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Epithelial cell renewal in the digestive gland and stomach of mussels: season, age and tidal regime related variations

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Summary. The natural variability in cell proliferation activity in the epithelium of the digestive gland and stomach was investigated in mussels, *Mytilus galloprovincialis* (Lmk), of different age and tidal level at different seasons. After treating mussels with the thymidine analogue bromodeoxyuridine (BrdU) for 6 hours, BrdU immunohistochemistry was performed every 2 hours for the next 36. The relative proportion of BrdU positive cells was quantified as BrdU labelling (‰). Marked seasonal differences were recorded in BrdU labelling, with much higher proliferating activity in summer than in autumn and winter. Cell proliferation seemed not to be significantly dissimilar between mussels of different age (size). In contrast, the digestive gland epithelium of mussels from intertidal and subtidal populations differed not only in the levels but also in the pattern of variation of BrdU labelling, which in intertidal mussels appeared to be modulated by photoperiod and tide, unlike in subtidal mussels, in which variations followed a circatidal pattern.

Key words: Bromodeoxyuridine immunohistochemistry, Digestive cell, Basophilic cell, Cell epithelial renewal, Circatidal rhythm, Size, Age, Season, Tide, Bivalve

Introduction

The digestive gland epithelium in mussels is comprised of two differentiated cell types, the so-called digestive and basophilic cells. Digestive cells possess a highly developed endo-lysosomal system and are responsible for the intracellular digestion of food materials. Basophilic cells have a well developed rough endoplasmic reticulum, which confers them their characteristic basophilia, and are less abundant than digestive cells. Marigómez et al. (1999) demonstrated by proliferating cell nuclear antigen (PCNA) immunohistochemistry that both digestive and basophilic cells are able to proliferate. The presence of mitotic figures in digestive cells and BrdU immunohistochemistry confirmed that cell renewal is produced by autologous division of mature digestive and basophilic cells (Zaldibar et al., 2004). Epithelial cell renewal in digestive diverticula is synchronised following a circatidal pattern, as indicated by changes in BrdU labelling (Zaldibar et al., 2004). Even when experiments were only carried out in subtidal individuals it was proposed that they were influenced by the tidal regime. It seems that cell renewal in the digestive gland epithelium is restricted to those periods in which, according to Izagirre (2002), the physiological activity of digestive cells (i.e., intracellular digestion) is reduced. Thus, gaining knowledge on the interactions between physiological activity and cell cycle in the digestive gland might be noteworthy to understand the dynamics of cell renewal in the digestive epithelium. In this context, a crucial question is to determine whether the circatidal pattern of proliferating activity found in the digestive gland cells is altered or modulated by natural factors (age, season, tidal regime) that affect the activity of the digestive gland. Digestive gland form and function, including timing and intensity of digestion and other physiological processes, vary according to these factors (Etxeberria et al., 1994; Cancio et al., 1999; Fernández-Reiriz et al., 2001; Wong and Cheung., 2001; Le Pennec and Le Pennec, 2002; Pazos et al., 2003). Moreover, mussels of different size (age) differ in the proliferation pattern in digestive gland cells. Indeed, PCNA immunoblotting suggested that the digestive gland cells of small (juvenile) mussels might posses a higher proliferating capacity than those of large (adult) ones (Marigómez et al., 1999).

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The distribution of proliferating cells in the digestive gland and stomach of mussels, *Mytilus galloprovincialis* (Lmk), of different sizes (small -juvenile- and large -adult-) collected from two different tidal zones (intertidal and subtidal) at three different seasons (winter, summer and autumn) was investigated by BrdU immunohistochemistry. The BrdU is an analogue of thymidine that during the S-phase of the cell cycle is incorporated in the DNA of proliferative cells. Mussels were treated with a pulse of 4 mg BrdU/l seawater for 6 h in the afternoon. The presence of BrdU was later detected immunohistochemically, and the number of proliferating cells (BrdU labelling) was recorded every 2 h for 36 hours.

Materials and methods

Chemicals

All chemical products were Sigma-Aldrich, St Louis, Missouri, USA unless specified otherwise.

Preliminary experiments

In order to compare cell proliferation in digestive gland and stomach between subtidal and intertidal mussels, *Mytilus galloprovincialis*, the BrdU pulse had to be restricted to the period of immersion for intertidal mussels (6 h). Since previous studies revealed a circatidal pattern of BrdU labelling after continuous waterborne exposure to BrdU, some preliminary experiments were designed to determine the best moment of the day to apply the BrdU pulse. With this purpose, 160 juvenile (<15 mm shell length) and 160 adult (35-45 mm shell length) subtidal mussels were collected from Plentzia (Bay of Biscay, 43° 24'N 2° 56'W) at low tide in July, transferred to the laboratory and distributed in 4 polyethylene aquaria (80 mussels in 15 l seawater) at 15°C with constant aeration. Seawater was sterilized by UV light and filtered through glass wool and active charcoal. Seawater was changed every 12 hours and mussels were fed (Marine Invertebrate Diet, Carolina, Burlington, North Carolina, USA) every 12 hours, together with seawater change. Two complementary experiments were carried out. In the first experiment (Day-pulse experiment), juvenile and adult mussels were exposed separately to 4 mg BrdU/l seawater for 6 h starting at 14:00. Further, mussels were placed in BrdU-free seawater and 5 specimens were sacrificed every 2 h during the next 36 hours. In the second experiment (Night-pulse experiment), the same BrdU pulse was applied during the night (between 24:00 and 6:00) and mussels were sacrificed every 2 h during the next 28 hours.

Core experiments

Mussels (n=320) were collected in Plentzia at low tide, transferred to the laboratory, and distributed in 4 polyethylene aquaria (15 l) at 15°C with constant aeration, in three different seasons: winter (March), summer (July) and autumn (October). Mussels of different age (juvenile: <15 mm shell length; adult: 35-45 mm shell length) were selected from both intertidal and subtidal populations, and thus four experimental sets (80 mussels each) were established for each season: adult subtidal (AS), adult intertidal (AI), juvenile subtidal (JS) and juvenile intertidal (JI) mussels. Seawater was sterilised with UV light and filtered through glass wool and active charcoal.

All experimental sets were treated with 4 mg BrdU/l seawater for 6 hours starting at 14:00, during the immersion period for intertidal mussels. Further, mussels were placed in BrdU-free seawater and 5 specimens were sacrificed at different time intervals in order to quantify BrdU labelling. Seawater was changed every 12 hours in AS and JS experimental sets. Mussels in AI and JI experimental sets were kept in intertidal conditions, being exposed to water and air at 6 h intervals according to the tidal regime of their source site. Feeding was synchronised and mussels from all the experimental sets were fed every 12 hours, together with seawater change, during the immersion period for intertidal mussels.

Immunohistochemistry of BrdU positive cells

Small pieces of digestive gland were fixed in Carnoy's fixative (60% absolute ethanol, 30% chloroform and 10% glacial acetic acid) at 4°C for 1 h, dehydrated in a graded series of ethanol and embedded in paraffin at 60°C. Sections, 3-4 μm thick, were obtained in an American Optical microtome and collected in previously silanised (3-aminopropyl-triethoxysilane) slides. Further, sections were dewaxed in xylene, hydrated in a graded series of ethanol and washed in phosphate-buffered saline buffer (PBS). In order to avoid endogenous peroxidase activity, sections were incubated for 5 min in a 3% H_2O_2 bath and washed again in PBS. DNA was denaturalised with 0.1 N HCl at 37°C for 1 h. After washing in PBS, sections were incubated at room temperature for 1 h in normal blocking serum (NBS) supplied in the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, California, USA) and washed again in PBS. Then sections were incubated in the specific antibody for BrdU (Roche, Basel, Switzerland; 1:10 in PBS with 0.1% bovine serum albumin), for 1 h. After washing the samples in PBS the immunocomplexes were visualized with the avidin-biotin-enzyme complex (ABC) method using the Peroxidase Vectastain Elite ABC Kit. Shortly, sections were incubated with biotinylated horse anti-mouse IgG secondary antibody diluted in PBS for 30 min and gently washed in PBS. Further, incubation in avidin-biotin-peroxidase complex (diluted in PBS) was carried out for 30 min. The visualisation of peroxidase activity was achieved using a chromogen solution containing 3-amino-9-ethylcarbazole in sodium acetate buffer (pH 5.2) and 0.015% H_2O_2. Finally, sections were

counterstained with hematoxylin (10 sec) washed with tap water and mounted in Kaiser's glycerol gelatine (Merck & Co., Inc., Whitehouse Station, New Jersey, USA). Micrographs were producing with an Olympus PM-20 camera attached to an Olympus BX50 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Quantification of BrdU positive cells

Quantification of BrdU positive cells was achieved by direct counting at the light microscope. In order to estimate the proportion of BrdU positive cells in the digestive epithelium, a total of 1000 cells were counted per sample, according to the sample size used in other studies (Okudela et al., 1999; Zaldibar et al., 2004). Preliminary histological observations of the mussel digestive gland revealed that above 20-30 cells form each tubule-like section of the digestive epithelium. Thus, in order to facilitate routine work, counting of BrdU positive cells was done in 40 randomly selected tubule-like sections (800-1200 cells) per digestive gland. In the stomach, counting was carried out following transects of 200 cells along the epithelium. This apparently small sampling size was established after preliminary observations that revealed a high proliferative capacity in the stomach epithelium. Results are presented as counts-per-thousand for comparison purposes.

Results

In the digestive epithelium both digestive and basophilic cells showed BrdU positive nuclei (Fig. 1C), though the majority of reactive cells were digestive cells (Fig. 1A,B). BrdU positive nuclei were more abundant in the stomach (Fig. 1D). Control sections incubated without primary antibody were completely devoid of any positive reaction (Fig. 1E).

Preliminary experiments

A comparable pattern of circatidal variation in BrdU labelling was recognized in digestive gland and stomach epithelia, although signal-to-noise ratio in the stomach

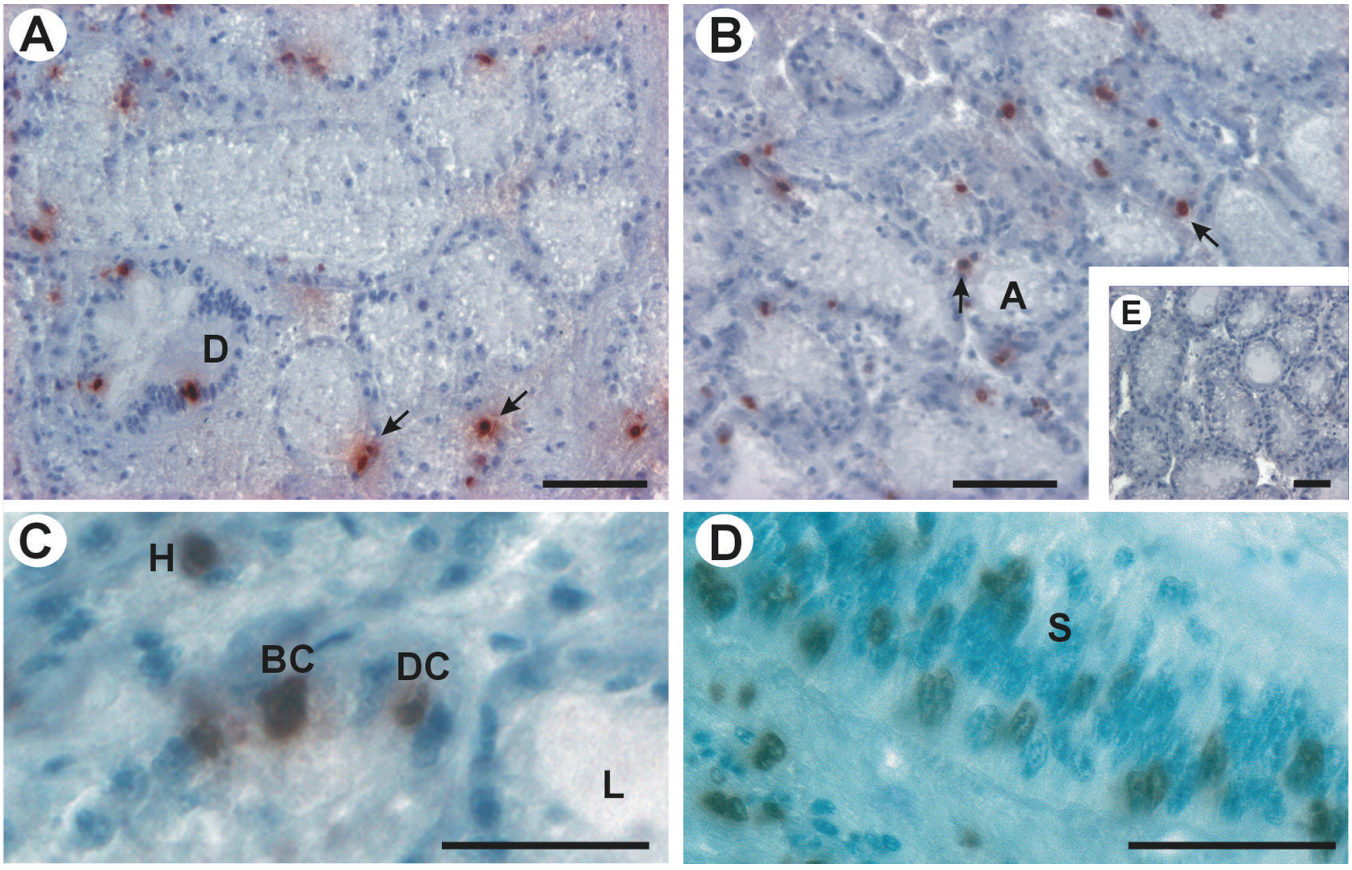

Fig. 1. Identification of BrdU positive cell in the digestive cells of alveolotubular units **(A-C)**, ducts **(A)** and stomach **(D)**. No BrdU positive cells are observed in control sections incubated without primary antibody **(E)**. D: duct epithelial cell; A: alveolotubular unit; H: hemocyte; BC: basophilic cell; DC: digestive cell; S: stomach epithelial cell; L: Lumen. Scale bars: 50 μm.

was low. Therefore, only the results corresponding to BrdU labelling in the digestive gland epithelium are presented below.

In the Day-pulse experiment, up to 33‰ of cells in the digestive epithelium appeared labelled with BrdU in large mussels, with a smaller labelling peak (21‰) in the small mussels (Fig. 2A,C). Results concerning quantification of BrdU labelling in the digestive epithelium basically corresponded to changes in the proliferation rate of digestive cells, since BrdU positive basophilic cells were only occasionally observed. In qualitative terms, exactly the same pattern of variation was found in both small and large mussels. In order to determine whether the variation pattern is governed by tidal regime (TR) or light/dark cycle (LDC), the average BrdU labelling corresponding to the periods of immersion (from low to high tide) and aerial exposure (from high to low tide) at both day and night was calculated (Table 1, Fig. 3). For this purpose, and considering the decay observed in BrdU labelling as the experiment progressed, only data within 12 hours after the BrdU pulse were considered for calculations. The maximum average BrdU labelling was found during immersion at night in both large and small mussels, with intermediate values during day immersion in small mussels (Fig. 3). Two-way ANOVA indicated that in adult mussels TR exerted a significant effect on average BrdU labelling, whereas in juvenile mussels only the

Table 1. Mean BrdU-labelling (‰) ± standard deviation of the digestive alveoli of mussel sampled at different photoperiod and tide conditions in the preliminary experiments.

DAY-PULSE	ADULTS			JUVENILES		
	Air-exposed	Submerged	Total	Air-exposed	Submerged	Total
Daylight	1±1.414	1±0.89	2	2.4±2.074	6.5±5.45	8.9
Dark	0.44±0.73	16.15±16.49	16.59	2.44±2.92	11.25±+9.02	13.69
Total	1.44	17.15	18.59	4.84	17.75	22.59

NIGHT-PULSE	ADULTS			JUVENILES		
	Air-exposed	Submerged	Total	Air-exposed	Submerged	Total
Daylight	3.75±2.5	2.8±0.83	6.55	1.33±1.53	1±1.41	2.33
Dark	2.42±2.71	4.75±3.24	7.17	5.91±5.90	7.9±5.51	13.81
Total	6.17	7.55	13.72	7.24	8.9	16.14

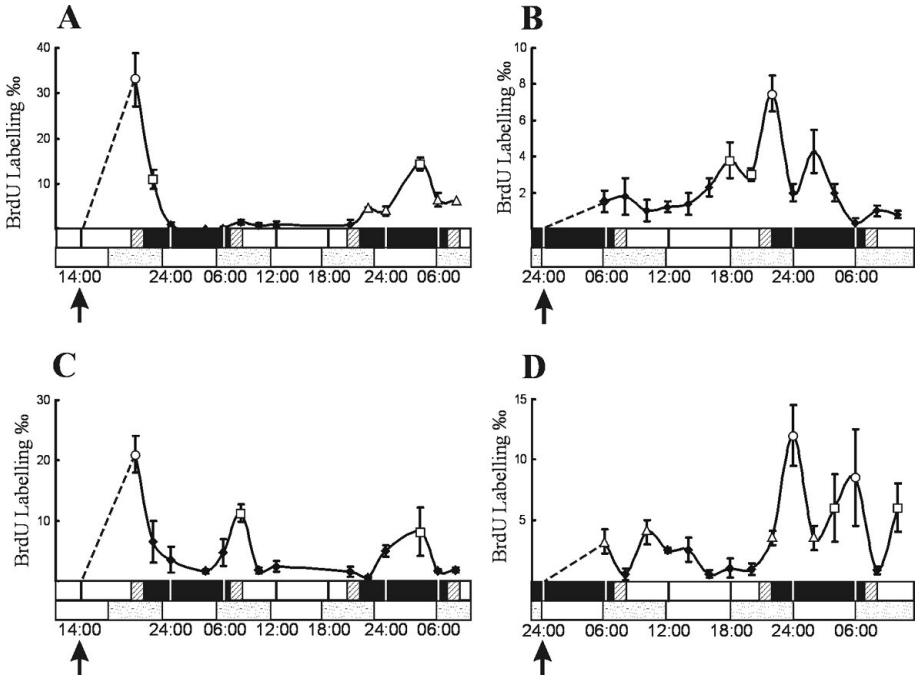

Fig. 2. BrdU labelling (‰ ± standard deviation) through experimental time (according to the sun) in the digestive gland of subtidal mussels maintained for 2 days under natural photoperiod (light/dark). **A.** Day-pulse adult mussels. **B.** Night-pulse adult mussels. **C.** Day-pulse juvenile mussels. **D.** Night-pulse juvenile mussels. Arrows indicate the moment in which BrdU was supplied. Horizontal bars indicate photoperiod (white, light, hatched twilight, black night) and tide-regime conditions (white, air-exposed; wavy, submerged) along the experimental period. The different dot shapes and fillings represent significantly dissimilar subsets of mean values according to the Duncan´s test ($p<0.05$).

interaction TR x LDC resulted to be significant (Table 2).

In the Night-pulse experiment, up to 7‰ of cells in the digestive epithelium appeared labelled with BrdU in large mussels, with a higher labelling peak (12‰) in the small mussels (Fig. 2B,D). In qualitative terms, exactly the same pattern of variation was found in both small and large mussels. As in the Day-pulse experiment, the average BrdU labelling values corresponding to the periods of immersion and aerial exposure at both daylight and night were calculated (Table 1, Fig. 3). For this purpose, and since no obvious peak values were observed in BrdU labelling during the next daylight period following the BrdU pulse, only data recorded during the second day were considered. Although the maximum average BrdU labelling was observed at night, especially in small mussels, no significant differences were found between experimental groups (Fig. 3). Accordingly, two-way ANOVA indicated that only LDC exert a significant effect on average BrdU labelling in small mussels, whereas in large mussels neither TR nor LDC or their interaction had a significant effect on average BrdU labelling (Table 2).

Table 2. Summary of the two-way ANOVA performed in order to determine the effect of the tidal regime and light/dark cycle and their interaction in the BrdU-labelling of digestive alveoli in mussels.

DAY-PULSE	Degrees of freedom	F	p(F)
Adults			
Tide Regime (TR)	1	4.091	0.05*
Light/Dark cycle (LDC)	1	3.348	0.08
TR x LDC	1	4.266	0.048*
Internal error	482.5		
Juveniles			
Tide Regime (TR)	1	8.943	0.06
Light/Dark cycle (LDC)	1	1.234	0.28
TR x LDC	1	1.189	0.029*
Internal error	862.92		
NIGHT-PULSE	**Degrees of freedom**	**F**	**p(F)**
Adults			
Tide Regime (TR)	1	0.412	0.53
Light/Dark cycle (LDC)	1	0.087	0.77
TR x LDC	1	2.347	0.14
Internal error	190.48		
Juveniles			
Tide Regime (TR)	1	0.507	0.48
Light/Dark cycle (LDC)	1	7.268	0.01*
TR x LDC	1	0.323	0.58
Internal error	620.46		
CONTINUOUS PULSE (1)	**Degrees of freedom**	**F**	**p(F)**
Adults			
Tide Regime (TR)	1	12.614	0.001*
Light/Dark cycle (LDC)	1	3.433	0.07
TR x LDC	1	2.399	0.13
Internal error	2159		
INTERTIDAL MUSSELS	**Degrees of freedom**	**F**	**p(F)**
Summer			
Tide Regime (TR)	1	2.115	0.15
Light/Dark cycle (LDC)	1	1.75	0.19
TR x LDC	1	0.002	0.96
Internal error	1452.07		
Autumn			
Tide Regime (TR)	1	3.362	0.07
Light/Dark cycle (LDC)	1	0.684	0.41
TR x LDC	1	2.283	0.13
Internal error	1750.11		

(1) After Zaldibar et al., 2004. p(F), Fisher's F ratio. Asterisks show statistically significant differences ($p<0.05$).

Core experiments

Season, age and tidal regime effects on cell proliferation

Several parameters were calculated in order to compare the rate of cell proliferation between mussels of two different ages collected in three seasons from two different tidal levels in an integrative way (Table 3). The total BrdU-labelling/day index was estimated on the basis of the number of BrdU-positive cells recorded in the maximum peak observed within the fist 12 hours after the pulse (A in Table 3) multiplied by 2 (B in Table 3) and by a correction factor (C in Table 3). The correction factor was applied considering that a 6 h BrdU pulse was used instead of continuous BrdU supply as in previous experiments (Zaldibar et al., 2004) for comparison purposes:

CF = 1.21 = BrdU-positive cells (12 hr CS)/ BrdU-positive cells (12 hr AP)

BrdU-labelling/day index = BrdU-positive cells (12 hr AP) x 2 x CF; where CF: Correction factor; CS: Continuous BrdU Supply; AP: after 6 hr BrdU pulse.

Finally, a rough estimate of the time in days

Table 3. Summary of BrdU-labelling (‰) quantified in core experiments.

	JUVENILES				ADULTS			
	A	B	C	D	A	B	C	D
WINTER								
INTERTIDAL	5.8	11.6	14.04	70	4	8	9.68	100
SUBTIDAL	6.25	12.5	15.12	66	4.25	8.5	10.28	95
SUMMER								
INTERTIDAL	10	20	24.2	40	17.25	34.5	41.74	24
SUBTIDAL	21	42	50.82	20	33	66	79.86	12
AUTUMN								
INTERTIDAL	10.66	21.32	25.79	38	18.5	37	44.77	22
SUBTIDAL	2.33	4.66	5.64	175	2.4	4.8	5.808	170

A: Maximum BrdU peak quantified along the first 12 hours of experiment. B: Amount of cells that enter S-phase if every day two BrdU peaks take place. C: Amount of cells that would enter S-phase in one day in case of continuous BrdU supply, estimated after the introduction of a correction factor according to the data presented by Zaldibar et al., (2004). D: Number of days required to fully renovate the digestive gland epithelium.

(epithelium renewal time; ERT) required for the complete renewal of the entire epithelium (D in Table 3) was made based on calculations already described above.

The proliferation activity in the digestive gland epithelium changed markedly between seasons, being highest in summer and lowest in winter, and between tidal levels, but also depended on the mussel age at certain seasons and tidal levels (Table 3). The highest values of BrdU-labelling/day index (C in Table 3) were recorded in summer, when ERT of the digestive gland epithelium (D in Table 1) was between 2 and 6 weeks, depending on the age and the tidal level. The lowest BrdU-labelling/day index, and hence the highest ERT values (around 6 months), were found in subtidal mussels in autumn. In winter, no differences were found in BrdU-labelling/day index between tidal levels but values turned out to be slightly higher in juvenile than in adult mussels (Table 3). Significant differences were found between subtidal and intertidal mussels in autumn since cell proliferation was relevant in intertidal mussels whereas it was almost absent in subtidal mussels. Particularly in summer, BrdU-labelling/day index was higher in adult mussels than in juveniles (Table 3).

Patterns of variation in epithelial cell proliferation

Cell proliferation activity in the digestive epithelium resulted negligible in mussels collected in winter as well as in subtidal mussels collected in autumn (Table 3), so that a pattern in epithelial cell renewal was not evidenced at all. Conversely, though distinct, consistent patterns of cell proliferation were found in subtidal mussels in summer and in intertidal mussels in summer and autumn (Figs. 2A,C, 4). Interestingly, distinct patterns were identified in the digestive gland epithelium of subtidal and intertidal mussels.

In subtidal mussels, both adult and young specimens exhibited the same pattern of variation in BrdU labelling, as described in the preliminary experiments (Day-pulse experiment; Fig. 2A,C). However, the pattern of variation in BrdU labelling in intertidal mussels was completely different, with only one peak per day and unchanged BrdU labelling values during high tide, irrespective of whether they were during the day or at night time (Figs. 4, 5; Table 1). In summer, up to 17.25‰ of cells in the digestive epithelium were BrdU positive. Labelling increased during morning aerial exposure (between 10:00 and 12:00) and remained almost unchanged to return to baseline levels before dusk aerial exposure, between 20:00 and 22:00 (Fig. 4A). In autumn, the maximum peak in BrdU labelling (18.5‰) corresponded to the morning aerial exposure (08:00), whereas the return to baseline levels was promptly accomplished during the morning, before the next immersion (Fig. 4B).

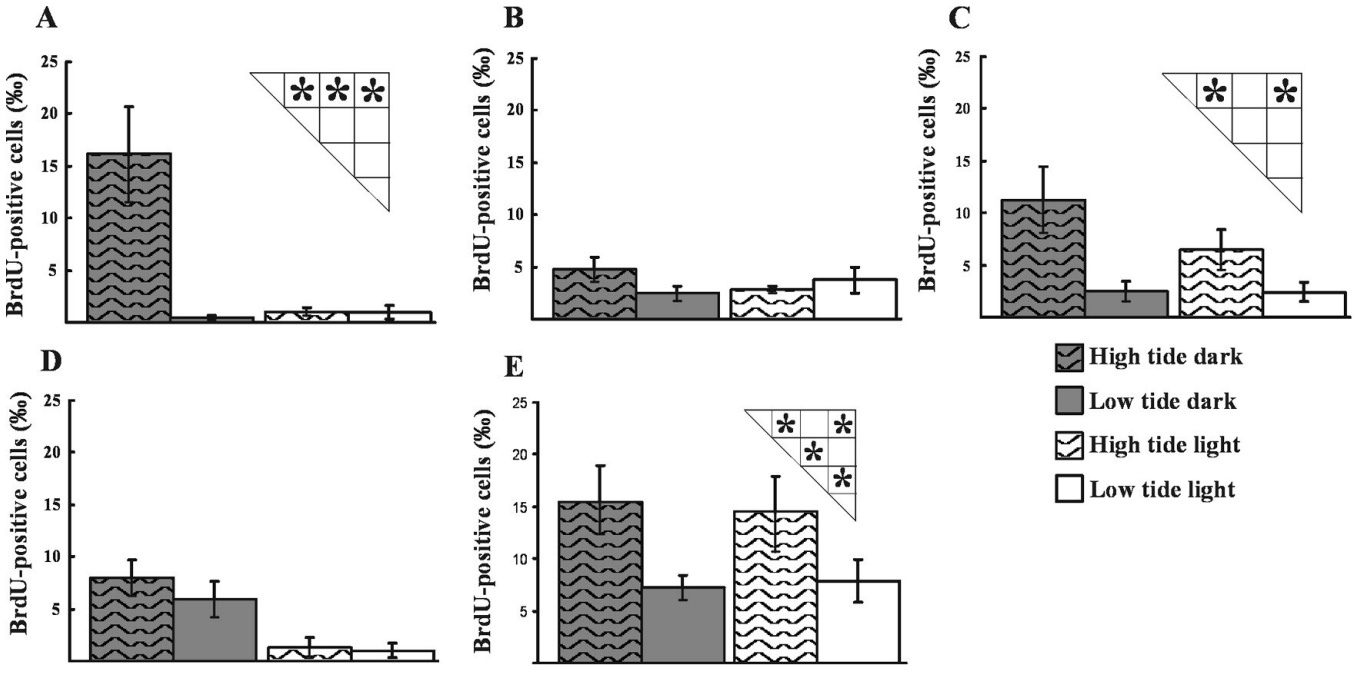

Fig. 3. Mean BrdU-labelling in the digestive alveoli of the mussels kept for 2 days under natural photoperiod (light/dark). **A.** Mature mussels in the day-pulse experiment. **B.** Mature mussels in the night-pulse experiment. **C.** Juvenile mussels in the day-pulse. **D.** Juvenile mussels in the night-pulse experiment. **E.** Mean BrdU-labelling in the continuous BrdU-pulse experiment (Zaldibar et al., 2004). Vertical segments show standard errors. Upper asterisks show significant differences between groups ($p<0.05$).

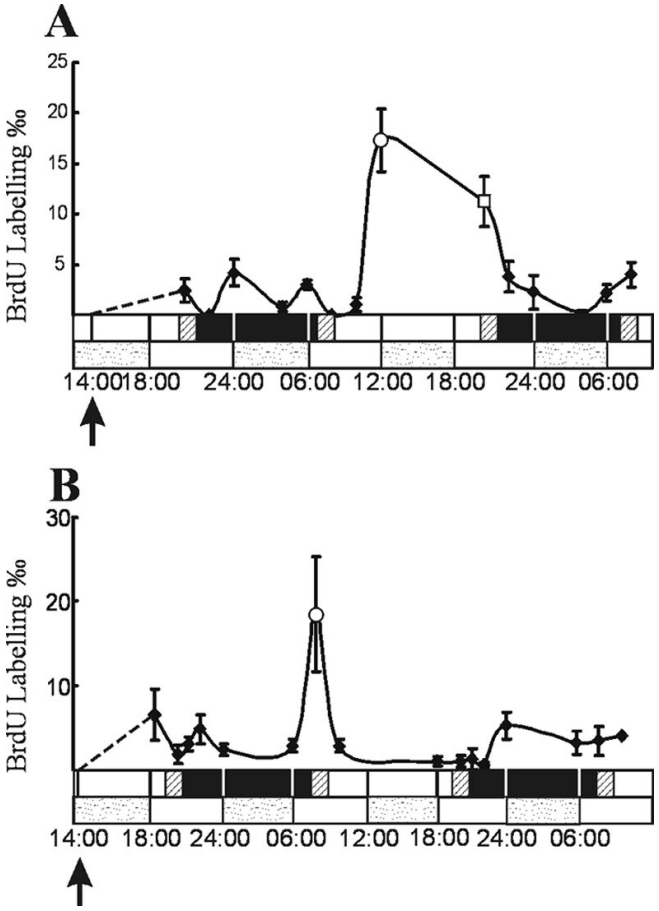

Fig. 4. BrdU labelling (‰ ± standard deviation) along the time (according to the sun) in the digestive gland of intertidal mussels maintained for 2 days under natural photoperiod (light/dark). **A.** Summer mature intertidal mussels. **B.** Autumn mature intertidal mussels. Arrows show the moment in which BrdU was supplied. Horizontal bars show light conditions (white: light, hatched: twilight, black: night) and tide conditions (white: air-exposed; wavy: submerged) along the experimental period. The different dot shapes and fillings represent significantly dissimilar subsets of mean values according to the Duncan´s test ($p<0.05$).

Discussion

Selection of BrdU Pulse to compare subtidal and intertidal mussels

In the Day-pulse experiment, the maximum BrdU labelling was found during immersion at night in both large and small mussels, with intermediate values during day immersion in small mussels. Tidal regime exerted a significant effect on BrdU labelling in young mussels, whereas in adults the interaction between tidal regime and dark/light cycle was significant. This pattern of variation in cell proliferation resembled the one reported in large subtidal mussels after continuous treatment with BrdU (Zaldibar et al., 2004). Probably, the decrease in BrdU availability due to a 6 h pulse, as opposed to the continuous BrdU addition that was employed in the previous report, would explain why the circatidal pattern of cell proliferation vanished beyond 12 h after the BrdU pulse, especially in adults, in which a limited BrdU supply would be more critical than in juveniles since BrdU labelling is more marked (higher maximum peaks). Under these conditions, it is likely that the BrdU levels available for mussels with this experimental setup were low (Potten et al., 1992; Candia Carnevali et al., 1997), but we were constrained by a balance between the need to use innocuous BrdU concentrations (see Zaldibar et al., 2004) and the limited time for waterborne exposure in the intertidal mussels that we planned to investigate in the present core experiments.

The pattern of variation in cell proliferation was different in the Night-pulse experiment. BrdU labelling only increased significantly during the night, with two consecutive maximum peaks, whereas only baseline levels were observed during the daylight period, even immediately after the BrdU pulse. It is worth noting that BrdU labelling is at minimum just after the BrdU pulse, unlike the situation in the Day-pulse experiment where maximum BrdU labelling was recorded at the first sampling, just after ceasing the BrdU pulse. In mammals, the presence of cell proliferation in digestive tract epithelia at night has been previously reported (Scheving et al., 1978; Smaaland, 1996). Although in marine molluscs certain cellular processes have been described to occur prominently at night and others during the day (Levy et al., 1994), it has been demonstrated that digestive cell proliferation follows a circatidal pattern in mussels (Zaldibar et al., 2004). Accordingly, the present results appear to indicate that tide is a major factor governing the cell proliferation cycle. The photoperiod might also modulate, to some extent, cell proliferation processes. Nevertheless, this photoperiod effect might well be an artifactual result due to the limited BrdU supply. In the Day-pulse experiment, major peaks, which were highest in adults in which the interaction TR x LDC was significant, were found during the first daylight period, in the first sampling after the BrdU pulse.

In order to compare the present results with those obtained after a continuous supply of BrdU under identical experimental conditions, the average BrdU labelling values corresponding to the periods of immersion and aerial exposure at both day and night were calculated with data obtained with mussels after continuous BrdU supply by Zaldibar et al. (2004), according to the calculations applied in the present study (Tables 1 and 2; Fig. 3E). As in the Day-pulse experiments, the tidal regime or the TR x LDC interaction exert a significant effect on the average BdU labelling.

Consequently, in view of the results of both preliminary experiments, it was decided to follow the procedure of the Day-pulse experiment for core experiments because: (a) more BrdU labelling was

recorded in the Day-pulse experiment; and (b) the variation pattern found in the Day-pulse experiment was more similar to that observed in previous experiments after continuous administration of BrdU (Zaldibar et al., 2004) than the variation pattern recorded after the Night-pulse experiment.

Seasonality in epithelial cell renewal

Cell proliferation rates were at their highest in summer time. Seasonal changes in enzyme activities (Cancio and Cajaraville, 1999; Le Pennec and Le Pennec, 2002), lipid contents (Pazos et al., 2003), peroxisomal parameters (Cancio et al., 1999) and digestive cell lysosomal structure (Etxeberria et al., 1994) have been reported in the digestive gland of mussels. Warmer temperatures and thus thermal acclimation together with increased food availability could be very important in digestive epithelium dynamics, resulting in activation of the digestive epithelium. Therefore, the increased proliferating activity of digestive epithelium cells during summer could be due to increased food availability, resulting from algae and phytoplankton blooms allowing an increase in somatic growth in mussels. Moreover, it could also be possible that an increased metabolic rate during summer would result in enhanced cell damage and increased cell turnover, as illustrated by the enhanced rates of DNA strand breaks and protein denaturation reported in tissues of summer vs. winter collected mussels (Hofmann and Somero, 1996; Shaw et al., 2000). Therefore, cell proliferation increase in the digestive gland during summer time might be related both to epithelial cell renewal and structure holding and to somatic growth. Likewise, during autumn and late winter months, the decrease in temperature and food availability and the subsequent decrease in metabolic rate, cell damage and cell turnover could explain the reduced proliferating rates described in this study. Similarly, Leibson and Frolova (1994) found a marked seasonal variation in cell proliferation in the digestive tract of *Cremomytilus grayanus* from the Sea of Japan, with maximum peaks of epithelial cell proliferation in May-June and minimum peaks in February-March. These authors relate the decrease in cell proliferation to low temperatures leading to a decrease in metabolic rate and feeding activity during winter months.

Variation with age in epithelial cell renewal

Although previous studies where PCNA immunochemistry was carried out demonstrated that the digestive gland of young mussels could be more proliferative than in large ones (Marigómez et al., 1999), this does not seem to be the case in the present study. In this way, small and large animals show similar cell proliferation rates. Even more, in summer, the BrdU-labelling/day index is higher in adults than in juveniles. It is likely that the sensitivity of PCNA and BrdU are different (Sarli et al., 1995; Muskhelishvili et al., 2003), which could explain these controversial results. Moreover, unlike PCNA, BrdU-labelling would depend on the availability of the probe after BrdU administration, which may differ between juveniles and adults, depending on variables such as feeding activity, assimilation efficiency and other physiological variables that change with mussel age (Thompson et al., 1974; Bayne et al., 1976). Thus, the same mode of waterborne pulse does not necessarily ensure that the digestive gland of young and adult mussels receives the same amount of BrdU or at the same time. Nevertheless, the results revealed the same pattern of variation in cell proliferation, at least in qualitative terms, which suggests that the amount of BrdU reaching the digestive epithelium was not a major limiting factor. In addition, the results obtained for PCNA were based on immunoblotting studies whereas the present BrdU results have been obtained through immuno-histochemistry. It cannot be discounted that the differences reported by Marigomez et al. (1999) after applying PCNA are not due to differences in the proliferation rate of digestive gland epithelial cells but other cell types included in the homogenate (i.e., hemocytes), whereas the present results are more concretely addressed to the proliferation activity of digestive cells.

Effect of tidal regime in epithelial cell renewal

In intertidal mussels, more S-phases are recorded during air-exposure, but no decrease in BrdU-labelling is observed during immersion. That would mean that during both emersion and immersion DNA replication progresses, but cell division is majorly present when animals are air exposed. It appears that cell division would never occur together with eating during high tide, while DNA synthesis could happen at the same time. The segregation among the time for eating and certain relevant physiological activities has been previously documented in marine molluscs (Susswein et al., 1983).

On the other hand, intertidal organisms may modify their metabolic pathways to support short or long periods of hypoxia, as it is the case in exposure to air twice a day. This is achieved through metabolic depression, switching to anaerobic pathways for energy generation and control of enzymes by transcriptional regulation or by reversible protein phosophorilation (Greenway and Storey, 2000; Ton et al., 2003; David et al., 2005; Papandreou et al., 2005). If the correct oxygen/supply demand is not fulfilled the number of cells needs to be reduced as well, so that the response to hypoxia can be described as an arrest of cell cycle or/and an apoptotic induction (Ton et al., 2003; Papandreou et al., 2005), which occurs under the control of a transcriptional activator, the hypoxia-inducible factor (HIF-1), in vertebrate species (Gracey et al., 2001, Ton et al., 2003; Papandreou et al., 2005). It is conceivable that exposure to the air and consequent hypoxia, or subsequent reoxygenation upon immersion, resulted in a shutdown of the DNA replication and the mitotic processes, but

this does not seem to be the case in the present study. Both processes would lead to a reduction of new BrdU incorporation into the cells and an accumulation of BrdU labelling of cells that experienced S-phase during immersion that has not been observed here. Although a HIF-1 like protein has been identified in the intertidal mollusc *Crassostrea virginica* (NCBI, CD648099), a recent study in *Crassostrea gigas*, which reports on a series of genes up- and down-regulated under severe hypoxia (David et al., 2005), does not suggest a regulation of the cell proliferatory process nor an enhanced apoptotic response.

Besides aerobic/anaerobic conditions, feeding activity is also a major factor influencing the physiology of marine mussels. Tidal regime has great effect in the form and function of the digestive epithelium of mussels, which functions in a different way depending on whether they are continuous feeders (subtidal mussels) or non-continuous feeders (intertidal mussels) (Owen, 1972; Robinson et al., 1981; Izagirre, 2002). Nevertheless, tide and photoperiod are closely related, at least concerning feeding activity in bivalves. Algae are mainly in the water column during daytime because of photosynthesis and daytime is the period when mussels feed, so night is the time when degenerated epithelia can be replaced. In the case of intertidal mussels, DNA replication can occur at any time, but mitosis only takes place when mussels are air-exposed, that is, when they are not feeding. Moreover, the digestive gland epithelium is highly dynamic and the shape, numbers and contents of the two cell types that conform it, change dramatically in few hours in response to food and other environmental factors (Thompson et al., 1974; Soto and Marigómez, 1997; Syasina et al., 1997). In this way, feeding rates and absorption efficiency are increased during low tide (Wong and Cheung, 2001), many digestive enzyme activities being activated upon immersion (Fernandez-Reiriz et al., 2001). So, by adjusting feeding rates and enzymatic activities, absorption can be maintained constant (Wong and Cheung, 2001). It has been suggested that a regeneration of the digestive epithelium occurs every feeding cycle, which is twice per day in intertidal mussels (Nelson and Morton, 1979). Thus, whereas in subtidal mussels digestive cell proliferation follows a circatidal pattern of variation (Zaldibar et al., 2004; present results), it is conceivable that in the more restricted conditions of intertidal mussels both tide and photoperiod govern the dynamics of digestive cell proliferation, so that increases in BrdU-labelling occur very intensely only once per day.

Concluding remarks

The most marked differences between experimental groups are detected with the variations in the tidal regime and season. After all, size seems not to have a great impact in the proliferative pattern and both small and large mussels showing a very similar proliferating pattern. The pattern of seasonal variability presently described, with highest cell proliferation rates in summer, is explained because summer is the period when increased somatic growth or enhanced cell turnover take place, and also because more digestion activity would cause digestive cells to be exhausted more rapidly. Concerning differences between mussels of different tidal regimes, it has been suggested that cell proliferation in the digestive gland epithelium takes place essentially when cells are not involved in intracellular digestion (Zaldibar et al., 2004), which is a cyclic process governed by food availability throughout the tidal cycle (Nelson and Morton, 1979; Morton, 1983). Thus, cell proliferation rate would be lower and more continuous in subtidal mussels that do not show peaks of digestive activitiy than in intertidal ones (Morton, 1983).

Epithelial turnover mediated by mature cell division in mussel digestive gland (Zaldibar et al., 2004) would imply a successful strategy to accomplish such variable cyclic patterns in cell proliferation, which would be hardly achieved if epithelial turnover were mediated by the activity of stem cell niches like in the digestive tract of other invertebrates (Illa-Bochaca and Montuenga, 2006).

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