Esterases of the flounder *(Platichthys flesus*, Pleuronectidae, Teleostei): Development of an identification protocol using starch gel electrophoresis and characterization of loci

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Summary. Fish esterases are among the most difficult enzymes to identify using starch gel electrophoresis because of the many loci that are simultaneously active, and especially because of duplication phenomena, satellite bands, and stain trails. In an attempt to simplify and clarify electropherograms, various staining and inhibitory methods were tested on esterases from the flounder *Platichthys flesus*. A range of migration and staining buffers and substrates were used, as well as chemical inhibition and heat inactivation. A combination of the methods made it possible to distinguish and characterize the five presumed esterase loci.
Key words. Esterases; enzymatic electrophoresis; enzymatic inhibitors; staining methodology; flounder genetics.

Among the thirty enzyme systems usually analyzed by starch gel electrophoresis in genetic studies of fish, esterases often involve identification problems. The difficulty is not due to the weakness of enzymatic activity but rather to the complexity of the electropherograms obtained. The enzymes are not very specific and can correspond to as many as 5 putative loci (depending on the organ) which are expressed simultaneously. Authors sometimes neglect to mention them in their results or express doubts with regard to the reliability of the identification or the genetic relevance of the patterns observed [1]. This situation may be further complicated by circadian changes in esterase activity, as described in the house cricket [2] and shrimp [3]. For example, Ward et al. [4] reported that in plaice *(Pleuronectes platessa)*, esterase electropherograms obtained with liver extracts were too complicated to be of use. Similar difficulties have been encountered in other organisms, e.g. the mouse [5] and the clam [6].

The initial objective of the present study was to develop a protocol for identifying esterases of the flounder *(Platichthys flesus)*. Since the difficulties involved are quite typical for this type of analysis, we consider that the method can be extrapolated to other species that pose similar identification problems.

The second objective was to characterize the esterase loci on the basis of their sensitivity to several inhibitors and to heat, and their specificity for different substrates.

Materials and methods

The species studied was the flounder *(Platichthys flesus)* from the Golfe du Lion (French Mediterranean coast). This teleostean belongs to the family Pleuronectidae. After capture, specimens were stored alive or on ice during transfer to the laboratory, where they were immediately dissected or frozen at −30 °C until later dissection.

The following organs were removed: 1 to 2 g of muscle and liver, and the eyes, kidney, spleen, stomach, heart and brain. The organs were homogenized in an equal volume of Tris-HCl-EDTA buffer, pH 6.8 (1.2 g/l Tris, 0.37 g/l disodic EDTA, 0.001 g/l NADP and HCl to adjust pH) and centrifuged at 15,000 r.p.m. for 30 min at 4 °C on a Sorval RC-5 centrifuge. The supernatant was stored at −60 °C until electrophoresis.

Electrophoresis was carried out on horizontal starch gels (12 %) [7]. The starch was produced at the Institut des Sciences de l'Evolution de Montpellier (France) by hydrolysis of potato flour. The following buffer systems were used:

1) The discontinuous 'TCBL' system consisting of TCBL 7.0 buffer for the gel (0.75 g/l Tris, 0.35 g/l citric acid, 0.11 g/l boric acid and 0.04 g/l LiOH, pH 7.0) and BL 7.0 buffer for the electrodes (27.2 g/l boric acid and 0.96 g/l LiOH, pH 7.0).

2) The discontinuous 'Poulik' system using TC 8.7 buffer for the gel (4.6 g/l Tris and 0.53 g/l citric acid, pH 8.7) and B 8.2 buffer for the electrodes (18.5 g/l boric acid and 2.4 g/l NaOH, pH 8.2).

3) The continuous 'TME' system comprising TME 6.9 for the electrodes (12.1 g/l Tris, 9.8 g/l maleic anhydride, 3.7 g/l EDTA and 2 g/l $MgCl_2$, pH 6.9) and an identical buffer diluted 10-fold for the gel (also pH 6.9).

Staining was performed with solutions consisting of various combinations of buffer, one or more substrates, and a salt.

1) The following staining buffers were used:
- 0.04 M phosphate, pH 7.0 (3.1 g/l monosodium phosphate and 2.8 g/l disodium phosphate),
- NaK 0.1 M phosphate, pH 6.5 (9.2 g/l monopotassium phosphate, and 4.8 g/l disodium phosphate),
- Na 0.1 M phosphate, pH 6.5 (10 g/l monosodium phosphate and 5.1 g/l disodium phosphate),
- 0.05 M acetate, pH 6.0 (6.8 g/l trihydrated sodium acetate)

2) Substrates for chromogenic staining were made up in the following stock solutions:
- Alpha naphthyl acetate or ANA (Sigma N6750),
- Beta naphthyl acetate or BNA (Sigma N6875),
- Alpha naphthyl propionate or ANP (Sigma N0500),
- Beta naphthyl propionate or BNP (Sigma N0501),

- Alpha naphthyl butyrate or ANB (Sigma N8000),
- Beta naphthyl butyrate or BNB (Sigma N8125),
- Alpha naphthyl oleate or ANO (Sigma N9875),
- Alpha naphthyl palmitate or ANPa (Sigma N9875).

These 8 substrates were in 1 % solution in acetone;
Substrates for fluorogenic staining were:

- 4 methyl umbelliferyl acetate or 4MUA (Sigma M0883),
- 4 methyl umbelliferyl propionate or 4MUP (Sigma 8633).

The two last substrates were in 0.5 % solution in acetone.
3) Staining salts were as follows:

- Fast Blue RR or FBRR (Sigma F375),
- Fast Garnett GBC or FG (Fluka 44710).

To further characterize esterases, inhibition experiments were performed. The following inhibitors were prepared in stock solutions and added to the staining buffer:

- S.S.S.-tributylphosphotri-thioate or DEF (Chem. Service PS562) in a 1 % solution in absolute alcohol,
- 1 % NaO_3 (Merck 6553) in water,
- 1 % $HgCl_2$ in water,
- 1 % PCMB (Sigma C4378) in acetone,
- 1 % PMSF (Merck 7347) in absolute alcohol,
- 1 % NaF (Sigma S1504) in water,
- 1 % Thimerosal (Sigma T5125) in water,
- 1 % Diisopropylfluore phosphate or DFP (Fluka 38399) in acetone,
- 0.5 % Eserine (Sigma E8625) in a phosphate buffer.

These inhibitors were first added to the basic staining buffer and the gels were incubated for 30 min. The other compounds of the staining solution (substrates, salts) were then added without removing the inhibitor, to avoid reactivation of the inhibited enzymes.
Heat inhibition was tested by heating the staining solution to the desired temperature. The buffer was poured onto the gel and the ensemble was placed in a drying oven for a specified time at the same temperature. Temperatures of up to 100 °C were tested, but it is likely that the indicated temperature was not actually reached by the enzyme because of the insulation of the bowl and gel. This is not important since the test was comparative and the experiments are reproducible.

Results

Test for esterase activity in the organs removed. Table 1 shows the relative esterase activity in the Poulik electrophoretic buffer system and standard staining, i.e. ANA + BNA, FG, 0.1 M NaK phosphate-buffer, pH 6.5. An unidentified diffuse zone of activity was also observed and tested.
Only kidney and liver showed sufficient activity in 3 zones, assumed to correspond to three loci: *Est-1, Est-2,* and *Est-3* (numbered according to increasing anodal rapidity). The liver was therefore taken as the reference organ for the first three loci. Muscle was the only organ tested in which locus *Est-4* was clearly expressed, and was taken as the reference organ for this locus.

Interpretation of the esterase electropherograms. The diffuse zone of activity, obtained especially with liver and spleen extracts, had the appearance of a badly localized migration trail, and could not be identified. There were no clear signs that it represented a distinct locus, and it may have consisted of modified esterase molecules.
EST-1: This activity zone corresponded to a locus encoding molecules that migrated rather slowly in all buffers. In the Poulik buffer system, three electromorphs appeared with very similar mobilities, which raised identification problems. In young flounder (less than 1 year), this esterase was easier to interpret because of the clarity of the electropherograms (probably due to the low fat content of the extracts). Lastly, identification of the esterase was often facilitated by the formation of echo spots just underneath (i.e. the cathode side; fig. 1), reproducing the allelomorph patterns of EST-1 with higher amplitude except when the echo spots were diffuse or of too low intensity.
EST-2: Proteins corresponding to this locus showed average migrating speed in the Poulik buffer system. As in the case of EST-1, it encoded three electromorphs with similar mobilities.
EST-3: Although the allelomorphs corresponding to this putative locus were clearly separated by the TCBL and TME buffer systems, the electropherograms were difficult to interpret, i.e. EST-3 isozymes coincided with EST-2 in the TCBL system and the results were very irregular in the TME system (figs 1A and 2).
Lastly, the enzyme patterns observed for EST-3 activity differed according to the buffer system. Individuals appearing as homozygotes in TME (one single spot) and TCBL buffers (one spot and very rarely a slight duplication) often showed two spots (but never more) in the Poulik system.
Individuals appearing as heterozygotes with TME and TCBL (exhibiting two different electromorphs) showed 2, 3 or 4 spots (but never less) in Poulik buffer. These observations were verified for several hundred individuals[8] as illustrated in figure 2 and interpreted in figure 3 (e.g. 2 spots in lanes 1 and 6; 3 spots in lanes 4 and 5; 4 spots in lanes 10 to 13).
No relationship could be found between phenotypes and parameters such as the age of the fish, the capture site, or sample 'aging' (which might have denatured the proteins). On the other hand, by using the TCBL system and inhibiting the other two esterases (EST-1 and EST-2), the electromorphs were completely separated. Several duplications could be seen, but the extra spots formed were extremely close, i.e. the distance between them was clearly less than that separating the two electromorphs (figs 2 and 3).
EST-4: This zone of esterase activity, which could be detected only in muscle extracts, was interpreted as a locus encoding two electromorphs with clearly different mobilities. However, the zone of EST-2 activity coincid-

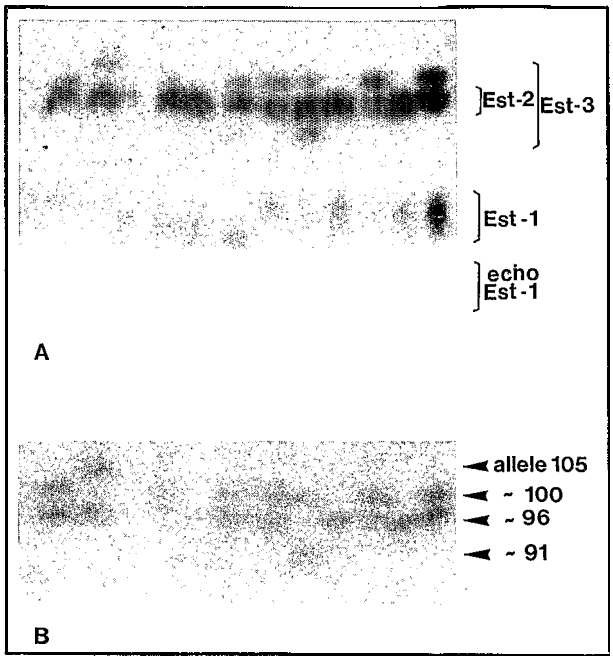

Figure 1. *A* Esterase electropherogram showing all enzyme activities in the liver. Note that the activity zone marked 'EST-2/EST-3' is unreadable because of superpositions. *B* By eliminating EST-2 activity, DEF allowed an unambiguous identification of genotypes comprising 4 electromorphs related here to alleles 91, 96, 100, and 105. Buffer used: TCBL.

ed with the slow electromorph of EST-4, regardless of the migration buffer used.

EST-4MU: This enzyme could only be revealed using substrates such as 4-methyl umbelliferate. It took the form of intense but irregular spots, half way between (with TCBL buffer) the activity zones of isozymes coded by loci *Est-1* and *Est-2*. This putative locus has always been found to be monomorphic in the flounder. In humans, it is called *Est-D* [9]. By analogy, some authors also use this terminology in fishes [4].

Development of a protocol assuring reliable identification. It was not difficult to interpret electropherograms processed to detect EST-4MU, apart from the irregular intensity of spots observed under UV light.

Liver extracts were first subjected to electrophoresis with three different migration buffer systems (TCBL, Poulik, and TME), followed by detection with the reference staining solution (1 ml of ANA + 1 ml of BNA, combined with 0.1 M NaK phosphate buffer, pH 6.5, and FG salt).

The following difficulties were encountered:

– TCBL produced a superposition of isozymes EST-2 and EST-3, and a mediocre appearance of EST-1 (fig. 1).

– Poulik buffer separated the three activity zones, but although EST-1 and EST-2 were of good quality, EST-3 appeared to be broken up into many spots (as many as 4 per individual, see figs 2 and 3).

– TME buffer produced irregular results (non-reproducible) in the case of EST-3. In the last buffer, the spots were round and diffuse (continuous buffer system) whereas they were flat and intense in the other two buffers (discontinuous buffer systems). Moreover, in many cases, the TME system produced electropherograms that were too diffuse and could not be interpreted for most of the extracts (the electropherogram shown in

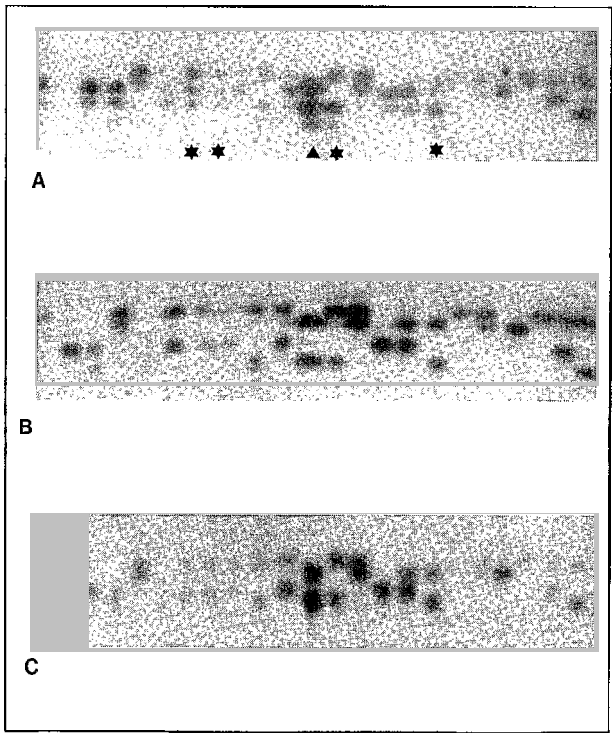

Figure 2. Effect of different buffers on the quality of the electropherogram. *A* Many duplications occurred in Poulik buffer, resulting in phenotypes with three spots (stars) or four spots (arrows). *B* Duplication was very limited in TCBL buffer, practically imperceptible on this photograph. *C* Identification was easy in TME buffer, but the results were very irregular. An inhibitor (DEF) was used in all cases.

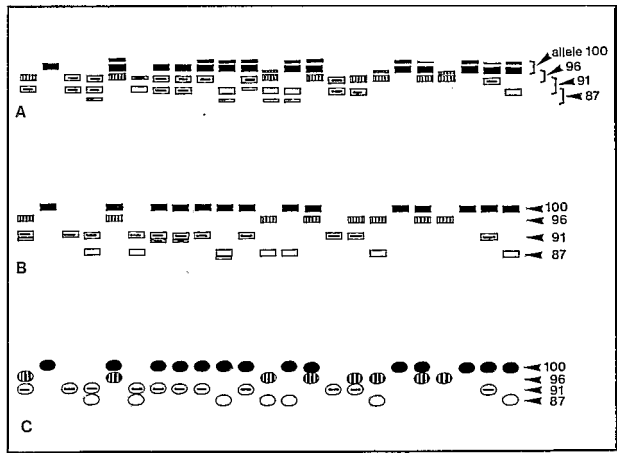

Figure 3. Schematic interpretation of figure 2. The confusion caused by duplication is due to the superposition of spots belonging to different allelomorphs. These allelomorphs, which are related here to alleles, are illustrated by different colors which reveal the behavior of each of the 4 alleles during the duplication phenomenon. *A* in Poulik buffer. *B* in TCBL buffer. *C* in TME buffer.

figure 2 is of exceptional quality and was not consistently obtained). Therefore, the TME buffer system was kept for comparison, but was not used for routine identification.

With muscle extracts, electropherograms stained for EST-4 were comparable to those stained for EST-3, but the electromorphs were not duplicated. Although the isozymes of EST-2 were less active in muscle, they also coincided with those of EST-4.

The next step involved testing chemical inhibitors capable of reducing the activity of EST-2 without suppressing those of EST-3 and EST-4. Eight inhibitors were tested on liver and muscle extracts with the TCBL and Poulik buffer systems. The standard staining solution was used. The tests could not be applied to EST-4MU. The results are shown in table 2. DEF showed the required qualities, i.e. it had no effect on EST-3 and EST-4 and strongly inhibited EST-2 (and EST-1).

Thus, the protocol for identifying flounder esterases consisted of two steps: a) interpretation of electropherograms stained for EST-1 and EST-2 in the Poulik buffer system, and b) interpretation of electropherograms stained for EST-3 and EST-4 in the TCBL buffer system, after inhibition by DEF. The best inhibition was obtained using 4 ml of 1 % DEF in 40 ml of buffer, incubating for 30 min, and adding substrate and salt without removing the inhibitor. It should be noted that DFP had the same effect but was not used for routine detection because of its high toxicity.

Characterization of the four esterase loci. Esterases are generally characterized according to their sensitivity to different inhibitors (table 2). In addition to the inhibition results described and used above, we observed that:

Table 1. Intensity of six zones of esterase activity in different organs (−: no activity; +: very weak activity; ++: clear activity; +++: strong activity; NT: not treated).

	trail	Diffuse *Est-1*	*Est-2*	*Est-3*	*Est-4*	*Est-4MU*
Muscle	−	++	++	+	+++	+
Liver	+++	+++	++	+++	−	++
Eyes	+	+++	+	−	NT	NT
Stomach	+	+++	+++	−	NT	NT
Gills	+	++	+	−	NT	NT
Spleen	+++	+++	++	−	NT	NT
Kidney	−	+++	++	+++	NT	NT
Brain	+	++	+	−	NT	NT
Heart	+	+++	++	−	NT	NT

Table 3. Substrate specificity of 5 esterase loci in two organs: muscle (M) and liver (L) (−: no activity; +: very weak activity; ++: clear activity; +++: strong activity).

Loci organs	*Est-1* M & L	*Est-2* M & L	*Est-3* L	*Est-4* M	*Est-4MU* L
ANA	+++	+++	+++	+++	−
BNN	+++	+++	+++	+++	−
4MUA	++	++	++	++	++
ANP	+++	+++	+++	+++	−
BNP	+++	+++	+++	+++	−
4MUP	++	++	++	++	++
ANB	+++	+++	+++	+++	−
BNB	+++	+++	+++	+++	−
ANO	−	−	−	−	−
ANPa	−	−	−	−	−

Table 4. Heat inhibition (−: no inhibition; +: weak inhibition; +++: total inhibition).

Time	Temperature	*Est-1*	*Est-2*	*Est-3*	*Est-4*
10 mn	80 °C	−	−	+	+
10 mn	95 °C	+	+	+++	+++
20 mn	100 °C	+++	+++	+++	+++

- NaF acted only in TCBL buffer, and the effect was indistinct and very weak,
- $NaVo_3$ had no effect,
- the effect of Thimerosal was similar to that of PCMB and
- PMSF acted the same as DEF.
- Only $HgCl_2$ discriminated EST-1, which was the only enzyme it inhibited.

None of the 10 substrates (table 3) distinguished esterases among themselves, except for *Est-4MU,* which was strictly specific for 4MU compounds. The specificity was the same for all the other enzymes tested: acetic and propionic compounds were susceptible to the enzymatic reaction, whereas the oleic and palmitic compounds were not transformed.

Heat sensitivity (table 4) was tested with standard staining, using the substrates ANA and BNA combined with FG in 0.1 M NaK phosphate buffer, pH 6.5. A large difference was found between esterases 1 and 2 (resistant) and esterases 3 and 4 (sensitive).

Discussion

Inhibition tests can rather accurately characterize esterases, according to the work of Aldridge[10] and Frankel[11]:

Table 2. Tests for inhibition by different chemical substances (−: no inhibition; +: slight inhibition; ++: clear inhibition; +++: total inhibition).

	TCBL 7 *Est-1*	*Est-2*	*Est-3*	*Est-4*	Poulik *Est-1*	*Est-2*	*Est-3*	*Est-4*
DEF	++	++	−	−	+++	+++	−	−
NaF	+	+	+	+	−	−	−	−
$HgCl_2$	+++	+++	+++	+++	−	+++	++	++
$NaVo_3$	−	−	−	−	−	−	−	−
Thiméros.	−	−	+	+	−	−	+	+
PCMB	−	−	+	+	−	−	+	+
PMSF	++	++	+	+	−	+	−	−
DFP	+++	+++	−	−	+++	+++	−	+
Esérine	−	−	−	−	−	−	−	−

- cholinesterases (including the pseudocholinesterases and acetylcholinesterases) are inhibited only by eserine, DEF, and DFP,
- carboxylesterases are inhibited only by DFP, and
- arylesterases are inhibited by PCMB, but resist the other inhibitors.

EST-1 and EST-2 were inhibited by DEF, DFP, and in a different manner by PMSF, and especially by mercuric chloride. They are thus clearly cholinesterases, but with properties that differ in some ways. These are not 'Aldridge cholinesterases'[10], considering the inactivity of eserine. It should also be added that they were remarkably heat-resistant.

EST-3 and EST-4 were inhibited only by PCMB, among the classically used inhibitors. They can therefore be classified as arylesterases.

Tests of specificity for different substrates provided no new information, whereas heat sensitivity tests confirmed the similarity of EST-1 to EST-2 and of EST-3 to EST-4.

It should be noted that esterases 1 and 2 were unequally affected by certain inhibitors (NaF, $HgCl_2$, and PMSF) depending on whether they migrated in Poulik or TCBL buffer. The results are thus relative, implying that identical protocols must be used to test for locus homologies between species.

The EST-3 patterns observed using the Poulik system (as many as four electromorphs for the same individuals) did not conform to the expected patterns for a single locus encoding a monomeric enzyme, which is the case for all esterases except the 4MU-specific 'EST-D'[12].

There are two possible alternative explanations: a) There could be more than one locus involved: two esterase loci encoding enzymes of close electrophoretic mobilities that were sufficiently separated only in the Poulik system could account for the number of spots (1 to 4) observed. b) Only one esterase locus is expressed, but non-genetic modifications occur, such as an alteration of mobility by fixation of small electrically charged carbohydrate moeities, which may only be present in the Poulik buffer.

According to hypothesis 'a', a first polymorph locus *Est-3* is active with the three buffers, and an additional one, *Est-3'*, is active only in Poulik buffer. This second locus must also be considered polymorphic because of the four-spot patterns observed in Poulik buffer corresponding to the heterozygous state of the two loci. One would expect to observe individuals exhibiting one spot in TCBL or TME buffers and three spots in Poulik buffer (homozygous for the first locus and heterozygous for the second one), but this was never observed in Poulik/TME-TCBL comparisons involving hundreds of individuals[8]. All individuals exhibiting one spot in TME and TCBL exhibited rarely one, and mostly two spots in the Poulik system. Such a duplication of spots conforms more closely with hypothesis 2.

Moreover, assuming allelic codominance at one single *Est-3* locus, Landaud[8] detected no departure from Hardy-Weinberg predictions for phenotypes observed in TME and TCBL. The tests were done with several samples comprising 30 to 100 individuals.

Since all patterns observed using TME and TCBL buffers conform to the expected patterns for a monomeric enzyme, and since the observed phenotype proportions conform to those expected for codominant alleles in the Hardy-Weinberg model, we assume that only one *Est-3* locus is involved. Consequently, we conclude that the protocol using TME or TCBL buffers is correct and that the Poulik system produces artefactual duplicates of electromorphs.

Conclusion

The main contribution of the present work is the development of a standard protocol for identifying multiple esterases. It involves:

a) the use of different migration buffers and inhibitors in the case of superpositions of different allelomorphs presumably related to different loci;

b) the use of a buffer rarely employed in the literature (TCBL buffer) whose properties are close to those of Poulik buffer, but which considerably reduces the duplication of heat-sensitive allelomorphs (essentially locus *Est-3* in the present study).

The second contribution is the use of inhibitors that reveal new distinctions among eserine-resistant cholinesterases, i.e. those that are sensitive to mercuric chloride (EST-2) and those that are not (EST-1).

In the present paper, we have been prudent with regard to interpreting electropherograms in terms of enzymatic loci. Reliable identification can only be obtained in experiments that test the Mendelian causality for the phenotypes observed. Laboratory cross-breeding has confirmed the Mendelian causality of the esterase phenotypes in the mouse[5] and of a cyprinid fish, the barbel (Berrebi, unpubl. data). In the flounder, as in all cases where reproduction cannot be easily conducted in the laboratory, we must generalize by analogy with the above-mentioned species.

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