The European Zoological Journal

ISSN: (Print) 2475-0263 (Online) Journal homepage: http://www.tandfonline.com/loi/tizo21

Seasonal changes in the liver of a non-hibernating population of water frogs, Pelophylax kl. esculentus (Anura: Ranidae)

D. Mentino, G. Scillitani, M. Marra & M. Mastrodonato

To cite this article: D. Mentino, G. Scillitani, M. Marra & M. Mastrodonato (2017) Seasonal changes in the liver of a non-hibernating population of water frogs, Pelophylax kl. esculentus (Anura: Ranidae), The European Zoological Journal, 84:1, 525-535, DOI: 10.1080/24750263.2017.1395482

To link to this article: https://doi.org/10.1080/24750263.2017.1395482

Published online: 03 Nov 2017.

The European Zoological Journal, 2017, 525–535
Vol. 84, No. 1, https://doi.org/10.1080/24750263.2017.1395482

Seasonal changes in the liver of a non-hibernating population of water frogs, *Pelophylax* kl. *esculentus* (Anura: Ranidae)

D. MENTINO, G. SCILLITANI *, M. MARRA, & M. MASTRODONATO

Department of Biology, Section of Animal and Environmental Biology, Laboratory of Histology and Comparative Anatomy, University of Bari Aldo Moro, Italy

(Received 28 July 2017; accepted 16 October 2017)

Abstract
Seasonal variation of liver glycogen, lipids and melanomacrophages were investigated in a non-hibernating population of *Pelophylax* kl. *esculentus* from Calabria by histochemical methods and computer-assisted image analysis. Twenty individuals of both sexes were sampled in a tank in Roseto Capo Spulico (Cosenza, Calabria) in four periods of the year 2016 (February, May, July, October). Portions of liver from each individual were included in paraffin for glycogen and melanomacrophages, and epoxydic resin-araldite for lipid analysis. Sections were stained with periodic acid-Schiff (PAS) for glycogen (with diastase-PAS as control) or osmium-tetroxide for lipids, or left unstained for melanomacrophages (appearing naturally black due to melanin). Image analyses were performed on 9–12 grayscale converted pictures per individual. Total areas per μm^2 of glycogen, lipids and melanomacrophages, as well as counts of lipid droplets and melanomacrophages and mean area of single lipid droplets and melanomacrophages, were measured. Statistical analyses were performed by analysis of variance (ANOVA) with bootstrap resampling. Significant variation among sampling periods was found for each variable. Glycogen and lipids co-vary, with higher values observed in October–February and lower values in May–July, whereas melanomacrophages reach a peak in May and have much lower values in the other months. It is concluded that, in the absence of a hibernating period, reproduction is the main force regulating the annual cycles of reserve storing and melanin production.

Keywords: Liver, seasonal cycle, non-hibernating, *Pelophylax* kl, *esculentus*, Calabria

Introduction

The liver performs several essential functions in the adaptation to the environmental changes that Anura face during their life cycle. The liver mass varies during the annual cycle up to three times and this variation is related to body shape and mass, environmental variables, reproduction, dormancy and even phylogenetic history (Withers & Hillman 2001). Liver involvement in several metabolic pathways is well known: in particular, it acts as an important storage system for glycogen and lipids (e.g. Crawshaw & Weinkle 2000). For both substances, seasonal variation in accumulation by the liver is reported, in particular in those species from temperate climates that undergo dormancy (e.g. Pasanen & Koskela 1974; Singh & Sinha 1989; Fenoglio et al. 1992; Dinsmore & Swanson 2008). Typically, reserves of glycogen and lipids increase in the period following reproduction and preceding dormancy. The increase of reserves between the emergence from dormancy and the starting of the breeding period depends on the reproductive strategy. From this point of view, anurans can be divided into two main categories, i.e. income and capital breeders. Income breeders feed after emergence and thus their reserves increase prior to breeding, whereas capital breeders do not feed and depend on the reserves accumulated prior to dormancy that consequently further decrease after emergence (i.e. Chen et al. 2011).

Additionally, the liver accumulates blood reserves to be released in stress conditions (Frangioni & Borgioli 1994) and hosts several melanomacrophage centers (Zuasti et al. 1998). Seasonal variation has also been documented for melanomacrophages, and appears to be correlated to glycogen and lipid storage in hepatocytes: in overwintering frogs, hepatocytes increase their glycogen and lipid concentration,

*Correspondence : G. Scillitani, Department of Biology, Section of Animal and Environmental Biology, Laboratory of Histology and Comparative Anatomy University of Bari Aldo Moro, Bari I-70125, Italy. Tel/Fax: +39 80 5443349 Email: giovanni.scillitani@uniba.it

Published online 03 Nov 2017

whereas melanomacrophages increase melanin content (Fenoglio et al. 1992).

Due to its key role in the accumulation and detoxification of xenobiotics, the amphibian liver can also undergo considerable variation when the animals are exposed to toxins and/or toxicants. In these conditions, liver mass can increase and histological alterations are observed, such as necrosis, infiltration, fibrosis, cytomegaly and variation in melanomacrophage number (Loumbourdis 2005; Păunescu et al. 2010; Jelodar & Fazli 2012; Thammachoti et al. 2012; Zhelev et al. 2015).

Both latitude and altitude can influence the seasonal patterns of reserve storage, as can be observed in those species having a wide geographic distribution (Pasanen & Koskela 1974; Irwin & Lee 2003; Jönsson et al. 2009; Chen et al. 2011; Costanzo et al. 2013). In general, more northerly populations store higher reserves than those found at lower latitudes, not only for energy purposes but also as a protection during freezing (e.g. Storey & Storey 2005).

The water frogs of the *Pelophylax esculentus* klepton system (including the parental species *P. lessonae* and its hybridogenetic hybrid *P. esculentus*: see Capula et al. 2007) are widespread along the Italian Peninsula, from the Alps to Sicily and from sea level to 800 m above sea level (Capula et al. 2007), and thus populations live in a variety of climate systems (e.g. Costantini et al. 2013). Therefore, populations living in colder climates hibernate in winter, whereas those living in warmer climates can be active all year long (Capula et al. 2007). Seasonal variation of both liver energy storage and melanomacrophages was documented by histological and histochemical methods in populations from Lombardy (Northern Italy) facing hibernation (Fenoglio et al. 1992; Barni et al. 1999). As regards the more southerly populations, data are fragmentary and papers describing variations in hepatocyte storage and melanomacrophages seldom give information about a possible hibernating and/or aestivating condition, or about the climatic features of the sampling site. Some analyses of seasonal variation were made using biochemical methods on glycogen (Scapin & Di Giuseppe 1994), lipids (Milone et al. 1978, 1983, 1990; Bruscalupi et al. 1989) and melanin (Sichel et al. 1981; Corsaro et al. 1990). Apparently, no author has studied the liver cycle by histochemical methods.

With all the preceding in mind, we thought it would be interesting to evaluate whether seasonal variation of liver glycogen, lipids and melanomacrophages takes place in a non-hibernating population of *Pelophylax* kl. *esculentus* from Calabria. Evaluations were made by histochemical methods and computer-assisted image analysis, made possible in recent times thanks to the availability of packages such as ImageJ (Rasband 2016). Then, we compared our data with those available from the literature to ascertain whether differences exist in respect to hibernating populations within the same species, and give possible explanations.

Material and methods

Animal collecting

Frogs were collected in Roseto Capo Spulico (Cosenza, Calabria: 39.971547°N, 16.618112°E), from a cubic tank at ground level (side = 2 m) built for irrigation purposes near a lemon orchard that is organically farmed, i.e. without using synthetic fertilizers and pesticides. The tank is constantly fed by a pipeline from the Ferro Stream (about 4 km south). The area has a climate of the hot-summer Mediterranean (Csa) subtype according to Köppen (e.g. Claps & Sileo 2001). The site was visited for some years prior to sampling and active frogs were observed in each month of the year. Breeding activities (courtship, mating and oviposition) were observed around April–May. Since genetic data about the possible presence of hybrids were not available, we attributed the frogs of this population to *P.* kl. *esculentus* on morphological grounds, i.e. the pigmentation of vocal sacs in males and the length and shape of the inner metatarsal tubercle (Capula et al. 2007).

Four collections of five adult frogs of both sexes (mean snout-vent length = 5.8 cm ± 1.5 cm standard deviation, SD) were made at intervals of about 90 days from the beginning of February (indicated as February, May, July and October, respectively) during 2016. Collecting periods are reported in the climatic chart of the site in Figure 1. Collecting was authorized by the Italian Ministry of Agricultural, Food and Forestry Policies and by the authorities of the Calabria Region.

Tissue sampling and processing

Animals were killed by cervical dislocation following EU Directive 2010/63/EU for animal experiments, and their livers were quickly removed. Samples were taken from the parenchyma of each of the two lateral main lobes of the liver and were treated for embedding in paraffin (for glycogen and melanomacrophage analysis) or epoxydic resin-araldite (for lipid analysis). For paraffin embedding, details are given in Scillitani et al. (2012). Serial sections 4 μm thick were cut by microtome. For epoxydic resin-araldite (M CY212 from TAAB, Aldermaston, UK) embedding, details are given in Scillitani et al. (2011).

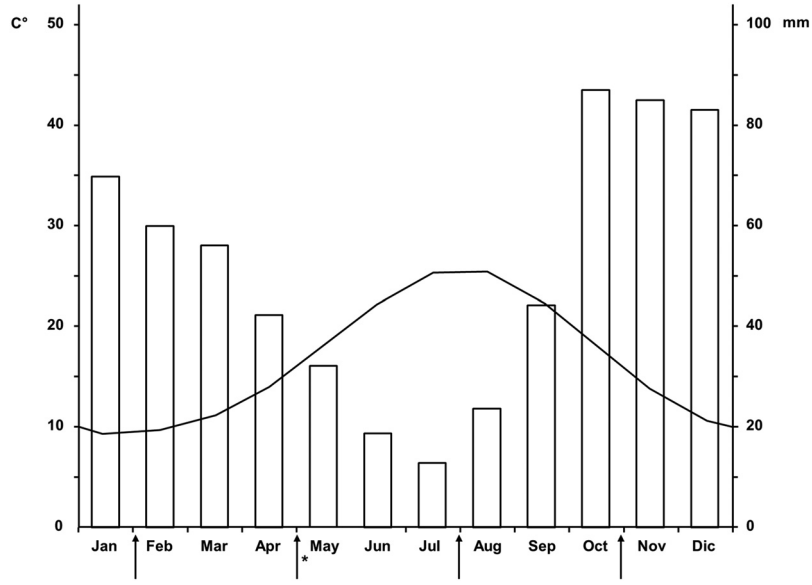

Figure 1. Sampling periods of *Pelophylax* kl. *esculentus* for liver analysis in the site of Roseto Capo Spulico (Cosenza, Calabria) projected onto a thermopluviometric diagram (data retrieved from the Meteorological Service of the Italian Air Force). Arrows: period of sampling; asterisk: beginning of breeding period.

Histological and histochemical staining

Paraffin sections for glycogen analysis were stained with periodic acid–Schiff (PAS) or with diastase-PAS (Lillie & Greco 1947) with minor modifications. For the diastase treatment, sections were incubated with 0.8% diastase in buffered saline (PBS) in a humid chamber at 37°C overnight. Negative staining after diastase demonstrated that PAS positivity was due mainly to glycogen. For melanomacrophage analysis the sections were not stained, since melanin renders the cells dark and thus they were easily detectable. Lipid droplets in the epoxidic resin-araldite sections were stained gray-brown with osmium tetroxide post fixation; thus, further staining was not required. All the reagents referred to in this section were from Sigma, St. Louis, USA.

Image analysis

Images were captured in bright light using an Eclipse E600 photomicroscope and a DMX1200 digital camera (Nikon Instruments SpA, Calenzano, Italy). Pictures of liver parenchyma were taken far from the main vessels to obtain the most homogeneous fields possible. Photos were shot at a resolution of 150 dpi under a magnification of 400× for lipids and glycogen and 200× for melanomacrophages, covering an area of about 33,610 μm^2 (400×) or 134,441 μm^2 (200×). Nine to 12 fields per individual were selected for the image analysis. Images were analyzed for each of the three parameters (glycogen, lipids, melanomacrophages) with the ImageJ software (Rasband 2016) through (1) conversion of the RGB image to grayscale, (2) isolation of the stained area by the threshold command, and (3) measurement of the resulting area, expressed in μm^2. Being approximately circular in section, both lipid droplets and melanomacrophages were counted by the Analyzing Particle procedure in ImageJ. Touching and/or merging of particles were reduced through background subtraction and watershedding. The mean area of single droplet/cell was computed by dividing the total area estimates by the number of droplets/cells.

Statistical analysis

The most common descriptive statistics (mean, SD, standard error, kurtosis, skewness) were computed from areas, counts and mean areas of single droplet/cell estimations for glycogen, lipids and melanomacrophages of each sample, and significant deviation from normality of distribution of data was estimated by Shapiro–Wilk tests. Since preliminary *t*-tests (not shown) did not find significant variation between sexes within the periods, they were clustered and treated as one sample. Due to violation of the assumption of normality for many samples, significant variation among the cited parameters was estimated by a one-way analysis of variance (ANOVA)

followed by resampling through bootstrap of F-stat values (10,000 iterations). Significance for probability computed from tests was set at $p < 0.01$. Statistical computations were generated by the Real Statistics Resource Pack software (Release 4.3) (Zaiontz 2015).

Results

Glycogen, lipid droplets and melanomacrophages showed significant variation among the four periods sampled, as shown in the results from the statistical analyses summarized in Tables I and II. Histopathologic alterations were not observed.

Table I. Descriptive statistics and Shapiro–Wilk tests for normality of distribution of liver parameters in *Pelophylax* kl. *esculentus*. Abbreviations: SD, standard deviation; W, value of estimator in Shapiro–Wilk test; *p*: probability.

Parameter	Value			
Month (number of pictures)	February (51)	May (51)	July (61)	October (51)
Glycogen: area per μm^2				
Mean (± SD)	2933.41 (± 379.74)	233.81 (± 89.93)	647.91(± 199.58)	1174.56(± 105.02)
Standard error	53.17	12.50	25.55	14.71
Kurtosis	−1.272	1.546	−0.521	0.907
Skewness	−0.354	1.133	0.574	−0.891
W (*p*)	0.905 (0.001)	0.948 (0.025)	0.944 (0.008)	0.925 (0.003)
Month (number of pictures)	February (60)	May (60)	July (45)	October (48)
Lipids: number of droplets				
Mean (± SD)	953.29 (± 452.12)	635.15 (± 302.79)	666.84 (± 410.83)	922.40 (± 362.88)
Standard error	65.26	39.09	61.24	52.377
Kurtosis	2.410	−0.680	2.629	0.241
Skewness	1.642	0.362	1.434	0.620
W (*p*)	0.814 (0.000)	0.968 (0.121)	0.886 (0.00)	0.960 (0.101)
Month (number of pictures)	February (60)	May (60)	July (45)	October (48)
Lipids: area per μm^2				
Mean (± SD)	1072.37 (± 485.17)	725.75 (± 448.66)	630.59 (± 384.93)	1040.15 (± 355.23)
Standard error	62.63	57.92	57.38	51.27
Kurtosis	−0.513	−1.598	0.085	0.566
Skewness	0.644	0.272	0.768	−0.045
W (*p*)	0.928 (0.002)	0.866 (0.000)	0.929 (0.009)	0.984 (0.755)
Month (number of pictures)	February (60)	May (60)	July (45)	October (48)
Lipids: area of single droplets in μm^2				
Mean (± SD)	1.47 (± 0.64)	0.94 (± 0.31)	1.03 (±0.51)	1.27 (± 0.74)
Standard error	0.08	0.04	0.07	0.11
Kurtosis	0.231	−0.193	5.123	21.504
Skewness	0.861	−0.006	1.699	4.059
W (*p*)	0.936 (0.004)	0.986 (0.724)	0.877 (0.000)	0.587 (0.000)
Month (number of pictures)	February (47)	May (59)	July (60)	October (60)
Melanomacrophages: number				
Mean (± SD)	8.20 (± 2.26)	25.90 (± 7.58)	15.18 (± 5.00)	7.66 (± 3.75)
Standard error	0.29	0.98	0.65	0.55
Kurtosis	−0.828	0.117	−0.637	8.887
Skewness	0.082	0.630	0.415	2.368
W (*p*)	0.947 (0.013)	0.960 (0.049)	0.959 (0.040)	0.795 (0.000)
Month (number of pictures)	February (47)	May (59)	July (60)	October (60)
Melanomacrophages: area per μm^2				
Mean (± SD)	33,904.73 (± 3012.95)	85,368.26 (± 11,592.59)	55,609.78 (± 9571.01)	15,822.73 (± 1794.93)
Standard error	392.25	1496.60	1235.61	261.82
Kurtosis	−1.109	8.886	−1.010	−0.278
Skewness	−0.326	2.538	−0.696	0.302
W (*p*)	0.937 (0.004)	0.755 (0.000)	0.862 (0.000)	0.973 (0.356)
Month (number of pictures)	February (47)	May (59)	July (60)	October (60)
Melanomacrophages: area of single cells in μm^2				
Mean (± SD)	3177.437 (± 592.78)	4693.21 (± 1848.35)	3734.54 (± 1094.22)	2359.33 (± 492.92)
Standard error	77.17	240.63	83.72	141.26
Kurtosis	−0.856	23.347	1.502	71.15
Skewness	0.544	4.737	1.5548	9.283
W (*p*)	0.900 (0.000)	0.413 (0.000)	0.767 (0.000)	0.755 (0.000)

Table II. Analysis of variance (ANOVA) tests with resampling for the liver parameters in *Pelophylax* kl. *esculentus* from Calabria. Abbreviations: df: degrees of freedom; F: value of F-statistics after resampling; MS: mean sum of squares; SS: sum of squares; *p*: probability.

Parameter	Sources	SS	df	MS	F	*p*
Glycogen: area per μm^2	Between groups	2.19×10^8	3	73,131,390.7	1454.865	0.000
	Within groups	10,556,021	210	50,266.770		
	Total	2.35×10^8	213	1,079,578.374		
Lipids: number of droplets	Between groups	4,240,457	3	1,413,486	9.725	0.000
	Within groups	28,632,033	197	145,340.3		
	Total	32,872,490	200	164,362.5		
Lipids: area per μm^2	Between groups	7,671,106	3	2,557,035	13.985	0.000
	Within groups	38,214,723	209	182,845.6		
	Total	45,885,829	212	216,442.6		
Lipids: area of single droplets in μm^2	Between groups	9.896016	3	3.298672	10.321	0.000
	Within groups	66.79549	209	0.319596		
	Total	76.6915	212	0.361752		
Melanomacrophages: number	Between groups	12,359.47	3	4119.824	157.405	0.000
	Within groups	5810.496	222	26.1734		
	Total	18,169.97	225	80.75542		
Melanomacrophages: area per μm^2	Between groups	1.4709×10^{11}	3	49,028,517,949	776.993	0.000
	Within groups	14,008,269,023	222	63,100,310.91		
	Total	1.6109×10^{11}	225	715,972,546.1		
Melanomacrophages: area of single cells in μm^2	Between groups	1.58×10^8	3	52,820,318	39.186	0.000
	Within groups	3.01×10^8	222	1,347,949		
	Total	4.59×10^8	225	2,031,210		

Glycogen variation is summarized in Figure 2 and in the histogram of the stained area in Figure 3. In February (Figure 2(a)) a maximum amount of glycogen is observed, that subsequently decreases in May (Figure 2(b)), whereas it starts increasing again in July (Figure 2(c)) and October (Figure 2(d)). Diastase rendered the hepatocytes PAS negative (an example is given in Figure 2(d), insert), confirming that PAS positivity is due mainly to glycogen.

Lipid droplet variation can be observed in Figure 4 and in the histograms representing variation in number of droplets (Figure 5(a)), total stained area (Figure 5(b)) and mean area of single droplets (Figure 5(c)). Similar to glycogen, the values of the three parameters are higher in February (Figure 4(a)), then decrease in May (Figure 4(b)), and a positive trend follows in July (Figure 4(c)) and October (Figure 4(d)).

The melanomacrophage cycle is shown in Figures 6 and 7(a–c), these being histograms summarizing numbers of cells, total area variation, and mean area of single cells, respectively. In February, small numbers of melanomacrophages are observed (Figure 6(a)), then a marked increase is observed in May (Figure 6(b)) and July (Figure 6(c)), whereas values are much lower in October (Figure 6(d)). Total area and mean area of single cell values show a similar trend.

Discussion

Seasonality in Anura involves several morpho-functional changes of most organs. The liver is a good example, since it plays a central role in a number of processes and metabolic pathways, allowing efficient adaptation to a variable environment. Cyclic storage of liver glycogen, lipids and melanin has been studied in populations that respond to extreme climatic conditions by dormancy, such as *P.* kl. *esculentus*, and was found to vary dramatically (e.g. Milone et al. 1978; Bruscalupi et al. 1989; Fenoglio et al. 1992; Scapin & Di Giuseppe 1994; Barni et al. 1999). Populations of the same species living in milder climates do not hibernate; thus, it might have been expected that their liver does not undergo changes of the same intensity observed in hibernating populations. The population of *P.* kl. *esculentus* in Roseto Capo Spulico contradicts this prediction, because it presents significant variation in the accumulation of hepatic glycogen, lipids and melanomacrophages during the annual cycle. The patterns of each of the cited components share some features with the hibernating populations of the same species, but differ in other features. The absence of histopathologic alterations and the organic farming of the surrounding area suggest that the variation in the cited parameters was not induced by toxicants.

Glycogen reserves in the population of Roseto Capo Spulico increase from summer to fall, similar to what is observed in hibernating populations (e.g. Fenoglio et al. 1992; Scapin & Di Giuseppe 1994). Different from the general hibernating condition (e.g. Dinsmore & Swanson 2008), the accumulated glycogen does not decrease from fall to winter, but

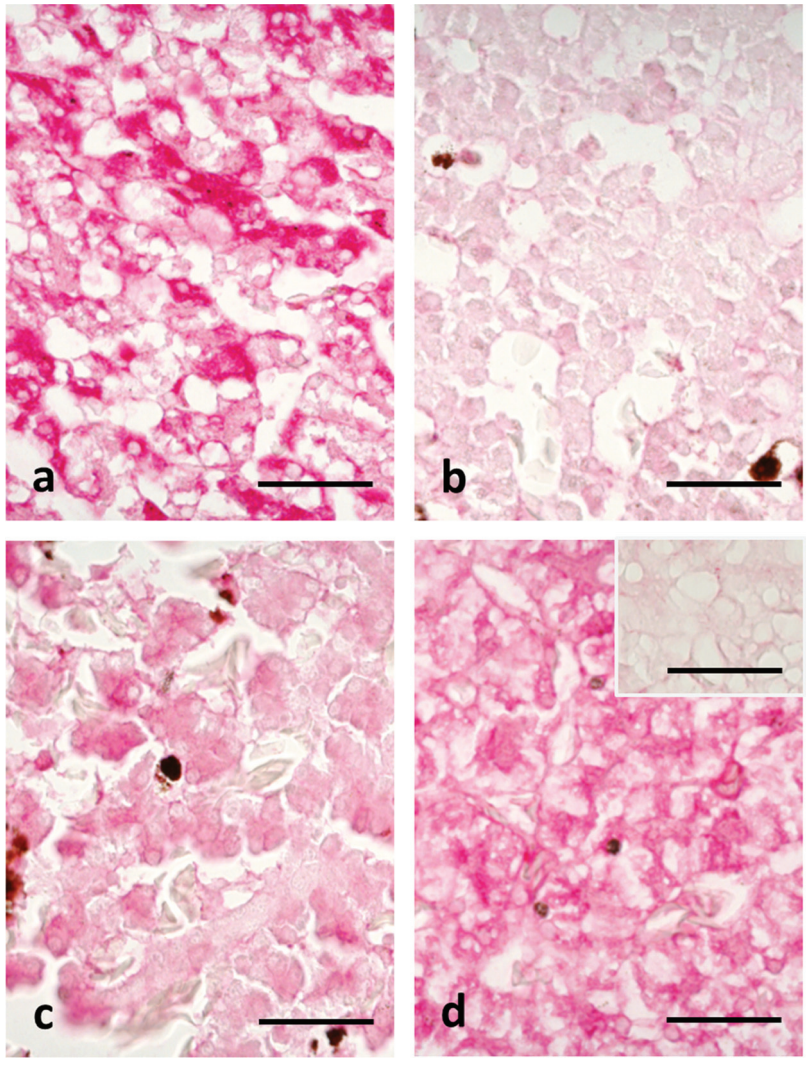

Figure 2. *Pelophylax* kl. *esculentus*, liver, PAS stain. Variation of glycogen (stained red) accumulation in the hepatocytes of individuals collected in the months of February (a), May (b), July (c) and October (d). Insert in (d): PAS positivity is not observed after diastase treatment. Insert in (d): Diastase-PAS. PAS: periodic acid–Schiff. Scale bars = 50 μm.

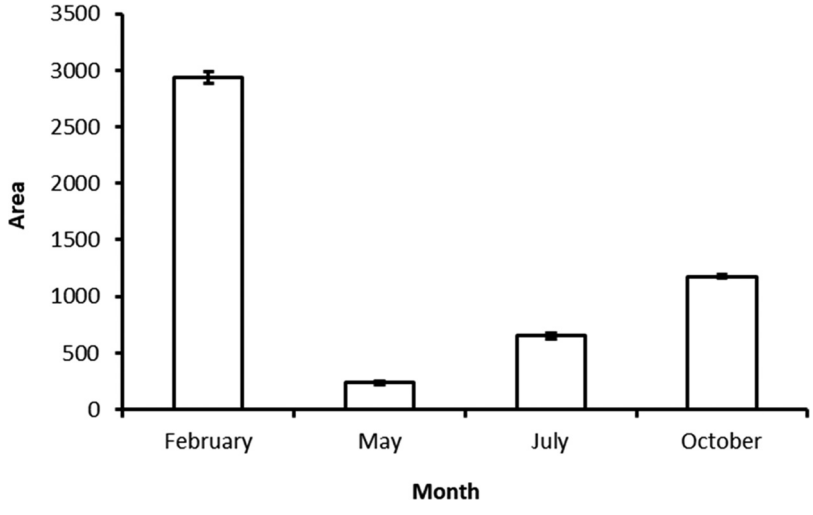

Figure 3. Histogram with standard errors of mean values of area per μm^2 of glycogen per sampled month in the hepatocytes of *Pelophylax* kl. *esculentus*.

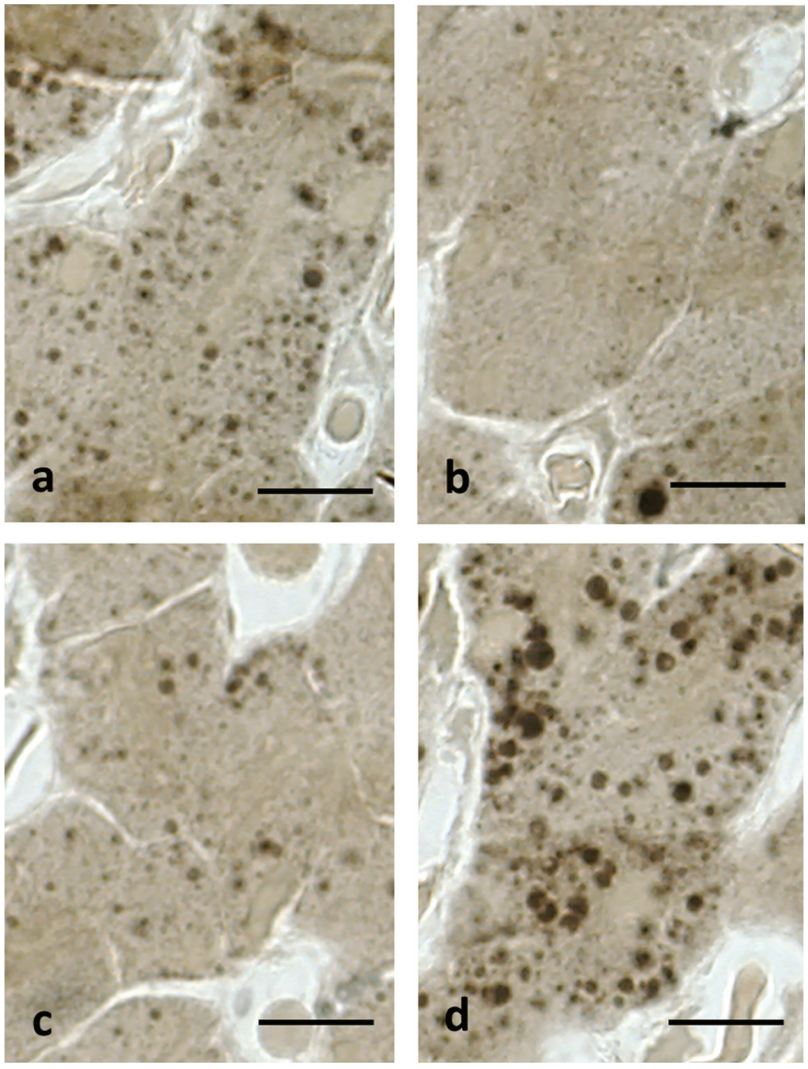

Figure 4. *Pelophylax* kl. *esculentus*, liver, osmium tetroxide stain. Variation of lipid droplet (stained gray-brown) accumulation in the hepatocytes of individuals collected in the months of February (a), May (b), July (c) and October (d). a–d: epoxidic resin-araldite embedding with osmium tetroxide postfixation. Scale bars = 10 μm.

keeps increasing until spring. The patterns of fall storage in hibernating species can follow two models, i.e. continuous increase or critical threshold (Dinsmore & Swanson 2008). In the first model, frogs store glycogen continuously over the fall, depending on the availability of food and favorable climatic conditions, whereas in the second they stop feeding when glycogen reaches a given level, sufficient to survive to hibernation. In the population of Roseto Capo Spulico, the first model seems to hold. As already noted, in hibernating populations with an income-breeder strategy feeding takes place after dormancy prior to reproduction (Chen et al. 2011). Thus, it would seem that since frogs in Roseto Capo Spulico do not hibernate and climate conditions there never become severe, they probably keep on being active and feed in winter, coupling the fall continuous-increase strategy with the spring income-breeder strategy. In this way, the animals can accumulate reserves for the reproductive season throughout the year. The period around reproduction is marked by massive glycogen depletion, and then storage restarts in summer, as has been observed in most spring-reproductive species (e.g. Fenoglio et al. 1992). Similar to the population in our study, Spornitz (1975) found that in the annual cycle of *Xenopus laevis*, the degradation of glycogen took place only in correspondence with vitellogenesis, which he explained by the absence of hibernation.

Lipids follow a cycle similar to that of glycogen: higher values are observed in February, then they

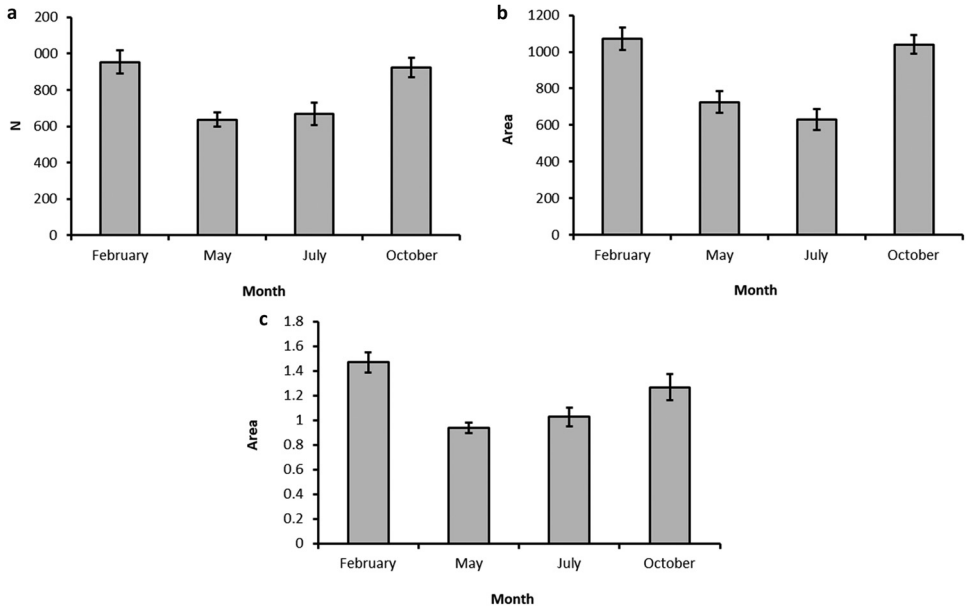

Figure 5. Histogram with standard errors of mean values of number of lipid droplets (a), area per μm^2 of lipids (b), and area of single lipid droplets in μm^2 (c) per sampled month in the hepatocytes of *Pelophylax* kl. *esculentus*.

decrease from May to July and rise again in October. Droplet numbers, total areas and mean areas of droplets appear to co-vary, indicating that the lipid storage is achieved by the increase of both droplet numbers and their size. The lipid cycle observed in Roseto Capo Spulico resembles that of other species facing dormancy (e.g. Byrne & White 1975; Fitzpatrick 1976; Sheridan 1994), but differs from that in other populations of *P.* kl. *esculentus*. Milone et al. (1978) and Bruscalupi et al. (1989) documented by biochemical methods the annual cycle of total lipid content in the liver in *P.* kl *esculentus* from Campania and Tuscany, respectively. They observed a peak in May–June, followed by a decrease in summer and a subsequent increase in fall–winter. In the Campania data set values kept the increasing trend in winter, then declined prior to May, whereas in Tuscany they declined throughout winter until May. Thus, the populations from Campania, Tuscany and Roseto Capo Spulico have different patterns in the lipid cycle; in particular, the last did not present the spring peak. Comparisons of these data are difficult, since they were collected in different decades, areas and climatic conditions by different methods. A possible explanation is that Milone et al. (1978) and Bruscalupi et al. (1989) quantified the total amount of lipids in liver homogenate by biochemical methods; thus, they did not evaluate the relative quantities present in hepatocytes, the vascular system or melanomacrophages. Our evaluation based on image analysis of histological sections focused on the lipids stored only in the hepatocytes, excluding those in the melanomacrophages and the vessels, which cannot strictly be regarded as storage centers. Indeed, the liver is only one of the sites involved in lipid storage in amphibians: considerable amounts are stored in the abdominal fat bodies, the carcass and the gonads (Fitzpatrick 1976). During the annual cycle, the lipid contents of these organs vary in relation to storage, degradation and synthesis processes linked to activities such as dormancy and reproduction, and considerable amounts are exchanged among regions by plasma transport, following routes that can vary seasonally (Singh & Sinha 1989; Milone et al. 1990). In *P.* kl. *esculentus* lipid storage is regarded to be a less important energy source than glycogen (Fenoglio et al. 1992); thus, its main involvement is in lipid metabolism rather than fuelling (Fitzpatrick 1976).

The trend of the annual cycle of melanomacrophages in the population of Roseto Capo Spulico is somewhat opposite to that of the glycogen and lipid storage, even if interpretation of the data is more difficult. Number of cells, total area and mean area of single cell values reach their maximum in May, then decrease in July and reach minimum values in October and February. The observed pattern is different to that observed in other populations of *P.* kl. *esculentus* (Sichel et al. 1981; Corsaro et al. 1990; Barni et al. 1999), in which higher numbers of melanomacrophages and melanin amounts are observed in fall/winter, and lower values are seen in spring/

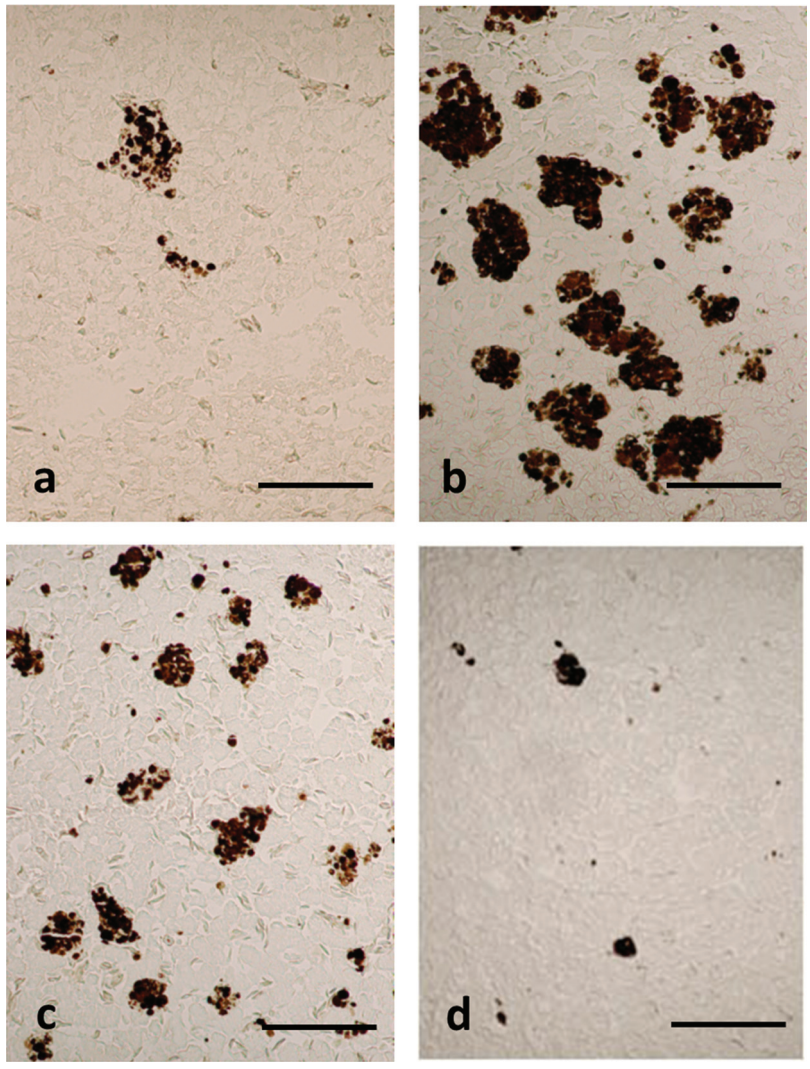

Figure 6. *Pelophylax* kl. *esculentus*, liver, no stain. Variation of melanomacrophage (appearing black due to melanin content) number in the liver of individuals collected in the months of February (a), May (b), July (c) and October (d). a–d: paraffin embedding without stain. Scale bars = 50 μm.

summer. In hibernating frogs, proliferation, hypertrophy and melanin synthesis increase the melanomacrophage area of the liver, and an inverse relation is observed in the glycogen storage by the hepatocytes (Barni et al. 1999). Melanomacrophages can play a role similar to that of hepatocytes, since they are involved in phagocytic and melanogenic processes (Zuasti et al. 1998; Purrello et al. 2001) and their melanins participate to inactivate cytotoxic substances such as ions and free radicals (Scalia et al. 1990). Thus, since during hibernation hepatocytes reduce their metabolism, the increase of melanomacrophages can help in keeping at a constant level the cited protective functions (Barni et al. 1999). The lack of dormancy in the population of Roseto Capo Spulico could explain why the increase of melanomacrophages is not observed, since hepatocytes do not reduce their metabolic activities. As far as the peak observed in May, it is probably linked to reproductive activities. As noted previously, in the Anura during the reproductive process the liver storage of glycogen, lipids and other substances is used for energetically costly activities (such as migration, calling, mating, searching for a deposition site) or in the synthesis of substances involved in the formation of gametes and their involucres (e.g. Assisi et al. 1999; Cadeddu & Castellano 2012; Cayuela et al. 2014; Mentino et al. 2014 and references therein). Some hepatic activities linked to reproduction, such as vitellogenesis, can lead to degenerative changes of hepatocytes following an apoptotic process (Assisi et al. 1999). Since melanins are known to mediate

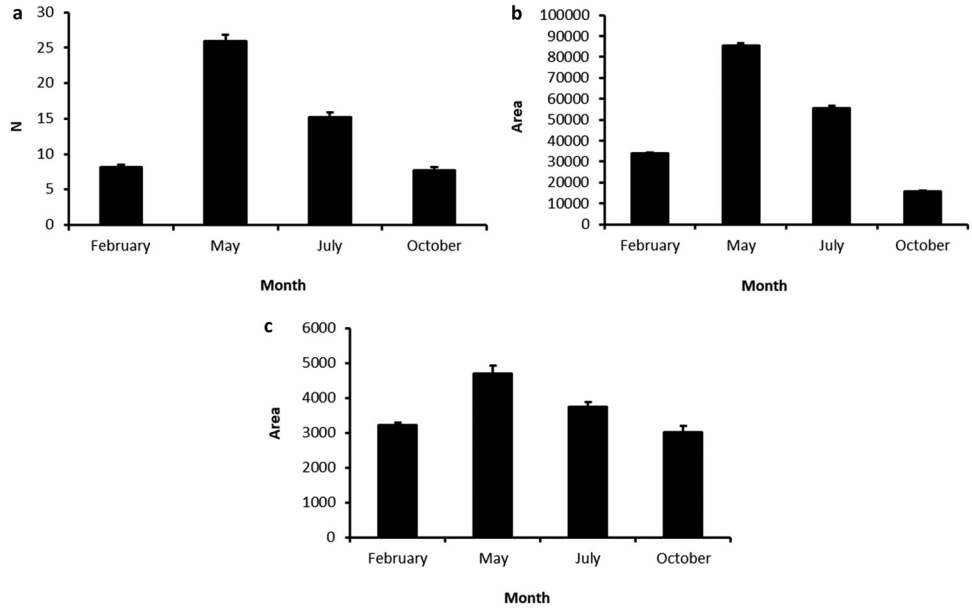

Figure 7. Histogram with standard errors of mean values of number of melanomacrophages (a), area per μm^2 of melanomacrophages (b), and area of single melanomacrophages in μm^2 (c) per sampled month in the liver of *Pelophylax* kl. *esculentus*.

apoptosis (Purrello et al. 2001; Barni et al. 2002), it could be hypothesized that the peak of melanomacrophages we observed in May is linked to programmed degenerative processes of the hepatocytes during reproductive, melanin-mediated activities.

In conclusion, the data in this study confirm the plasticity of liver and its capability to adapt to extremely different climatic conditions even among populations of the same species. Marked seasonality was observed even in a non-hibernating population, in which the main forces driving storage variation seem to be linked to reproduction. Further studies should investigate the molecular mechanism responsible for the functional shifts of hepatocytes and melanomacrophages and how environmental factors affect them.

ORCID

G. Scillitani http://orcid.org/0000-0002-5888-7906

References

Assisi L, Autuori F, Botte V, Farrace MG, Piacentini M. 1999. Hormonal control of "tissue" transglutaminase induction during programmed cell death in frog liver. Experimental Cell Research 247:339–346. doi:10.1006/excr.1998.4358

Barni S, Bertone V, Croce AC, Bottiroli G, Bernini F, Gerzeli G. 1999. Increase in liver pigmentation during natural hibernation in some amphibians. Journal of Anatomy 195:19–25. doi:10.1046/j.1469-7580.1999.19510019.x

Barni S, Vaccarone R, Bertone V, Fraschini A, Bernini F, Fenoglio C. 2002. Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. Journal of Anatomy 200:185–194. doi:10.1046/j.0021-8782.2001.00011.x

Bruscalupi G, Castellano F, Scapin S, Trentalance A. 1989. Cholesterol metabolism in frog (*Rana esculenta*) liver: Seasonal and sex-related variations. Lipids 24:105–108. doi:10.1007/BF02535245

Byrne JJ, White RJ. 1975. Cyclic changes in liver and muscle glycogen, tissue lipid and blood glucose in a naturally occurring population of *Rana catesbeiana*. Comparative Biochemistry and Physiology. A Comparative Physiology 50:709–715.

Cadeddu G, Castellano S. 2012. Factors affecting variation in the reproductive investment of female treefrogs, *Hyla intermedia*. Zoology 115:372–378. doi:10.1016/j.zool.2012.04.006

Capula M, Sacchi R, Razzetti E. 2007. *Pelophylax* klepton *esculentus* (Linnaeus, 1758). In: Lanza B, Andreone F, Corti C, Razzetti E, editors. Fauna d'Italia. 42. Bologna MA: Amphibia. Bologna: Calderini. pp. 386–392.

Cayuela H, Besnard A, Bonnaire E, Perret H, Rivoalen J, Miaud C, Joly P. 2014. To breed or not to breed: Past reproductive status and environmental cues drive current breeding decisions in a long-lived amphibian. Oecologia 176:107–116. doi:10.1007/s00442-014-3003-x

Chen W, Zhang L-X, Lu X. 2011. Higher pre-hibernation energy storage in anurans from cold environments: A case study on a temperate frog *Rana chensinensis* along a broad latitudinal and altitudinal gradients. Annales Zoologici Fennici 48:214–220. doi:10.5735/086.048.0402

Claps P, Sileo C. 2001. Caratteri termometrici dell'Italia meridionale. L'Acqua 5:23–31.

Corsaro C, Scalia M, Sinatra F, Sichel G. 1990. Circannual rhythm of the melanin content in frog liver (*Rana esculenta* L.). Pigment Cell Research 3:120–122. doi:10.1111/pcr.1990.3.issue-2

Costantini EAC, Fantappié M, L'Abate G. 2013. Climate and pedoclimate of Italy. In: Costantini EAC, Dazzi C, editors. The soils of Italy. Dordrecht: Springer Science+Business Media. pp. 19–37.

Costanzo JP, do Amaral MC, Rosendale AJ, Lee Jr. RE. 2013. Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog. Journal of Experimental Biology 216:3461–3473. doi:10.1242/jeb.089342

Crawshaw GJ, Weinkle TK. 2000. Clinical and pathological aspects of the amphibian liver. Seminars in Avian and Exotic Pet Medicine 9:165–173.

Dinsmore SC, Swanson DL. 2008. Temporal patterns of tissue glycogen, glucose, and glycogen phosphorylase activity prior to hibernation in freeze-tolerant chorus frogs, *Pseudacris triseriata*. Canadian Journal of Zoology 86:1095–1100. doi:10.1139/Z08-088

Fenoglio C, Bernocchi G, Barni S. 1992. Frog hepatocyte modifications induced by seasonal variations: A morphological and cytochemical study. Tissue Cell 24:17–29. doi:10.1016/0040-8166(92)90077-K

Fitzpatrick LC. 1976. Life history patterns of storage and utilization of lipids for energy in amphibians. American Zoologist 16:725–732. doi:10.1093/icb/16.4.725

Frangioni G, Borgioli G. 1994. Hepatic respiratory compensation and blood volume in the frog (*Rana esculenta*). Journal of Zoology 234:601–611. doi:10.1111/j.1469-7998.1994.tb04867.x

Irwin JT, Lee JRE. 2003. Geographic variation in energy storage and physiological responses to freezing in the gray treefrogs *Hyla versicolor* and *H. chrysoscelis*. Journal of Experimental Biology 206:2859–2867. doi:10.1242/jeb.00500

Jelodar HT, Fazli H. 2012. Monthly changes in condition, hepatosomatic index and bioavailability in frogs (*Rana ridibunda*). Research Journal of Biology 2:9–14.

Jönsson KI, Herczeg G, O'Hara RB, Söderman F, Ter Schure AFH, Larsson P, Merilä J. 2009. Sexual patterns of prebreeding energy reserves in the common frog *Rana temporaria* along a latitudinal gradient. Ecography 32:831–839. doi:10.1111/j.1600-0587.2009.05352.x

Lillie RD, Greco J. 1947. Malt diastase and ptyalin in place of saliva in the identification of glycogen. Stain Technology 22:67–70. doi:10.3109/10520294709108296

Loumbourdis NS. 2005. Hepatotoxic and nephrotoxic effects of cadmium in the frog *Rana ridibunda*. Archives of Toxicology 79:434–440. doi:10.1007/s00204-005-0652-x

Mentino D, Mastrodonato M, Rossi R, Scillitani G. 2014. Histochemical and structural characterization of egg extra-cellular matrix in bufonid toads, *Bufo bufo* and *Bufotes balearicus*: Molecular diversity versus morphological uniformity. Microscopy Research and Technique 77:910–917.

Milone M, Caliendo MF, Rastogi RK, Chieffi G. 1983. Seasonal lipid composition in the liver, fat body and gonads of *Rana esculenta*. Italian Journal of Zoology 50:227–234.

Milone M, Caliendo MF, Rastogi RK, Varriale B, Pierantoni R, Chieffi G. 1990. Temporal pattern of labeling of liver, blood, fat body and testis lipids in *Rana esculenta*. Italian Journal of Zoology 57:125–130.

Milone M, Iela L, Esposito V, Rastogi RK, Chieffi G. 1978. Annual variations in the total lipid and protein content of the liver, fat body, ovary and plasma of the female frog (*Rana esculenta* L.). Journal of Endocrinology 78:165–169.

Pasanen S, Koskela P. 1974. Seasonal and age variation in the metabolism of the common frog, *Rana temporaria* L. in northern Finland. Comparative Biochemistry and Physiology. A Comparative Physiology 47:635–654.

Păunescu A, Ponepal CM, Drăghici O, Marinescu AG. 2010. Liver histopathologic alterations in the frog *Rana (Pelophylax) ridibunda* induce by the action of reldan 40EC insecticide. Analele Universităţii Din Oradea – Fascicula Biologie 17:166–169.

Purrello M, Scalia M, Corsaro C, Di Pietro C, Piro S, Sichel G. 2001. Melanosynthesis, differentiation, and apoptosis in Kupffer cells from *Rana esculenta*. Pigment Cell Research 14:126–131.

Rasband W. 2016. Image J. Bethesda, MD: US National Institutes of Health. Available: https://imagej.nih.gov/ij/. Accessed Jul 2017 26.

Scalia M, Geremia E, Corsaro C, Santoro C, Baratta D, Sichel G. 1990. Lipid peroxidation in pigmented and unpigmented liver tissues: Protective role of melanin. Pigment Cell Research 3:115–119.

Scapin S, Di Giuseppe G. 1994. Seasonal variations of glycogen synthase and phosphorylase activities in the liver of the frog *Rana esculenta*. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 107:189–195.

Scillitani G, Mentino D, Liquori GE, Ferri D. 2012. Histochemical characterization of the mucins of the alimentary tract of the grass snake, *Natrix natrix* (Colubridae). Tissue Cell 44:288–295.

Scillitani G, Moramarco AM, Rossi R, Mastrodonato M. 2011. Glycopattern analysis and structure of the egg extra-cellular matrix in the Apennine yellow-bellied toad, *Bombina pachypus* (Anura: Bombinatoridae). Folia Histochemica Et Cytobiologica 49:306–316.

Sheridan MA. 1994. Regulation of lipid metabolism in poikilothermic vertebrates. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 107:495–508.

Sichel G, Brai M, Palminteri M, Sciuto S. 1981. Seasonal dependence of ESR features of frog melanins. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 70:611–613.

Singh RP, Sinha RC. 1989. Seasonal changes in energy reserves in the common frog, *Rana tigrina*. Japanese Journal of Physiology 39:969–973.

Spornitz UM. 1975. Studies on the liver of *Xenopus laevis*. I. The ultrastructure of the parenchymal cell. Anatomy and Embryology 146:245–264.

Storey KB, Storey JM. 2005. Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: The wood frog. In: Benson E, Fuller B, Lane N, editors. Life in the frozen state. Boca Raton: CRC Press. pp. 243–274.

Thammachoti P, Khonsue W, Kitana J, Varanusupakul P, Kitana N. 2012. Morphometric and gravimetric parameters of the rice frog *Fejervarya limnocharis* living in areas with different agricultural activity. Journal of Environmental Protection 3:1403.

Withers PC, Hillman SS. 2001. Allometric and ecological relationships of ventricle and liver mass in anuran amphibians. Functional Ecology 15:60–69.

Zaiontz C. 2015. Real statistical analysis using Excel. Available: http://www.real-statistics.com/. Accessed Jul 2017 26.

Zhelev Z, Popgeorgiev G, Mehterov N. 2015. Changes in the hepatosomatic index and condition factor in the populations of *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria. Part II. Bulgarian Journal of Agriculture Science 21:534–539.

Zuasti A, Jimenez-Cervantes C, Garcia-Borron JC, Ferrer C. 1998. The melanogenic system of *Xenopus laevis*. Archives of Histology and Cytology 61:305–316.