sciences*, *93*(5), 1924–1929. https://doi.org/10.1073/pnas.93.5.1924

Chivers, D. P., & Brown, G. E. (1996). The Evolution of Chemical Alarm Signals: Attracting Predators Benefits Alarm Signal Senders. *The American Naturalist*, *148*(4), 649–659. https://doi.org/10.1086/285945

Cortright, D. N., Goosens, K. A., Shonee Lesh, J., & Seasholtz, A. F. (1997). Isolation and Characterization of the Rat Corticotropin-Releasing Hormone (CRH)-Binding Protein Gene: Transcriptional Regulation by Cyclic Adenosine Monophosphate and CRH [1]. *Endocrinology*, *138*(5), 2098–2108. https://doi.org/10.1210/endo.138.5.5128

Dautzenberg, F. M., & Hauger, R. L. (2002). The CRF peptide family and their receptors: Yet more partners discovered. *Trends in Pharmacological Sciences*, *23*(2), 71–77. https://doi.org/10.1016/S0165-6147(02)01946-6

Denver, R. J. (1988). Several hypothalamic peptides stimulate in vitro thyrotropin secretion by pituitaries of anuran amphibians. *General and Comparative Endocrinology*, *72*(3), 383–393. https://doi.org/10.1016/0016-6480(88)90160-8

Denver, R. J. (1997). Proximate Mechanisms of Phenotypic Plasticity in Amphibian Metamorphosis. *American Zoologist*, *37*(2), 172–184. https://doi.org/10.1093/icb/37.2.172

Deussing, J. M., & Chen, A. (2018). The Corticotropin-Releasing Factor Family: Physiology of the Stress Response. *Physiological Reviews*, *98*(4), 2225–2286. https://doi.org/10.1152/physrev.00042.2017

Doyon, C., Gilmour, K. M., Trudeau, V. L., & Moon, T. W. (2003). Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. *General and Comparative Endocrinology*, *133*(2), 260–271. https://doi.org/10.1016/S0016-6480(03)00195-3

Ferrari, M., Wisenden, B., & Chivers, D. (2010). Chemical ecology of predator–prey interactions in aquatic ecosystems: A review and prospectus The present review is one in the special series of reviews on animal-plant interactions. *Canadian Journal of Zoology*, *88*, 698–724. https://doi.org/10.1139/Z10-029

Glennemeier, K. A., & Denver, R. J. (2002). Role for corticoids in mediating the response ofRana pipiens tadpoles to intraspecific competition. *Journal of Experimental Zoology*, *292*(1), 32–40. https://doi.org/10.1002/jez.1140

Hauger, R. L., Grigoriadis, D. E., Dallman, M. F., Plotsky, P. M., Vale, W. W., & Dautzenberg, F. M. (2003). International Union of Pharmacology. XXXVI. Current Status of the Nomenclature for Receptors for Corticotropin-Releasing Factor and Their Ligands. *Pharmacological Reviews*, *55*(1), 21–26. https://doi.org/10.1124/pr.55.1.3

Hauger, R. L., Millan, M. A., Lorang, M., Harwood, J. P., & Aguilera, G. (1988). Corticotropin-Releasing Factor Receptors and Pituitary Adrenal Responses during Immobilization Stress. *Endocrinology*, *123*(1), 396–405. https://doi.org/10.1210/endo-123-1-396

Hsu, S. Y., & Hsueh, A. J. W. (2001). Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nature Medicine*, *7*(5), 605–611. https://doi.org/10.1038/87936

Huising, M. O., & Flik, G. (2005). The Remarkable Conservation of Corticotropin-Releasing Hormone (CRH)-Binding Protein in the Honeybee (Apis mellifera) Dates the CRH System to a Common Ancestor of Insects and Vertebrates. *Endocrinology*, *146*(5), 2165–2170. https://doi.org/10.1210/en.2004-1514

Jp, H., D, A., & C, P. (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology*, *61*(2). https://doi.org/10.1159/000126839

Kats, L. B., & Dill, L. M. (1998). The scent of death: Chemosensory assessment of predation risk by prey animals. *Écoscience*, *5*(3), 361–394. https://doi.org/10.1080/11956860.1998.11682468

Lewis, K., Li, C., Perrin, M. H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T. M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P. E., & Vale, W. W. (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proceedings of the National Academy of Sciences*, *98*(13), 7570–7575. https://doi.org/10.1073/pnas.121165198

Lima, S. L., & Dill, L. M. (1990). Behavioral decisions made under the risk of predation: A review and prospectus. *Canadian Journal of Zoology*, *68*(4), 619–640. https://doi.org/10.1139/z90-092

Manzon, R., & Denver, R. (2004). Regulation of pituitary thyrotropin gene expression during Xenopus metamorphosis: Negative feedback is functional throughout metamorphosis. *Journal of Endocrinology*, *182*(2), 273–285. https://doi.org/10.1677/joe.0.1820273

Mathis, A., & Smith, R. J. F. (1993). Fathead minnows, Pimephales promelas, learn to recognize northern pike, Esox lucius, as predators on the basis of chemical stimuli

from minnows in the pike's diet. *Animal Behaviour*, *46*(4), 645–656. https://doi.org/10.1006/anbe.1993.1241

McCoy, M. W., Touchon, J. C., Landberg, T., Warkentin, K. M., & Vonesh, J. R. (2012). Prey Responses to Predator Chemical Cues: Disentangling the Importance of the Number and Biomass of Prey Consumed. *PLOS ONE*, *7*(10), e47495. https://doi.org/10.1371/journal.pone.0047495

McEwen, B. S. (2007). Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. *Physiological Reviews*, *87*(3), 873–904. https://doi.org/10.1152/physrev.00041.2006

Munoz, N. E., & Blumstein, D. T. (2012). Multisensory perception in uncertain environments. *Behavioral Ecology*, *23*(3), 457–462. https://doi.org/10.1093/beheco/arr220

Narayan, E. J., Cockrem, J. F., & Hero, J.-M. (2013). Sight of a Predator Induces a Corticosterone Stress Response and Generates Fear in an Amphibian. *PLoS ONE*, *8*(8), e73564. https://doi.org/10.1371/journal.pone.0073564

Nicolaides, N. C., Kyratzi, E., Lamprokostopoulou, A., Chrousos, G. P., & Charmandari, E. (2015). Stress, the Stress System and the Role of Glucocorticoids. *Neuroimmunomodulation*, *22*(1–2), 6–19. https://doi.org/10.1159/000362736

Potter, E., Behan, D. P., Linton, E. A., Lowry, P. J., Sawchenko, P. E., & Vale, W. W. (1992). The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proceedings of the National Academy of Sciences*, *89*(9), 4192–4196. https://doi.org/10.1073/pnas.89.9.4192

Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P. E., & Vale, W. (1994). Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proceedings of the National Academy of Sciences*, *91*(19), 8777–8781. https://doi.org/10.1073/pnas.91.19.8777

Radulovic, M., Hippel, C., & Spiess, J. (2003). Corticotropin-releasing factor (CRF) rapidly suppresses apoptosis by acting upstream of the activation of caspases: CRF

suppresses activation of caspases. *Journal of Neurochemistry*, *84*(5), 1074–1085. https://doi.org/10.1046/j.1471-4159.2003.01594.x

Reyes, T. M., Lewis, K., Perrin, M. H., Kunitake, K. S., Vaughan, J., Arias, C. A., Hogenesch, J. B., Gulyas, J., Rivier, J., Vale, W. W., & Sawchenko, P. E. (2001). Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proceedings of the National Academy of Sciences*, *98*(5), 2843–2848. https://doi.org/10.1073/pnas.051626398

Rivier, C., & Vale, W. (1983). Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature*, *305*(5932), 325–327. https://doi.org/10.1038/305325a0

Romero, L. M. (2004). Physiological stress in ecology: Lessons from biomedical research. *Trends in Ecology & Evolution*, *19*(5), 249–255. https://doi.org/10.1016/j.tree.2004.03.008

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, *21*(1), 55–89. https://doi.org/10.1210/edrv.21.1.0389

Scherer, A. E., Garcia, M. M., & Smee, D. L. (2017). Predatory blue crabs induce stronger nonconsumptive effects in eastern oysters Crassostrea virginica than scavenging blue crabs. *PeerJ*, *5*, e3042. https://doi.org/10.7717/peerj.3042

Scherer, A. E., & Smee, D. L. (2016). A review of predator diet effects on prey defensive responses. *Chemoecology*, *3*(26), 83–100. https://doi.org/10.1007/s00049-016-0208-y

Seasholtz, A. F., Burrows, H. L., Karolyi, I. J., & Camper, S. A. (2001). Mouse models of altered CRH-binding protein expression. *Peptides*, *22*(5), 743–751. https://doi.org/10.1016/S0196-9781(01)00387-4

Seasholtz, A., Valverde, R., & Denver, R. (2002). Corticotropin-releasing hormone-binding protein: Biochemistry and function from fishes to mammals. *Journal of Endocrinology*, *175*(1), 89–97. https://doi.org/10.1677/joe.0.1750089

Selye, H. (1936). A Syndrome produced by Diverse Nocuous Agents. *Nature*, *138*(3479), Article 3479. https://doi.org/10.1038/138032a0

Selye, H. (1956). *The stress of life* (pp. xvi, 324). McGraw-Hill.

Supekar, S. C., & Gramapurohit, N. P. (2017). Can embryonic skipper frogs (Euphlyctis cyanophlyctis) learn to recognise kairomones in the absence of a nervous system? *Journal of Biosciences*, *42*(3), 459–468. https://doi.org/10.1007/s12038-017-9688-3

Supekar, S. C., & Gramapurohit, N. P. (2020). Does temporal variation in predation risk affect antipredator responses of larval Indian Skipper Frogs (*Euphlyctis cyanophlyctis*)? *Canadian Journal of Zoology*, *98*(3), 202–209. https://doi.org/10.1139/cjz-2019-0118

Tata, J. R. (1993). Gene expression during metamorphosis: An ideal model for post-embryonic development. *BioEssays*, *15*(4), 239–248. https://doi.org/10.1002/bies.950150404

Tonon, M.-C., Burlet, A., Lauber, M., Cuet, P., Jégou, S., Gouteux, L., Ling, N., & Vaudry, H. (1985). Immunohistochemical Localization and Radioimmunoassay of Corticotropin-Releasing Factor in the Forebrain and Hypophysis of the Frog *Rana ridibunda*. *Neuroendocrinology*, *40*(2), 109–119. https://doi.org/10.1159/000124063

Ulrich-Lai, Y. M., & Ryan, K. K. (2014). Neuroendocrine Circuits Governing Energy Balance and Stress Regulation: Functional Overlap and Therapeutic Implications. *Cell Metabolism*, *19*(6), 910–925. https://doi.org/10.1016/j.cmet.2014.01.020

Vale, W., Spiess, J., Rivier, C., & Rivier, J. (1981). Characterization of a 41-Residue Ovine Hypothalamic Peptide That Stimulates Secretion of Corticotropin and β-Endorphin. *Science*, *213*(4514), 1394–1397. https://doi.org/10.1126/science.6267699

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M. H., Lewis, K., Sutton, S., Chan, R., Turnbull, A. V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P. E., & Vale, W. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature*, *378*(6554), 287–292. https://doi.org/10.1038/378287a0

Weil-Malherbe, H., Axelrod, J., & Tomchick, R. (1959). Blood-Brain Barrier for Adrenaline. *Science*, *129*(3357), 1226–1227. https://doi.org/10.1126/science.129.3357.1226

Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1401), 1205–1208. https://doi.org/10.1098/rstb.2000.0668

Yao, M., Westphal, N. J., & Denver, R. J. (2004). Distribution and Acute Stressor-Induced Activation of Corticotrophin-Releasing Hormone Neurones in the Central Nervous System of Xenopus laevis. *Journal of Neuroendocrinology*, *16*(11), 880–893. https://doi.org/10.1111/j.1365-2826.2004.01246.x