Reçu le 14 juin 1989.

Use of the biochemical analysis of muscle proteins to help the classification of polychromic individuals of the genus Symphodus

BY

B. FOCANT (1), C. MICHEL (2) and P. VANDEWALLE (3)

[(1) *Biologie Cellulaire et Tissulaire, Institut d'Anatomie;* (2) *Unité de Recherches en Ethologie Marine, Aquarium et* (3) *Laboratoire de Morphologie Fonctionnelle, Institut de Zoologie, Université de Liège; Liège, Belgique*]

(2 figures)

Among Labrid fishes (Wrasses), polychromy is very frequent. Indeed, a few species of *Symphodus* show individuals with a peculiar colouration.

On one hand, electrophoretic analysis of muscular proteins (myosin and parvalbumins) reveals no differences between the normal individuals and the coloured morphs of the three species. On the other hand, these analyses display disparities between fishes of the two subgenus (*Crenilabrus* and *Symphodus*). Biochemical characters seem to have evolved in the same way that morphological and behavioural ones : the subspecies based only on differences in colouration are not confirmed but the differences between subgenus are revealed.

Introduction

Among fish muscle proteins, sarcoplasmic parvalbumins are acid low molecular weight (12 KD) calcium-binding proteins relatively abundant (0.5 mM/kg of fresh muscle) in fast white muscle (FOCANT & PECHÈRE, 1965; HAMOIR & GERARDIN-OTTHIERS, 1980). They can exist in 5 isoforms differing by their electric charge and usually characteristic of the species (BUSHANA RAO *et al.*, 1969; PECHÉRE *et al.*, 1973).

The main myofibrillar protein, the myosin, is an hexamere and is composed of 2 heavy chains (HC, 220 KD) and of 3 types of light chains (LC_1, LC_2, Lc_3, 16 to 25 KD) in fish white muscles (GAZITH *et al.*, 1970; HURIAUX & FOCANT, 1977). The analysis of myosin light chains from various marine (FOCANT *et al.*, 1976; FOCANT *et al.*, 1985; FOCANT & PEQUEUX, 1985) and fresh water (HURIAUX & FOCANT, 1985; ROWLERSON *et al.*, 1985; FOCANT & VANDEWALLE, 1989) fish species also puts forwards a variability as much in electric charge as in molecular weight of these small subunits according to the species.

Using the high resolution polyacrylamide gel electrophoretic methods, we succesfully scanned the polymorphism of these proteins to discriminate closely related species of mediterranean *Serranidae* and *Gobiidae* (FOCANT *et al.*, 1988; FOCANT & JOYEUX, 1988).

B.F. and P.V. are « Research Associate » of the « Fonds National de la Recherche Scientifique » of Belgium.

In this paper we compare by these analytical methods, the differently coloured morphs of 3 species of the *Labridae* family, *Symphodus (Crenilabrus) ocellatus* (Forsskal, 1775), *Symphodus (Crenilabrus) roissali* (Risso, 1810) and *Symphodus (Symphodus) rostratus* (Bloch, 1797) belonging to 2 distinct subgenus according to QUIGNARD (1966).

The colouration variability among Fishes was for systematicians an obstacle to an appropriate description and classification of numerous specimens. This phenomenon is usual in the genus *Symphodus* (European Wrasses) : many species exhibit a low percentage of specimens with a basal colouration radically different from typical ones. These specimens were often collected in 'Varieties' and, in some cases, wrongly considered as subspecies on the basis of morphological observations of very few specimens. Their unity was already verified during behavioral observations especially in the reproduction period by the interfecondity of these varieties or subspecies (MICHEL *et al.*, 1987).

Material

S. ocellatus : 2 specimens (N°1 and 2) with usual brown colouration (8.0 and 7.0 cm long) were compared to a red coloured, *bertini* subspecies (GARNAUD, 1970), specimen (N° 3, 3.4 cm long).

S. roissali : 2 specimens (N° 4 and 5) with usual brown colouration (7.8 and 6.6 cm long) were compared to a green coloured, *tigrinus* variety (QUIGNARD, 1966), specimen (N° 6, 7.8 cm long).

S. rostratus : 2 brown specimens (N° 7 and 8, 11.5 and 10.8 cm long) and 2 green ones (N° 9 and 10, 7.2 and 8.5 cm long) were compared.

All these fishes were catched around the submarine and oceanographic research station (STARESCO) of the University of Liège (Belgium) at Calvi, Corsica (France). Dorso-lateral muscle portions of 0.1 to 0.5 gr were immediately dissected from MS 222 killed fishes, finely cut up and kept in 10 volumes of a neutral buffered glycerol solution (Tris-Cl 0.01 M, KCl 0.05 M, DTT 0.01 M, NaN_3 0.005%, pH 7.5) at − 18°C for 4 months.

Methods

A centrifugation (18.000 × *g*, 30 min) of muscle samples separates a supernatant containing the sarcoplasmic proteins including parvalbumins (PA) and a myofibrillar fraction from which the actomyosin complex with its associated proteins is extracted by an high ionic strenght solution and purified by precipitation with water at pH 6.3 (HURIAUX & FOCANT, 1977).

To allow the analysis of myosin light chains (LC), the actomyosin complex is dissolved before the electrophoretic separation in a denaturing solution of urea 8 M (Tris 0.02 M, glycine 0.12 M, urea 8 M, beta-mercaptoethanol 3%, pH 8.6) or SDS (Tris-Cl 0.0625 M, SDS 2%, glycerol 10%, beta-mercaptoethanol 5%, pH 6.8, 3 min heating at 100° C).

We used two types of vertical slab polacrylamide gel (18 × 8 × 0.2 cm) electrophoresis (PAGE) giving complementary results :

— Glycerol-or urea 8 M-PAGE at pH 8.6 (HURIAUX & FOCANT, 1977).

— SDS-PAGE at pH 8.8 according to LAEMMLI (1970).

After completion of the electrophoretic run, proteinic bands are stained with Coomassie Brillant Blue R-250.

Results

When the sarcoplasmic proteins are submitted to glycerol or urea 10% polyacrylamide gel electrophoresis, the low molecular weight and highly negatively charged PA are well separated in front of other components (Fig. 1a & b). At the level of PA bands (arrow), we observe 2 isoforms (types I and II, Carp PA being

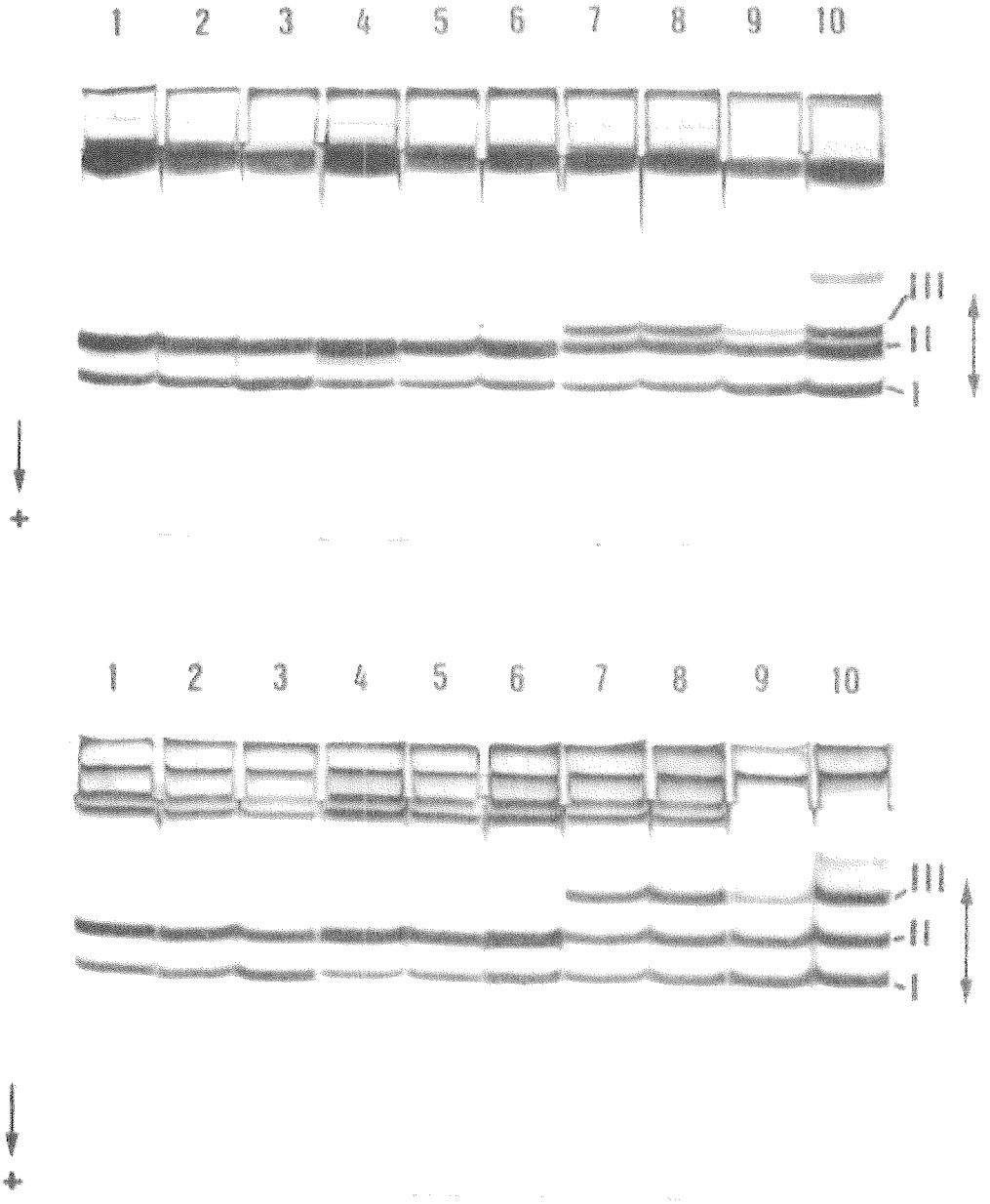

FIG. 1. *Electrophoretic separation of parvalbumins from S. occellatus* (1 and 2), *S. ocellatus « bertini »* (3), *S. roissali* (4 and 5), *S. roissali « tigrinus »* (6), *S. rostratus* (brown) (7 and 8) and *S. rostratus* (green) (9 and 10).
a : on urea-PAGE,
b : on glycerol-PAGE.
The arrow shows the migration area of parvalbumins.

used as references) in *S. ocellatus* and *S. roissali* dans 3 isoforms in *S. rostratus* (types I, II and III). No qualitative differences can be noticed between normal specimens and their « variants » with peculiar colouration. The glycerol gel (Fig. 1b) appears to have an higher resolving power for the slower migrating isoform of *S. rostratus*. Quantitative variations of each isoform between the different species, subspecies of varieties, estimated by densitometry (Table I), are slight but reproductible. In *S. ocellatus* the « red » or « bertini » variety does not contain the same isoform (I and II) proportions than the 2 « brown » specimens. Only a certain variability appears in *S. roissali*. In *S. rostratus* the 2 « brown » specimens are identical; in one « green » specimen the amount of slow PA isoform looks lower and in the other the isoforms proportions slightly differ.

TABLE I. *Percentage of parvalbumin isoforms in muscles from S. ocellatus* (1 and 2), *S. ocellatus « bertini »* (3), *S. roissali* (4 and 5), *S. roissali « tigrinus »* (6), *S. rostratus (brown)* (7 and 8) and *S. rostratus (green)* (9 and 10), *calculated from urea-and glycerol-PAGE.*

PA GEL	*S. ocellatus*			*S. roissali*			*S. rostratus*			
type	brown		red	brown		green	brown		green	
	1	2	3	4	5	6	7	8	9	10
III urea							31.2	26.9	13.2	22.4
glyc							34.7	34.4	20.5	33.4
II urea	67.1	65.1	52.2	75.6	69.5	70.2	40.7	40.9	39.5	39.4
glyc	64.8	62.9	51.5	75.6	67.4	68.1	35.0	35.5	35.8	31.7
I urea	32.9	34.9	47.8	24.4	30.5	29.8	28.1	32.2	47.3	38.2
glyc	35.2	37.1	48.5	24.4	32.6	31.9	30.3	30.1	43.7	34.9

PA : Parvalbumin.

In denaturing solutions containing urea or SDS, the actomyosin dissociates mainly in actin, myosin heavy and light chains.

When the mixture is analysed on urea-PAGE, myosin LC and tropomyosin (TM) only enter in the gel. Other proteins such as myosin HC and actin remain at the top of the gel due to their mass (Fig. 2a). On SDS-PAGE (Fig. 2b) the components separate according their molecular weight : the faster bands are the myosin LC (16 to 25 KD) followed by TN (18 to 37 KD), TM (36 KD), actin (43 KD) and myosin HC (220 KD) (HURIAUX & FOCANT, 1977). All fishes examined present on both gel types the same LC complement : no specific variations can be recorded.

Discussion

The comparison of myosins does not reveal any significant differences between colour morphs of a same species nor between the three species examined. This unity is not so far a determinant factor in the assembling or separation of the colour morphs. It should be noted that we have not always discovered differences between myosins of closely related species (FOCANT *et al.*, 1986).

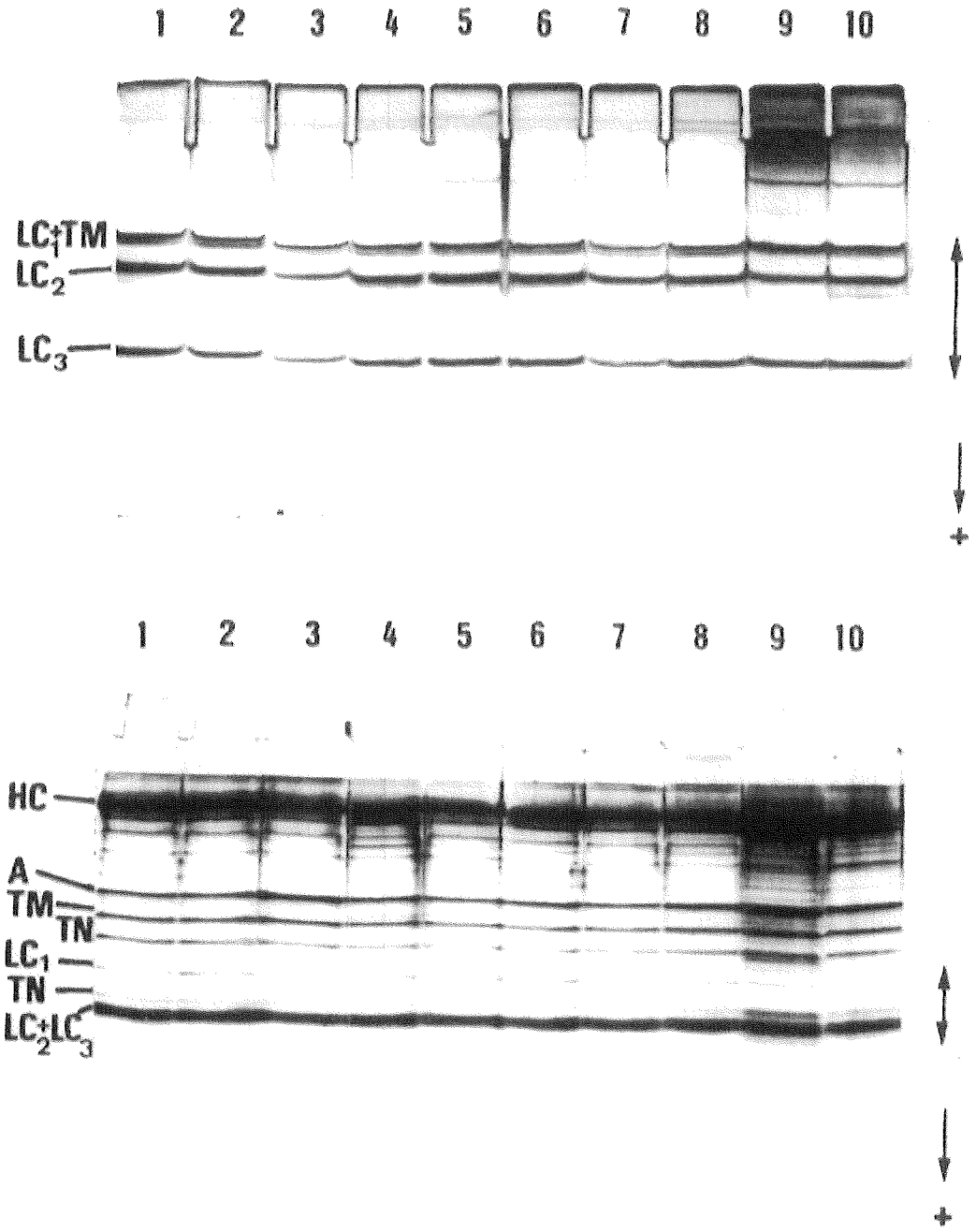

FIG. 2. *Electrophoretic separation of myosin light chains from S. occellatus* (1 and 2), *S. ocellatus « bertini »* (3), *S. roissali* (4 and 5), *S. roissali « tigrinus »* (6), *S. rostratus* (brown) (7 and 8) and *S. rostratus* (green) (9 and 10).
a : on urea-PAGE,
b : on SDS-PAGE.
The arrow shows the migration area of myosin light chains. See text for the abbreviations.

On the other hand, opposite to myosin, the PA reveal important disparities between species : *Symphodus (Symphodus) rostratus* differs significantly from the other two species, *Symphodus (Crenilabrus) ocellatus* and *Symphodus (Crenilabrus) roissali.* This biochemical disparity supports the other differences previously established and

which have notably led to the separation of the two subgenus (QUIGNARD, 1966). The morphological particularities, the biology, the social structures and the reproductive stategies of *Symphodus (Symphodus) rostratus* set up a lot of parameters isolating them from the other species of the genus.

The qualitative analysis of PA isoforms is not more successfull to detect differences between colour morphs and does not allow their separation. Meanwhile, we point out some quantitative divergences between the different PA isoforms, for example, between « brown » and « red » varieties of *Symphodus (crenilabrus) ocellatus*. This quantitative aspect should be regarded with caution from the systematic point of view because the isoform proportions may slightly vary among the representatives of a given species according to their age and their size (FOCANT & PECHÈRE, 1965) or to the examined trunk muscle part (HURIAUX *et al.*, 1988). The problem should be reinvestigated on a larger number of specimens.

To conclude about specific problems linked to colour morphs (« polychromy »), if the biochemical analysis does not give proofs for the assembling of these morphs, it does not furnish sufficient supports to their separation. An extensive structural analysis of their proteins may provide more convincing data.

Résumé

Au sein de la famille des Labridés, plusieurs espèces de *Symphodus* (Crénilabres) sont caractérisés par l'existence d'individus qui diffèrent par leur coloration. Une étude électrophorétique des myosins et parvalbumines des individus types et des formes colorées de trois espèces *(Symphodus (Crenilabrus) ocellatus, Symphodus (Crenilabrus) roissali, Symphodus (Symphodus) rostratus)* a été entreprise pour évaluer la validité systématique de cette polychromie.

Aucune différence électrophorétique n'a pu être mise en évidence entre individus types et formes colorées dans les trois espèces.

Par contre, les variations électrophorétiques des parvalbumines renforcent les observations comportementales et les particularités morphologiques qui ont conduit à séparer les Crénilabres en deux sous-genres (*Crenilabrus* et *Symphodus*).

Acknowledgments — This work was supported by the *Fonds National de la Recherche Scientifique, Belgium, contract F.R.S.M. n°* 3.4516.89 and F.R.F.C. n° 2.9005.84. The authors wish to thank Dr. BAY, Dr. WERNERUS and the team of the oceanographic station STARESO (Calvi, France) for the catch of the fishes.

References

BRUSHANA RAO, K. S. P., FOCANT, B., GERDAY, CH. & HAMOIR, G. (1969) *Comp. Biochem. Physiol.* **30**, 33-48.
FOCANT, B. & PECHERE, J. F. (1965) *Arch. int. Physiol. Biochim.* **73**, 334-354.
FOCANT, B. & PEQUEUX, A. (1985) *Bull. Soc. Roy. Sci. Liège* **1**, 55-64.
FOCANT, B. & JOYEUX, J. C. (1988) *Rapp. Comm. int. Mer Medit.* **31** (2), 258.
FOCANT, B. & VANDEWALLE, P. (1989) *Ann. Mus. Roy. Afr. Centr. Sc. Zool.* **257**, 81-84.
FOCANT, B., HURIAUX, F. & JOHNSTON, I. A. (1976) *Int. J. Biochem.* **7**, 129-133.
FOCANT, B., BENMOUNA, H. & VANDEWALLE, P. (1985) *Arch. int. Physiol. Biochim.* **93**, B83.
FOCANT, B., BENMOUNA, H. & VANDEWALLE, P. (1986) *Rapp. Comm. int. Mer Medit.* **30**, 225.
FOCANT, B., VILADIU, C. & VANDEWALLE, P. (1988) *Rapp. Comm. int. Mer Medit.* **31**, 257.
GARNAUD, J. (1970) *Bull. Inst. Océanogr. Monaco* **69**, n° 1411, 12 p.
GAZITH, J., HIMMELFARB, S. & HARRINGTON, W. F. (1970) *J. Biol. Chem.* **245**, 15-22.
HAMOIR, G. & GERARDIN-OTTHIERS, N. (1980) *Comp. Biochem. Physiol.* **65B**, 199-206.
HURIAUX, F. & FOCANT, B. (1977) *Arch. int. Physiol. Biochim.* **85**, 917-929.
HURIAUX, F. & FOCANT, B. (1985) *Comp. Biochem. Physiol.* **82**, 737-743.

HURIAUX, F., VANDEWALLE, P. &FOCANT, B. (1988) *Arch. int. Physiol. Biochim.* **96**, B44.
LAEMMLI, U. K. (1970) *Nature (London)* **227**, 680-685.
MICHEL, CH., LEJEUNE, P. & VOSS, J. (1987) *Rev. Franç. Aquariol.* **1-2**, 1-84.
PECHERE, J. F., CAPONY, J. P. & DEMAILLE, J. (1973) *Syst. Zool.* **22**, 533-548.
QUIGNARD, J. P. (1966) *Naturalia Monspeliensia, sér. Zool.* **5**, 7-248.
ROWLERSON, A., SCAPOLO, P. A., MASCARELLO, F., CARPENE, E. & VEGETTI, A. (1985) *J. Muscle Res. Cell. Mortil.* **6**, 601-640.

B. FOCANT
Institut d'Anatomie, Université de Liège
20, Rue de Pitteurs
B-4020 Liège