

## **LEUCISCUS PYRENAICUS FOR SCEs TESTING OF GENETIC DAMAGE ON AQUATIC ECOSYSTEMS AND FISH FARMS**

### **EMPLEO DE *LEUCISCUS PYRENAICUS* EN EL TEST SCEs PARA DETECTAR DAÑOS GENÉTICOS EN ECOSISTEMAS ACUÁTICOS Y PISCIFACTORIAS**

Lobillo, J. and J.V. Delgado

Facultad de Veterinaria, Departamento de Genética, 14005-Córdoba, España, Tlno. 218708, Fax 218666, e-mail: id1debeg@lucano.uco.es

#### **ADDITIONAL KEYWORDS**

Genotoxicity, Sister chromatid exchange, Monitoring pollutants.

#### **PALABRAS CLAVE ADICIONALES**

Genotoxicidad, Intercambio de cromátidas Hermanas, Detección de contaminantes.

#### **SUMMARY**

The Sister Chromatid Exchange (SCEs) for monitoring water pollutants was studied in five (wild and cultured) fish species of southern Spain. *Leuciscus pyrenaicus* was the most useful for biological monitoring of mutagens in polluted waters and the best choice for the standardization of the Brdu method and so, contribute to the evaluation of genotoxic contaminants in order to prevent catastrophes in fish populations, economic losses in fish farms and ingestion of contaminated fish.

económicas en piscifactorias o ingestión de peces contaminados.

#### **INTRODUCTION**

The increasing contamination of the aquatic environment affect the aquatic ecosystems and in a indirect way, mainly through ingestion of contaminated fish, the human health (De Flora *et al.*, 1991).

The SCE test, a very sensitive indicator of chromosomal damage involves exposure of rapidly dividing cells to the thymidine analogue, 5,2, Bromodesoxiuridine (Brdu) for two rounds of DNA replication. The analogue produces one unifilary and one bifilary substituted chromatids, which stain differentiatly when treated with various dyes, giving one lightly and one darkly stained chromatids. The cells showing differently stained

#### **RESUMEN**

La inducción de intercambios entre cromátidas hermanas para el testaje de contaminantes en el agua, se estudió en cinco especies (silvestres o cultivadas) de peces del sur de España. *Leuciscus pyrenaicus* podría ser adecuada para el testaje biológico de mutágenos en aguas contaminadas y para la estandarización del método de marcaje con Brdu y así contribuir a aclarar el papel de los agentes genotóxicos en la prevención de catástrofes en poblaciones de peces, graves pérdidas

chromatids are named M2 metaphases or M2 cells (Latt *et al.*, 1981). Exchanges can be detected by the presence of a piece of darkly stained material attached to a lightly stained chromatid and vice-versa (figure 1).

This paper's goal is to determine a fish species optimal for genotoxic evaluation of water pollutants, as measured by the SCE test.

#### MATERIALS AND METHODS

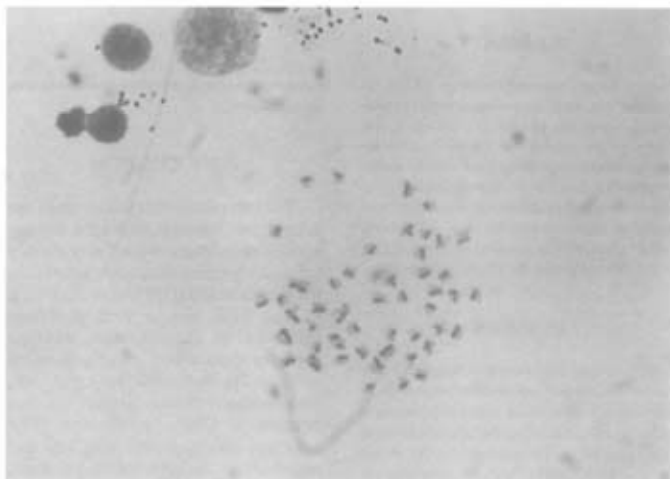
Specific genetic and handling criteria used to evaluate the Brdu-labelling-

method in *Leuciscus pyrenaicus* and proposed for the future application of the SCEs test under field conditions, are summarized by Lobillo (1993) as follows:

a) Specific genetic criteria: tissular response to Brdu treatment, including: optimum M2 and mitotic indexes and Brdu-dose-dependent significant increases in SCE rates.

b) Karyotype criteria: optimum number and size of chromosomes for a fast and easy visualization of SCEs.

c) Handling criteria: facility in capture, transport survival, maintainance and adaptation to specific laboratory



*Figure 1. M2 cells of Leuciscus pyrenaicus stained with the FPG technique, showing the sister chromatids differentiation and sister chromatid exchanges. (Células M2 de Leuciscus pyrenaicus teñidas con la técnica FPG, mostrando la diferenciación e intercambio de las cromátidas hermanas).*

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conditions and low stress susceptibility.

### SPECIES AND PRELIMINARY PROCEDURES:

The following species have been used: *Liza Aurata* (Pisces, Mugilidae): 28 specimens between 30-50 g *Ciprinus Carpio* (Pisces, Ciprinidae): 5 specimens weighting 400-500 g and 12 between 15-30 g *Fundulus heteroclitus* (Pisces, Ciprinodontidae): 41 specimens between 5-12 g *Rutilus alburnoides* (Pisces, Ciprinidae): 40 specimens between 3-8 g *Leuciscus pyrenaicus* (Pisces, Ciprinidae): 150 specimens between 2-13 g. The two first species (from Mugilidae and Ciprinidae families) are considered cosmopolitan and are frequently cultured. The third species is a foreign killifish located in slightly saltish and hypersaline waters in Cádiz Gulf where they are found in large populations. *Rutilus alburnoides* and *Leuciscus pyrenaicus* are autochthonous species of the Ciprinidae family from continental waters of southern Spain.

Capture sites and methods are summarized in **table I**. Taxonomic keys of Lotina and Hormaechea (1979) were used for rapid identification.

Transportation procedures were carried out according to Vollmann-Schipper (1978). Captured fish were introduced into opaque plastic cylindrical tanks with 50-100 l of water, under continuous aeration.

Previous to experimental assays, the fish were maintained in 70 l storage aquariums.

Under experimental conditions, each group of fish (three per treatment) was kept in 25 l assay aquariums. Before and during the experiments, water was biologically and chemically filtered with superposed plaques (containing reducer

bacterial inoculates) at the bottom and active charcoal in external filters and was maintained at a continuous ventilation flow rate of 2 l/min and constant temperature of 24-25°C.

The photoperiod was variable. From January to June, aquariums were exposed to natural light with increasing photoperiod. From July to September, they were exposed to a constant period of 14 light-hours/day using artificial light originated by two Silvania Gro-lux lamps with 30 watts each one (1000 lux). Before experiments the fish were well-fed with a commercial diet containing 34 p.cent protein.

### BRDU-DIFFERENTIAL TECHNIQUE:

#### *Variables in study:*

For the standardization of the Brdu-labelling method in *Leuciscus pyrenaicus*, five variables were defined:

- M2 Index (discontinuous variable): Percentage of metaphases that undergo two rounds of DNA replication (two cellular cycles) in the presence of Brdu. This variable was defined as a function of the Brdu dose and treatment time (time during which cells are exposed to Brdu).

- Mitotic index (discontinuous variable): number of metaphases per one hundred interphase cells. Also, a function of the Brdu dose and the treatment time.

- Sister chromatid differentiation or SCD (subjective variable): Chromatic contrast level between sister chromatid of each chromosome. Major contrast implies optimum SCEs visualization.

- Chromosome denaturalization or DN (subjective variable): level of chromosomal structure alteration. A low DN implies optical resolution of entire

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**Table I.** Capture methods and general characteristics of sampling sites for each species. (Métodos de captura y características generales de los lugares de muestreo para cada especie).

Specie	Sampling sites	Water salinity	Ecosystem	Capture method
<i>L. aurata</i>	Cádiz	Hypersaline Saline	Stuary Harbour	Beach seines Hand-pole lines
<i>C. carpio</i>	Córdoba Coria del Río	Non-saline Non-saline	Guadalquivir river	Hand-pole lines Bottom trawls
<i>F.h. macrolepidotus</i>	Cádiz	Hypersaline	Stuary	Jigging lines
<i>R. aburnoides</i>	Córdoba	Non-saline	Mascatomiza small stream	Electric fishing (Cowx, 1990)
<i>L. pyrenaicus</i>	Córdoba	Non-saline	Pedroches small stream	Electric fishing

chromosomic structure, avoiding wrong SCEs scores.

-  $SCEs/M2/Individual$  (continuous variable): this is the main variable and is mathematically defined as a weighted mean:

Mean  $SCEs/M2/individual = SCEs =$

$$= \frac{(M2_{20h} \times 0) + (M2_{24h} \times 1) + (M2_{28h} \times 2) + \dots + (M2_{32h} \times n)}{\sum (M2)}$$

These five variables were measured at each experimental step in order to define the experimental objectives until the optimum base-line SCE frequency was estimated.

**Experimental objectives (see table II):**

In the first experimental step, optimum treatment time was estimated: exposure time to Brdu from which the maximum M2 index is obtained. High M2 indexes permit easy and rapid

measurement of SCE rates and optimum estimation of the cell-cycle time under the specific experimental conditions.

Cell-cycle estimation (Kligerman *et al.*, 1982 and Giles *et al.*, 1988), is the half-time of the maximum yield of M2 cells (or maximum M2 index).

The experimental design to reach this objective is as follows: At time 0, the fish receive one intraperitoneal (IP) injection of 0.5 mg of Phytohemagglutinin-M (Sigma) in PBS solution/gram of body weight (bw). Phytohemagglutinin-M (PHA-M) has been used on fish to stimulate the cellular division (Giles *et al.*, 1988 ; Pendás and Lobillo 1991). At different treatment times (20, 24, 28 and 32 hours before sacrifice), a constant Brdu (Sigma) dose of 0.5 mg/g of bw. in PBS solution, is inoculated IP simultaneously with a second dose of PHA-M to each group of fish (three individuals per group). Two hours before sacrifice, the fish receive a colchicine (Sigma) inoculation in PBS solution at a

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constant dose of 0.35 µg/g of bw. The total assay time from the first inoculation of PHA-M was 48 hours.

In the second experimental step the optimum Brdu dose was estimated: It is defined as the dose at which the optimum values for defined variables are simultaneously obtained: the lowest possible SCEs rate and DN grade, and the greatest possible percentage of M2 and mitotic index and a high grade of sister chromatid differentiation (SCD).

The experimental design to reach this second objective is as follows: at constant treatment time, increasing doses of Brdu (from 0.1 mg to 1.1 mg of Brdu/gram of bw.) were inoculated IP into each group of fish. PHA-M and colchicine were inoculated in the same way as described in the first step.

In the third experimental step the Brdu dose was adjusted. At the optimum treatment time, the fish were injected with a medium dose of Brdu, according to the optimum interval dose obtained in the second step. PHA-M and colchicine inoculations were injected in the same way as above.

Finally, in the fourth step, the baseline frequency of SCEs was estimated: the mean of SCEs between individuals at optimum Brdu dose and at treatment time.

Experimental design: at optimum treatment time, different groups of fish were inoculated at constant and optimum Brdu dose.

### *Statistical analysis*

The computer program SAS (1987) was used as a specific statistical analysis. Values of the five variables previously described were registered so as to define the experimental objectives.

The number of animals tested per treatment was three, and the number of cells examined per animal was between 50-100, according to *Gene Toxicological Work Group* (Latt *et al.*, 1981).

*Proc-Univariate-SAS* was carried out to verify the adjustment of the SCEs variable to a Normal Distribution. *Proc-means-SAS* was also used to obtain the descriptive statistics of the SCEs variable.

*Proc-GLM-SAS* for unbalanced models was used to study the effects of variation sources on the SCEs variable. This procedure applies the parametric ANOVA and the Tukey's multiple comparison test.

*Proc-NPAR1WAY-SAS* was used for discontinuous variables to compare the levels of a specific variation factor. This procedure uses a non parametric ANOVA, the Wilcoxon test (Kruskal-Wallis ANOVA by ranks).

Finally, Brdu-dose-response curves were plotted for each case using a regression analysis. The best fitness equation was estimated by PROC-NLIN.

### *Chromosome preparations*

Colchicination time was normally estimated at two hours at about 25°C. After this time, the fish were sacrificed. The cephalic kidney, an haemopoietic organ with a high mitotic index, was removed, transferred to 5 ml of hypotonic medium (0.56 p.cent KCl) and homogenized in a conical glass. The cellular suspension was transferred into a cylindrical glass and kept there for 95-100 minutes at 17-18°C. After hypotonic shock the suspension was prefixed by the addition of 1 ml of Carnoy fixer (1:3. Acetic/Methanol). The suspension was slightly mixed and then centrifuged at

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**Table II.** Summary of the Experimental and treatment conditions for each group of fish. (Resumen de las condiciones Experimentales y de tratamiento para cada grupo de pez).

Specie	N	T	S	Photoperiod	1	2
<i>L. aurata</i>	2n=48	26-28	40	Decreasing (oct.-dec.)	20-24-28 hs (0.8)	0.4-0.6-0.8 mg/g (24)
<i>C. carpio</i>	2n=100	26-28	4	Increasing (april-may)	20-24-28-32- 42-52 hs (0.8)	unobtainable
<i>R. alburnoides</i>	2n=45-60 3n=65-80	26-28	4	Increasing (Jan.-april)	8-16-24 hs (0.8)	0.4-0.6-0.8 mg/g (16)
<i>L. pyrenaicus</i>	2n=50	24-25	4	Increasing (Jan.-june)	20-24-28-32 hs (0.5)	0.1-0.3-0.5-0.7-0.9- 1.1 mg/g (24 or 28)
<i>F.h. macrolepidotus</i>	2n=48	25	4 10	Increasing (Jan.-june)	12 to 200 hours 0.5	0.1-0.2-0.5-0.7-0.8 mg/g (12 to 200)

1 First experimental objective. Brdu treatment times at constant Brdu dose (mg/g) indicated in parenthesis.  
2 Second experimental objective. Brdu optimal dose at constant treatment time (h) indicated in parenthesis.  
N=Chromosome number; T= Water temperature °C; S= Salinity (g/l).

1500 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended with 4 ml of Carnoy. The suspension was then left for at least 12 hours at 4°C. Then it was centrifuged again and the fixative replaced at least twice prior to slide preparation.

The slides were obtained by applying the suspended cells (4-5 drops) onto a clean slide using a Pasteur pipette.

#### Staining procedure

After air drying the slides were stained with the Fluorescence Plus Giemsa (FPG) method of differential staining (Perry and Wolff, 1974; modified for fish by Giles *et al.*, 1988; Lobillo 1993). The slides were introduced into a staining jar with 100

ml of Carnoy and stored at 4°C for 12 hours. They were then washed in 0.5 x SSC (pH 7.05) and immersed in Hoechst 33258 solution (1mg in 1 ml of methanol plus 100 ml of 0.5 x SSC, pH 7.05) for 30 minutes. After rinsing in distilled water, they were covered with a thin layer of 0.5 x SSC and a coverslide, sealing the edges with a colour smalt. The slides were then exposed to a two 15-W Sylvania Germicidal Lamp at a distance of 7.5 cm for 90 minutes. After exposure, the coverslides were removed and then rinsed in distilled water and treated with 0.5 x SSC for 60 minutes at 50-55°C before staining in 4 p.cent solution Giemsa in Sorensen buffer, pH 6.85 for 10-20 minutes. The washed slides were covered with a thin layer of

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Sorensen and a coverslide and observed under an optical microscope for SCEs scoring.

Both the chromosome preparation and staining procedures were carried out in the dark or under very low light conditions, in order to avoid direct contact of natural light with BrdU-substituted-chromosomes. Experimental and treatment conditions for each group of fish are summarized in **table II**.

### RESULTS AND DISCUSSION

The obtained results showed in **table III** are discussed for each specie:

#### *LIZA AURATA*

Individuals of this species gave a medium-low response to BrdU-labelling method, with maximum M2 index between 15-35 p.cent and BrdU-treatment time at 24 hours. Optimal averages of M2 cells are necessary to make the SCEs scoring easier, more feasible and rapid (Hoeven *et al.*, 1982). Scoring of SCEs in some individuals was difficult because of low M2 index.

*L. aurata* has a medium chromosome number (2n=48), compared with other species such as *U. limi* (2n=22) or *N. rackowi* (2n=18), used successfully in genetic toxicology (Kligerman, 1979, Alink *et al.*, 1980; Hoeven *et al.*, 1982).

At optimal BrdU dose and treatment time, a base-line frequency of 2.73 0.3482 SCEs/M2/individual was obtained. This is the lowest frequency obtained from the species studied and could be explained by different metabolization capacities varying among species and also as a function of the medium-high temperature established

during assays with *L. aurata* (26-28°C). Base-line frequency is similar to that obtained by Lobillo *et al.*, (1990) in the same species and under similar experimental conditions.

Difficult capture and handling, the lowest survival percentages during travelling and laboratory conditions, and high stress susceptibility during manipulations operations (inoculations, etc) make this species inappropriate for our work conditions. But considering the suitable response to BrdU-labelling method, it could be used in laboratories near coasts.

#### *CIPRINUS CARPIO*

*C. carpio* is included in Post (1964) classification as a non- suitable species for studies of genetic toxicology. Very high chromosome number, (between 100-104) with little chromosomes and many microchromosomes make the scoring of SCEs, chromosome bands, etc., very tedious. Results obtained with BrdU technique show a maximum yield of M2 cells of 12 p.cent at 24 hours. Except in *F.h. macrolepidotus*, this percentage was the lowest obtained.

Kligerman (1979) also found a low percentage of M2 cells in *U. limi* (Pisces, Ciprinodontidae), between 20-30 p.cent in intestinal epithelial cells and 9-20 p.cent in kidney lymphocytes. However this author did not apply any mitotic index increasing methods such as the ones used in this paper. Pha-M stimulation method applied in *C. carpio*, doesn't show expected optimal results. Although the mitotic index was the highest found among the species used in this study, the M2 index doesn't increase comparatively. This fact seems to show that PHA-M response in renal lymphocytes

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**Table III.** Summary of the results obtained in the present study. (Resumen de los resultados obtenidos en el presente estudio).

	<i>L. aurata</i>	<i>C. carpio</i>	<i>R. alburnoides</i>	<i>L. pyrenaicus</i>	<i>F.h.macrolepidotus</i>
<b>Handling criteria</b>					
Capture difficulty	Difficult	Difficult	Very easy	Very easy	Easy
Travel Survival	Very low	Medium	Medium	Very high	Very high
Adaptation to laboratory	Very low	High	Very high and fast	Very high and fast	Very high and fast
Stress susceptibility	Great	Medium	Medium	Low	Lowest
<b>Genetic criteria</b>					
Maximum mitotic index	0.5 p.cent	1 p.cent	0.5 p.cent	0.7 p.cent	0.1 p.cent
Maximum M2 index	15-35 p.cent at 24 hours	12 p.cent at 24 hours	15-40 p.cent at 16 hours	48-63 p.cent at 28 hours	0 p.cent
Optimal (mg/g) Brdu dose	0.8	0.8	0.8	0.6	unobtainable
3	2.73 0.35	unobtainable	3.2841 0.10	3.482 0.465	unobtainable

3= Third Experimental Objective: Base-line frequencies of SCEs (SCEs/M2/individual).

phocites of *C. carpio* was optimal, but Brdu was not sufficiently incorporated to DNA, so that the cellular progression was not easily detected. It could be explained by specific conditions that could alter the carrier, uptake, excretion and metabolism of Brdu. These factors would be varying mainly as a function of medium-high temperature in assays with *L. aurata* and would determine the very low intracellular uptake and Brdu incorporation to DNA.

***F.H. MACROLEPIDOTUS***

No expected results were found for

this species after Brdu-labelling method. At wide intervals of Brdu doses and treatment times it was not possible to evidence any M2 cells. Under the same conditions and in order to perform experiments in water with different salinities, the mitotic index was the lowest obtained.

Experimental designs in this species were based on references by some authors that used other Ciprinodontides. These authors found that long Brdu treatment times were necessary to obtain the maximum yield of M2 cells. As Ciprinodontidae species tested seem to



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coincide in very large cell-cycle times (as a function also of temperature), consequently Kligerman and Bloom (1976) reported optimal treatment time of 120-156 hours in intestinal epithelium cells of *U. limi* and Alink *et al.*, (1980) found 240 and 120 hours in branquial epithelium cells of *U. pygmea* at 12 and 20°C, respectively.

In spite of the long treatment times tested (12-200 hours) and optimal temperature of 25°C, we didn't find any M2 cells. Possible explanations are widely discussed by Lobillo (1993). This author refers to a low lymphopoyesis rate in cephalic kidney as evidenced by renal smears stained with metachromatic dyes. The presence of tissular barriers such as the renal porta system in eurihaline species such as *F.h. macrolepidotus*, and increases of renal activity in fresh-water conditions because of osmotic regulations, could also accelerate the excretion of substances intraperitoneally administrated. Other physiological factors could act on Brdu levels or intracellular concentrations.

### *RUTILUS ALBURNOIDES*

Brdu technique applied to this species showed good results. Also were optimal: its effective capture using electric fishing methods, high survival percentage during transportation and easy adaptation to laboratory conditions.

*R. alburnoides* has a great genetic interest because of the presence of diploid and triploid individuals in natural populations. Collares-Pereira (1984) explain this phenomena due to hibridization of this specie with *L. pyrenaicus*.

But *R. alburnoides* shows some disadvantages compared to *L. pyrenaicus*. Triploid individuals of this specie

(about 80 p.cent in natural populations) has a mean value of 75 chromosomes, with large variation: less than 70 and more than 76 (Collares-Pereira, 1984). A high number and too much variability in chromosome numbers make the SCEs scoring very tedious and slow. Moreover chromosome size of *R. alburnoides* is comparatively shorter than *L. pyrenaicus*. This problem could be avoided if we changed the SCEs/M2 variable to the SCEs/Chromosome, although we would lose additional time in scoring the chromosomes per metaphase.

*R. alburnoides* is also highly sensitive to handling operations. This problem is unusually present in *L. pyrenaicus*. Optimal Brdu dose for *R. alburnoides* was found in a previous paper by our team (Lobillo *et al.*, 1991). At medium-high temperature conditions of 28°C this optimal dose was 0,8 mg/g. At this temperature the M2 index at optimal treatment time of 16 hours was more variable and shorter (15-40 p.cent), compared with *L. pyrenaicus* (48-63 p.cent). High variability on M2 index in *R. alburnoides* was referred to by Lobillo (1990) and could be explained by the temperature effects on metabolic activity and more specifically on cellular division synchronism (Giles *et al.*, 1989; Lobillo, 1990; ).

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Results exposed in table III seem to show that *L. pyrenaicus* is the best choice for standarization of the Brdu method and posterior genotoxic assays. The main reasons for this choice are: easy and efficient capture by electric fishing method; high survival percentage during transportation; easy and rapid adaptation to aquaria conditions; very low stress

susceptibility; constant chromosomal formulae equal to  $2n=50$ ; chromosome size superior than that of *R. alburnoides*; optimal response to Brdu treatment with maximum percentages of M2 cells (48-63 p.cent) and low interindividual variability; short time per assay, taking only 28 hours; optimal body weight and size, about 10-15 g and 10 cm, respectively. This implies easy handling during inoculation process and less cost due to the low amount of administered substances.

*L. pyrenaicus* is included in the group of fish with medium-high chromosomal number. Presently day, authors are using several species with chromosome numbers much higher compared with limitations referred to by Post (1964) about fish species with suitable karyotypes for toxicological studies. In practical situations quality criteria of optical observation and chromosome structure resolution are more important than chromosomal number criteria.

Compared with other species selected to measure genetic damage by water pollutants in natural waters and aquaculture farms, this species has an important role over *U. limi*, the most commonly used specie of fish in the SCE test. Optimal treatment time with Brdu in *L. pyrenaicus* was found to be 28 hours at 24-25°C using renal lymphocytes as target cells. In *U. limi* the optimal time was obtained as 120-156 hours at 20°C on intestinal cells (Kligerman *et al.*, 1976). Moreover, M2 index in *L. pyrenaicus* is very high (48-63 p.cent) if compared with the results obtained for *U. limi* (30 p.cent maximum).

According to our results, *L. pyrenaicus* seems to be the optimal species to assess the SCE test in fish.

Further intensive studies such as exposure to well-known genotoxic agents revealed a suitable end-point and high sensitivity of the SCE test in this species for detecting and monitoring genotoxic agents in laboratory conditions (Lobillo, 1993). SCEs test in *L. pyrenaicus* could become an integral and important piece of genotoxic control programs under both laboratory and field (continental waters only) conditions.

Efforts to evaluate the feasibility of fish cytogenetics assays for assessing genotoxic exposure in natural or farm fish populations, have been limited. Alink *et al.*, (1980) reported increase frequencies of chromosome breaks and SCEs, respectively in *U. pygmea* exposed to Rhine water in Flow-through aquaria. The reports of SCE induction in English sole (Stromberg *et al.*, 1981) and of chromosomal aberration induction in a feral goby in India (Krisnaja and Redge, 1982) are found in this context. Subsequent studies by others have continued to validate the mundminow (*Umbra* sp.) SCE assay for laboratory testing of chemicals for genotoxic activity (Vigfusson *et al.*, 1983). But the fact that *Umbra* species may not be readily available in areas where studies are desired and they don't have a good adaptation, are two great limitations.

The easy availability of *L. pyrenaicus* in continental waters of the Spanish southwest, and optimal and very sensitive response to Brdu technique indicate:

1. The suitability of the SCE test in *L. pyrenaicus* for detecting and monitoring genotoxicants under laboratory conditions (Lobillo, 1993).

2. SCEs test in this species present

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clear advantages for the development of procedures to assess genetic damage in fish exposed *in situ* to point sources of aquatic pollution and so, the evaluation of the role of genotoxic contaminants in epizootic neoplasia in fish populations as referred to by Maddock *et al.*, (1986) and the prevention of serious ecological catastrophes, economic losses in fish

populations and the ingestion of contaminated fish.

3. The Brdu-method applied in *L. pyrenaicus* and other fish species is also useful in estimating the cell-cycle times (Lobillo, 1993). The cell-cycle knowledge is a previous and very interesting datum for the development of several zootecnic operations in fish production.

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