

Increase in liver pigmentation during natural hibernation in some amphibians

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ABSTRACT

The amount/distribution of liver melanin in 3 amphibian species (*Rana esculenta*, *Triturus a. apuanus*, *Triturus carnifex*) was studied during 2 periods of the annual cycle (summer activity–winter hibernation) by light and electron microscopy, image analysis and microspectrofluorometry. The increase in liver pigmentation (melanin content) during winter appeared to be correlated with morphological and functional modifications in the hepatocytes, which at this period were characterised by a decrease in metabolic activity. These findings were interpreted according to the functional role (e.g. phagocytosis, cytotoxic substance inactivation) played by the pigment cell component in the general physiology of the heterothermic vertebrate liver and, in particular, in relation to a compensatory engagement of these cells against hepatocellular hypoactivity during the winter period.

Key words: Melanin; seasonal variation.

INTRODUCTION

In heterothermic vertebrates, extracutaneous melanin-containing cells may be found in various tissues and organs such as the kidney, liver, spleen, lungs, etc. (Oppel, 1889, 1900; Verne, 1926; Sichel, 1988; Cicero et al. 1989; Zuasti et al. 1990). In particular, the pigment cells of the liver are localised mainly in the sinusoid walls and possess autonomous melano-synthetic activity (Scalia et al. 1988). These cells are considered to belong to the reticulohistiocytic system (also defined as the mononuclear phagocytic system) and to derive from Kupffer cells based on their localisation and phagocytic capacity. For these reasons they are also defined melanomacrophages (Agius, 1980; Zuasti et al. 1990; Christiansen et al. 1996; Rund et al. 1998). The melanins of the liver pigment cells seem to play an important role as scavengers of cytotoxic substances such as ions and free radicals (Sichel, 1988; Zuasti et al. 1989).

Moreover, the liver melanin content has been found not to be stable during the year in amphibians (Berg, 1914; Kremer 1932; De Robertis 1938; Corsaro et al. 1977, 1990) and to increase with age in reptiles (Christiansen et al. 1996).

In previous studies, we demonstrated marked morphological and functional changes (e.g. decrease in metabolism and increase of inclusion material) in the hepatocytes in some amphibian species during the annual cycle, particularly in relation to the conditions of natural hibernation (Barni & Bernocchi, 1991; Fenoglio et al. 1992; Barni et al. 1994). On the basis of these findings, it seemed interesting to examine some modifications of the pigmentary component that, in the liver of amphibians, has been demonstrated to be functionally coupled with liver parenchymal cells (Sichel et al. 1987). We report here some data on hepatic melanin accumulation during adaptation to natural hibernation in an anuran species (*Rana esculenta*) and 2 urodele species (*Triturus a. apuanus* and *Triturus carnifex*).

MATERIAL AND METHODS

Animal collection

Adult male individuals (6 for each species) of *Rana esculenta*, *Triturus a. apuanus* and *Triturus carnifex* were caught in their natural environment in June/July (mean environmental temperature 21.4 °C) and December/January (mean environmental temperature 0.3 °C) of 2 successive years. These 2 periods of the year were selected on the basis of previous investigations on morphological and functional changes in hepatocytes (Fenoglio et al. 1992). *Rana esculenta* (total length 40–65 mm, weight 7–28 g) were collected during summer from rice field drains and during winter from terrestrial hibernation sites in the countryside near Pavia (northern Italy). *Triturus a. apuanus* (total length 70–85 mm, weight 2.5–3.5 g) and *Triturus carnifex* (total length 110–130 mm, weight 8.5–12.5 g) were collected in permanent ponds at 700 m above sea level in the Appennine mountains (province of Pavia, northern Italy). The animals were killed under anaesthesia with MS 222 (Sandoz) and the livers immediately removed and excised for optical, ultrastructural and microspectrofluorometric studies.

Image analysis of pigment amount/distribution

Liver samples frozen by liquid nitrogen and stored at –80 °C were cut in 8 µm thick sections by a Reichert-Jung 2800 Frigocut cryostat. Weakly haematoxylin-eosin stained specimens were submitted to the evaluation of pigment content expressed as relative size (percentage) of the area of the liver sections occupied by the melanic material. The measurements, performed by a VIDAS 2.1 (Zeiss-Kontron) image analysis system in randomly chosen microscope fields (30 for each individual), both in the parenchymal and haematopoietic regions of the liver, were expressed graphically as mean values ± s.d. The changes between active and hibernating phases, in the same animal species, were checked by Student's *t* test. A value of $P < 0.05$ was accepted for statistical significance.

Microspectrofluorometry of tissue autofluorescence

Autofluorescence emission spectral analysis was performed on cryostat sections of liver parenchyma, both in the pigmented (melanomacrophage) and unpigmented (hepatocyte) areas, by means of a Leitz microspectrograph equipped with an optical multichannel analyser (OMA III, PAR-Princeton

Instruments, Princeton NJ, USA). Measurements were performed under epi-illumination conditions using a TK 405 nm dichroic mirror. A 100 W mercury lamp was used as an excitation source. The excitation wavelength, at 366 nm, was selected by means of an interference filter (366 nm, Hbw = ± 5 nm, T = 40%). The fluorescence signal of the sample, collected by the microscope optics, was displayed linearly by a grating and focused onto a 512 intensified-photodiode linear array, as a detector. The emission spectra were recorded in the 420–700 nm spectral range. Measuring time did not exceed 1.0 s, an interval time during which no spectral distortion attributable to a photo-decomposition effect was observed.

Transmission electron microscopy

Fragments of liver parenchyma were fixed for 3 h at 4 °C in 1.5% glutaraldehyde buffered at pH 7.4 with 0.07 M cacodylate solution, washed in 0.1 M cacodylate buffer containing 7% sucrose, postfixed for 1 h at 4 °C in 1% osmium tetroxide buffered at pH 7.4 with 0.1 M phosphate solution, dehydrated in graded ethanol solutions, and embedded in Epon 812. Semithin sections (1 µm) were stained with 1% borated methylene blue. Ultrathin sections (~60 nm thick) were contrasted with saturated uranyl acetate in 50% acetone and Reynold's lead citrate solution. The specimens were examined in a Zeiss TEM 900 electron microscope at 80 kV.

RESULTS

By light and electron microscopy it was observed that the liver pigment cells, also named melanin-containing cells or melanomacrophages, were localised both in the parenchymal area, at sinusoidal level, and in the haematopoietic component of the liver (Figs 1*a, c*; 2*a, b*). In the haematopoietic tissue, which in the newt liver is mainly localized in the perihepatic subcapsular region (Fig. 1*a*), a close contact between pigment cells, which were dendritic-shaped, and myeloid cells was found (Fig. 2*b*).

Differences in the amount and distribution of the liver melanin, quantified by the image analysis technique were found between the amphibian species considered and as a consequence of the transition from active to hibernating phase (Figs 1, 3). During the same phase of the annual cycle (either activity or hibernation) the extension of liver pigmented areas, as expressed by the melanin content, was found to be

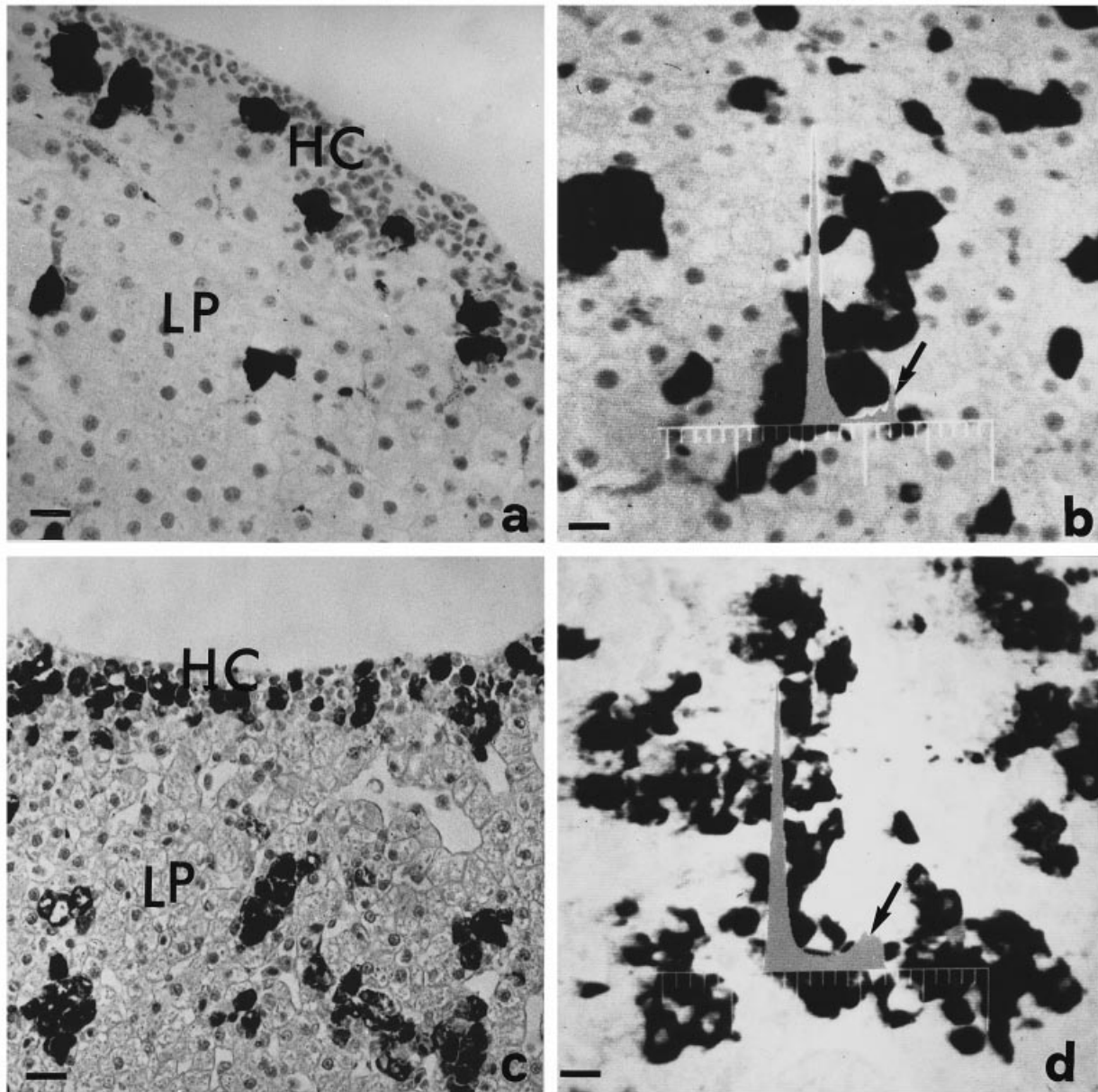


Fig. 1. Differing content/distribution of the melanin containing cells in the liver of *Triturus carnifex* during the active (*a, b*) and hibernating (*c, d*) phases. In *b* and *d*, the computerised images of the grey level distribution histograms have been superimposed on the tissue section images (the arrows label the part of histograms related to the pigmented area); LP, liver parenchyma; HC haematopoietic subcapsular tissue. (Bars: *a, b*, 30 μm ; *c, d*, 25 μm).

much higher (about 9 times) in newts (*Triturus carnifex*, *Triturus a. apuanus*) than in the frog (*Rana esculenta*) (Fig. 3). In winter, the amount of melanin in the liver of the 3 amphibian species examined was found to increase significantly both in the parenchymal and the haematopoietic areas (Figs 1, 3). Nevertheless, this increase was more intense in the frog (about 200%) than in newts (about 40%).

Ultrastructural observations revealed that during hibernation, pigment cells contained melanosomes that were less homogeneous in size and more scattered, with large amorphous cytoplasmic areas (Fig. 2*c, d*).

Conversely, during the active phase, numerous phagosomes, also containing melanosomes, were found (Fig. 2*c*).

The microspectrofluorometric analysis of the pigment cells confirmed the heterogeneity of the melanosome composition. The autofluorescence emission covered the 500–600 nm spectral range and no differences of the pattern were found either in relation to the animal species or to the annual period (Fig. 4). On the other hand, evident differences of the autofluorescence properties were detected in the parenchymal liver component in the 3 amphibian

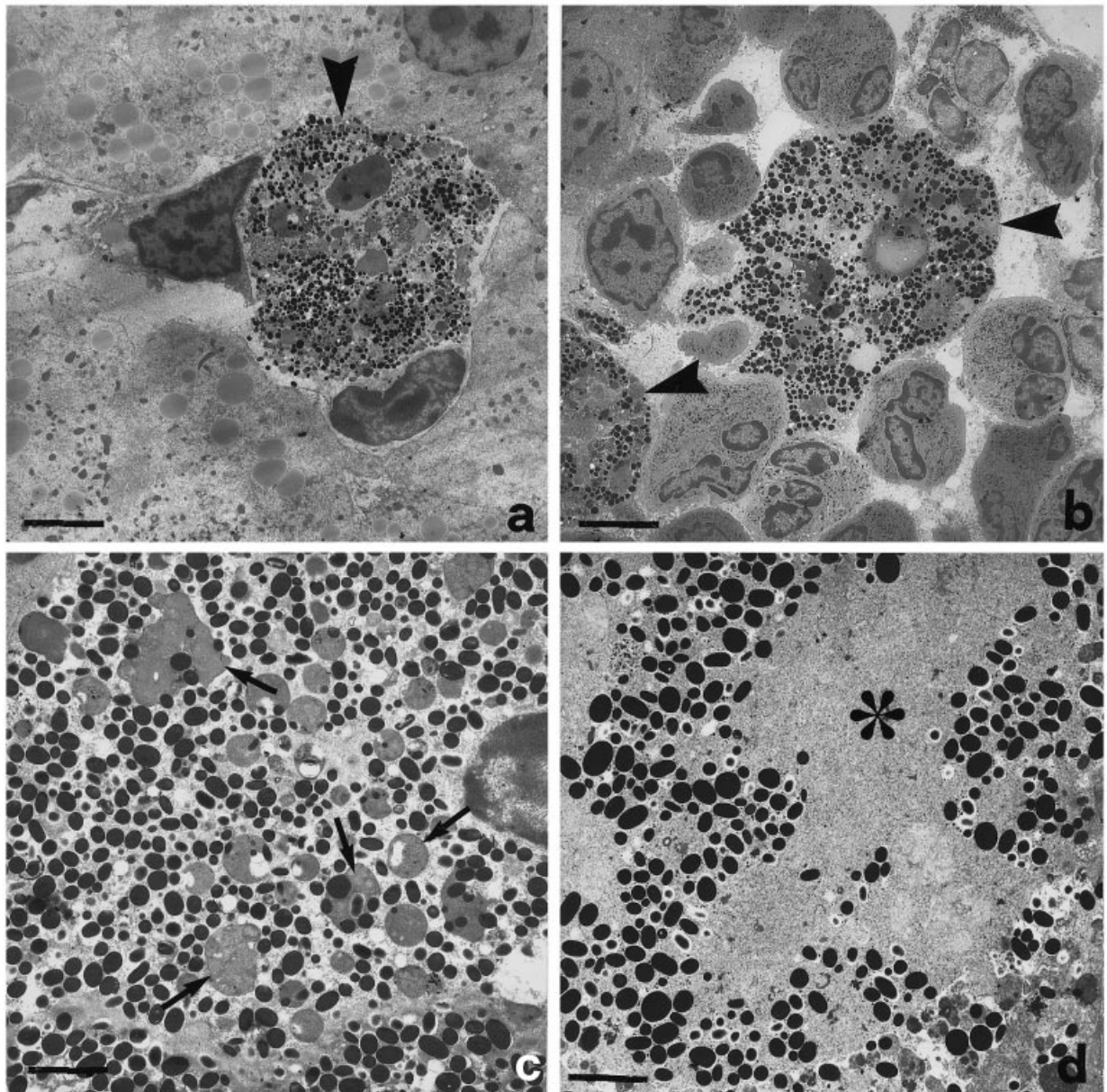


Fig. 2. Electron micrographs of the liver of *Triturus a. apuanus*. (a, b) Pigment cells (arrowheads) in the parenchymal (sinusoid) and haematopoietic (subcapsular space) areas respectively. (c, d) Differing distributions of melanosomes in the pigment cells during activity (c) and hibernation (d). The arrows indicate the presence of numerous phagocytic bodies during the active phase (c); the asterisk labels a wide amorphous area of the cytoplasm during winter (d). Bars: a, b, 10 μ m; c, 2.5 μ m; d, 2 μ m.

species: the most intense spectral modifications in relation to hibernation were found in *Rana esculenta* (Fig. 4).

DISCUSSION

The morphological and morphometric analysis performed on the amphibian liver during the 2 characteristic phases of the annual cycle (activity and hibernation) has provided additional information on the role of the melanin pigment cells in the hepatic physiology of heterothermic vertebrates.

From a comparative point of view, an intriguing observation was the different melanin content between the frog (*Rana esculenta*) and newts (*Triturus a. apuanus*, *Triturus carnifex*). This situation could be explained by taking into account (1) some features of the pigment cells in the liver of heterothermic vertebrates and (2) some morphological and functional differences between the hepatocytes of frogs and newts. The pigment cells present in the liver of fishes, amphibians and reptiles belong to the extracutaneous pigment system (Breathnach, 1988); they derive from Kupffer cells and show melanosynthetic activity

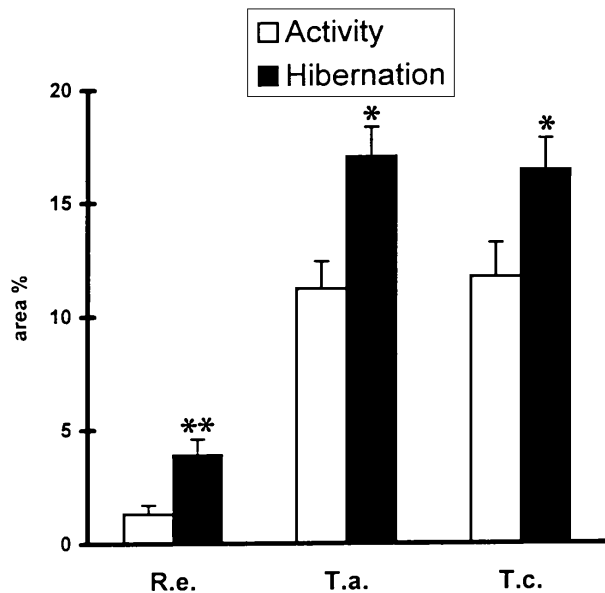


Fig. 3. Pigment content of the liver parenchyma in *Rana esculenta* (R.e.), *Triturus a. apuanus* (T.a.) and *Triturus carnifex* (T.c.) during the 2 phases of the annual cycle. The data are expressed as mean \pm S.D. * $P < 0.01$, ** $P < 0.001$ (Student's *t* test).

(Scalia et al. 1988; Sichel, 1988). The physiological role of these cells in the liver parenchyma of lower vertebrates, in addition to their phagocytic activity (Sichel et al. 1997), is to act, thanks to the defensive properties of melanin, against endogenous and exogenous cytotoxic substances (Scalia et al. 1990; Sichel et al. 1994). This provides an alternative to enzymatic activity (e.g. superoxide dismutase, catalase, etc.), also performed in hepatic cells (Barni et al. 1994). An increase in liver melanomacrophages has also been found in association with a variety of infective diseases (Vogelbein et al. 1987).

A close functional integration over some biochemical mechanisms is thus present between pigment cells and hepatocytes. As newt hepatocytes, compared with frog hepatocytes, are characterised by a wide range of cytoplasmic inclusion (e.g. glycogen masses, lipid droplets), a reflection of metabolically active cytoplasm (e.g. ribosomes, endoplasmic reticulum, mitochondria, etc.) (Barni & Bernocchi, 1991; Fenoglio et al. 1992; Barni et al. 1994), it is possible that this morphological and functional situation could be balanced by a larger number of pigment cells that, in certain metabolic activities, may replace the hepatocytes. In this connection, an inverse correlation between melanin content and mitochondrial superoxide dismutase activity has been established in the liver of various vertebrate taxa (Sichel et al. 1987).

The increase in the pigmented area in the liver during hibernation is a consequence of different mechanisms that involve melanosynthesis, hypertro-

phy and proliferation of melanohistiocytic cells during prehibernation (Barni et al. 1997). This extension of liver pigmentation, in the sequence of active vs hypoactive phases, appears to correlate with functional modifications in the morphology of the liver parenchymal cells that during the winter are characterised by a decrease in metabolic activity and an increase in storage of trophic material, which is more intense in the frog than in newts (Barni & Bernocchi 1991; Fenoglio et al. 1992; Barni et al. 1994). Significant changes of the general metabolic activity have been found during the annual cycle particularly in anurans (Smith, 1950; Mizell, 1965; Rosenkilde & Jorgensen, 1977).

The prominent morphological changes found in hepatocytes during the frog annual cycle were confirmed by the results obtained with microspectrofluorometric analysis. The autofluorescence spectral shapes, recorded on liver parenchymal cells in the 2 annual periods, change in the relative amplitude of the emission in the 440–520 nm range that is known to be mostly attributable to endogenous fluorophores involved in cell metabolism, such as nicotinamide adenine dinucleotide and flavine adenine dinucleotide (Aubin, 1979; Croce et al. 1996); this indicates a decrease in the energy metabolism engagement of hepatocytes during winter.

Microspectrofluorometric analysis performed on pigment cells confirmed the heterogeneity of the melanosome composition. The autofluorescence emission covered the 500–600 nm spectral range and appeared to be a combination of several emission bands. The relative amplitudes of these emission bands varied, indicating the presence of a highly heterogeneous group of substances differing in basic structure, degree of polymerisation and in the nature and amount of material included in the polymerising mass (Wolman, 1980). We also believe it is possible that changes in liver pigment content during hibernation are not accompanied by various structural modifications of the melanin polymer as revealed by the spectral analysis of the autofluorescence. Nevertheless, a more detailed analysis of autofluorescence patterns, mainly based on microspectrofluorometric and ratio imaging techniques, is required to define the relationship between melanosome autofluorescence properties and the annual cycle phase. Evidence of seasonal modifications of melanin has already been obtained, although in isolated material from frog liver, by electron spin resonance analysis (Sichel et al. 1981).

A different functional involvement of the liver pigment cells during the annual cycle may be deduced

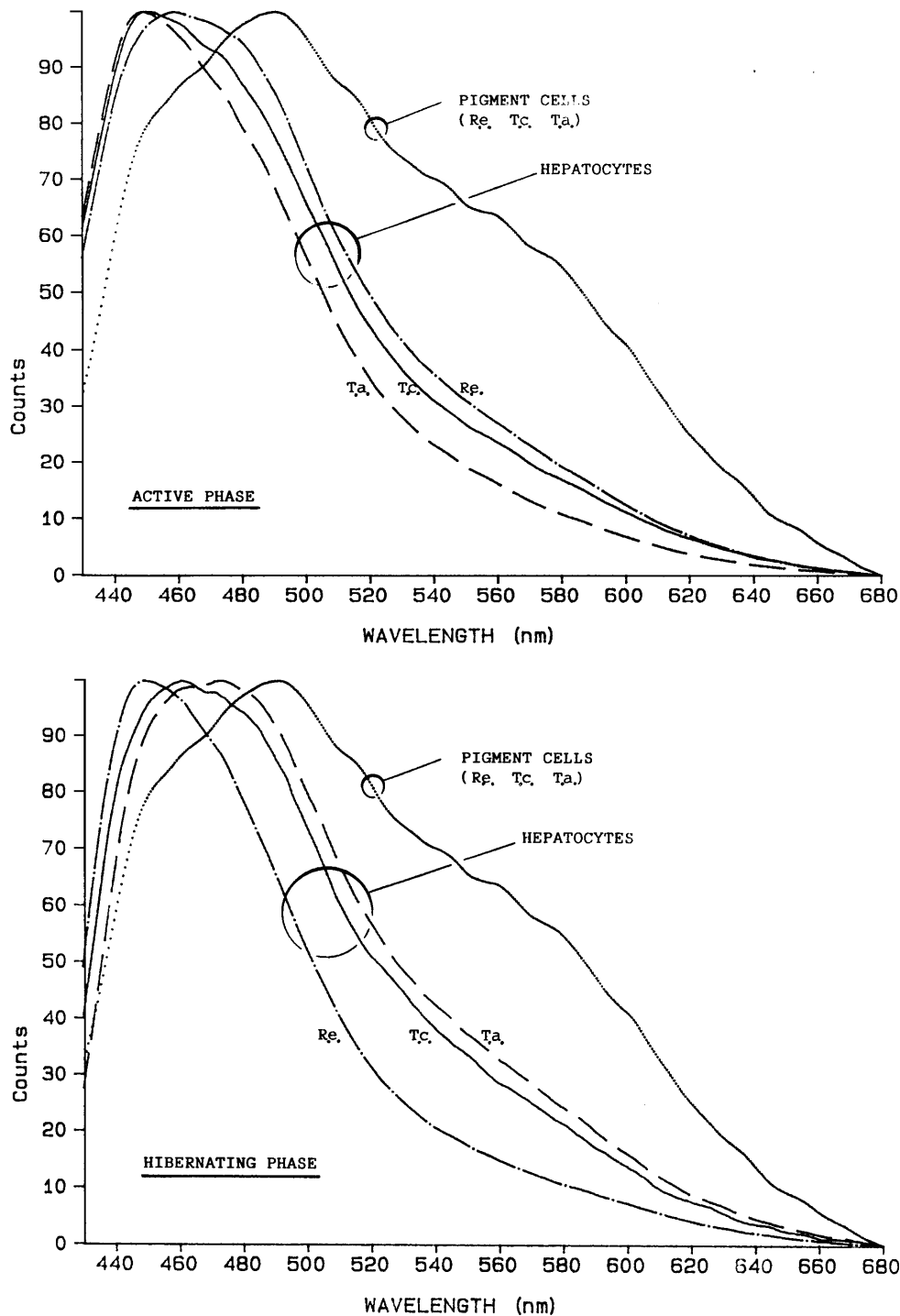


Fig. 4. Examples of autofluorescence emission spectra of the liver pigment cells and hepatocytes of *Rana esculenta* (R.e.), *Triturus a. apuanus* (T.a.) and *Triturus carnifex* (T.c.) during the 2 periods of the annual cycle. Note the unchanged pattern of the pigment autofluorescence emission spectra in the 3 amphibian species and in relation to the 2 periods.

from the larger number of cytoplasmic phagosomes present during the active phase as an expression of destruction and recycling of endogenous material (e.g. more active red cell removal and melanosome degradation) (Barni et al. 1993; Sichel et al. 1997).

In conclusion, the results of the present study can be interpreted by considering the role of pigment cells (melanomacrophages) in the changes of the liver

physiology of heterothermic vertebrates as a consequence of marked modifications of the environmental conditions (e.g. temperature, food availability) during the annual cycle (seasonal changes). The modifications in the melanin content and consequently in the melanomacrophages is certainly in connection with morphological and functional plasticity of the hepatocellular component.

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