

## Article

# Cellular Stress Responses of the Endemic Freshwater Fish Species *Alburnus vistonicus* Freyhof & Kottelat, 2007 in a Constantly Changing Environment

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**Abstract:** Herein we investigated the cellular responses of the endemic fish species *Alburnus vistonicus* Freyhof & Kottelat, 2007, under the variation of several physico-chemical parameters including temperature (°C), salinity (psu), dissolved oxygen (mg/L), pH and conductivity (µS/cm), which were measured in situ. Monthly fish samplings (October 2014–September 2015) were conducted in Vistonis Lake in northern Greece, a peculiar ecosystem with brackish waters in its southern part and high salinity fluctuations in its northern part. Fish gills and liver responses to the changes of the physico-chemical parameters were tested biochemically and histologically. Heat shock protein levels appeared to be correlated with salinity fluctuations, indicating the adaptation of *A. vistonicus* to the particular environment. The latter is also enhanced by increased Na<sup>+</sup>-K<sup>+</sup> ATPase levels, in response to salinity increase during summer. The highest mitogen activated protein kinases phosphorylation levels were observed along with the maximum mean salinity values. A variety of histological lesions were also detected in the majority of the gill samples, without however securing salinity as the sole stress factor. *A. vistonicus* cellular stress responses are versatile and shifting according to the examined tissue, biomarker and season, in order for this species to adapt to its shifting habitat.

**Keywords:** Vistonis Lake; physico-chemical parameters; gills; liver; HSPs; MARKs; Na<sup>+</sup>-K<sup>+</sup> ATPase; histology

## 1. Introduction

The ecological stability of inland and transitional waters is more vulnerable to exogenous pressures (e.g., climate change) compared to marine ecosystems, as the physico-chemical characteristics of freshwater environments can be highly variable by season and by water body [1]. Moreover, these aquatic ecosystems, and especially lakes, are subject to constant anthropogenic pressures, with the impact of degradation of their ecological value [2]. The majority of published reports focus on individual parameters as a single stress factor [1,3,4]. However, in order to assess the risk of extinction and to secure populations of endemic inland fish species, it has been proposed to study the combined effect of stressors (e.g., increase in temperature and pH change) on the regulation of gene expression and enzymatic activity [1,5].

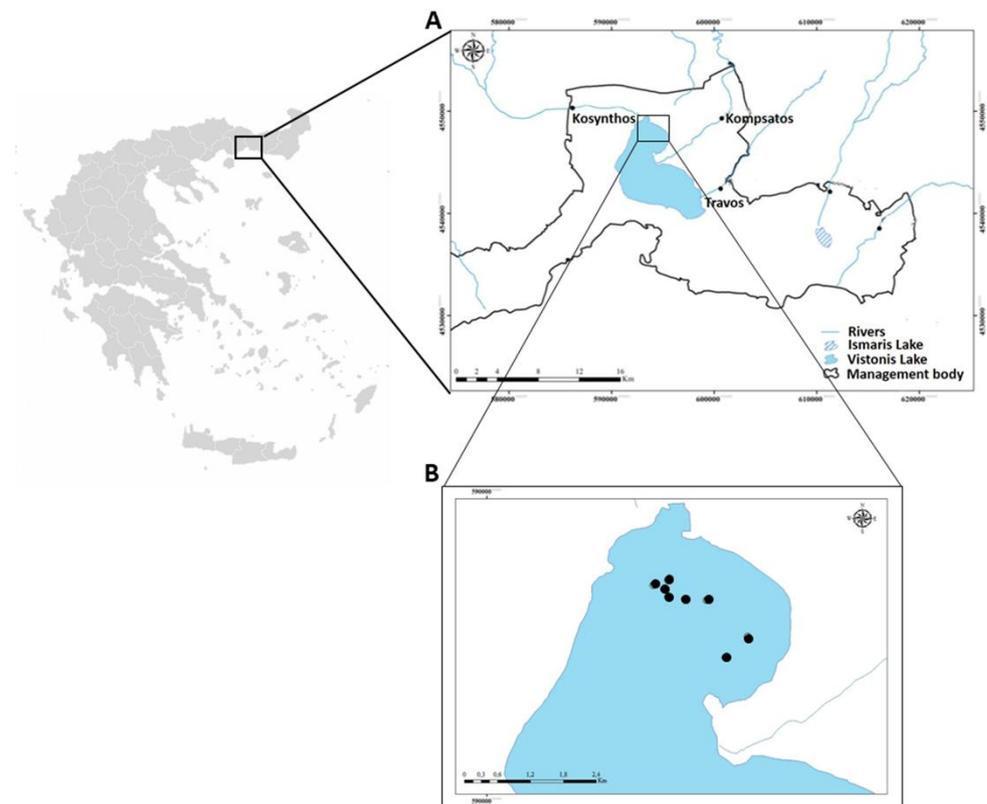
Fluctuations of the physico-chemical parameters may affect freshwater organisms on several levels of biological organization [6]. The fish responses towards environmental stressors are widely assessed in gills and liver due to the great sensitivity of these tissues in external stimuli [7]. Cellular Stress Response (CSR) represents a highly evolutionarily conserved physiological mechanism for handling cellular stress, due to changes in the environment. The response to the stress factor will determine the adaptation or not of the organism to the change [8]. CSR fundamental components might include the induction of macromolecules for protection and repair, as well as apoptosis, especially if cellular tolerance limits are exceeded. For instance, Heat shock proteins (HSPs) are widely used as a measure of cellular stress in fish [9,10], for a number abiotic factors [11], such as changes in salinity [12], pH and CO<sub>2</sub> [13], as well as environmental pollutants such as heavy metals [14], industrial waste [15] and pesticides [16]. Moreover, members of the Mitogen Activated Protein Kinases (MAPKs) superfamily are also related to various stressors, such as thermal stress, salinity fluctuations, as well as chemical agents [17–22]. It should be noted that the study of Na<sup>+</sup>-K<sup>+</sup> ATPase is of utmost importance in experiments with salinity as the main stress factor [23], since this protein is mostly located in osmoregulation tissues, such as fish gills [24–26].

The freshwater fish species *Alburnus vistonicus* Freyhof & Kottelat, 2007 commonly known as Vistonis shemaja, is found in the basins of Vistonis Lake, and in the basins of Ismaris Lake and Filiouris River [27,28]. The species is classified as “critically endangered” (CR) ([www.iucnredlist.org](http://www.iucnredlist.org), accessed on 1 January 2008) and it is protected by national and international law. Specifically, it is included in the Annex II of the Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora and it is also included in the Red Book of Threatened Species of Greece [29] and in the Berne Convention. Therefore, its conservation is of significant importance. Aspects of species biology and ecology, such as growth and reproductive biology, were studied recently [28,30,31]. Although the lacustrine environment is known to constantly change and affect inhabiting fish species ecology [32–35], the effect of Vistonis Lake’s continuously changing physico-chemical parameters on the biochemical and physiological responses of *A. vistonicus* remains unknown.

## 2. Materials and Methods

### 2.1. Study Area

Vistonis Lake (Figure 1) is a natural, hypereutrophic, shallow lake in northern Greece and is considered as a peculiar ecosystem. The lake’s northern part contains low salinity water, due to the freshwater inflows from the Kosynthos, Kompsatos and Travos Rivers. Meanwhile, its southern part receives seawater inflows from the North Aegean Sea, via a narrow artificial channel, the Porto Lagos Lagoon [36]. The distinctive hydrology of the system results in brackish waters in its southern part, while in the northern part the salinity levels are fluctuating, following the seasonal variations of the freshwater inflows from the rivers. This phenomenon is even more pronounced during the dry period, when the freshwater inflows from the rivers are minimized, resulting in increased salinity levels, even in the northern part of the lake [28,30,36].



**Figure 1.** Map of the study area (Vistonis Lake, Northern Greece) (A) and sampling stations indicated with black dots (B) (Reprinted with permission from ref. [30]. Copyright 2108, Copyright Owners Assoc. Professors Dimitra Bobori and Efthimia Antonopoulou and Dr. Emmanouil Tsakoumis).

## 2.2. Sample Collection—Ethical Approval

Fish samplings were performed on a monthly basis throughout a full calendar year (October 2014–September 2015). In January 2015, due to flooding events in the study area, the access to Vistonis Lake was not feasible and therefore no samples are available from this month. Fish samplings were performed in the northern part of the lake, where water exhibits lower salinity levels, using Nordic-type benthic multi-mesh gill-nets following the requirements set by EU [37,38] in accordance with the EU (Directive 63/2010) legislation for the protection of animals used for scientific purposes. All necessary permissions were provided by the Management Body of the Delta Nestos and Lakes Vistonida—Ismarida. During each sampling, five individuals were collected and were all used for both histopathological and biochemical analysis (45 individuals in total). On the sampling days, fish were removed from the water and were immediately anesthetized by immersion in water with an overdose of concentrated solution of buffered ethyl 3-aminobenzoate methanesulfonate (MS-222). Five minutes after cessation of respiration, fish were removed from water and were euthanized, according to the protocols of the Canadian Council of Animal Care (Euthanasia of finfish) and the American Veterinary Medical Association, aiming to minimize fish psychophysical stress. Dead fish were placed on ice and from each sampled individual, one pair of gills was immediately fixed in 10% buffered formalin and stored for histopathological analysis. For the biochemical analysis, and immediately after dissection, the other pair of gills and liver tissue were dissected and retained in dry ice, until their transfer to the laboratory, where the samples were stored at  $-80^{\circ}\text{C}$  until further analysis. Fish gills are, besides their respiratory function, one of the most important osmoregulatory organs, playing a critical role in ionic regulation [39]. The responses of fish gills under the variation of the physico-chemical parameters can be tested either histologically or on

biochemical level [40–42]. Moreover, the liver is also considered as an early stress indicator compared to other fish tissues, as shown by several researchers [21,43–45].

Parallel to fish sampling, water physico-chemical parameters such as temperature (°C), salinity (psu), dissolved oxygen (DO) (mg/L), pH and conductivity ( $\mu\text{S}/\text{cm}$ ) were also recorded at eight stations in the lake using a portable multi sensor (AquaRead, AP-2000) (Figure 1). Recording of the physico-chemical parameters was performed at each sampling station, both at the surface and above the lake's bottom ( $n = 16$  records per sampling).

### 2.3. Analytical Procedures

#### 2.3.1. SDS-PAGE and Immunoblot Analysis

Frozen gill and liver samples were homogenized in 3 mL/g of cold lysis buffer [20 mM  $\beta$ -glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM benzamidine, pH 7, 200 mM leupeptin, 10 mM transepoxy succinyl-L-leucylamido-(4 guanidino) butane, 5 mM dithiothreitol, 300 mM phenyl methyl sulfonyl fluoride (PMSF), 50  $\mu\text{g}/\text{mL}$  pepstatin, 1%  $v/v$  Triton X-100], and extracted on ice for 30 min. The samples were then centrifuged ( $10,000\times g$ , 10 min, 4 °C) and the supernatants were collected and boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris-HCl, 13%  $v/v$  glycerol, 133 mM DTT, 10%  $w/v$  SDS, 0.2%  $w/v$  bromophenol blue). Protein concentrations were determined using the BioRad protein assay (BioRad, Hercules, CA, USA). Thereafter, equivalent amounts of proteins (50  $\mu\text{g}$ ) were separated either on 10% and 0.275% ( $w/v$ ) acrylamide and bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45  $\mu\text{m}$ , Schleicher and Schuell, Keene, NH, USA). All nitrocellulose membranes were dyed with Ponceau stain in order to assure a good quality of transfer and equal protein loading. Non-specific binding sites on the membranes were blocked by 5% ( $w/v$ ) non-fat milk in TBST [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% ( $v/v$ ) Tween 20], during a 30–45 min incubation at room temperature. Subsequently, the membranes were further incubated overnight with primary antibodies, including polyclonal rabbit anti-HSP70 (Cat. No. 4872, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-HSP90 (Cat. No. 4874, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-Na, K-ATPase (Cat. No. 3010, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-p38MAP kinase (Cat. No. 9212, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-HSP60 (Cat. No. 12165, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-phospho-p38 MAPK (Cat. No. 4511, Cell Signaling, Beverly, MA, USA) and monoclonal rabbit anti-phospho-p44/42 MAPK (Cat. No. 4370, Cell Signaling, Beverly, MA, USA) and monoclonal rabbit anti-p44/42 MAPK (Cat. No. 4695, Cell Signaling, Beverly, MA, USA). The next day and after washing in TBST (3 time periods for 5 min each), the blots were incubated with horseradish peroxidase-linked secondary antibodies and washed again in TBST (3 time periods for 5 min each). The bands were then detected, using enhanced chemiluminescence (Chemicon, MA, USA) and were exposed to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, Media Cybernetics/Image Studio Lite Software Ver 5.2).

#### 2.3.2. Histopathological Analysis

Samples were fixed in 10% buffered formalin and dehydrated in graded series of ethanol and then immersion in xylol, and embedding in paraffin wax followed. Thin sections of 5–7  $\mu\text{m}$  were mounted, deparaffinized, rehydrated, stained with Hematoxylin-Eosin, mounted with Cristal/Mount and examined for alterations with a microscope (Bresser Science TRM 301) under total magnification of  $100\times$  and  $400\times$ . A digital camera adjusted to the microscope (Bresser MikroCam 5.0 MP) was used for acquiring histopathological photomicrographs.

### 2.4. Statistical Analysis

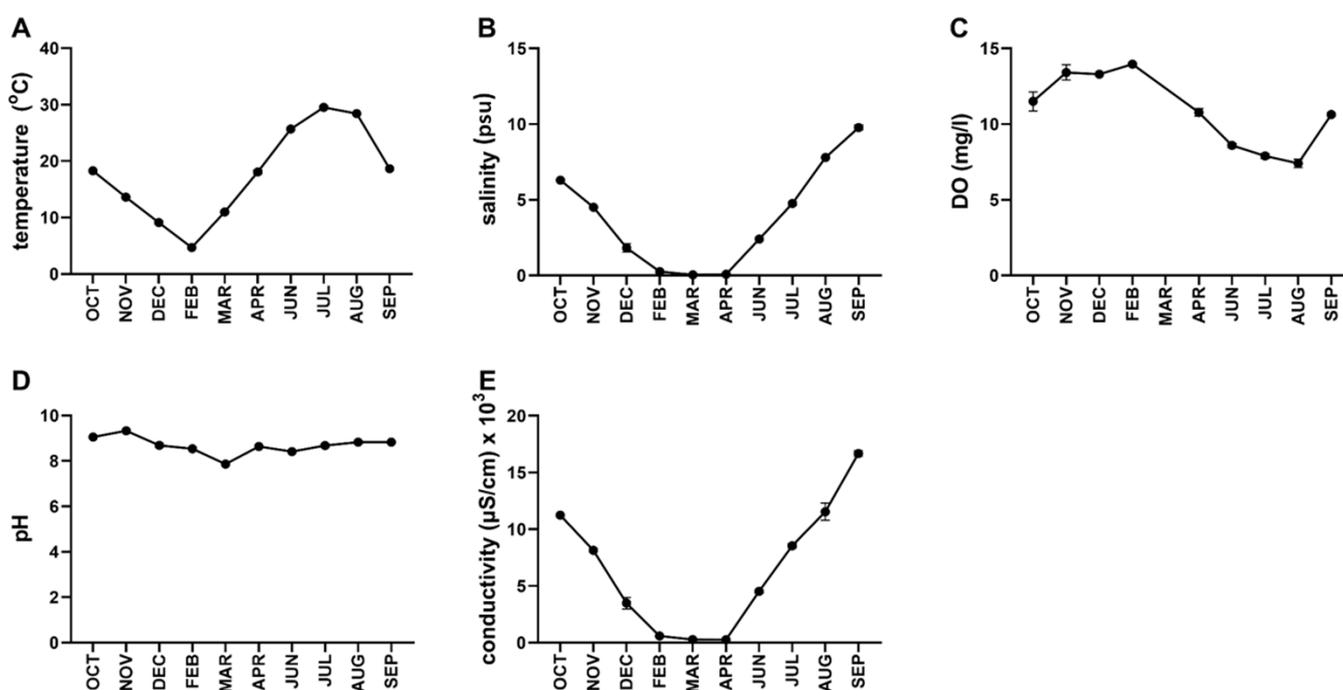
Data are expressed as mean  $\pm$  standard deviation (SD) of  $n = 5$  biological samples. Significant differences ( $p < 0.05$ ) between the monthly expression levels of the proteins

analyzed were conducted by one-way analysis of variance (ANOVA), using SPSS version 27 (SPSS, Inc., Chicago, IL, USA). Further elaboration of the potential physiological and environmental codependency was assessed by Principal Component Analysis (PCA), using the FactoMineR package in R [46]. Graphs were prepared using GraphPad Prism 9 (San Diego, CA, USA).

### 3. Results

#### 3.1. Water Parameters

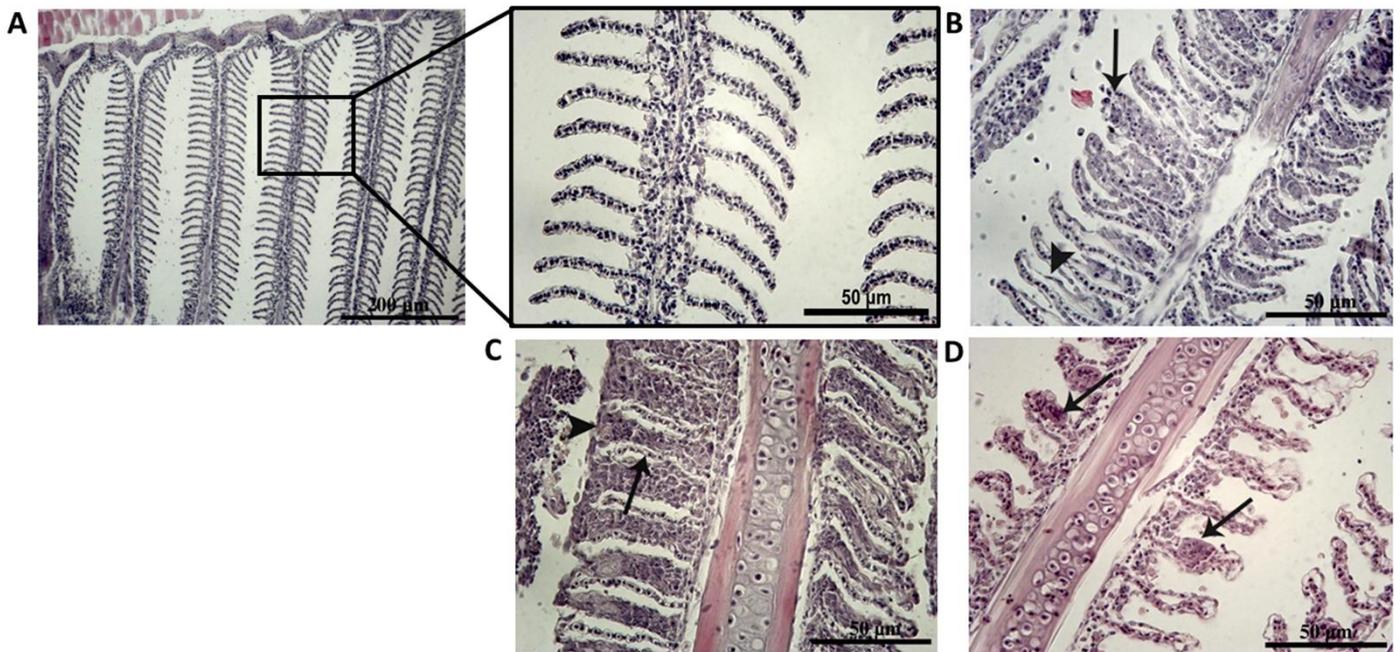
The variation of the levels of the water parameters recorded in Vistonis Lake throughout the sampling period are shown in Figure 2. Temperature levels exhibited seasonal fluctuation, with the lowest value (4.7 °C) recorded in February and the highest (29.5 °C) in July (Figure 2A). The lowest salinity values (0.044 psu) were recorded in March and the highest (9.77 psu) in September (Figure 2B). Dissolved oxygen (DO) values also showed seasonal variation, with the lowest concentration (7.4 mg/L) observed in August and the highest (13.96 mg/L) in February (Figure 2C). The pH levels were quite stable throughout the sampling period (Figure 2D). The variation of conductivity followed a similar pattern to that of salinity, since during the colder months low levels were recorded, in contrast to the warmer months, when the levels were higher (Figure 2E).



**Figure 2.** Mean values ( $\pm$ SD) of (A) temperature (°C), (B) salinity (psu), (C) dissolved oxygen (DO, mg/L), (D) pH and (E) conductivity ( $\mu$ S/cm) in Vistonis Lake (Northern Greece), during the sampling period (October 2014–September 2015). No sampling was conducted in January 2015.

#### 3.2. Gills Histopathology

Several histological lesions were found in the gill samples by the histopathological examination (Figure 3). Specifically, epithelium detachment at the secondary lamella (edema) (Figure 3B), hyperplasia of the primary lamella (Figure 3B,C), aneurysms in the secondary lamella (Figure 3D), hyperplasia of the edge of the secondary lamella, hypoplasia of the secondary lamella and hyperplasia of the secondary lamella (Figure 3C) were the histological lesions detected in the gills of the species (Table 1).



**Figure 3.** Histological lesions detected in the gills of the freshwater fish species *Alburnus vistonicus* in Vistonis Lake (Northern Greece), during the period October 2014–September 2015. (A) November sampling. Normal gills architecture. (B) April sampling. Hyperplasia of the primary lamella (arrow) and epithelium detachment (edema) of the secondary lamella (arrowhead). (C) April sampling. Hyperplasia of the primary lamella (arrowhead). Arrow shows a secondary lamella. (D) June sampling. Aneurysm of the secondary lamella (arrows).

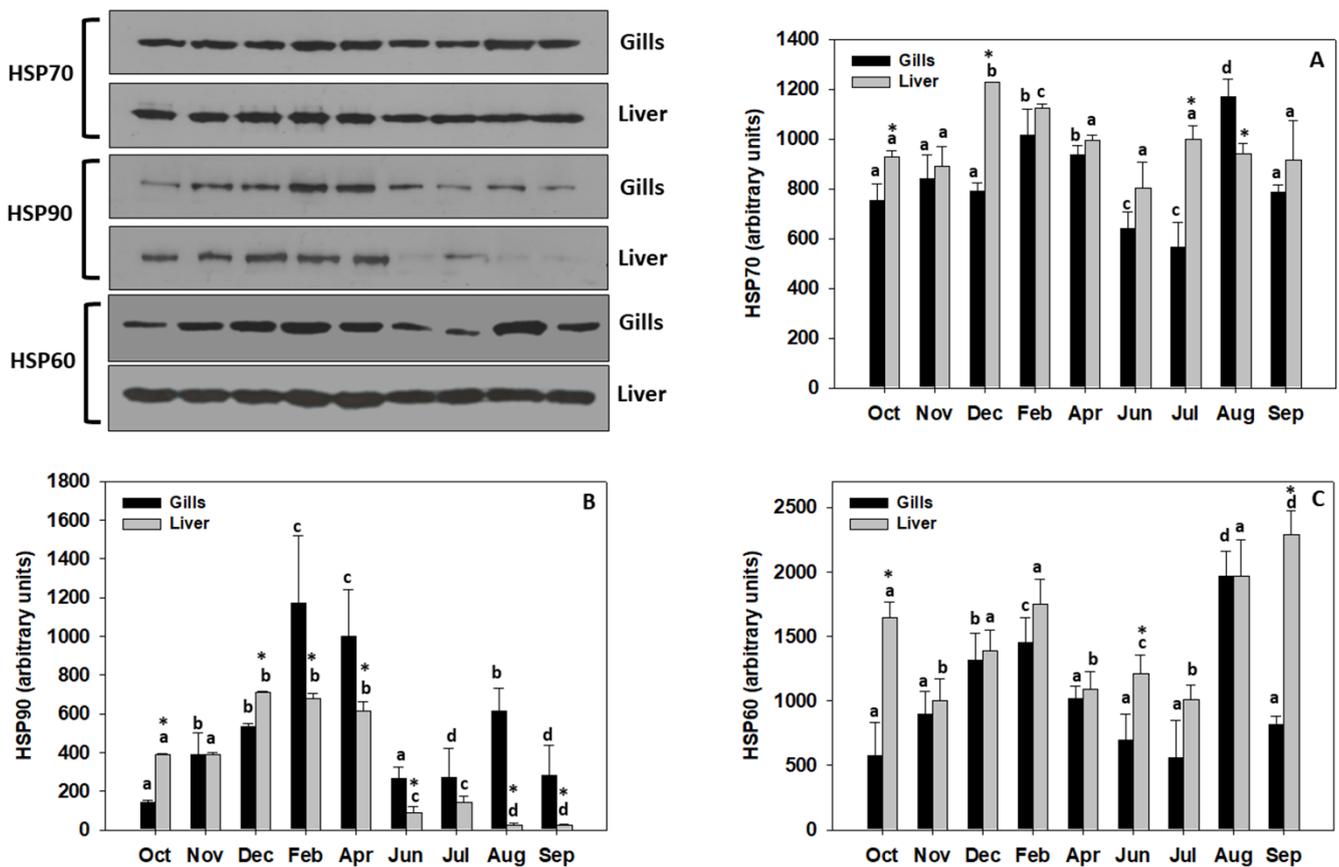
**Table 1.** Presence (+) and absence (–) of histological lesions detected in gill samples of the freshwater fish species *Alburnus vistonicus* in Vistonis Lake (Northern Greece), during the period October 2014–September 2015 (no samples were obtained in January 2015 due to unfavorable weather conditions).

Histological Lesion	OCT	NOV	DEC	FEB	MAR	APR	JUN	JUL	AUG	SEP
Epithelium detachment (edema) at the secondary lamella	+(3)	-	+(4)	+(2)	+(3)	+(3)	+(2)	-	+(2)	+(4)
Hyperplasia of the primary lamella	+(3)	-	+(3)	+(2)	+(4)	+(2)	+(2)	+(1)	+(1)	+(3)
Hyperemia (aneurysm) of the secondary lamella	+(2)	-	-	-	-	+(2)	+(1)	+(1)	-	-
Hyperplasia of the edge of the secondary lamella	-	-	-	-	-	-	-	-	-	-
Hypoplasia (small size) of the secondary lamella	-	-	-	-	-	-	-	+(1)	-	-
Hyperplasia of the secondary lamella	-	-	-	+(2)	+(3)	-	-	+(1)	-	-

The number of fish in which lesions are observed is mentioned in parentheses.

### 3.3. HSPs

HSPs expression levels in the gills and liver of *A. vistonicus* are shown in Figure 4. In the gills, HSP70 levels exhibited their lowest in July and their highest in August, when they were also significantly higher compared to all the rest samplings (Figure 4A). In the liver, HSP70 lowest expression levels were observed in June and the highest in December, when they were also significantly higher compared to the rest of the samplings, with the exception of those in February (Figure 4A).



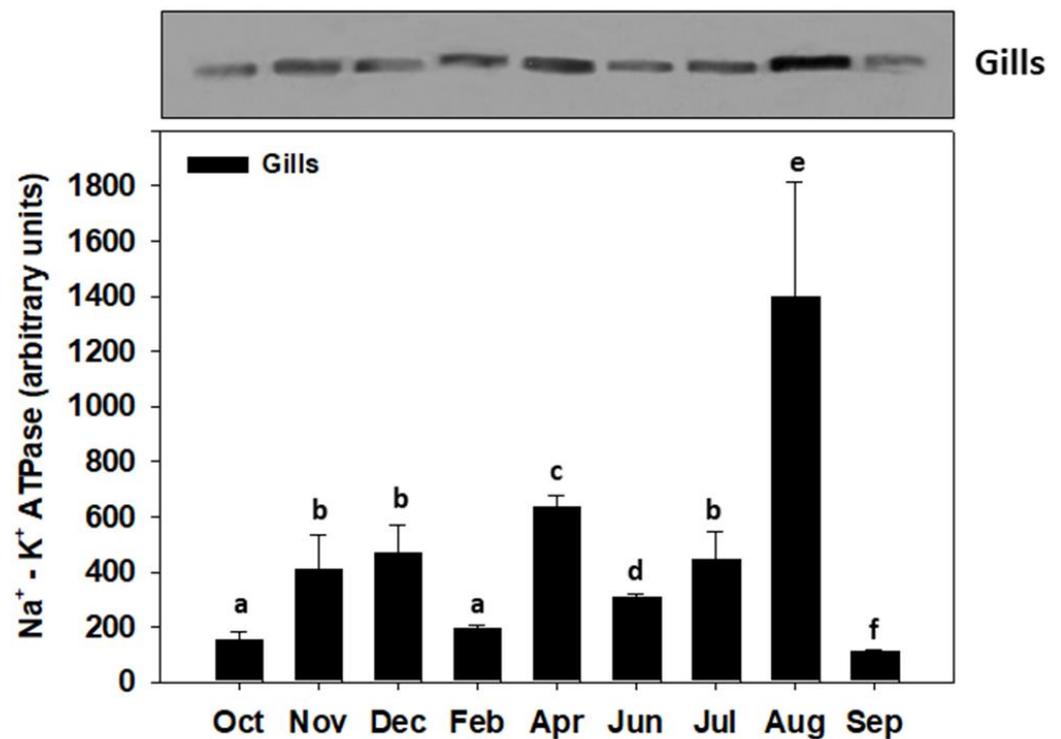
**Figure 4.** (A) HSP70, (B) HSP90 and (C) HSP60 levels (mean ± SD) in the gills and liver ( $n = 5$ ) of *Alburnus vistonicus* in Vistonis lake (Northern Greece), during the sampling period (October 2014–September 2015). Samplings were performed on a monthly basis. Representative blots are presented. Significant differences ( $p < 0.05$ ) are presented as: lower case letters—between sampling months and \*—between gills and liver. No sampling was conducted in January 2015.

HSP90 levels in the gills exhibited seasonality, with their minimum in October and maximum in February. The expression levels in February were significantly higher compared to those in the rest of the months (Figure 4B). In the liver, HSP90 minimum expression levels were observed in August, while the maximum in December. HSP90 levels exhibited seasonality, with higher values during the winter and autumn months and lower during the summer ones (Figure 4B).

HSP60 minimum expression levels in the gills were found in July and their maximum in August, when they were also significantly higher compared to the rest months (Figure 4C). In the liver, HSP60 levels exhibited their lowest in July. Protein expression levels were significantly lower in April, June, July and November compared to those in October, December, February, August and September. Moreover, HSP60 levels in September were significantly higher compared to those from the rest months, with the exception of August (Figure 4C).

### 3.4. $Na^+ - K^+$ ATPase

$Na^+ - K^+$  ATPase was examined only in gills as shown in Figure 5. The lowest levels were observed in September and the maximum in August, which was also significantly higher compared to those from the rest months (Figure 5).

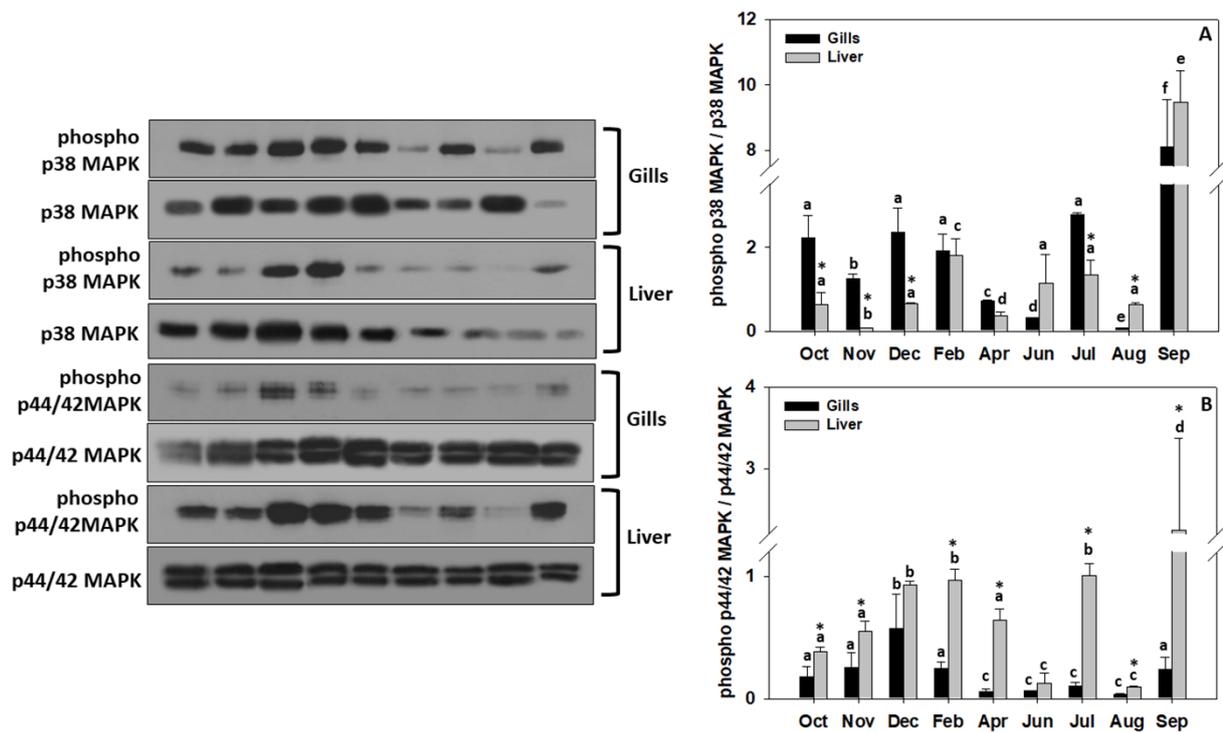


**Figure 5.** Na<sup>+</sup>-K<sup>+</sup> ATPase levels (mean ± SD) in the gills ( $n = 5$ ) of *Alburnus vistoncus* in Vistonis lake (Northern Greece), during the sampling period (October 2014–September 2015). Samplings were performed on a monthly basis. Representative blot is presented. Significant differences ( $p < 0.05$ ) between sampling months are presented as lower case letters. No sampling was conducted in January 2015.

### 3.5. MAPKs

Phospho p38 MAPK/p38 MAPK ratio exhibited its highest levels, for both tissues (gills and liver) in September, which were significantly higher compared to those of the rest months (Figure 6A). In the gills, significantly lower ratio levels were observed in August compared to the other sampling months, while the ratio levels were also significantly different in the samples from April, June and August to those from all the rest sampling months (Figure 6A). In the liver, significantly lower ratio levels were observed in November compared to the rest sampling months, while those in February were also significantly higher than those from October, November, December, April and August (Figure 6A).

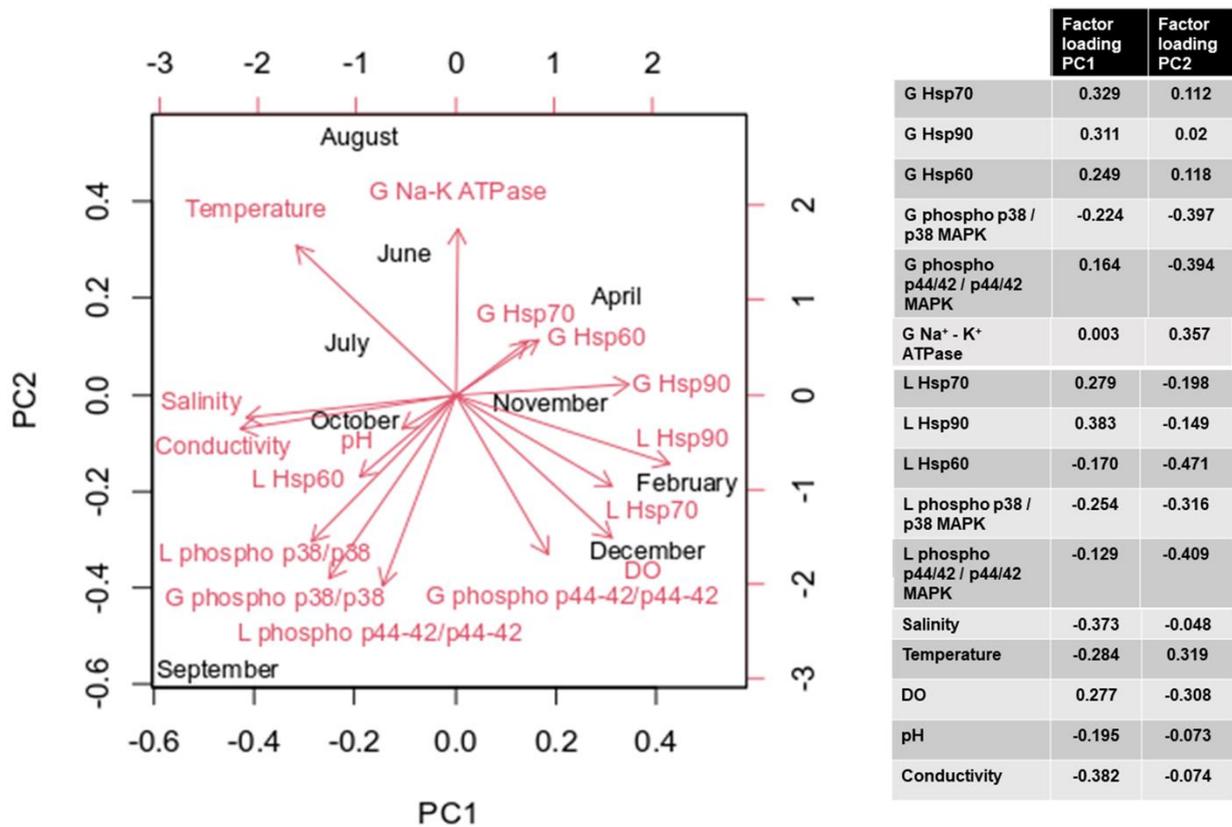
Phospho p44/42 MAPK/p44/42 MAPK ratio in the gills exhibited its minimum levels in August and maximum in December. Phosphorylation ratio levels in December were also significantly higher compared to those from the rest months. Phosphorylation ratio levels showed seasonality, with those in the dry season months (April–August) being significantly lower than those from the wet season months (October–February) (Figure 6B). In the liver, phosphorylation ratio levels exhibited mild fluctuations throughout the sampling period, with the exception of August and September, when the minimum and maximum ratio levels were found, respectively (Figure 6B).



**Figure 6.** Phosphorylation ratio levels (mean  $\pm$  SD) of (A) p38 MAPK (phospho p38 MAPK/p38 MAPK) and (B) p44/42 MAPK (phospho p44/42 MAPK/p44/42 MAPK) in the gills and liver ( $n = 5$ ) of *Alburnus vistonicus* in Vistonis lake (Northern Greece), during the sampling period (October 2014–September 2015). Samplings were performed on a monthly basis. Representative blots are presented. Significant differences ( $p < 0.05$ ) are presented as: lower case letters—between sampling months and \*—between gills and liver. No sampling was conducted in January 2015.

### 3.6. Multivariate Analysis

PCA applied on the bioindicators of cellular stress responses and the average values of the water physico-chemical parameters measured in Vistonis Lake extract two significant components (PC1 and PC2) that explained 61.32% of the total variance (35.21% for PC1 and 26.11% for PC2). HSP70 in the gills and HSP90 in the liver and gills were positively correlated to PC1 axis while salinity and conductivity were negatively related (Figure 7 and inserted table). Accordingly, HSP60 in the liver, phospho p38 MAPK/p38 MAPK and phospho p44/42 MAPK/p44/42 MAPK in both tissues and dissolved oxygen (DO) were negatively related to PC2 axis, while  $\text{Na}^+\text{-K}^+$  ATPase and temperature had positioned on the positive part of the axis (Figure 7 and inserted table).



**Figure 7.** Principal Component Analysis biplot of the CSR biomarkers (G = gills, L = liver) measured in *Alburnus vistonicus* ( $n = 45$ , 5 fish per month) and mean values of the physico-chemical parameters measured in Vistonis Lake (northern Greece) during the sampling period (October 2014–September 2015). In the inserted table the factor loadings of each parameter on the two principal components (PC1 and PC2) are presented.

#### 4. Discussion

The present study aimed to provide some insights into the physiological responses of the endemic freshwater fish species *A. vistonicus* in Vistonis Lake, in relation to the continuous fluctuation of the abiotic parameters in the lake.

Temperature values exhibited seasonal fluctuations, a phenomenon that is generally observed in aquatic freshwater ecosystems [47–49]. As expected from previous studies and due to the hydrology of the lake [50], salinity values followed a seasonal pattern, with low values recorded during the cold months December–February and higher during the hot months June–August. The periodic inflow of seawater in coastal lakes, such as Vistonis Lake, is a common, but alarming phenomenon, since even small increases in salinity might cause serious defects in the biodiversity of the habitats and threaten the survival of the species [51]. DO and pH values did not show any strong fluctuations and were ranging among the permitted limits for cyprinids [52]. pH values were constantly higher than 7, reflecting the alkaline character of the natural freshwater systems in Greece, and conductivity values were high, especially during the dry period, due to the seawater inflows in the lake [53].

The peculiar hydrology of Vistonis Lake system, leading often to salinization, especially during the dry season, has resulted in species population decline, threatening thus its survival [28]. Species with restricted distribution, such as *A. vistonicus*, may be especially prone to extinction than others with wider distribution range [54]. Therefore, the importance for acute implementation of management plans for the protection and preservation of the species has already been recommended [28,30]. Our results show that biomarkers of cellular stress responses could also be included in future management plans of the species, for achieving a better and more complete assessment of the biology of the species.

#### 4.1. HSPs' Induction

HSP60 and HSP70 expression levels, both in the gills and in the liver, were maintained at high levels throughout the year. During colder and warmer months, when salinity and temperature showed minimum and maximum values, respectively, both HSP60 and HSP70 in the gills exhibited their highest levels. This was also depicted in the two-dimensional plot of the results of PC analysis (Figure 7). Generally, HSP members in both gill and liver samples were positioned on the positive part of PC1, where the samples of the cold months are placed, having a negative correlation to salinity and conductivity. It has been demonstrated that several molecular chaperones are upregulated in fish gills when exposed to stress provoked by salinity variations [18,55,56]. Specifically, in black sea bream *Mylio macrocephalus* (Basilewsky, 1855), HSPs (HSP70, HSP90 and HSP60) were induced after acclimation to both plasma-hyperosmotic and plasma-hypoosmotic salinity [57]. According to Protas et al. [58], low conductivity appears to elicit responses in fish similar to heat stress response [59–61]. In fact, the above claim has been confirmed by Rohner et al. [62], who showed that low water conductivity values were accompanied by increased HSP90 expression in the fish species *Astyanax mexicanus* (De Filippi, 1853). These indications are very similar to the ones obtained in the present work, since increased levels of HSP members in the gills were mostly negatively correlated to conductivity, as shown by the PCA. We cannot support with certainty whether salinity is the single stress factor, as PCA has shown that, in gill samples, HSP levels are positively related to temperature, since they are positioned on the positive part of the PC2 axis. However, the opposite stands for HSP measured in liver samples that were placed on the negative part of PC2. (Figure 7). Because cold and heat stress destabilizes the hydrophobic interactions of polypeptide chains, leading to non-functional proteins, HSPs provide protection against protein structural changes [63–66]. Similar results of Heat Shock Response (HSR) as a means of fish acclimatization to cold [67–70] as well as to increasing ambient temperatures were reported by Feidantsis et al. [71], who investigated seasonality effects in the gilthead sea bream *Sparus aurata* (Linnaeus, 1758). Regarding HSP90, its expression levels exhibited seasonality in both gills and liver. Specifically, in the liver, its levels were consistently high during the colder and low in the warmer months. It has been argued that HSP induction in fish has a protective role against a number of environmental stressors [72,73]. However, according to Iwama et al. [8], HSPs increased levels in fish may be more of a stress indication rather than a measure of its degree. Differences observed between gills and liver HSR can be attributed to the well-known tissue specificity observed in fish species (e.g., [13,71]).

#### 4.2. MAPKs Phosphorylation

MAPKs involvement in HSP induction has been shown in various tissues of *S. aurata* [22,71]. In some published reports, MAPKs activation by various stressors has been suggested [17–19,74,75]. However, data on the seasonal pattern of their activation in fish are very limited (e.g., [71]). According to Kültz and Avila [18], MAPKs phosphorylation is related to the process of osmoregulation of large fish, especially in gill cells. The above observation seems to depict part of the results of the present study, since in September, along with the maximum mean salinity values, the highest MAPKs phosphorylation levels were observed. Specifically, p38 MAPK higher activation levels were observed, both for gills and liver in the same period. The latter coincides well with increased water conductivity levels and increased p38 MAPK phosphorylation in the gills in December and February. However, literature concerning the relation between MAPK pathway and water conductivity is extremely limited. In contrast, p44/42 MAPK, both in the liver and the gills, exhibit their higher phosphorylation levels in September, and are probably more a result of high summer temperatures and less of high salinity levels. Seasonal activation of the MAPK members was also observed in several examined tissues of *S. aurata* after exposure to increased environmental and laboratory temperature, as a response to heat stress [13,22,71]. However, on the PCA biplot a different pattern is evident. MAPKs measured in both liver and gill

samples were placed close to September samples when salinity values were the highest, having negative correlation with temperature.

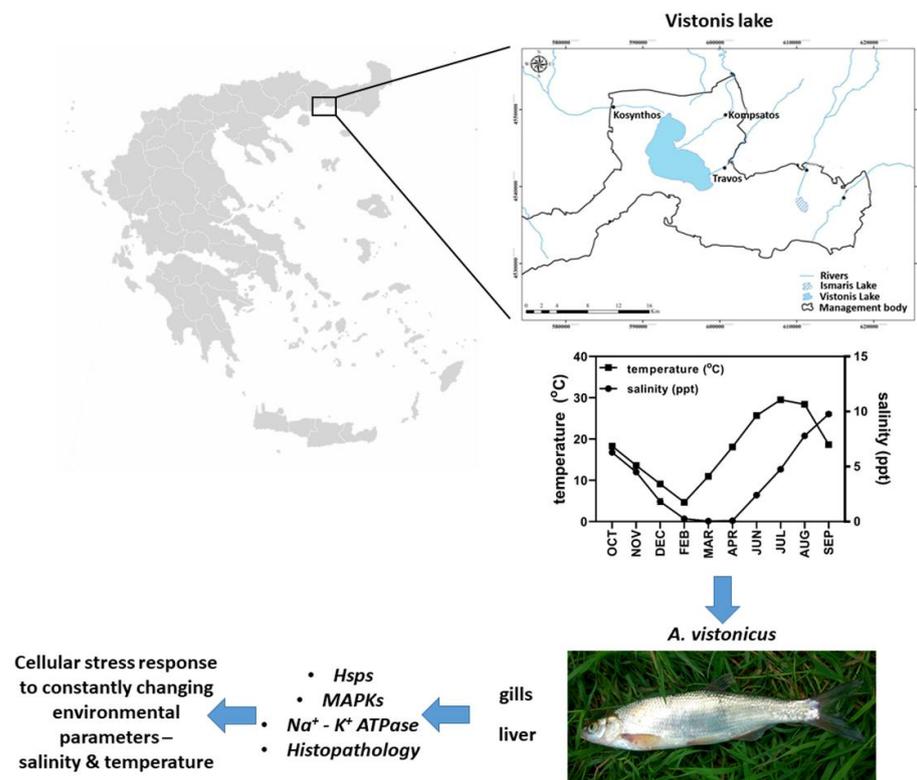
#### 4.3. Gills' Physiology

*A. vistonius* belongs to the Cyprinidae family [76]. Cyprinids show lack of secondary lamellae in the gills [77,78] under normoxic water ( $DO > 3$  mg/L) and temperatures below 20 °C. This strange structure makes them strikingly different from most other fish species. The specific gill structure (known as “hyperplasia of the primary lamella”) was more or less observed at all sampling months, except November (Table 1). The DO values during the sampling period were above 8 ml/L. So, the observed “hyperplasia of the primary lamella” may be a normal *A. vistonius* gill structure and not a gill lesion. During the sample period the salinity of the lake was very high ( $>3.5$  psu) for 5 months (October, November, July, August and September) and the pH was over 8.5 (except March). These conditions could be described as “stress conditions” for the fish and could explain the observed histological lesions to the gills.

According to Tine et al. [12], HSP70 high expression levels are accompanied by increased levels of  $Na^+ - K^+$  ATPase in fish, as a response to hyper-osmotic stress. Similarly, Herrera et al. [79] have also observed that  $Na^+ - K^+$  ATPase in the gills of Senegalese sole *Solea senegalensis* (Kaup, 1858) has been only increased when fish were transferred from seawater to 55 g/kg salinity, and not from seawater to low 5 g/kg salinity. These data indicate that active ion transport is only significantly increased when the proper mechanisms are already in place i.e., when ionic and osmotic gradients are not reversed. In fact, this observation could be a possible explanation for the survival of fish in habitats with extreme salinity values. In the case of Vistonis Lake, the results presented herein, exhibited a similar pattern regarding HSP70 expression levels and  $Na^+ - K^+$  ATPase in the gills, since both exhibited their maximum levels during increasing ambient water temperature, when mean salinity values increased (also confirmed by PCA analysis). This observation could be an indication of the adaptability of the fish to the particular lake ecosystem, with  $Na^+ - K^+$  ATPase responding to the increase in salinity, balancing the ion gradient between plasma and the extracellular environment with active  $Cl^-$  excretion [23,80,81].

#### 5. Conclusions

In conclusion, it seems that *A. vistonius* can still cope with the constantly changing environment of Vistonis Lake. The latter is concluded by the fact that *A. vistonius* CSR is versatile and varies according to the examined tissue, biomarker and season, in order for this species to adapt to its shifting habitat. Therefore, HSPs' induction,  $Na^+ - K^+$  ATPase activity and MAPKs activation seem to consist of a successful adaptive mechanism (Figure 8). However, additional studies on the species' physiological responses, including additional biomarkers, would contribute both to safer conclusions and to further understanding of the species' physiology. At the same time, the estimation of the range, as well as the lower and upper limits, of each environmental parameter, where critically endangered *A. vistonius* exhibits its optimal physiological capacity (e.g., Oxygen- and capacity-limited thermal tolerance hypothesis according to Pörtner et al. [82]) is of great importance. However, further research of the potential correlation between the changing physico-chemical parameters and the synergistic response of different proteins, along with expansion of histological studies, will contribute to understanding the physiology of the endangered species *A. vistonius*.



**Figure 8.** Summarized model of cellular stress responses of the freshwater fish species *Alburnus vistonicus* in Vistonis Lake (Northern Greece) under the seasonal variation of the physico-chemical parameters.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to local authorities’ privacy restrictions.

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