



## Changes in expression of GFAP in ganglion of *Procambarus virginalis* under conditions of temperature stress

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The issue of neurophysiological reactivity of invertebrates, especially invasive species, in response to environmental stress factors, in particular temperature changes, still remains poorly studied. Due to the genetic homogeneity of the offspring, the marbled crayfish *Procambarus virginalis* (Lyko, 2017) (Decapoda, Cambaridae) is unique for studying these issues – a parthenogenetic invasive species that demonstrates high adaptability to various environmental conditions. This work presents studies on changes in GFAP expression in the nervous system of *P. virginalis* under the influence of extremely low water temperatures for this species (+9 °C) and elevated temperatures (+23 and +26 °C) in order to assess the glial response to stress. The suprapharyngeal ganglion was isolated, cytoskeletal protein extraction was performed, and GFAP immunoblotting using polyclonal antibodies was performed. Quantitative assessment of GFAP expression was performed by densitometry. No statistically significant changes in the protein content in the cytoskeletal fraction of the nervous tissue of crayfish were detected. In the control group, weak expression of GFAP was detected, the highest level of expression was observed in group 2 – cold stress, in which a large number of shortened GFAP polypeptides (~ 35 kDa) was also detected. In the control, the intensity of the 49 kDa band was  $0.12 \pm 0.05$  a.u. The largest increase in GFAP content compared to the control was detected in group 2 under cold stress –  $0.38 \pm 0.07$  a.u. Temperature stress induces glial cell activation in *P. virginalis*, similar to reactive gliosis in vertebrates. GFAP expression and its proteolytic changes can be used as early indicators of neurostress in invertebrates. These results emphasize the feasibility of using GFAP as a biomarker for assessing neurotoxic effects of the environment in crustaceans. Monitoring GFAP levels in marbled crayfish is promising for assessing the impact of environmental factors on their adaptive capabilities.

**Keywords:** Decapoda; Cambaridae; protein; brain; crayfish; invertebrates; glial cells; adaptation.

### Introduction

Adaptation of invasive hydrobionts to new environmental conditions has attracted considerable scientific interest. The ability of a species to successfully colonize waterbodies with a stable ecological regime depends on its physiological and biochemical plasticity. The marbled crayfish *Procambarus virginalis* (Lyko, 2017) is an invasive species that has recently appeared in Ukrainian freshwater ecosystems (Novitsky & Son, 2016). It is the first known decapod species to reproduce parthenogenetically, being represented by triploid individuals (Scholtz et al., 2003; Martin et al., 2010). Due to the genetic identity of individuals, low environmental demands, and high reproductive potential, *P. virginalis* serves as a suitable model for studying adaptation mechanisms at various levels of biological organization (Jimenez & Faulkes, 2010; Lykholat et al., 2023).

It is known that the optimal temperature range for marbled crayfish is between +18 and +25 °C; however, they are capable of tolerating water temperatures below +8 and above +30 °C for extended periods (Vogt et al., 2015; Kaldre et al., 2015). Our observations confirm that at water temperatures above +20 °C, marbled crayfish exhibit the most active growth and reproduction, whereas at temperatures below +15 °C, the rates of growth and development significantly decrease. Specifically, when the temperature drops below +15 °C, crayfish cease egg-laying, and at water temperatures below +10 °C, molting usually stops. At the same time, marbled crayfish show considerable cold tolerance: they can survive temporary cooling to +6 °C for several months (although growth is halted) and can even endure short-term exposure to around 0 °C. Nevertheless, such extreme water temperature conditions may potentially induce physiological stress and affect biochemical parameters essential for the viability and survival of the species, as prolonged maintenance outside the optimal range increases mortality.

Glial fibrillary acidic protein (GFAP) is a tissue-specific intermediate filament protein of the cytoskeleton in astrocytic glial cells. It is

known that astrocytes are the most sensitive to adverse factors. In neurobiological research, GFAP is considered a molecular index of neurotoxicity and a marker of nervous tissue damage. GFAP expression is a characteristic indicator of astrocytic response to injury caused by unfavorable factors of various origins (Sharma et al., 1992; O'Callaghan & Sriram, 2005; Zhang et al., 2010; Kochanek et al., 2011; Wagner & Zitelli, 2013; Tikhomirov et al., 2016).

The determination of GFAP content has been carried out in various animal species. It is believed that the astrocytic response to different stress factors is non-specific, and the intensity of GFAP synthesis is determined mainly by the dose and duration of exposure rather than by the nature of the factor itself. For example, in the rat brain, prolonged heating (+38 °C for 4 hours) induces a significant increase in GFAP expression, i.e., reactive gliosis (Sharma et al., 1992).

It has been established that astrocytes of marsh frogs *Pelophylax ridibundus* (Pallas, 1771) can respond to environmental toxicants by employing a typical astroglial reaction. GFAP expression and its fragmentation in *P. ridibundus* serve as valid biomarkers of industrial pollution exposure (Hahut, 2021).

The results of a comparative analysis of GFAP in the brain of the monkey goby *Neogobius fluviatilis* (Pallas, 1814) from the coastal waters of the Kerchenska Bay and a conditionally clean site of the Vorskla River revealed a statistically significant increase in GFAP levels in the industrially polluted region. The elevation of GFAP indicates the development of astroglial gliosis as a result of metabolic disturbances that may be caused by petroleum hydrocarbon contamination. The presented data on cytoskeletal disruptions in astrocytes in the brain of sand gobies from the Kerchenska Bay suggest the manifestation of a sublethal biological effect of pollutants in aquatic organisms inhabiting this area. Previous studies have shown that the detected cytoskeletal rearrangements in the fish brain arise under the influence of adverse anthropogenic environmental factors. This supports the use of GFAP as a reliable and valid marker of toxic effects caused by pollutants, which can be applied for early detection of damage and for the

development of effective strategies to mitigate pathogenetic disturbances (Sukhareenko, 2014).

An increase in water temperature can induce neurophysiological stress in aquatic invertebrates. In an experimental study on the Chinese mitten crab *Eriocheir sinensis* H. Milne-Edwards, 1853, individuals were maintained for several weeks under elevated temperature conditions, simulating heat stress. The researchers recorded significant neurotoxic effects, including structural damage in the cerebral ganglion (brain), activation of apoptosis in neurons, and changes in the expression of neuroreceptors (Zhang et al., 2025).

Decreasing water temperature also affects the neurophysiology of poikilothermic invertebrates. Experiments with the Californian red swamp crayfish *Procambarus clarkii* (Girard, 1852) demonstrated that prolonged cold acclimation (maintenance at +10 °C for several weeks) significantly alters physiological functions, notably slowing synaptic transmission (Zhu et al., 2018).

Recent experimental studies on the effects of chronic thermal stress on GFAP levels in decapod crustaceans reveal limited but significant trends. Therefore, a comparative analysis of the impact of various adverse factors (thermal, toxic, etc.) on GFAP metabolism is relevant for understanding the universal mechanisms of the neuronal-glial response to stress.

The aim of the study was to investigate the effects of extremely low and elevated temperatures on GFAP expression levels in the nervous system of marbled crayfish *P. virginalis*. This will allow assessment of whether GFAP can serve as an indicator of neurostress in invertebrates and how similar their responses are to astrocytic reactions in vertebrates.

The relevance of studying the effects of thermal stress on GFAP levels in crustaceans is driven by important scientific and practical factors. In the context of global climate change, the frequency of prolonged temperature extremes in aquatic ecosystems is increasing, directly affecting the physiology of hydrobionts and, consequently, the neurophysiological stability of species (Baydas, 2002; Forster, 2024). Secondly, GFAP serves as a marker of the functional state of glial cells, which play a key role in maintaining homeostasis and neuroprotection of the nervous system; studying changes in its level allows assessment of the extent of neurophysiological stress responses. Crustaceans of the order Decapoda are important model organisms in both neurobiology and ecotoxicology, and GFAP-like proteins in their nervous system demonstrate sensitivity to external influences, particularly mechanical and, presumably, thermal stress. Investigating such responses not only expands fundamental knowledge about invertebrate adaptive mechanisms but also has practical significance for evaluating the resilience of biological resources to stressors and developing strategies for their protection under climate change conditions (Sukhareenko, 2014; Zhu et al., 2018; Zhang et al., 2025).

## Material and methods

The experiment involved 24 mature *P. virginalis* individuals, which were divided into 4 groups (n = 6). The temperature regimes during the exposure period were as follows: Group 1, control – +20 °C (normothermic conditions, optimal temperature); Group 2, cold stress – +9 °C (low water temperature); Group 3, heat stress I – +26 °C (elevated water temperature); Group 4, heat stress II – +23 °C (moderately elevated temperature). The experiment lasted for 7 days. Upon completion, crayfish were dissected to isolate the supraesophageal ganglion. The ganglion tissues were immediately homogenized in a cold hypotonic buffer (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 2 mM DTT; 0.2 mM PMSF; 3 mM Na<sub>3</sub>N) at a ratio of 1:10 (weight / volume). Differential centrifugation was used to obtain subcellular fractions: initially centrifuged at 1,000 g (10 min, +4 °C) to remove the nuclear pellet; the supernatant was then centrifuged at 10,000 g (20 min) to obtain the S1 fraction (cytosol) and pellet P2, which was further extracted with 0.1% Triton X-100 and centrifuged again (15,000 g, 30 min) to yield the Triton-insoluble cytoskeletal fraction (designated S3). Total protein content in each fraction was determined by the Bradford method and expressed in mg/ml to control the uniformity of protein loading on the gel.

**Immunoblotting of GFAP.** Protein samples (100 µg protein per lane) were subjected to denaturing electrophoresis in a gradient 7–18% polyacrylamide gel (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS). The separated proteins were transferred onto a nitrocellulose membrane (0.45 µm pore size) by semi-dry blotting. The membrane was blocked in 5% nonfat dry milk (37 °C, 1.5 hours), followed by incubation with the primary rabbit antibody Anti-GFAP (Santa Cruz Biotechnology, USA, no. sc-9065, dilution 1:2000) at +4 °C overnight. After washing with phosphate-buffered saline containing 0.05% Tween-20 (PBST), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Goat Anti-Rabbit IgG, Abcam, USA, 1:5000) at 37 °C for 90 minutes. Immune complexes were detected using enhanced chemiluminescence (ECL), and the luminescence was recorded on X-ray film. For each sample, the intensity of the GFAP band with a molecular weight of 49 kDa was measured, as well as low-molecular-weight GFAP fragments (30–45 kDa, if present). Quantitative analysis was performed by densitometry of the scanned gel images using Image 2000 software (Bio-Techne Corp., USA). The obtained optical density values were normalized to the mean value of the control group (group 1), which was assigned a value of 1.0 arbitrary units (a.u.).

For the histological assessment of the nervous tissue in the studied crustaceans, transverse sections of the supraesophageal ganglia were prepared. Sectioning was performed using the automated microtome “PM100A” (Ukraine/China, 2022). The resulting tissue sections were stained with hematoxylin and eosin. The stained histological slides were examined under a MICROmed Fusion FS-7620 light microscope (MICROmed, China, 2024), and microphotographs were captured using a digital USB camera with a SIGETA M3CMOS 25000 microscope adapter (25.0 MP; manufacturer: SIGETA, Ukraine/China, 2023). Image acquisition was carried out using ToupView software, version 3.5 (ToupTek Photonics, China, 2023).

Histological analysis was descriptive and based on the qualitative evaluation of tissue structure without morphometric quantification. Special attention was paid to the general architecture of the ganglia, neuronal morphology, the condition of the neuropil, and the presence of cellular inclusions. The description of cellular structures was guided by published crustacean histology atlases (Shields & Boyd, 2014; Marenkov et al., 2018).

Manipulations with animals were conducted in compliance with bioethical principles according to the Regulations on the Ethics Committee (Bioethics) (2012), normative document of the Ministry of Education and Science, Youth and Sport of Ukraine, Order No. 1287 dated 19.11.2012.

To assess the significance of differences between groups, one-way analysis of variance (ANOVA) was applied. Differences were considered statistically significant at  $P < 0.05$ . Results are presented as mean ( $\bar{x}$ ) ± standard deviation (SD).

## Results

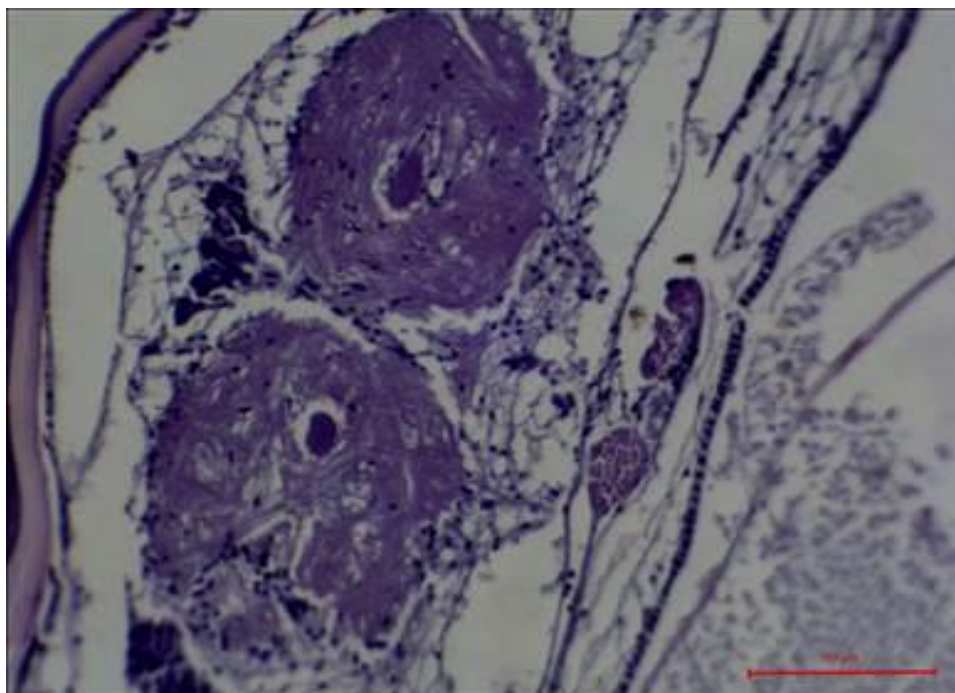
Based on the results of the histological examination of nervous tissue, no neuronal pathologies were detected in any of the experimental groups. The micrographs show a transverse section of the supraesophageal ganglion with a characteristic structure: large neurons with distinct nuclei and nucleoli are located at the periphery, while the central region contains the neuropil, where nerve fibers pass and synaptic zones are formed (Fig. 1). In all examined samples, the neurons exhibited strongly basophilic cytoplasm and clearly visible nucleoli, indicating high metabolic activity. Connective tissue surrounding the ganglion provides structural support and protection. Also visible are long parallel structures – axons – extending from the ganglion into the peripheral regions of the body.

The absence of pathological changes may indicate that the temperature ranges used in the experiment were not sufficiently extreme to significantly affect the structure of nervous tissue during the experimental period.

The total protein content in the cytoskeletal fraction of the nervous tissue of *P. virginalis* didn't undergo significant changes under different temperature conditions (Fig. 2). The results of the study indi-

cate a tendency to increase in the amount of total protein in the experimental groups compared to the control, but the data were not statisti-

cally significant since the content of total protein fluctuated in a wide range.



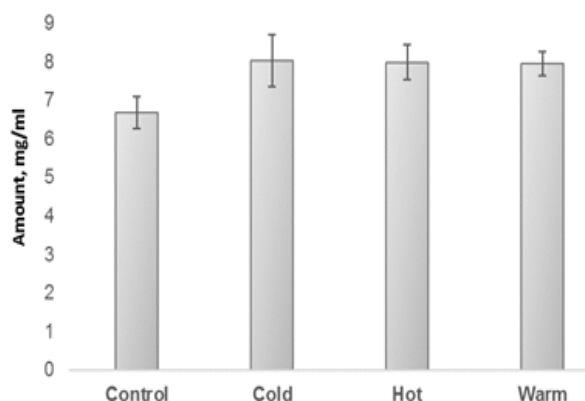
**Fig. 1.** Histological structure of the supraesophageal ganglion of the marbled crayfish (*Procambarus virginalis*) in the region of the antennal base: bar = 50  $\mu$ m

In the control group, the concentration of total protein was  $6.7 \pm 0.4$  mg/mL, while in the experimental groups it ranged from  $7.9 \pm 0.3$  to  $8.0 \pm 0.7$  mg/mL. Therefore, the effect of temperatures did not lead to significant changes in the level of total protein in the cytoskeletal fraction of the crayfish ganglion.

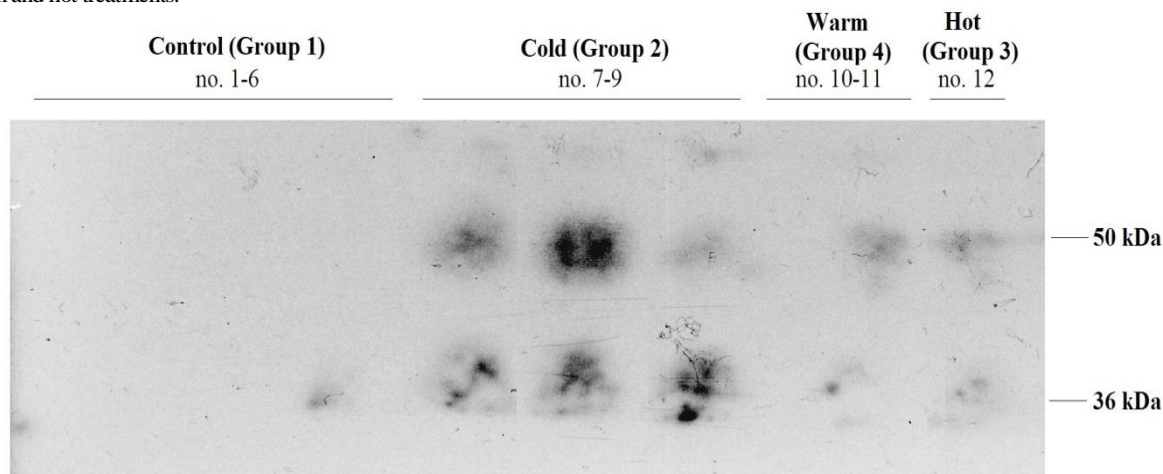
Figure 3 presents the results of western blot analysis of the content of the cytoskeletal fraction of GFAP in the nervous tissue of *P. virginalis* under different temperature conditions.

In the control group, weak expression of GFAP was detected, represented by faint signals. The highest level of expression was observed in group 2 (cold stress, +9  $^{\circ}$ C), where intense bands in the 49 kDa range were recorded, indicating a significant increase in GFAP synthesis in response to cold stress.

It is possible that the cold treatment induced a glial response known as reactive astrocytosis. Along with the upregulation of the intact GFAP subunit, large amounts of truncated GFAP polypeptides (~35 kDa) were detected in the cold-treated samples. Significant, but not as pronounced, changes in GFAP content were also induced by the warm and hot treatments.



**Fig. 2.** Total protein content in the cytoskeletal fraction of nervous tissue of *Procambarus virginalis*: control – +20  $^{\circ}$ C; cold stress – +9  $^{\circ}$ C; hot stress – +26  $^{\circ}$ C; warm stress – +23  $^{\circ}$ C



Fraction S3

**Fig. 3.** Western blot analysis of GFAP (S3) in the nervous tissue *Procambarus virginalis*

In the control group, the GFAP content was low: the intensity of the 49 kDa band barely exceeded the noise level ( $0.12 \pm 0.05$  a.u.). The effect of cold temperature (+9 °C) led to a sharp increase in GFAP expression in the ganglia of the crayfish: the relative intensity of GFAP increased more than threefold compared to the control ( $0.38 \pm 0.07$  a.u.;  $P < 0.05$ ). An increase in water temperature was also accompanied by an increase in the GFAP content in the nervous system of the crayfish. In particular, at +23 °C, the GFAP level increased approximately 1.5 times ( $0.18 \pm 0.06$  a.u.), and at +26 °C – 2 times ( $0.24 \pm 0.08$  a.u.) relative to the control. Although these changes were less pronounced than with cold exposure, the difference between groups 3–4 and the control was also statistically significant ( $P < 0.05$ ).

## Discussion

The nervous system of crustaceans (Decapoda) is composed of a series of segmental ganglia with a branched neuropil and is surrounded by glial cells. Environmental temperature changes lead to significant physiological alterations in these poikilothermic animals. Elevated temperatures induce heat stress, which activates the synthesis of heat shock proteins (HSPs) in both neurons and glia (Rochelle et al., 1991; Callinan et al., 2003). High temperatures cause characteristic morphological damage to the nervous system in decapod crustaceans, including membrane disruption, cytoplasmic vacuolization, neuropil degradation, and neuronal death, often accompanied by glial cell death or activation (Callinan et al., 2003). In contrast, moderate cooling merely slows neuronal function and helps preserve tissue architecture (neurons and ganglia remain morphologically intact). Glial responses generally occur only under conditions of significant damage (activation, GFAP alterations, phagocytosis) and are minimally affected by cold exposure (Zhu et al., 2018).

The results obtained indicate that temperature stress significantly affects the expression of glial fibrillary acidic protein in invertebrates. In particular, cold exposure causes a pronounced astroglial response – an increase in the level of GFAP in the ganglia of the marbled crayfish, which can be interpreted as reactive gliosis. Similarly, in higher animals, it is known that various stress factors activate astrocytes, increasing the synthesis of GFAP. Thus, in rats, an increase in body temperature to +38 °C induces hypertrophy and activation of astrocytes after a few hours, which is manifested by increased expression of GFAP in various parts of the brain (Sharma et al., 1992).

Toxic effects are also often accompanied by changes in GFAP: researchers have found that intoxication of rats with an organic solvent (toluene) causes reactive gliosis with increased GFAP levels, while administration of melatonin attenuates this astrocytic response (Baydas et al., 2003).

Even chronic exposure to heavy metals can affect GFAP metabolism. In particular, cadmium *in vitro* reduces the content of the soluble form of GFAP in astrocytes, which is interpreted as a sign of glial cell damage (this effect is prevented by the antioxidant curcumin) (Baydas et al., 2003). Thus, in vertebrates, GFAP is widely used as an indicator of neurotoxic effects and astrocytic stress.

Another important observation is the appearance of GFAP fragments at elevated temperatures. Fragmentation of this protein may be a consequence of protease activation during intense stress and leads to reorganization of the astrocytic cytoskeleton. Similar phenomena have been recorded in other models: in the brain of snakes under chronic environmental pollution, abnormally high levels of fragmented GFAP polypeptides (35–47 kDa) were observed, which was accompanied by an increase in the total content of GFAP in astrocytes. (Gasso et al., 2010).

The study authors interpreted this as a sign of oxidative stress and neurotoxicity, emphasizing that disruption of the glial cytoskeleton structure could serve as a reliable biomarker of the harmful effects of pollutants (Gasso et al., 2020).

In general, GFAP is today considered a promising biomarker of damage to nervous tissue in various pathologies and influences – from traumatic brain injury to the effects of neurotoxins. (Jung et al., 2007). Our results confirm that this marker can informatively reflect the state of the nervous system in invertebrates as well. In particular,

the presence of GFAP and its dynamics have been shown for other crustaceans: in the freshwater crayfish *Procambarus clarkii* (Girard, 1852), the level of GFAP in the ganglia exhibits circadian rhythms, changing throughout the day (Rodríguez-Muñoz & Escamilla-Chimal, 2015), which indicates that GFAP expression in glial cells can dynamically change in response to endogenous (circadian) factors or environmental signals (e.g., changes in lighting).

This indicates a functional role of the glial cytoskeleton in the nervous system of crustaceans, similar to that in mammals. The increase in GFAP we detected in *P. virginalis* under the influence of heat and cold stress can be considered as a manifestation of a universal protective reaction of glial cells to negative factors. Astrocyte-like glial cells of the marbled crayfish respond to stress similarly to vertebrate astrocytes, which emphasizes the conservativeness of cellular mechanisms of neuroresistance.

It is worth noting that cold stress was a stronger stimulus for glial response than moderate heating. This may be due to the fact that *P. virginalis* is a tropical-temperate species and is more tolerant to temperature increase (up to a certain limit) than to cooling. According to the literature, at temperatures below +6–8 °C, marbled crayfish significantly reduce their vital activity, and prolonged exposure to such conditions can lead to disruption of neurophysiological processes (Kaldre et al., 2015).

The data obtained on a sharp increase in GFAP at +9 °C are consistent with this, indicating the occurrence of neuroload (neuronal stress) in cold conditions. In contrast, a moderate increase in temperature (+23–26 °C) causes glial activation, but to a lesser extent, which may reflect the adaptive capabilities of crayfish to warm conditions close to optimal.

Therefore, GFAP synthesis may occur in an inducible manner, reflecting changes in the phenotype of ganglion astrocytes.

## Conclusions

The intensity of GFAP signals indicates the strongest response of the cytoskeletal system of nervous tissue to cold stress, while heat stress causes less pronounced changes in the nervous system of *P. virginalis*.

Increased GFAP expression and its fragmentation indicate the activation of glial (astrocyte-like) cells in the nervous system of marbled crayfish in response to extreme temperature exposure. This glial response is analogous to vertebrate reactive gliosis, highlighting the universality of neuroglial stress responses. The results obtained indicate that GFAP synthesis can occur in an inducible manner, reflecting changes in the phenotype of ganglionic astrocytes. The sharp astroglial response to suboptimal temperatures can be considered as a cellular response of the nervous tissue of *P. virginalis* to environmental stress factors and could potentially lead to local adaptation of the invasive species.

GFAP can serve as an informative biomarker of neurotoxicity and cellular stress in invertebrates. Monitoring GFAP levels in marbled crayfish is promising for assessing the impact of environmental factors on their nervous system and adaptive capabilities. Further studies are planned to determine whether the change in GFAP levels is accompanied by functional consequences for the *P. virginalis* nervous system (e.g., changes in behavioral responses, neurosecretion, etc.), and whether these changes are reversible upon return to optimal temperature.

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