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RESEARCH ARTICLE

Seasonal cellular stress phenomena and phenotypic plasticity in land snail *Helix lucorum* populations from different altitudes

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ABSTRACT

Temperature, a major abiotic environmental factor, regulates various physiological functions in land snails and therefore determines their biogeographical distribution. Thus, species with different distributions may present different thermal tolerance limits. Additionally, the intense reactivation of snail metabolic rate upon arousal from hibernation or estivation may provoke stress. Land snails, *Helix lucorum*, display a wide altitudinal distribution resulting in populations being exposed to different seasonal temperature variations. The aim of the present study was to investigate the expression of heat shock proteins (Hsps), mitogen activated protein kinases (MAPKs) and proteins that are related to apoptosis (Bcl-2, ubiquitin), that have 'cytoprotective' roles and are also considered to be reliable indicators of stress because of their crucial role in maintaining cellular homeostasis. These proteins were assessed in *H. lucorum* individuals from two different populations, one at Axios (sea level, 0 m) and the other at Kokkinopilos (Olympus, 1250 m), as well as after mutual population exchanges, in order to find out whether the different responses of these stress-related proteins depend solely on the environmental temperature. The results showed seasonally altered levels in all studied proteins in the hepatopancreas and foot of snails, both among different populations and between the same populations exposed to varying altitudes. However, individuals of the same population in their native habitat or acclimatized to a different habitat showed a relatively similar pattern of expression, supporting the induction of the specific proteins according to the life history of each species.

KEY WORDS: Species distribution, Temperature variation, Tissue-specific response, Oxidative stress, Vertical distribution

INTRODUCTION

Ambient temperature affects the functions of all organisms through changes in the rates of physiological and biochemical processes, and influences species distribution (Somero, 2010; Pörtner, 2012). Temperature can also determine species habitat suitability and biogeographical distribution. The thermal tolerance of ectotherms is proportional to the magnitude of temperature variation in their habitat, which often increases with latitude or altitude (Huey and Stevenson, 1979; Kingsolver and Huey, 2008). The climate variability hypothesis suggests that thermal limits and tolerance differ greatly between ectotherms with different latitude or altitude distributions. Thus, species or populations evolved wider thermal tolerance breadth in environments with more variable temperatures, versus thermal specialization in thermally stable environments (Pallarés et al., 2019). Within species distributed over a wide latitudinal range, higher latitude individuals experience lower than optimal temperatures, whereas those at lower latitudes experience average temperatures closer to the upper thermal limit (Pörtner, 2002; Righton et al., 2010). A corollary of this hypothesis is that an organism's thermal limits are adapted to the climate extremes that they experience. However, many ectotherms display phenotypic plasticity to compensate for drastic changes in environmental conditions (Seebacher et al., 2015), thus avoiding stress phenomena and protein malfunction. Plastic responses occur on a short-term scale (reversible changes within an individual, i.e. phenotypic flexibility) or on a long-term scale (irreversible changes).

Several studies have shown significant metabolic differences and thermal tolerance limits in land snail species occupying different geographic habitats (Staikou, 1999; Köhler et al., 2009; Scheil et al., 2011; Mizrahi et al., 2012a,b; Gaitán-Espitia et al., 2013a,b; Staikou et al., 2016, 2017; Schweizer et al., 2019). In temperate regions, land snails undergo yearly activity and dormancy cycles (estivation and/or hibernation) in response to environmental stimuli such as high or low temperature, and humidity (Staikou et al., 1989; Giokas et al., 2005; Staikou and Koemtzopoulos, 2019). During hibernation, land snails face low and sometimes subzero temperatures. Metabolic depression and hypometabolism are common responses to temperature extremes, enabling land snails to survive under unfavorable environmental conditions such as cold and frost, or heat and drought (Storey and Storey, 1990, 2004, 2010; Guppy and Withers, 1999). Nevertheless, these physiological and behavioral responses can differ greatly between populations inhabiting different climatic environments (Schweizer et al., 2019).

One of the hallmark responses and measures of thermal sensitivity to changes in ambient temperature is heat shock protein (Hsp) induction (Hofmann, 2005; Gerber et al., 2016). Hsps stabilize and/or refold proteins against denaturing stresses (Tomanek, 2010) and, thus, they are ecologically and evolutionarily important in thermal adaptation, setting thermal tolerance limits and improving animal tolerance of thermal stress (Feder and Hofmann, 1999; Sørensen et al., 2003; Tomanek, 2010). Organisms occupying extreme environments can employ a 'preparative defense' strategy by maintaining high constitutive levels of Hsps as a protection mechanism against periods of extreme and/or unpredictable stress events, including temperature extremes or oxidative stress (Somero, 2020).

'Preparation for oxidative stress' (POS) (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998, 2015; Ramos-Vasconcelos and

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Hermes-Lima, 2003; Nowakowska et al., 2010, 2011) implies that land snails are prepared in a hormetic way to defend themselves against reactive oxygen species (ROS) production and oxidative stress during reoxygenation when they arouse from metabolic depression (estivation or hibernation) (Nowakowska et al., 2014; Oliveira et al., 2018). This further supports the interrelationship between Hsps and oxidative stress in several snail species under thermal stress (Mizrahi et al., 2010, 2012a,b; Scheil et al., 2011; Dieterich et al., 2013; Troschinski et al., 2014). Similarly, recovery from winter hibernation causes increased oxidative stress and Hsp expression in water frogs, thus stimulating an elevation of effective organ antioxidant defenses (Hermes-Lima and Storey, 1996; Hermes-Lima et al., 1998; Bagnyukova et al., 2003; Feidantsis et al., 2012a).

Land snail *Helix lucorum* Linnaeus 1758 populations have been found in Greece's northern part (Staikou et al., 1988) between sea level, 0 m, and up to 1300 m in continental mountainous habitats (Paiko mountain and Olympus mountain; A.S., K.F., M.H. and B.M., personal observations). This altitudinal difference results in particular climatic types. Sea-level populations cope with high temperatures and summers with severe drought, whereas mountain populations are confronted with temperatures below 0°C for long periods of the year, whereas high temperatures, favoring their growth and reproduction, last only from late spring to summer and early autumn. Several studies conducted in Mediterranean-type climates suggest that summer drought, in combination with high temperatures, shapes the activity cycles and metabolic responses of land snail populations (Michaelidis and Pardalidis, 1994; Michaelidis et al., 2008; Kotsakiozi et al., 2012). However, little is known regarding seasonal patterns of the above-mentioned stress phenomena and phenotypic plasticity in land snails from populations with different vertical distributions. The present study aimed to study seasonal cellular stress phenomena in two native populations of *H. lucorum* from different altitudes, one from the Axios area (sea level, 0 m) and the other from Kokkinopilos (Olympus Mountain, 1250 m). We analyzed Hsp70 and Hsp90 expression. As members of the mitogen activated protein kinase (MAPK) family are involved in Hsp expression (Sheikh-Hamad et al., 1998; Uehara et al., 1999; Rafiee et al., 2003; Feidantsis et al., 2012b) and are modulators of other cellular processes including gene expression related to oxidative stress (Oliveira et al., 2018), we also assessed p38 MAPK, JNK and p44/42 MAPK phosphorylation. Anti-apoptotic and ubiquitination responses were also investigated in an effort to relate the heat shock response (HSR) to a potential risk of apoptosis (Gerber et al., 2016; Hoyeck et al., 2019). Ubiquitination regulates protein degradation, apoptosis, autophagy and cell cycle progression (Orlowski, 1999; Bader and Steller, 2009). Moreover, groups of native populations were reciprocally transplanted to the other test site in order to examine how phenotypic plasticity in cellular stress responses is modulated in populations as a result of the altitudinal gradient. Although transplantation experiments have been conducted in marine organisms (e.g. Bams, 1976; Beaumont et al., 1993, Cochard and Devauchelle, 1993; Mackie and Ansell, 1993; Riveros et al., 2002), to our knowledge this is the first study regarding a land species.

MATERIALS AND METHODS

Chemicals

All biochemicals were purchased from Sigma (Darmstadt, Germany), Cell Signaling (Beverly, MA, USA) and Bio-Rad (Hercules, CA, USA). All other chemicals were obtained from Sigma, Merck (Darmstadt, Germany) and Applichem (Gatersleben, Germany) and were of analytical grade.

Animals and experimental procedures

Adult snails were collected in early October 2018 from two native populations of *H. lucorum*, living at different altitudes, one from the Axios (henceforth referred as 'coast') area (sea level, 0 m; 40.73923, 22.66075) and the other from Kokkinopilos (henceforth referred as 'mountain') (Olympus Mountain, 1250 m; 40.09548, 22.25245). Each population was separated into two groups. One group from each population remained in its original habitat, whereas the second group was reciprocally transplanted (Axios to Kokkinopilos and Kokkinopilos to Axios). Both native and reciprocally transplanted populations were maintained in wire cages, established at the natural habitat of the snail population, which contained plants usually consumed by snails; surplus food was always available. The cages were immersed in the soil and covered with wide plastic mesh (mesh size 2 cm) to prevent snails from escaping, and at the same time to maintain the surrounding environmental conditions inside the cages. However, a slight temperature increase inside the cages, although unlikely, cannot be completely excluded. The cages were separated into two compartments, one for the native population and the second for the transplanted snails. Each compartment of each cage measured 1.5×1×1 m^3 (length×width×height) and contained ∼100 individuals, a density similar to that usually observed in natural populations of the species and that would not cause adult mortality (Staikou and Lazaridou-Dimitriadou, 1989) (Fig. 1). Snails were both naturally and additionally fed with commercial vegetables and their activity was recorded at regular intervals throughout the year. Transplanted groups were acclimatized for 15–20 days in their new habitat (the coast group to mountain conditions, and the mountain group to the coast conditions) before the beginning of sampling. A total of seven samples were taken throughout one year, starting from mid-November 2018 and ending in mid-October 2019. At each sampling occasion, 8–10 individuals were removed from each of the four groups. Samples from hepatopancreas and foot muscle were quickly removed, immediately frozen in liquid nitrogen and then transferred at −80°C to the laboratory for biochemical analysis. Meteorological data for both regions were obtained from the Department of Meteorology and Climatology (sea level, 0 m), School of Geology, Aristotle University of Thessaloniki.

Analytical procedures

SDS-PAGE and immunoblot analysis

The preparation of tissue samples for SDS-PAGE and immunoblot analysis are based on well-established protocols. Specifically, in the present study, equivalent amounts of protein (50 µg) were separated on slab gels containing 10% (w/v) acrylamide and 0.275% (w/v) bisacrylamide or 15% (w/v) acrylamide and 0.33% (w/v) bisacrylamide, respectively (depending on the molecular weight of the proteins to be detected) and subsequently transferred electrophoretically onto nitrocellulose membranes (0.45 µm; Schleicher and Schuell, Keene, NH, USA). All nitrocellulose membranes were dyed with Ponceau stain in order to ensure good transfer quality and equal protein loading. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies: monoclonal mouse anti-heat shock protein, 70 kDa (cat. no. H5147, Sigma); monoclonal mouse anti-heat shock protein, 90 kDa (cat. no. H1775, Sigma); monoclonal rabbit anti-phospho p44/42 MAPK (Thr202/Tyr204) (cat. no. 4376, Cell Signaling Technology); polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180-Tyr182) (cat. no. 9211, Cell Signaling Technology); monoclonal mouse anti-phospho-SAPK-JNK (Thr183-Tyr185) (Cell Signaling Technology); and polyclonal rabbit anti-bcl2 (cat.

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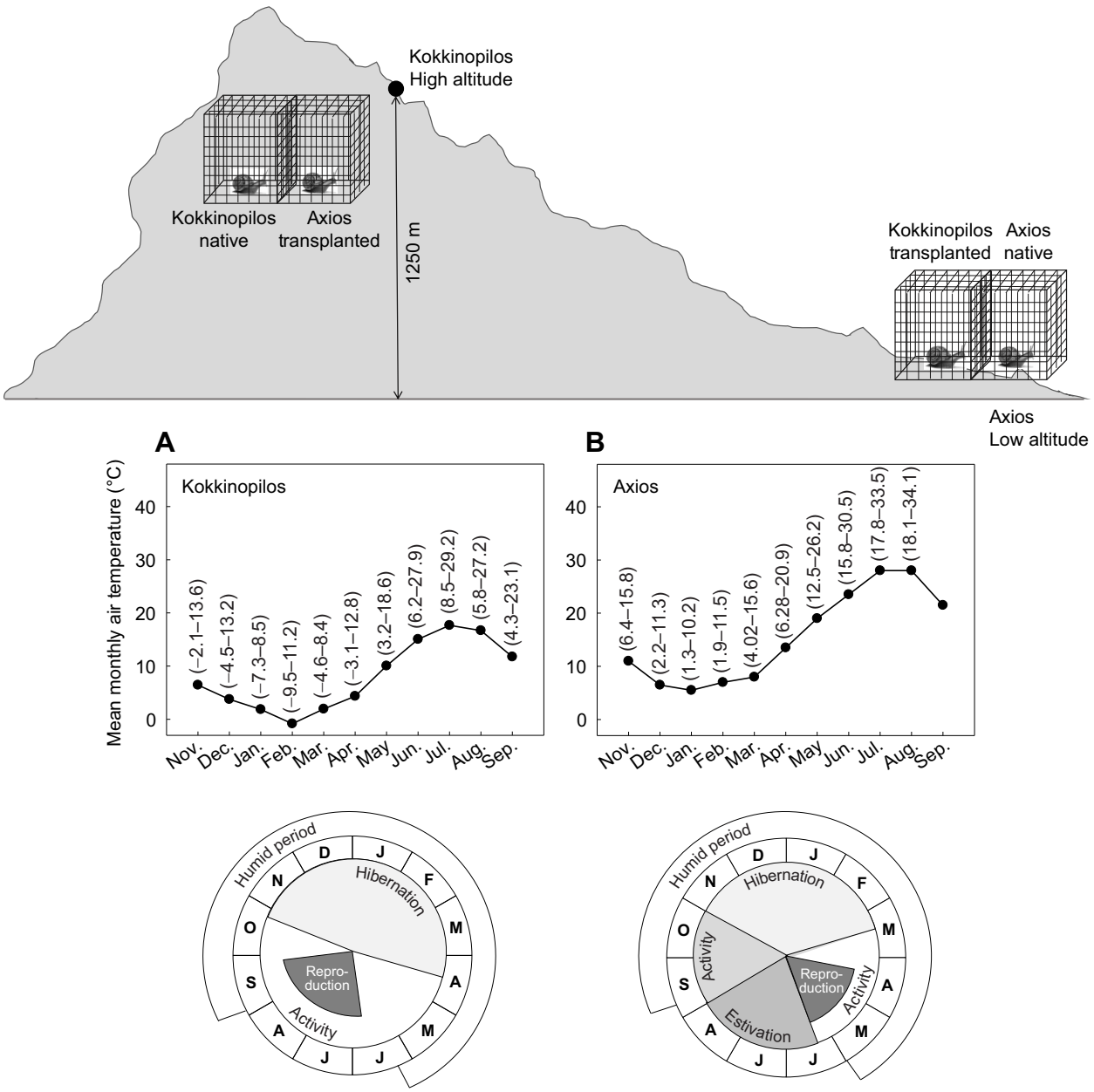

Fig. 1. *Helix lucorum* study populations. Top: two native and two reciprocally transplanted populations of *H. lucorum* were settled at Axios (0 m, coast) and | Kokkinopilos (1250 m, mountain) in cages for a year. Bottom: mean monthly ambient temperature (with the mean monthly temperature range in parentheses) and snail activity recorded monthly at (A) Kokkinopilos and (B) Axios.

no. 7973, Abcam, Cambridge, MA, USA). Antibodies were diluted as recommended by the manufacturer's guidelines. After washing in TBST (3 times, 5 min each), the blots were incubated with horseradish peroxidase-linked secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody, cat. no. 7074, and anti-mouse IgG, HRP-linked Antibody, cat. no. 7076, Cell Signaling Technology), washed again in TBST (3 times, 5 min each), and the bands detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, GraphPad).

Quantitative immunochemical assay for ubiquitin conjugates

Quantification of ubiquitinated proteins was assessed following a solid-phase immunochemical assay as described by Feidantsis et al. (2015). The antibody used was polyclonal anti-ubiquitin rabbit antibody (cat. no. 3936, Cell Signaling Technology). After washing in TBST (3 times, 5 min each), the blots were incubated with horseradish peroxidase-linked secondary antibody (Anti-mouse IgG, HRP-linked Antibody, cat. no. 7076, Cell Signaling Technology), washed again in TBST (3 times, 5 min each), and the bands detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, GraphPad).

Morphometric characteristics

At each sampling occasion, 8–10 individuals were removed from each of the four groups, and from each individual, measurements of diameter (D), height (H), shell and body weight [W (S+B)] and

body weight [W(B)] were recorded (Table S1). Additionally, snail behavior was visually monitored and recorded.

Statistics

General linear model ANOVA (GLM) (independent variables: population of origin, experimental site and time) and one-way ANOVA were performed to detect significant differences at 5% (P<0.05) probability level (SPSS Scientific Inc. Software, version 21). GLM analysis was annually and also semiannually (periods November–February, and March–October) performed in order to examine in detail the effect of season.

Principal component analysis (PCA) in the FactoMineR package in R was employed to assess patterns of possibly correlated variables, and more specifically to detect how cellular stress responses varied between temperatures, samplings and populations.

RESULTS

Air temperature, behavioral characteristics and morphometric parameters of the study populations

Seasonal mean monthly air temperature variations are shown in Fig. 1. A gradual decrease was observed from November to February, when the lowest mean temperature (1°C) was recorded in the mountain (Fig. 1A). Air temperature increased gradually after March and reached the highest mean value in mid-July (17.6°C). At the coast (Fig. 1B), temperature decreased from November to January (5.2°C). Thereafter, temperature rose again in March until the highest mean temperatures were recorded between the end of July and early August (28.2°C).

According to our field observations, native *H. lucorum* snails in the mountain started burying into the soil and forming a thick epiphragm in the middle of October and began to arouse in mid-April (Fig. 1A). For coast individuals moved to mountain, an epiphragm was observed a bit later, at the beginning of November, and they also started to bury into the soil for hibernation. These individuals also showed arousal from hibernation after March. Native mountain snails were active throughout the rest of the year and reproduced in the middle of summer to the beginning of autumn (Fig. 1A). In contrast, native coast snails reproduced just after exit from hibernation in spring and entered estivation in the summer (Fig. 1B).

Seasonal HSR

Hsp70 levels in *H. lucorum* hepatopancreas followed a similar pattern to that of temperature and were higher in the coast population compared with corresponding levels in the mountain population (Fig. 2A,B). The pattern of Hsp70 levels in the coast population transplanted to high altitude was not similar to that exhibited by the coast population (Fig. 2C). When individuals from the mountain were transplanted to the coast, Hsp70 levels were found to be higher than the corresponding ones in the native population, and a gradual increase was observed by mid-February, remaining through March (Fig. 2D).

In contrast to findings for individuals from the mountain population, Hsp70 levels, after an initial decrease from November to December, remained stable in the foot throughout the year despite temperature fluctuations (Fig. 2G,H). When the coast population was transplanted to the mountain, it exhibited fairly similar changes in Hsp70 levels to those of the coast population. However, the levels of the transplanted population were higher than those of the native one except for July and October, when they dropped significantly below coast levels (Fig. 2I). Different patterns from those of the native population were also exhibited when individuals from the mountain were transplanted to the coast (Fig. 2J). After arousal early in March, Hsp70 levels recovered to those measured in November in the native coast population (Fig. 2H). In contrast, in the native mountain population, the corresponding levels increased beyond the levels determined in November, early in July (Fig. 2H).

In contrast to Hsp70, differences determined in hepatopancreas Hsp90 levels between the two native populations were smaller and fewer (Fig. 3A,B). Until February, the two populations showed a different pattern of changes, with Hsp90 levels in the coast population increasing and those in the mountain one decreasing, but rose in both populations later in the year. Fig. 3C shows that Hsp90 levels were constant for both groups from November to February. Thereafter, an increase in Hsp90 levels was observed in the coast snails that were transplanted to the mountain that may be due to winter cold stress but levels declined again with the arrival of spring. The coast group showed elevated Hsp90 levels in July, which remained high through October, whereas the coast snails transplanted to the mountain did not. In March, Hsp90 levels of the transplanted population were higher compared with those of the native population (Fig. 3C). In mountain, and mountain to coast transplanted populations, Hsp90 levels followed temperature changes. However, in October, the native population exhibited significantly higher levels compared with the transplanted one (Fig. 3D).

In the foot of the coast population, Hsp90 levels increased with environmental temperature increases, while in the mountain population, they sharply increased with the initial temperature rise (Fig. 3G,H). Overall, Hsp90 levels in the foot were significantly higher for the coast population. For coast to mountain transplanted snails, the initial decrease in environmental temperature resulted in a strong initial increase in Hsp90 compared with the coast population (Fig. 3I). A similar pattern was observed for mountain, and mountain to coast transplanted populations (Fig. 3J). The initial movement to the warmer environment led to a much higher Hsp90 content in the mountain group that was transplanted to the coast in November/December but in the warmer seasons, Hsp90 levels in the two populations were fairly similar to each other (Fig. 3J). This is also supported by the reciprocal transplantation experiments showing that coast individuals did not upregulate Hsp90 during summer when transplanted to high altitude (Fig. 3I).

Seasonal MAPK activation

In the hepatopancreas of the coast population, phospho-p38 MAPK content declined in February and increased in the spring months. Thereafter, it exhibited a declining trend from May through to October. By contrast, the mountain population showed the highest phospho-p38 MAPK content in both December and May, and in general levels were significantly higher compared with the coast population (Fig. 4A,B). p38 MAPK phosphorylation in the coast population transplanted to the mountain was significantly higher in November and October, but lower in March to May, compared with the coast population (Fig. 4C,F). For the mountain population transplanted to low altitude, p38 MAPK activation levels were generally lower, compared with those of the mountain population (Fig. 4D,E).

In the foot of both native populations, after a significant increase in December, p38 MAPK phosphorylation was maintained at relatively low levels in the cold months but thereafter peaked in the warmer months, coinciding with snail arousal from hibernation and activity. Overall, the mountain population exhibited higher phosphorylated levels compared with the coast population, although the opposite was observed during October (Fig. 4G,H). The coast

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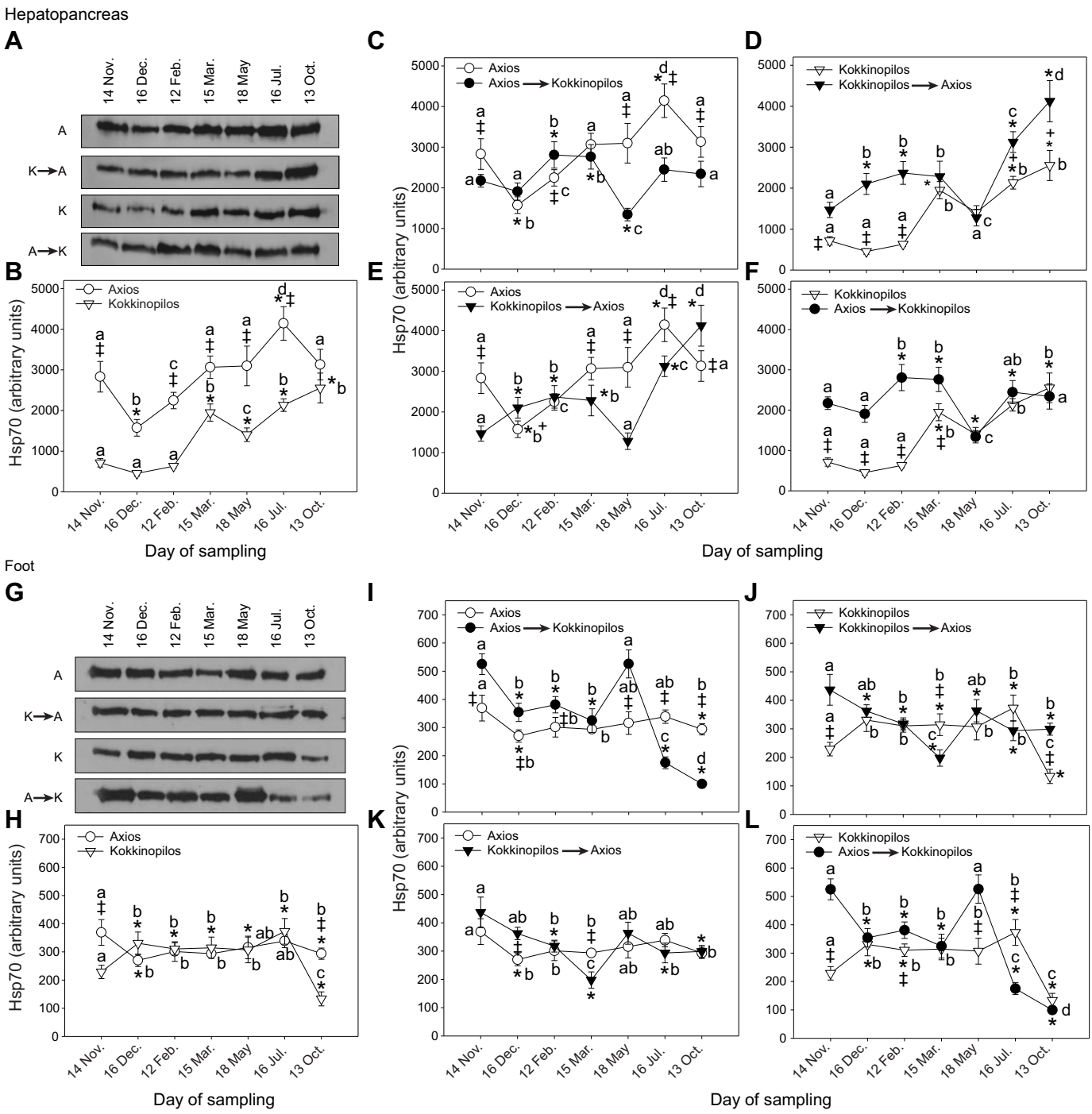

Fig. 2. Seasonal variation in Hsp70 levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of Hsp70 in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

population transplanted to the mountain exhibited higher levels of phospho-p38 MAPK compared with native one. After an initial decrease during the subsequent 2 months, levels increased again in March, remaining high by May. In contrast to the native population, however, p38 MAPK phosphorylation levels declined during seasonal warming (Fig. 4I,L). In contrast, the mountain population transplanted to the coast exhibited higher p38 MAPK phosphorylation from November to February, and after a sharp decrease in March remained at relatively high levels during seasonal warming (Fig. 4J,K).

p44/42 MAPK phosphorylation in the hepatopancreas of the native populations initially declined in December/February for coast snails but increased in mountain snails in December (Fig. 5A,B). Levels in both populations were low in February (hibernation period), peaked

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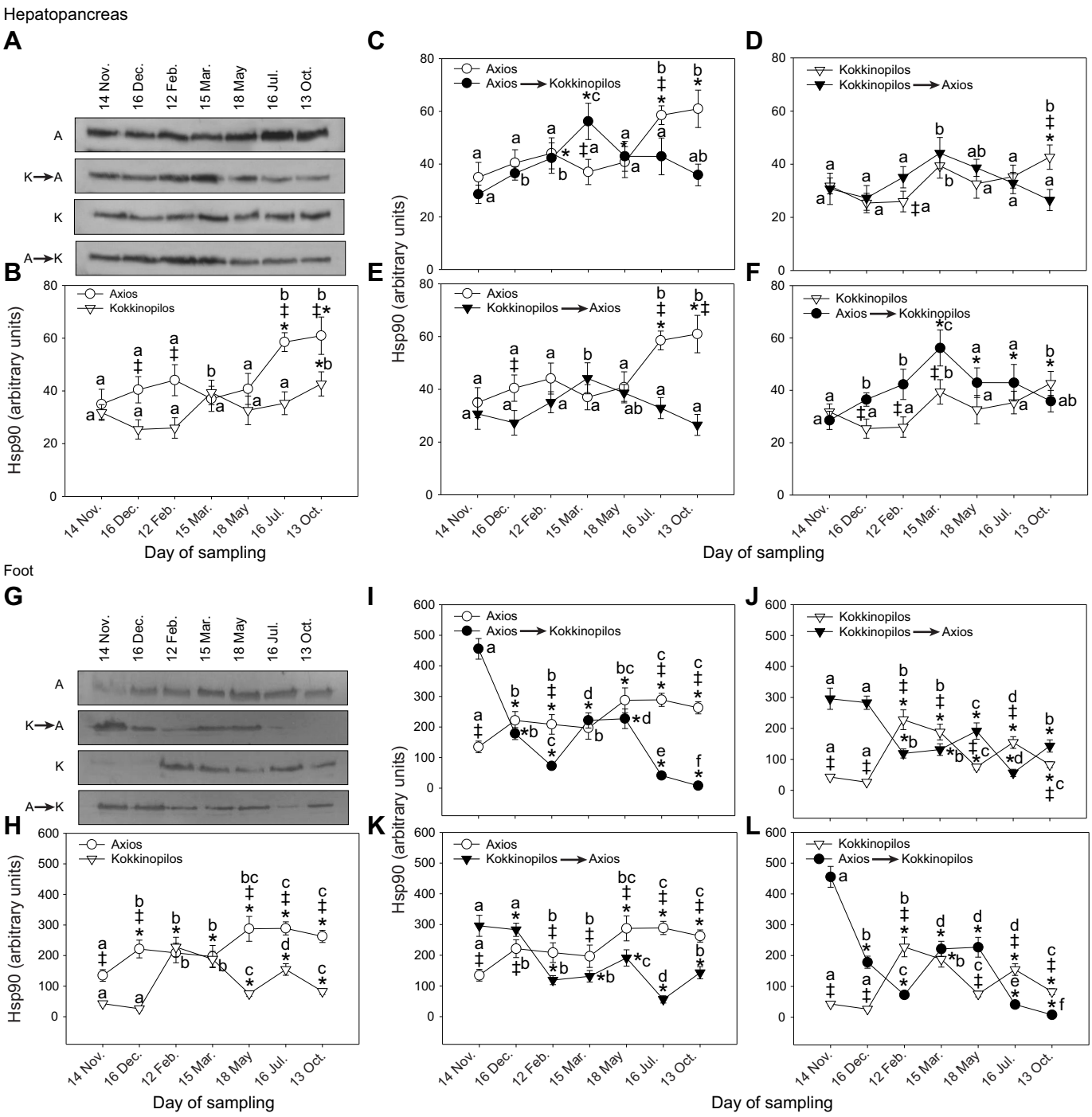

Fig. 3. Seasonal variation in Hsp90 levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of Hsp90 in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

in March (arousal and activation), and decreased through to July (estivation). p44/42 MAPK phosphorylation levels in May and July were significantly higher in the coast population compared with that of the mountain population. For the coast native population transplanted to the mountain, the temperature decrease during winter provoked a reduction in p44/42 MAPK phosphorylation until February and then an increase in March (Fig. 5C,F). p44/42 MAPK phosphorylation in the mountain population transplanted to the coast seemed to follow the same pattern of changes as those observed for the native coast population (Fig. 5D,E).

In the foot of both native populations, p44/42 MAPK phosphorylation was maintained at low levels during the cold months. Thereafter, during snail arousal, phosphorylation levels increased significantly, with the coast population exhibiting

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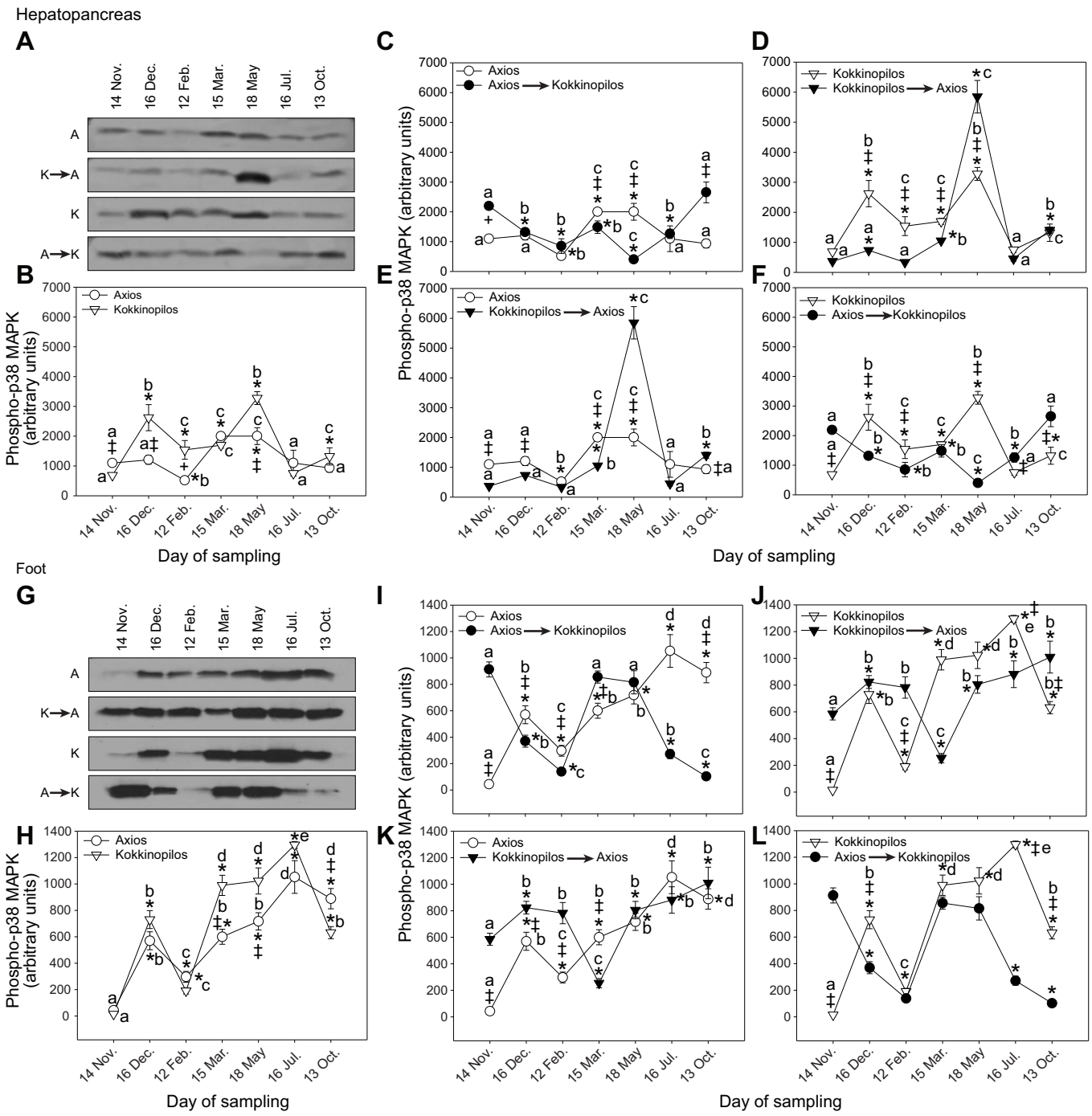

Fig. 4. Seasonal variation in p38 MAPK phosphorylation levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of phosphorylated p38 MAPK in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

comparatively higher levels of phosphorylation, which peaked in May (Fig. 5G,H). When the native coast population was transplanted to the mountain, it maintained levels of p44/42 MAPK phosphorylation similar to those determined for the native mountain population, and same pattern of seasonal changes (Fig. 5I, L). In contrast, a different pattern was observed for the mountain population compared with the mountain population transplanted to the coast, with the latter exhibiting higher levels in December and May (Fig. 5J,K).

JNK phosphorylation in the hepatopancreas of the coast population increased significantly only in March, and gradually declined after May. However, in the mountain population, highest levels were observed in December (hibernation), and in March (arousal and activity) (Fig. 6A,B). In general, JNK phosphorylation was higher in

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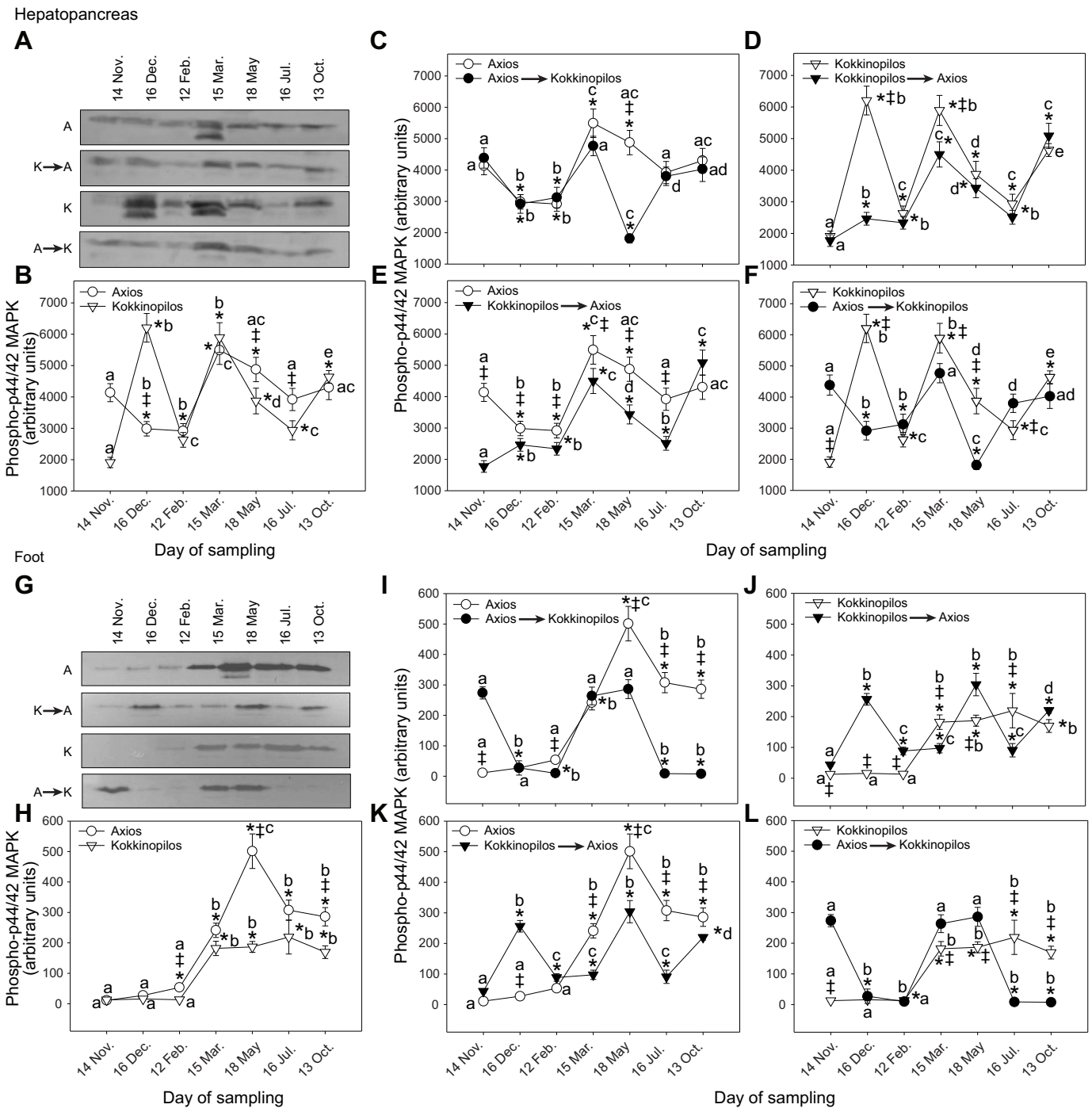

Fig. 5. Seasonal variation in p44/42 MAPK phosphorylation levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of phosphorylated p44/42 MAPK in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05) ; *significant difference from November; ‡significant difference between samplings from the two populations.

the coast population compared with the mountain one. The coast population transplanted to the mountain showed seasonal changes by March similar to those observed to the native one. Thereafter, and after a sharp decrease in May, JNK phosphorylation recovered to levels of the coast population (Fig. 6C,F). When the mountain population was transplanted to the coast, the pattern of JNK phosphorylation was similar to that observed for the coast population (Fig. 6D,E).

Regarding the foot of both native populations, JNK phosphorylation was maintained at low levels over the cold months but increased as temperatures rose, coinciding with snail arousal from hibernation and activity (Fig. 6G,H). However, JNK phosphorylation levels were generally higher in the coast population. Whereas JNK phosphorylation followed an similar increasing pattern in both the coast population and the

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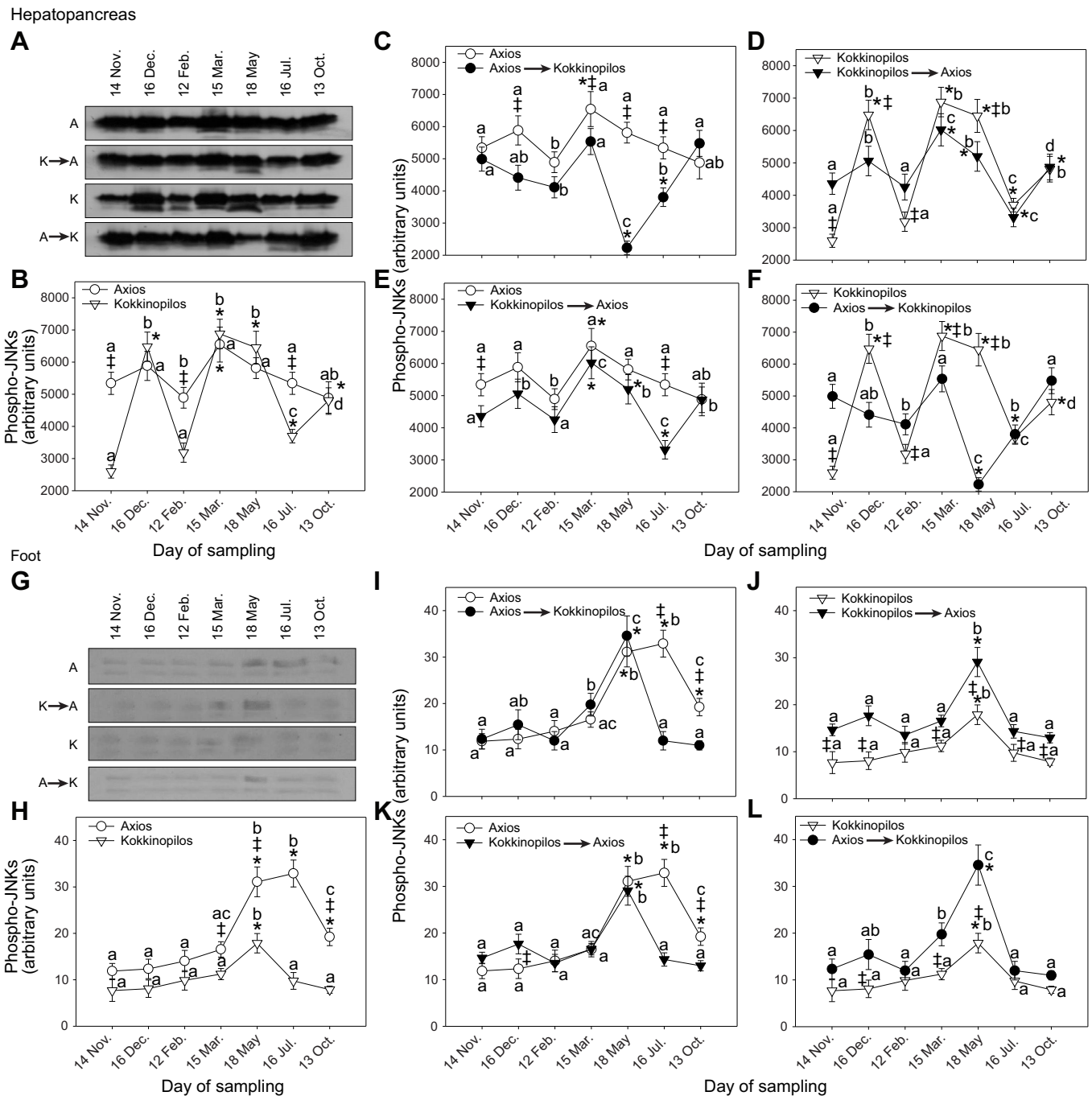

Fig. 6. Seasonal variation in JNK phosphorylation levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of phosphorylated JNK in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

coast population transplanted to the mountain, levels of the transplanted population dropped significantly after May, whereas those in the native population remained high (Fig. 6I,L). The mountain population and the mountain population transplanted to the coast exhibited a similar pattern of JNK phosphorylation, with levels increasing in May and thereafter decreasing. However, the transplanted population exhibited higher levels compared with the mountain population (Fig. 6J,K).

Seasonal ubiquitination

In general, ubiquitin conjugate levels were higher in the hepatopancreas of the coast population compared with those of the mountain population (Fig. 7A,B). Compared with the coast, however, ubiquitin conjugates remained at low levels by July in the mountain population, thereafter increasing by October. In contrast, they decreased significantly by February in the coast population, but recovered to November levels by May, declining during estivation

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(July–October). However, ubiquitination decreased significantly when the coast population was transplanted to the mountain (Fig. 7C) and it fluctuated at low levels similar to those determined for the mountain population (Fig. 7F). When the mountain population was transplanted to cost, the snails exhibited changes in ubiquitin conjugates similar to those observed for the coast population (Fig. 7D,E).

In contrast to findings for the hepatopancreas, foot ubiquitin conjugates were similar for the populations during the first months (Fig. 7G,H). Thereafter, ubiquitin conjugates increased from February to March in the coast population, remaining at high levels by July, and declining by October. Such an increase was observed in the mountain population, which 2 months later followed the same pattern of changes as those of the coast

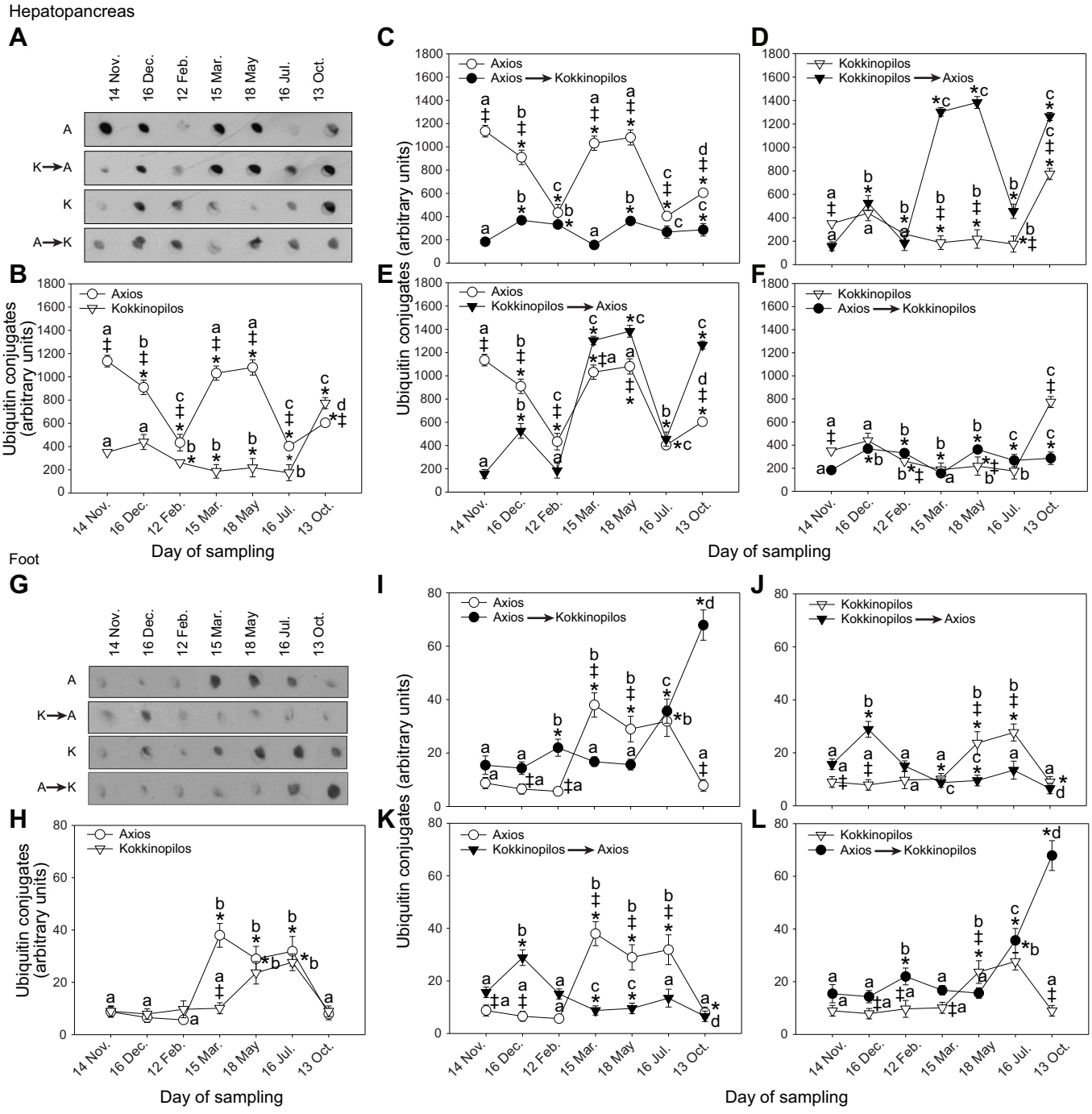

Fig. 7. Seasonal variation in ubiquitin conjugate levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of ubiquitin conjugates in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

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population (Fig. 7H). However, when the coast population was transplanted to the mountain, ubiquitin conjugates increased after March and remained at high levels to October (Fig. 7I,L). Compared with the mountain population, the mountain population transplanted to coast exhibited higher levels in November to February, while the opposite was observed from May to July (Fig. 7J,K).

Seasonal Bcl-2 patterns

In general, Bcl-2 levels were higher in the hepatopancreas of the coast population compared with the mountain one. After an initial decrease in hepatopancreas Bcl-2 levels in December and February, when coast snails hibernate, Bcl-2 levels increased during activity and estivation in March and July, respectively. In contrast, in the mountain population, Bcl-2 levels initially decreased between

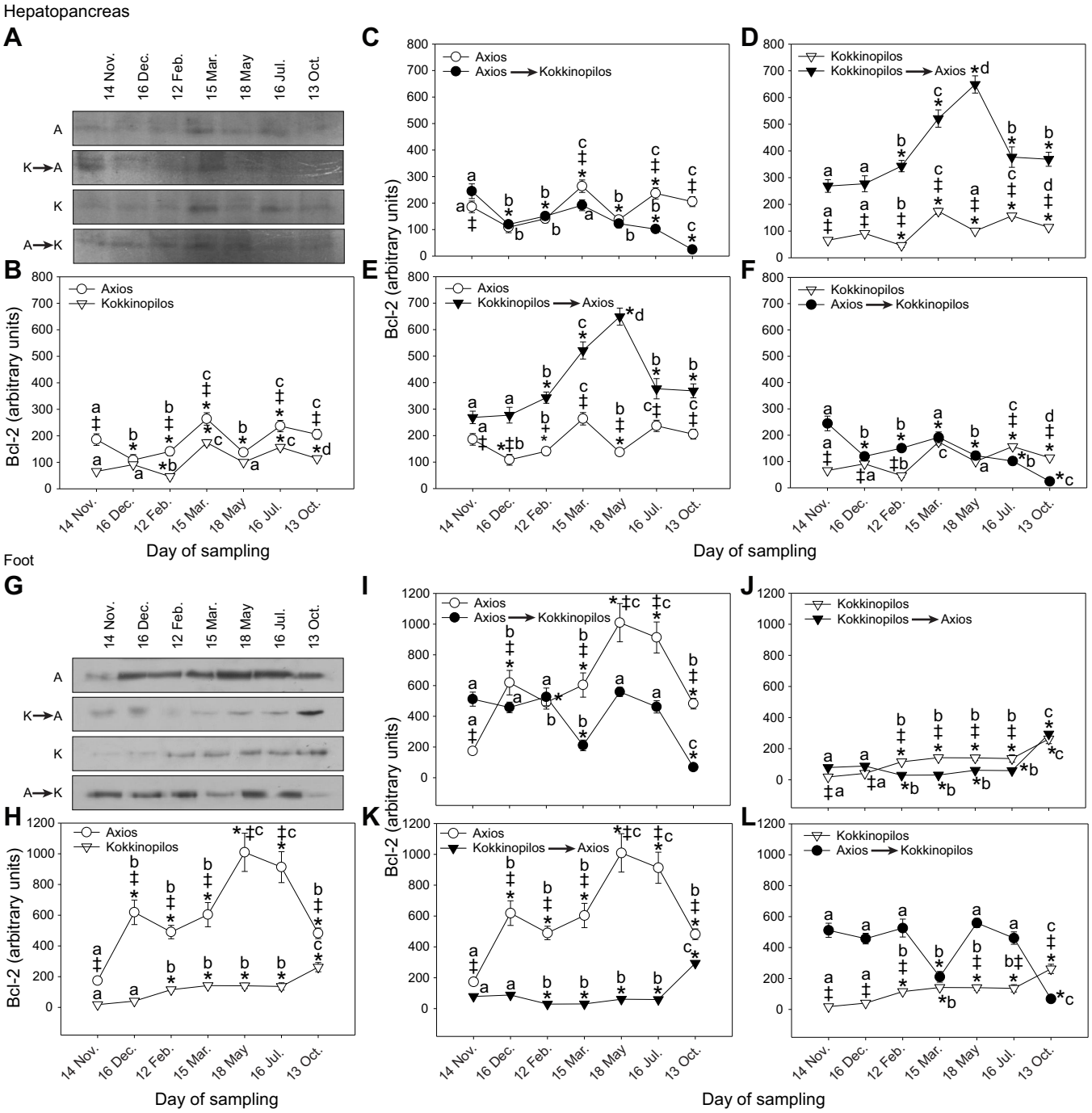

Fig. 8. Seasonal variation in Bcl-2 levels in hepatopancreas (top) and foot (bottom) of *H. lucorum* from two native and two reciprocally transplanted populations. (A,G) Representative immunoblots of Bcl-2 in populations of snails at the two study sites (Axios, A, coast; and Kokkinopilos, K, mountain), and transplanted between the sites as indicated. (B–L) Comparisons are presented between populations at Axios and Kokkinopilos (B,H), Axios and Axios→Kokkinopilos (C,I), Kokkinopilos and Kokkinopilos→Axios (D,J), Axios and Kokkinopilos→Axios (E,K) and Kokkinopilos and Axios→Kokkinopilos (F,L). Values are means±s.d.; n=8. Lowercase letters indicate significant differences between samplings of the same population (P<0.05); *significant difference from November; ‡significant difference between samplings from the two populations.

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Table 1. Percentage variation of snail biochemical responses according to population of origin, experimental site and time, and interactions between these variables

	Tissue sample	Population of origin	Experimental site	Time	Population of origin×experimental site	Population of origin×time
Hsp70	Hepatopancreas	24.35	16.32	16.35	22.34	21.54
	Foot	29.67	13.46	14.55	25.68	16.64
Hsp90	Hepatopancreas	30.22	11.76	11.22	26.47	20.33
	Foot	19.78	16.78	16.55	21.88	25.01
Phospho-p38 MAPK	Hepatopancreas	18.78	16.78	15.33	19.89	29.22
	Foot	16.72	21.33	22.34	18.98	20.63
Phospho-p44/42 MAPK	Hepatopancreas	16.71	16.99	21.01	21.88	23.41
	Foot	16.77	17.81	21.33	22.44	21.65
Phospho-JNK	Hepatopancreas	17.88	19.99	21.33	21.55	17.74
	Foot	31.22	17.01	15.66	17.88	18.23
Ubiquitin	Hepatopancreas	22.33	18.78	16.55	21.65	20.69
	Foot	34.55	13.2	12.34	29.98	9.93
Bcl-2	Hepatopancreas	28.77	14.22	14.56	28.9	13.55
	Foot	16.78	18.91	22.31	19.88	22.12

November and February, but rose again during arousal in March and remained elevated (Fig. 8A,B). While individuals from the coast population and the coast population transplanted to the mountain exhibited a similar Bcl-2 pattern from November to May, the latter population exhibited a decreasing trend (Fig. 8C,F). However, when mountain population was transplanted to the coast, the snails exhibited higher levels of Bcl-2 and a marked increase after arousal, in early March (Fig. 8AD,E).

Similar to findings in the hepatopancreas, higher levels of Bcl-2 were determined in the foot of the coast population compared with the mountain one (Fig. 8G,H). For the coast population, Bcl-2 levels increased significantly as ambient temperature dropped until February. With warming spring temperatures, Bcl-2 increased during arousal, remaining at high levels in July and declining thereafter by October. Although Bcl-2 levels exhibited a gradual increase in the mountain population, they remained at markedly lower levels throughout the year compared with those of the coast population. In contrast, compared with the native population, Bcl-2 levels were higher in November in the transplanted coast population and, apart from a decrease in March and October, remained at the same levels (Fig. 8I, L). Bcl-2 levels did not change significantly even when the mountain population was transplanted to the coast (Fig. 8J,K).

Contribution of variables to biochemical responses

Table 1 depicts the percentage variation of the examined biochemical responses explained by each variable and interactions between variables. Splitting our dataset in two semiannual groups revealed no significant differentiation of the obtained results in relation to the initial annual results (data not shown). Table 2 exhibits the overall effect of all variables and their interactions. All variables (population of origin, experimental site and time) and interactions between variables (population of origin×experimental site, and population of origin×time) were statistically significant.

Table 2. Results of general linear model (GLM) ANOVA analyses

	d.f.	Type III SS	*F*	*P*
Population of origin	3	0.345	5.167	0.012*
Experimental site	3	0.203	4.123	0.023*
Time	3	0.422	6.552	0.005*
Population of origin × experimental site	3	0.195	2.331	0.031*
Population of origin × time	3	0.302	4.766	0.017*

*Statistically significant effect.

Multivariate analysis reveals strong seasonal correlations with cellular stress responses

PC1 explained 36.33% of the variance. The physiological variables that were positively correlated with PC1 scores were p38 MAPK, JNK and p44/42 MAPK with ubiquitin conjugates in the hepatopancreas, forming clusters with habitats and seasons at: the coast in March, the mountain in March and May, the mountain to coast in October, March and May. Bcl-2 and both Hsps in the hepatopancreas form clusters in the coast to mountain in March and in the coast in May. The physiological variables that were positively correlated with scores on PC2, which explained 20.01% of the variance, were one cluster with MAPKs and Bcl-2 in the hepatopancreas at the coast in October and July, and Hsps at the coast to mountain in October, and the mountain to coast in December. The cumulative value of PC1 and PC2 was 56.34% (Fig. 9).

DISCUSSION

Air temperature patterns show clear seasonal differences between the two sampling locations. The much lower temperatures of the mountain compared with the coast are reflected in the snails' behavioral strategies from the corresponding populations as far as entrance/arousal from hibernation or estivation is concerned. It is clear that mountain snails face annual cold challenges whereas coast snails deal mainly with warm challenges**.** These differences in the two habitats are reflected in the seasonal patterns of the proteins examined in the present study, indicating major differences in biochemical and physiological responses in order to cope with cold or warm challenges. Specifically, the data obtained in the present study, show clear seasonal patterns in Hsp expression (Figs 2 and 3), MAPK phosphorylation (p38 MAPK, p44/42 MAPK and JNK; Figs 4, 5 and 6, respectively), apoptosis-related Bcl-2 (Fig. 7) and ubiquitin conjugates (Fig. 8) in *H. lucorum* from the low and high altitude populations. These patterns seem to be closely related to *H. lucorum* population habitat and thermal history. As depicted in Table 2, according to the GLM analysis, the effect of all factorial parameters measured herein is significant in snail physiological performance.

Seasonal patterns of Hsp expression

Compared with individuals from the mountain, coast individuals maintained relatively high Hsp70 constitutive levels (Fig. 2A–C,E, G–I,K), indicating that species adapted to higher temperature niches were more heat tolerant and showed higher upper thermal limits

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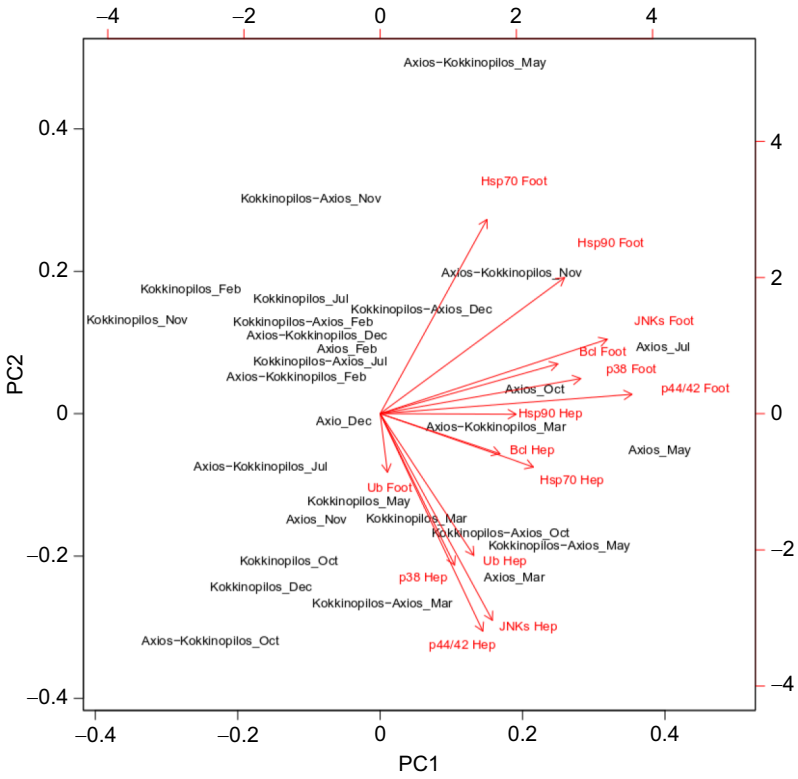

Fig. 9. Variable correlations with each of the first two principal components (PCs) in the multivariate analysis. The principal components analysis (PCA) was generated from the complete biochemical dataset. Parameters with red vector arrows were included as PCA construction predictors.

of protein (including Hsp) synthesis (Tomanek and Somero, 1999; Mizrahi et al., 2010, 2012a,b; Kotsakiozi et al., 2015). This adjustment of Hsp expression due to long-term acclimation to environmental temperature has also been shown in *Drosophila melanogaster* (Bettencourt et al., 1999). Organisms occupying extreme and unpredictable environments use a 'preparative defense' strategy involving maintenance of high Hsp constitutive levels (Somero, 2020), thus allowing an immediate response to the potentially damaging effects of heat stress. This response would be much more rapid than that required for *de novo* Hsp production (Sørensen et al., 2003).

During entrance into hibernation, Hsp70 levels decreased significantly, but only in the coast population. As Hsp maintenance at high levels is costly, we suggest that it is an energy-saving strategy during hibernation and winter hypometabolism (November to December). By contrast, mountain snails generally showed lower but constant Hsp70 levels throughout the winter months, compared with coast snails, indicating a reduced need for Hsp action. Another significant difference between the two populations was observed during the snails' preparation for arousal, which is characterized by metabolic reorganization and gradual reactivation of several physiological processes, including heart rate and respiration (Storey and Storey, 1990). Preparation for arousal started after December in the coast population, and after February in the mountain population (<0°C). After arousal, snail activity was observed early in March for the coast population and early in April for the mountain population. In both cases, snails face the impacts of ROS accumulation resulting from intense reoxygenation (Hermes-Lima et al., 1998, 2015). Accordingly, the Hsp70 increase during arousal is in agreement with the POS defense hypothesis. In the aestivating land snail species *Otala lactea*, lipid peroxidation, indicating oxidative stress and tissue damage, was significantly enhanced in the hepatopancreas at the onset of arousal from dormancy. Antioxidant enzyme activity was higher in the hepatopancreas and foot muscle of aestivating snails than in active snails (Hermes-Lima and Storey, 1995). Similar results were found in the land snail *Cornu aspersum* during experimental estivation and arousal cycles (Ramos-Vasconcelos and Hermes-Lima, 2003). Overall, POS is a well-documented phenomenon related to snail arousal (Hermes-Lima and Storey, 1995, 1996; Hermes-Lima et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2010, 2011, 2015). This further supports the interrelationship between Hsp expression and oxidative stress in several snail species under thermal stress (Mizrahi et al., 2010, 2012a,b; Scheil et al., 2011; Dieterich et al., 2013; Troschinski et al., 2014).

As coast individuals experience higher temperatures at low altitude, their recovered Hsp70 levels after arousal (early March) could serve as a preparatory strategy in order to defend against high temperature impacts, whereas there is a need for further upregulation of the stress protein machinery for the mountain population. The latter is supported by the fact that Hsp70 levels in mountain snails tend to reach close to those of coast snails in the summer. Such a phenotypic response is further supported by the reciprocal transplantation experiments, where there was no Hsp70 upregulation when individuals from the coast were transplanted to the mountain (Fig. 2C,F,I,L), whereas those transplanted from the mountain to the coast exhibited further Hsp70 upregulation at the coast (Fig. 2D,E,J,K).

As coast snails face intense thermal challenges during summer, a second phase of Hsp70 upregulation, which is in line with the POS defense hypothesis, was observed in July mainly. In contrast to mountain snails, they enter the hypometabolic state of estivation during summer and are reactivated early in September when conditions become favorable again. Moreover, the data obtained from the reciprocal transplantation clearly show phenotypic Hsp70 expression plasticity and indicate that the coast thermal regime

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determines Hsp70 expression patterns. Specifically, when snails from the coast were transplanted to the mountain, there was no further increase in Hsp70 expression in the hepatopancreas after May (Fig. 2C,F), but when individuals from the mountain were transplanted to the coast, a strong Hsp70 upregulation occurred which increased to higher levels than those in the native population (Fig. 2D,E). Probably the stress levels needed to induce Hsp synthesis are directly related to the habitat of the organism under study (Feder and Hofmann, 1999; Kotsakiozi et al., 2015).

Compared with findings in the hepatopancreas, our results indicated a different seasonal role of Hsp70 and probably less demand for molecular chaperoning in the foot. Hsp70 levels, after an initial drop from November to December in the coast population and after an initial increase in the mountain population, were maintained at an even level to July (Fig. 2H). These results, also observed after reciprocal transplantation (Fig. 2I–L), indicate that Hsp70 steady levels are maintained in the foot in both populations over the largest part of year.

In contrast to Hsp70, Hsp90 resting levels were similar in the hepatopancreas of the two native populations. While they showed an opposite direction of change during the hibernation months, from February onwards a gradual increase in coast snail Hsp90 levels and maintenance of levels in mountain snails was exhibited (Fig. 3B). These differential responses are probably related to specific cellular and physiological processes during and after overwintering, especially warm season effects at the coast. This is supported by the significant increase in Hsp90 levels from May to July in the coast population.

The seasonal changes in Hsp90 levels in the foot from coast and mountain snails indicate a similar pattern and probably similar physiological role to the hepatopancreas during and after overwintering (Fig. 3H–L). An increase in Hsp90 during cooling might be related to cold hardiness when snails face low or subzero temperatures and is seen only among mountain snails. Habitat and body size may be involved in freeze tolerance in several land snail species (Ansart and Vernon, 2003, 2004; Ansart et al., 2010). Phenotypic responses during cooling involve transcriptional processes resulting in Hsp expression and metabolic reorganization, leading to cell preservation in freeze-tolerant animals (Zhang et al., 2018; Des Marteaux et al., 2019; Storey and Storey, 2019). However, Hsps, and especially Hsp90, may play an important role in osmoregulation during estivation (Mizrahi et al., 2010, 2015; Arad et al., 2010). Coast individuals face desiccation challenges, as they enter estivation during summer. Accordingly, further upregulation of Hsp90 and maintenance in *H. lucorum* tissues from the coast during summer, and Hsp90 maintenance in coast individuals transplanted to the mountain, seem to be in agreement with this hypothesis (Fig. 3H–L). However, except for oxidative stress, several factors, such as increased protein synthesis accompanying arousal and the shift to growth, reproductive and behavioral processes, are involved in Hsp regulation. Moreover, morphological divergence (e.g. shell color diversity) between populations of the same species indicates that different Hsp expression patterns can occur (Köhler et al., 2009; Mizrahi et al., 2010, 2015; Dieterich et al., 2013; Di Lellis et al., 2012).

Seasonal patterns of MAPK activation

MAPK activation plays a crucial cytoprotection role by mediating a vast number of cellular responses including gene transcription, cytoskeletal organization, metabolite homeostasis, cell growth and apoptosis in response to many different extracellular signals (Kyriakis and Avruch, 1996, 2001; Cowan and Storey, 2003). MAPKs are characterized as cellular sensors because they transduce external signals into cellular responses. MAPK levels in *H. lucorum* showed distinct seasonal periods and were tissue specific (Figs 4–6). Activation of all MAPKs exhibited two major peaks in the hepatopancreas of mountain snails, one in December and the second from mid-March to early May (Figs 4B, 5B and 6B). The first phase of activation coincides with the hibernation and hypometabolism period. In line with these data, our previous investigation showed that hibernation caused significant increases in JNK and p38 MAPK phosphorylation in *H. lucorum* heart and ganglia (Michaelidis et al., 2008). The mountain population, compared with the coast population, faces subzero temperatures during winter and the reciprocal transplantation shows clearly that subzero temperature is a strong environmental factor triggering MAPK activation. We do not know whether MAPKs are activated below a threshold low temperature. However, it is indicated that under such thermal threats (subzero temperatures) the phenotypic plasticity of the MAPK signaling cascade might contribute to physiological and biochemical remodeling and cell protection in land snail tissues.

Signal transduction pathways and their regulatory effects on gene expression have been shown to be pivotal in meeting challenges associated with hibernation in ground squirrels and bats (Cowan and Storey, 2003; Mamady and Storey, 2006; Morin et al., 2008; Wu et al., 2013). Biggar et al. (2015) reported that each MAPK subfamily responded differently during torpor and each showed organ- and tissue-specific patterns of response. It has been reported that phosphorylated p38 MAPK could trigger the expression of numerous downstream genes, which encode products that protect the whitefly from adverse cold stress effects or elicit rapid cold hardening (Iwata et al., 2005; Fujiwara and Denlinger, 2007). Greenway and Storey (1999, 2000) suggested specific roles for p44/42 MAPK and p38 MAPK in response to freezing or anoxia in frogs and turtles.

The second phase of MAPK activation coincides well with arousal and it takes place in both the hepatopancreas and foot, suggesting a correlation between ROS production and MAPK activation. Previous investigations showed significant MAPK activation and antioxidant defense in water frog tissues after arousal from hibernation, strongly supporting the above assumption (Feidantsis et al., 2012a, 2013). However, some members of the MAPK family, such as p44/42 MAPK and JNKs, exhibited 2- or 3-fold higher activation in the foot of snails from the coast compared with the mountain population during arousal (Figs 4–6, lower panels). This might be correlated with the sharp increase in ambient temperature at the coast, resulting in acceleration of metabolic reorganization and rate during arousal. Additionally, as the snails at the coast will face higher temperatures during seasonal warming, maintenance of activated MAPKs at high levels, mainly in the foot, may be involved in cytoprotection by modulating expression of different genes. JNK and p38 MAPK activation is in most cases associated with the promotion of apoptosis, whereas p44/42 MAPK activation is generally associated with protection (Xia et al., 1995; Yu et al., 2013).

Evidence for seasonal apoptotic and anti-apoptotic responses

The above-mentioned responses may be related to several cellular stress phenomena, including apoptotic processes. A cellular process that is closely related to apoptosis is the activation of the ubiquitin pathway. Several studies have reported that the ubiquitin–proteasome system has an important role to play in the apoptotic

pathway (Orlowski, 1999; Bader and Steller, 2009). Ubiquitination is crucial for cellular processes, such as protein degradation, apoptosis, autophagy and cell cycle progression. Our results show a marked increase in the levels of ubiquitin conjugates in hepatopancreas of *H. lucorum* from the coast, indicating increased protein degradation during arousal from overwintering, probably related to oxidative stress (Fig. 7B–F), which was not that obvious in the foot (Fig. 7H–L). It has been reported that a sudden ROS increase during reperfusion can temporarily overwhelm the cellular antioxidant system and cause irreversible damage to DNA, proteins and lipids, and alter cell viability and integrity (Storey and Storey, 2004). The results from both hepatopancreas and foot tissues, including those obtained from reciprocal transplantation, indicate that habitat determines ubiquitination levels, which seems to be more potent in the tissues of snails from the coast during and after arousal. As reported elsewhere, both JNK and p38 MAPK activation may be involved in apoptotic phenomena (Dhanasekaran and Reddy, 2008; Redza-Dutordoir and Averill-Bates, 2016). Both MAPK members are markedly increased during seasonal arousal from hibernation and/or estivation, strongly suggesting a correlation with ROS production. Rouble et al. (2013) reported that activation of the anti-apoptotic pathway may be central to energy conservation during hypometabolism in mammalian hibernation and play a cytoprotective role that ensures long-term cell and macromolecular viability under anoxic versus aerobic recovery conditions. Moreover, Hockenbery et al. (1993) found that Bcl-2 prevents cells from H_2O_2 and oxidative stress-induced death. Our results, however, indicate that the significance of anti-apoptotic pathways and Bcl-2 is tissue specific in *H. lucorum*, with phenotypic plasticity being habitat dependent (Fig. 8). The marked increase in the levels of Bcl-2 in the hepatopancreas of mountain snails when transplanted to the coast strongly supports the above assumption (Fig. 8D). However, the anti-apoptotic pathway seems to be more significant in the foot either during winter or after arousal in snails from the coast (Fig. 8H). Similarly, Gerber et al. (2016) reported enhanced anti-apoptotic responses during anoxia and recovery in a freeze-tolerant wood frog, *Rana sylvatica*. Moreover, the further Bcl-2 increase during summer indicates its anti-apoptotic role during estivation. A recent investigation suggested that microRNAs regulate survival mechanisms by targeting the Akt and p44/42 MAPK signaling pathways, as well as myosin genes in land snails during estivation (Hoyeck et al., 2019). The marked increase in the levels of p44/42 MAPK in the foot of coast snails during arousal and summer is strong evidence regarding the correlation between p44/42 MAPK signaling pathways and Bcl-2. In support of this, the reciprocal transplantation experiments showed that when snails from the coast are transplanted to the mountain, there is no increase in the levels of p44/42 MAPK (Fig. 5) and Bcl-2 (Fig. 8) during summer.

The decrease of ubiquitin conjugate levels during the summer indicates reduction of protein degradation during estivation, which is consistent with the reduction of protein recycling during hypometabolism (Storey and Storey, 2004). However, the elevated ubiquitin conjugates in October in the tissues of mountain snails is evidence for the activation of the corresponding pathway. In line with this response, JNK and p44/42 MAPK exhibited a similar pattern of changes but mainly in the hepatopancreas, indicating their probable involvement in cellular reorganization and preparation of snails to enter hibernation.

Conclusion

Overall, our results are in agreement with the hypothesis of a preparatory strategy for defense against oxidative stress and that ectotherms from higher altitude are more sensitive to changes in temperature, whereas populations inhabiting niches where higher temperatures prevail maintain higher levels of constitutive Hsps at all times. Moreover, the reciprocally transplanted snails indicate a phenotypic plasticity of most biochemical factors studied as a response to environmental stress in the corresponding habitat, which is also suggested by the results of the PCA analysis. However, a few of them exhibited the same pattern of changes regardless of the acclimatization habitat (as shown by the high percentage of the variable ‘population of origin’ explaining these results), and it seems to be tissue specific. Specifically, steady Hsp70 levels in the foot when the mountain population was transplanted to the coast, steady Hsp90 levels in the hepatopancreas when both native snail populations were reciprocally transplanted, and similar JNK activation levels in the foot of both populations when these were transplanted indicate a genetic predisposition. Additionally, similar foot ubiquitination levels between the mountain population moved to the coast and the original mountain population, and the same Bcl-2 levels in the hepatopancreas when the coast snails were transplanted to the mountain might suggest a genetic basis supporting these biochemical responses. Several studies have showed genetic differences in thermal tolerance and Hsp70 expression among populations of *Drosophila* spp., indicating that high temperature in nature may be an important selective factor (Krebs and Feder, 1997; Sørensen et al., 2001; Zatsepina et al., 2001). As we do not know whether these responses could be attributed to the snails’ genetically inherited traits, this should be addressed in future studies focusing on species and the genetic diversity of their populations.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.S., B.M.; Investigation: A.S., K.F., O.G., M.N.B., M.H., B.M.; Data curation: A.S., K.F., B.M.; Writing - original draft: A.S., K.F., B.M.; Writing - review & editing: A.S., K.F., K.B.S., B.M.

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