Intestinal response to prolonged fasting and subsequent feeding in Mallard *Anas platyrhynchos*

SYLVIE GEIGER, CHRISTEL WAGNER, JEAN-HERVÉ LIGNOT, YVON LE MAHO & JEAN-PATRICE ROBIN

Institut Pluridisciplinaire Hubert Curien, Département Écologie, Physiologie et Éthologie, UMR 7178 Centre National de la Recherche Scientifique-Université de Strasbourg, 23 rue Becquerel, F-67087 Strasbourg Cedex 2, France.
E-mail: sylvie.geiger@c-strasbourg.fr

Abstract

In numerous species, prolonged fasting occurs during food shortage periods or when feeding competes with activities such as reproduction, migration or hibernation. Efficient energy saving, which is a key factor to adapt to this challenge, is partly achieved by an atrophy of the intestines. In this study, morphological and functional responses of the Mallard's intestine during the metabolic phases of prolonged fasting (a phase of protein sparing followed by a phase of protein wasting) and subsequent feeding were determined. Morphometry of the intestinal villi and cell replication within intestinal crypts were examined. Prolonged fasting induced a significant (33%) atrophy of the villi but there were no changes in cell proliferation rate inside the crypts. These events are not phase-dependent, unlike in laboratory rats where cell proliferation is increased in the crypts during the protein wasting phase. Mallard do not rely on such mechanisms, but re-feeding remained successful. The absorption of nutrients in the jejunum seemed to be restored rapidly as the length of the villi increased by one-third and the proportion of cells in DNA synthesis increased by 70% within 24 h of subsequent feeding.

Key words: birds, cell proliferation, intestinal crypts, intestinal villi, jejunum.

Over the course of their life-time, organisms may experience seasonal fluctuations in energy availability. Enforced or spontaneous prolonged total fasts may occur during food shortage periods, for example during cold spells, or when feeding competes with activities such as reproduction, migration or hibernation (Mrosovsky & Sherry 1980; Piersma 2002). Ducks can experience such energetic challenge during the wintering period and may even starve to death (Dobinson &

Richards 1964; Owen & Cook 1977; Suter & Van Eerden 1992; Pawlina *et al.* 1993). In birds and mammals, long-term fast is characterised by three successive metabolic phases: first glycogen stores are exhausted (phase I). This is then followed by a long period of protein sparing and preferential mobilisation of fat stores (phase II), and last the net protein catabolism is increased when a lower threshold in the lipid stores is reached (phase III) (Le Maho *et al.* 1981; Robin *et al.* 1988; Cherel & Groscolas 1999). One of the main adaptations to prolonged fasting is a reduction in energy expenditure (Le Maho *et al.* 1981; Cherel & Groscolas 1999). This is achieved by a decrease of body temperature and an atrophy of the digestive system (Cherel & Groscolas 1999; Dunel-Erb *et al.* 2001; Karasov *et al.* 2004). However, in a mammal model cellular proliferation has been shown to increase in the gut during phase III (Habold *et al.* 2004), despite an increased atrophy (diminution of villi length and of epithelium width) of this organ (Dunel-Erb *et al.* 2001). This phenomenon has been interpreted as an anticipatory mechanism for post-fast feeding (Habold *et al.* 2004). The aim of this study was to investigate whether this anticipatory mechanism occurred in other vertebrates such as birds. To this end, the morphology and replication rate of cells in the jejunum throughout prolonged fasting, from the fed state to phase III, were determined for Mallard *Anas platyrhynchos*. Furthermore, the reversibility of the phenomenon over a short-term re-feeding period was assessed.

Materials and methods

Study species

The study was undertaken in February and March 2008 on 36 captive female Mallard (*Anas platyrhynchos*) obtained from the registered breeding field station "Canarderie de la Ronde" (Céré-la-Ronde, France). The work was performed under government licence and complied with current legislation (Decret 87–848, Journal Officiel, October 1987, pp. 12245–12248) on animal experimentation in France. The protocol was approved by the Departmental Veterinary Services and complied with the "Principles of Animal Care" publication no. 86–23, revised in 1985 by the National Institute of Health.

Upon arrival in the laboratory, the birds were placed in outdoor aviaries under ambient temperature and natural photoperiod. They were fed *ad libitum* with a balanced commercial food (Standard duck food 7751, Sanders Corporation) and provided with running water. During an adaptation period (*c.* 2 weeks) the birds were weighed (± 1 g) daily between 08:00–10:00 h, until body mass stabilised.

Experimental groups

Prior to nutritional manipulation, the birds were assigned to one of four experimental groups, with 7–10 birds per group. Average initial body mass and size (tarsus length) did not differ significantly between groups ($F_{3,32}$ = 0.071, n.s.; $F_{3,32}$ = 0.220, n.s., respectively) (Table 1). The metabolic status at sacrifice for the different groups was as follows: 1) fed *ad libitum* (Fed), 2) fasting up to the transition from phase II to III (PII), 3)

Table 1. Body mass and structural measurement profiles of Mallard in the four groups (Fed, PII, PIII and R1). Fed = fed *ad libitum*; PII = fasted up to the end of phase II of fasting; PIII = fasted up to entrance into phase III of fasting; R1 = fasted up to entrance into phase III and then fed again for 1 day. Values are means ± s.e. Body mass variation in PII and PIII groups was the percentage of initial body mass lost during fasting. In R1 birds it corresponded to the percentage of body mass lost during the fast recovered during re-feeding.

	Fed	**P II**	**P III**	**R1**
No. of Mallard (*n*)	8	9	10	9
Initial Body Mass (g)	966 ± 33	986 ± 45	986 ± 36	973 ± 45
Final Body Mass (g)	N/A	594 ± 13	543 ± 12	631 ± 24
Body Mass Variation (%)	N/A	–39 ± 3	–45 ± 1	+23 ± 4
Tarsus length (mm)	54.8 ± 0.6	54.4 ± 0.7	54.4 ± 0.6	54.9 ± 0.9

fasting up to the protein wasting stages (phase III: PIII), and 4) fasting up to phase III then fed *ad libitum* for one day (R1). In these last two groups, the duration of the fast (20.0 ± 2.2 days for PIII; 20.6 ± 2.4 days for R1) was not significantly different, (t_{17} = 0.354, n.s.). In this experiment, fasting means that the food was withdrawn. This was done to mimic the extreme situation birds may encounter during severe cold spells (see Dobinson & Richards 1964; Suter & Van Eerden 1992). However, birds were put down or re-fed while in a reversible stage of fasting, *i.e.* when birds were no more than 2–3 days in phase III of fasting (see Robin *et al.* 1991). The metabolic stages of fasting (phases II or III) were assessed from the changes in the specific daily body mass *d*m/m*d*t, *i.e.* the body mass loss per unit of time per unit of body mass. This index has been shown to reflect modifications in body fuel utilisation throughout fasting (Le Maho *et al.* 1981; Robin *et al.* 1988).

Preparation of the intestine samples

After weighing the birds, an injection (50 mg/kg) of 5-Bromo-2' deoxy-Uridine (BrdU) dissolved in distilled water was given in the abdomen. BrdU, a labelled nucleotide, was incorporated into the DNA of phase S cells (phase of synthesis of DNA) and after immuno-histological labelling, was used as a marker of cell proliferation. The birds were put down 1 h later. The digestive track was removed by dissection and laid out on a plate placed on crushed ice and the jejunum was dissected within 33 ± 5 min.

Histological preparation

Three to five pieces of 0.5 cm of jejunum were put in a solution of 0.5%

Glutaraldehyde and 2% Paraformaldehyde in a 0.1 mol/L cacodylate buffer (pH 7.5) for 2 h at room temperature. Samples were washed three times in the cacodylate buffer for 10 min to remove traces of fixators. They were then dehydrated with successive baths of 50% and 70% dilution of ethanol in distilled water. The samples were then placed for 30 min in two successive baths containing equal parts of LR White® resin and 70% ethanol, and then two parts of LR White® resin to one part of 70% ethanol. Finally, the samples were placed in two baths of pure LR White® resin, one for 1 h at room temperature, and one overnight at 4°C. The objective of these treatments is to replace the water of a high hydrated tissue with resin to harden it for cutting and conservation. The samples were included in capsules with pure LR White® resin and put in an incubator at 50°C for 18–24 h to allow the resin to harden. The samples included in the resin were cut at 0.35 µm with an ultra-microtome (Ultra-cut; Reichert-Jung) and mounted on poly-L-lysined slides.

Morphometrical measures and mitosis estimation

Jejunal sections were stained with methylene-blue and were observed under a Zeiss Axioplan microscope equipped with a camera. Microphotographs were taken and analysed using a PC-based image analysis and morphometry system (SigmaScan Pro Jandel Scientific, SPSS, Chicago). Height of the villi and epithelial thickness, which are morphometrical indicators of functionality of the intestine, were measured. The height of the villi was measured from the limit with the crypts to the top of the villus, and the epithelial thickness at two-thirds of the villus height. Only villi that were cut in their mid-line, from tip to base, were considered. Between 2–18 villi, and between 3–24 measures of epithelial thickness, according to the quality of the section, were realised per individual.

In the crypt region, where cell proliferation occurred in birds and mammals, between 100–150 nuclei were observed per animal and the proportion of those in mitotic status (*i.e.* with condensed DNA) was determined. The proportion of mitosis (division phase) and phase S (phase of DNA synthesis) within the crypts are indicators of cell dynamics in the intestine.

BrdU immuno-labelling

After being hydrated in a phosphate-buffered saline solution, jejunal sections were plunged into a 2N chlorhydric acid solution at 37°C for 30 min for denaturising the DNA and then treated with 0.1% trypsin for 45 min at 37°C. The sections were then incubated for 20 min at room temperature with 5% normal goat serum for blocking non-specific sites. Anti-BrdU (Sigma Chemicals) was added (dilution 1/10) and the incubation continued for 2 h at 37°C. Finally, sections were treated with a secondary antibody (goat anti-mouse IgG) combined with Fluoresceine Iso Thio Cyanate (FITC) (dilution 1/10) for 2 h in the dark at 37°C. Counter-staining was performed using 4',6' Di Amidino-2-Phenyl Indole (DAPI) 2 µg/mL (30 min in the dark at room temperature). Slides were mounted with Fluoromount® and analysed with a fluorescence microscope equipped with a camera (Zeiss, Axioplan 2). Pictures were

taken at 400–500 nm and superposed using Photoshop. The same experimentator (SG) observed between 300–400 nuclei per animal in the crypt region and calculated the labelling ratio.

Statistics

Mean values are presented with ± standard errors (s.e.). Statistical analyses were conducted using SPSS 16.02 (SPSS Inc., Chicago, Ill., USA). Friedman Repeated Measures Analysis of Variance on ranks and Dunn's *post hoc* test were used to compare dm/mdt values during prolonged fasting in the different groups. A Generalised Linear Model (GzLM) was used to test for morphometric measures (villi and epithelium) according to two factors, individuals and treatment (Fed, phase II, phase III, R1). A General Linear Model (GLM) was used for proliferation values (Phase S and mitosis) and normality was tested on residuals (Kolmogorov-Smirnov, n.s.). Bonferroni tests were used as *post hoc* tests; results of *post hoc* tests are presented as difference of means (Δm) ± standard errors. Significance levels were set at $P < 0.05$.

Results

Validation of the experimental design

During phase II, dm/mdt values were maintained at a low level (20–25 g/day/kg) but they increased significantly (2.5–2.7 fold) during phase III ($\chi^2_7 = 35.63$, $P < 0.001$ and $\chi^2_7 = 37.80$, $P < 0.001$) in the PIII and R1 groups, respectively.

Body mass modifications

Mallard lost 39–45% of their initial body mass in phases II and III, respectively. During the first day of re-feeding 23% (± 4%) of the lost body mass was regained (Table 1).

Jejunal morphometry

The jejunal villi lengths were different across treatments ($\chi^2_3 = 26.306$, $P < 0.001$; Fig. 1a). There was a significant ($\Delta m = 133.9 \pm 33.7$, Bonferroni adjusted $P < 0.001$) decrease in villi length (33%) between the fed and the fasted to phase II condition. However, no significant changes ($\Delta m = 5.4 \pm 21.9$, Bonferroni adjusted $P = 1.000$) were recorded between phase II and phase III animals. After one day of re-feeding, villi length increased significantly ($\Delta m = 52.9 \pm 17.5$, Bonferroni adjusted $P < 0.05$) and one-third of the fasting-induced length reduction was restored.

Jejunal epithelium thickness did not vary significantly during fasting and re-feeding ($\chi^2_3 = 7.502$, n.s., Fig. 1b).

Jejunal proliferation

The BrdU labelling in crypt cells measured cell proliferation by quantifying BrdU incorporation into the newly synthesised DNA of replicating cells. The proportion of labelled cells were different among treatments ($F_{3,26} = 13.586$, $P < 0.001$). There were no significant differences between fed, phase II and phase III groups (Fed/Phase II: $\Delta m = 4.9 \pm 3.9$, n.s., Fed/Phase III: $\Delta m = 5.0 \pm 3.9$, n.s., Phase II/PhaseIII: $\Delta m = 10.0 \pm 3.8$, Bonferroni adjusted, n.s.), but the proportion of labelled cells was significantly raised by 70% after one day of re-feeding (Phase III/R1: $\Delta m = 23.9 \pm 3.9$, Bonferroni adjusted $P < 0.001$).

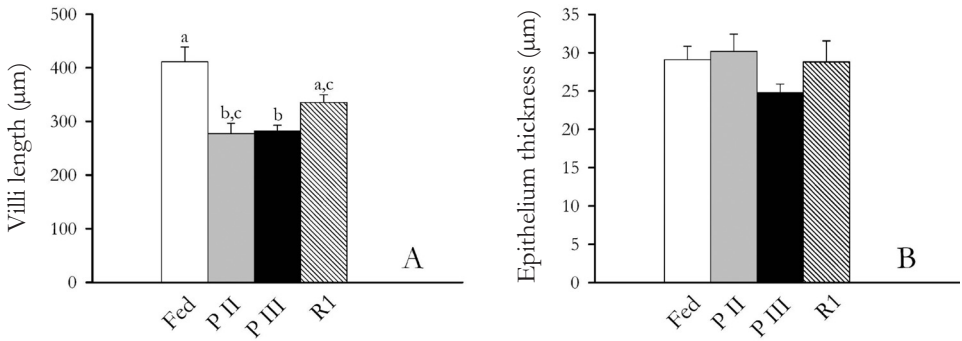

Figure 1. Jejunal morphometry recorded for the four groups of Mallards (mean values ± s.e). A = jejunal villi length (µm); the overall difference between classes was significant (GzLM, $P = 0.001$). B = epithelium thickness of jejunum (µm); the overall difference between classes was not significant (GzLM, $P = 0.058$, n.s.). Means sharing common letters do not differ significantly from each other (Bonferroni tests, $P > 0.05$).

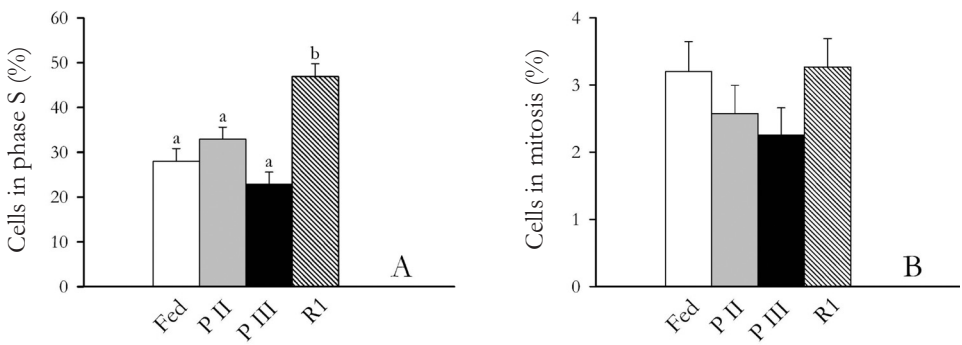

Figure 2. Cell proliferation in the jejunum recorded for the four groups of Mallards (mean values ± s.e). A = percentage of cell in phase S in jejunal crypts (%); the overall difference between classes was significant (GLM, $P = 0.001$). B = percentage of mitosis in jejunal crypts (%); the overall difference between classes was not significant (GLM, $P = 0.266$, n.s.). Means sharing common letters do not differ significantly from each other (Bonferroni tests, $P > 0.05$).

The proportion of cells in mitosis did not differ significantly among experimental groups ($F_{3,32} = 1.381$, n.s.).

Discussion

The Mallard in this study showed a similar pattern of body mass loss modifications during fasting to those previously described for birds in the same metabolic phases (Le Maho *et al.* 1981; Cherel *et al.* 1988; Robin *et al.* 1988, 1998). Despite an atrophy of jejunal villi in fasting phases, Mallard's intestines do not show any change in cell proliferation through prolonged fasting

from fed state to phase III. Conversely, villi length and DNA synthesis in the crypts increase after one day re-feeding.

Several studies have shown that the digestive system presents an important phenotypic flexibility according to different phases of the biological annual cycle. In birds, specific morphologic adaptations have been reported in relation to seasonal migrations. For example, the mass of the stomach and the intestines of the Eared Grebe *Podyceps nigricollis*, the Bristle-thighed Curlew *Numenius tahitiensis* and the Bar-tailed Godwit *Limosa lapponica* are reduced before migration departure, when food ingestion is diminished (Jehl 1997; Piersma 1998).

Furthermore, some of the adjustments observed at the cellular intestine level have also been described in the absence of food ingestion. The digestive tract is progressively atrophied during prolonged fasting among all vertebrates, from snakes to mammals (reviewed in Wang *et al.* 2006). In mammals, cell proliferation has been shown to decrease during phase II of fasting (Habold *et al.* 2004) but an increase in the cell proliferation has been reported at the end of the prolonged fast connected with hibernation in Ground Squirrel *Citellus undulatus*, just before arousal (Kruman *et al.* 1988), and during phase III of fasting in rats *Ratus norvegicus*, *i.e.* when re-feeding becomes a priority (Habold *et al.* 2004). This phenomenon has been interpreted as an anticipation of re-feeding (Habold *et al.* 2004), as it corresponds with an increase of the locomotor activity (Koubi *et al.* 1991; Robin *et al.* 1998). Yet, no data allows generalising the mammalian gut anticipation of re-feeding in birds. Gut cell proliferation within the crypts is diminished in domestic hens *Gallus gallus domesticus* during phase II fasts (Yamauchi *et al.* 1996; Shamoto *et al.* 2000) but no data were available concerning phase III. From this study, the intestines atrophied during prolonged fasting but no significant changes in the cell proliferation could be detected whatever the measured parameter. Importantly, no morphological or functional changes between phase II and phase III could be recorded. Thus, it is not possible to confirm in fasting Mallard the phenomenon described in rats as anticipation for re-feeding (Habold *et al.* 2004). In wintering areas, when Mallard and more generally ducks experience long-term starvation during cold spells (Dobinson & Richards 1964; Suter & Van Eerden 1992), the atrophy of the intestine might participate to the reduction of the metabolism and thus to energy sparing. It is also a part of the reduction of the mass load before a winter displacement if the bad nutritional conditions persist (Owen & Cook 1977). Nevertheless, the active jejunal cell proliferation should allow a rapid restoration of the digestive capacity together with the restoration of the body reserves. Indeed, the atrophy of the intestine still remains reversible as the villi length is markedly increased after only one day of re-feeding.

To conclude, our study suggests that different strategies of digestive adjustments during prolonged fasting might exist among vertebrates. However, further studies are needed to confirm these preliminary observations and to compare the relative efficiency of the adjustments in the

intestinal mucosa in terms of restoration of the absorptive functionalities of this tissue after a prolonged fast.

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