Effects of season and reproductive state on lipid intake and fatty acid composition of gastrointestinal tract contents in the European hare

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Abstract We investigated lipid content and fatty acid (FA) composition of gastrointestinal tract contents in free-living, herbivorous European hares (Lepus europaeus). Mean crude fat content in hare stomachs and total gastrointestinal (GI) tracts was higher than expected for typical herbivore forages and peaked in late fall when hares massively deposited body fat reserves. Changes of FA proportions in different parts of the GI-tract indicated a highly preferential absorption of polyunsaturated fatty acids (PUFA). A further reduction of PUFA content in the caecum, along with the appearance of odd-chained FAs in caecum, caecotrophes, and colon content, pointed to a biohydrogenation of PUFA in the hare’s hindgut. GI-tract contents showed significant seasonal changes in their FA composition. Among PUFA, α-linolenic acid peaked in spring while linoleic acid was predominant in late summer and fall, which probably reflected changes in the plant composition of forage. However, independent of seasonal changes, GI-tracts of lactating females showed a significantly (+33%) higher content of linoleic acid, a FA that is known to increase reproductive performance in European hares. This finding suggests that lactating females actively selected dietary plants rich in linoleic acid, a PUFA that may represent a limited resource for European hares.

Keywords Brown hare · Essential fatty acids · Polyunsaturated fatty acids · Caecotrophy · Nutrient content · Reproduction

Abbreviations
FA Fatty acids
PUFA Polyunsaturated fatty acids
SFA Saturated fatty acids
MUFA Monounsaturated fatty acids
GI Gastrointestinal
CT Caecotrophes
SI Small intestine
DM Dry mass

Introduction

The dry matter fat content in typical food plants of herbivorous mammals, e.g. on pastures, is typically less than 3.5% (Palmquist and Jenkins 1980). Thus, dietary fat may represent a limited resource for these species. Expectedly then, herbivores such as horses and cattle are highly susceptible to variation in dietary fat content, which can, for instance, profoundly affect milk fatty acid (FA) composition during lactation (e.g. Hoffman et al. 1998; Bargo et al. 2003; Chilliard et al. 2000). In the herbivorous European hare (Lepus europaeus), dietary fats also play a crucial role for reproduction. Lactating females rapidly increase food consumption in response to low dietary fat content, but still
transfer less milk energy (per unit body weight) to young than animals on a high fat diet (Hackländer et al. 2002; Valencak and Ruf 2009). To some degree, variation in the fat content of natural diets may be buffered by the deposition of body fat stores after the reproductive season, which occurs in both sexes (Fig. 1; see also Flux 1967; Zörner 1996). Body fat stores are, however, depleted over summer, which in males may be due to increased activity associated with male–male aggression and in females involves direct transfer of lipid stores to the mammary gland (Valencak et al. 2009). Consequently, dietary fat content may have a strong impact on reproductive success in this species either directly or via its effects on the turnover rate of body fat stores.

Natural diets of hares may, however, differ not only in total fat content but also in their FA composition. Arguably, this variation should be particularly important when it concerns the two major plant PUFA linoleic acid (C18:2n-6) and ω-linolenic acid (C18:3n-3). This is because linoleic acid and ω-linolenic acid, in contrast to saturated (SFA) and monounsaturated fatty acids (MUFA), cannot be synthesized de novo in mammals, but must be obtained from the diet. Among other functions, PUFA can also affect reproductive performance. Recently, Tufarelli et al. (2010) found that dietary PUFA composition in European hares, particularly n-6 PUFA supplementation, significantly increased the number of successfully weaned young compared with females on an isocaloric MUFA supplemented diet. An important role of n-6 PUFA uptake has been also suggested for muscular performance and maximum running speed in mammals (Ruf et al. 2006), another trait that directly affects fitness in species such as hares, which evade predators by fast speed.

Despite this evidence for an important role of dietary fat content and composition, relatively little is known about fat intake, digestion and FA uptake in the GI-tract of European hares. Therefore, we analysed contents of all parts of the GI-tract of free-living hares, collected at all four seasons. We were particularly interested to see whether the well-known seasonal cycle of body fat deposition and depletion is merely due to differences in energy expenditure or is also reflected in seasonal changes of food quality, namely dietary fat content.

Further, contents of hare stomachs and small intestines should provide an index of not only total fat intake but also of the uptake and absorption of PUFA, relative to SFA and MUFA. This is because hares are hindgut fermenters in which—in contrast to ruminants—little or no hydrogenation of unsaturated FAs by intestinal microorganisms should be expected before their absorption (Doreau and Ferlay 1993; Hoffman et al. 1998). Consequently, determining the FA composition of GI-tract contents should allow us to see (i) if there are significant seasonal changes of FA composition, (ii) if there is any indication for preferential uptake of any FA classes or specific FA and (iii) if there is evidence for a preferential absorption of certain FA. Finally, we intended to examine whether any of the variables investigated, in particular possible preferences for certain FA, may differ between sexes and, in particular, may deviate in lactating females that have dramatically increased energy demands (Hackländer et al. 2002; Valencak et al. 2009).

Materials and methods

Animals and sample collection

To determine seasonal changes in the size of body fat deposits intraperitoneal fat (but not gonadal deposits) were removed from a total of 1120 European hares, and fresh weight was determined to the nearest 0.1 g. Hares were shot by local hunters in four regions of Lower Austria (48°11′N, 16°42′E, elevation 140 m) between 2001 and 2005. Since more hares were shot during the hunting season, compared to the breeding season, sample sizes varied (Fig. 1).

GI-tracts were collected from a subsample of hares shot in February (N = 33), May (N = 36), August (N = 32) and November (N = 39) in the year 2004 only. Hare carcasses were transported to the lab immediately after the kill and kept in a cold room (+5°C) prior to dissection on the same day to minimise post mortem autolysis of the digestive tract. Typically, animals were shot in the morning, before noon. Animals (N = 140) were classified into two age classes, subadults (N = 35) and adults (N = 105),

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**Fig. 1** Seasonal change of intraperitoneal fat content (w/o gonadal fat depots) in free-living European hares (both sexes) shot at bi-monthly intervals in Lower Austria. The numbers above the symbols indicate sample sizes. Means ± 95% confidence intervals.
according to the weight of the dried eye-lens (Suchentrunk et al. 1991). The mean body weight was 3963 ± 641 g.

The GI-tract was divided into stomach, small intestine (SI), caecum and colon (Nickel et al. 1984). Full weights and weights of the content of each part were recorded, and contents were stored at −18°C for later nutrient and FA analyses. Clearly distinguishable soft, pale agglomerates found in the stomach content were considered caecotrophes (CT) and analysed separately (N = 18). All data on stomach contents thus refer to the content of this organ after removal of all CT.

Lipid and fatty acid analyses

GI-tract contents were dried at 60°C, and 1 g of the each sample was further dried for 2 h at 105°C to determine dry matter. Crude fat was analysed by Soxhlet’s procedure extraction (using petrol ether as solvent) from 1 g of the sample, and the difference in weight before and after extraction was calculated.

To determine FA contents, we used FA methyl ester (FAMES) FA transesterification (Garces and Mancha 1993), separation, identification and quantification, based on peak areas obtained by gas chromatography (Eder 1995). A quantity of 300 mg of GI-tract content sample was used to analyse FAs composition. The sample was heated for 2 h with a reagent mixture containing methanol: toluene: 2,2-dimethoxypropane: H2SO4 (39:20:5:2, by volume). Two phases were formed, the upper one containing the FAMES ready for GC analysis (Perkin Elmer AutoSystem XL with Autosampler and FID; Norwalk, USA) using a capillary column (HP INNOWax, 30 m × 0.25 mm; Hewlett Packard, USA). The methyl esters of SFA (C14:0, C15:0, C16:0, C17:0 and C18:0), MUFA (C16:1 and C18:1) and PUFA (C18:2n-6 and C18:3n-3) were separated under gas chromatographic conditions and identified comparing their retention time with those of standards (Sigma-Aldrich, St. Louis, USA).

Statistical analyses

All statistical analyses were carried out using the statistical package R (R development core team 2010). To lower the problems caused by a multiplicity of tests (if data on all measured FAs were compared individually), we reduced statistical analyses to the following response variables: Total lipid content, proportions of SFA, MUFA and PUFA among FAs, and proportions of linoleic acid and ω-linolenic acid within the PUFA class. To test for differences between samples collected from different parts of the GI-tract (of the same animals), we used repeated measurement ANOVAs by computing linear mixed effects models using library nlme (Pinheiro et al. 2009). In these models, GI-tract section was entered as a fixed factor and animal ID as the random factor.

To obtain estimates of potentially absorbable nutrients, we subsequently computed means of all of the above response variables over all GI-tract sections. This computation of means also included colon contents because not only soft but also hard faeces are a potential nutritional source in leporids, including hares (Hirakawa 2001). None of the results was, however, significantly altered by inclusion or exclusion of colon contents. We tested for possible effects of body weight, sex, age, season and lactation among females, on these mean contents using simple linear model ANOVAs.

Residuals from all models were inspected for non-normality (Shapiro-Wilks tests) and heterogeneity of variances (Levene tests), but none of these tests indicated significant problems with the model structure. Starting with full models including all predictor variables, we reduced model structures in a stepwise procedure by eliminating non-significant terms. This included age-class in all cases, which therefore is not further mentioned. For all ANOVAs we computed marginal (Type III) sums of squares. We report degrees of freedom, F and P values from ANOVAs for non-significant terms obtained from full models, and for significant terms obtained from final, reduced models. In all graphs, data are presented as means ± 95% confidence intervals to ease comparisons between subsamples.

Results

Lipid content

Total lipid content differed significantly between all fractions collected from the GI-tract (F4,138 = 364.178; P < 0.001). As illustrated in Fig. 2, lipid content significantly increased between stomach and small intestine, and was significantly lower in all posterior fractions. Comparing means from all GI-tract fractions, we found that lipid content was significantly different between seasons (F3,139 = 13.2; P < 0.001). Mean lipid content showed an early peak in May and reached maximum values in November (Fig. 3). Lipid content was unaffected by body weight (F1,135 = 1.92; P = 0.167), sex (F1,135 = 0.001; P = 0.966) and age (F1,135 = 1.94; P = 0.165). Also, among females, mean lipid content of GI-tract contents was not different between lactating and non-lactating animals (F1,80 = 0.004; P = 0.945).

Fatty acids

Fatty acid composition of lipids drastically changed between contents of different sections of the GI-tract...
PUFA content significantly decreased along the GI-tract ($F_{4,403} = 1059.8; P < 0.001$), while SFA content increased by a similar extent ($F_{4,403} = 786.7; P < 0.001$). The proportion of MUFA increased only slightly but significantly ($F_{4,403} = 119.4; P < 0.001$) between stomach and posterior fractions, compared to the changes in PUFA and SFA content (Fig. 4a).

Among PUFA, there were significant differences between the GI-tract fractions for both linoleic acid (C18:2n-6; $F_{4,403} = 326.8; P < 0.001$) and α-linolenic acid (C18:3n-3; $F_{4,403} = 465.6; P < 0.001$). However, while α-linolenic acid continuously decreased between stomach and caecum, linoleic acid proportion increased from stomach to small intestine and decreased only in the posterior GI-tract fractions (Fig. 4b). Mean proportions of all individual FA determined are given in Table 1.

Season had a strong effect on mean GI-tract contents of PUFA ($F_{3,139} = 101.0; P < 0.001$), SFA ($F_{3,139} = 75.2; P < 0.001$) and MUFA ($F_{3,139} = 23.6; P < 0.001$). PUFA content was significantly increased in May and August, compared to February and November (Fig. 5a), and the inverse pattern was found for SFA content. Virtually, the same pattern of seasonal changes was found if stomach contents alone, rather than total GI-tract contents were analysed (not shown). The fact that changes in PUFA content mirrored those in SFA content was due to a strong negative correlation between these FA classes ($R = -0.945, P < 0.001$). In comparison, correlations of MUFA with both SFA ($R = 0.346, P < 0.001$) and PUFA ($R = -0.634, P < 0.001$) were much weaker. All major FA classes were unaffected by body weight, sex and age of the animals, or by lactation among females ($F < 2.1, P > 0.15$ in all cases).

The proportions of both linoleic acid ($F_{3,139} = 65.8; P < 0.001$) and α-linolenic acid ($F_{3,139} = 67.0; P < 0.001$)
significantly changed between seasons, with α-linolenic acid reaching a peak in spring (May), and linoleic acid peaking in late summer (August; Fig. 5b). Interestingly, even after statistically adjusting for seasonal differences, mean linoleic acid content was significantly (33%) higher in lactating than in non-lactating females (Fig. 6; \(F_{1,80} = 12.1; P < 0.001\)). In contrast, α-linolenic acid content was not elevated in lactating females (\(F_{1,80} = 0.41; P = 0.64\)). Neither of the two major PUFA was affected by any of the other variables investigated.

**Discussion**

**Crude fat content of GI-tract contents**

Mean crude fat content in the of hare stomach contents varied around approximately 46 mg g\(^{-1}\) (i.e. 4.6%; Fig. 2), reaching a peak of 5.4% in November, and that of the entire GI-tract between approximately 4.5 and >6%, depending
on the season (Fig. 3). This is somewhat higher than previous determinations of crude fat proportions from pastures, which typically range from 1.5 to 4% of DM (Palmquist and Jenkins 1980; Kuntz et al. 2006; Buccioni et al. 2008; Leiber et al. 2008). Although up to 50% of forage ether extracts can consist of non-fatty acid lipid components (waxes, chlorophyll, etc.; Palmquist and Jenkins 1980), the relatively high proportion of crude fat in European hare ingesta may indicate a preferential intake of plant parts rich in fat. Previous botanical analyses of hare stomach contents (Reichlin et al. 2006) have in fact shown clear preferences for several plant species (e.g. *Glycine max*, *Papaver spp.*, *Trifolium ssp.*, *Panicum miliaceum*, *Medicago sativa*) which either in general, or for certain parts, are characterised by above average fat content (*Glycine max* seeds, 18%; sprouts, 10%; *Papaver* seeds, 46-49%; *Trifolium*, up to 6%; *Panicum*, 4%; *Medicago*, 5%; CropIndex (2011), data converted to % of DM). However, the data currently available on nutrient composition of different parts of plants (sprouts, leaves, inflorescences, seeds)—especially in weeds—are insufficient to unambiguously demonstrate selective feeding, based on stomach content analyses. Clearly, controlled experimental trials would be needed to confirm such a dietary selection. It should be noted, however, that the ability to select certain dietary nutrients as well as specific plant parts (e.g. Milton 1979; Berteaux et al. 1998; Bryant and Kuropat 1980) is well documented for other mammals. In European hares, such a preference, especially during the late-autumn fattening phase (Fig. 1), clearly would make sense given the strong impact of dietary fat on reproductive performance in this species (Hackländer et al. 2002; Valencak et al. 2009).

The comparison of crude fat contents in different parts of the GI-tract (Fig. 2) does, however, further support the view of a dietary preference for fatty plant parts. This is because mean crude fat content in the SI reached 8.8%, which may be partly due to a rapid passage of highly digestible, fat-rich dietary particles from the stomach to the SI, or a facilitated removal of low-fat, fibrous particles. Note that the majority of hares in this study were shot several hours after sunrise (i.e. their last food intake; Hirakawa 1994) which provides sufficient time for such a passage (especially in lagomorphs in which the passage rate of food is quite rapid; Hirakawa 2001). In that case, the crude fat content of the diet may well have significantly exceeded that of stomach contents.

Theoretically, part of the increase in crude fat content between stomach and SI could have been due to the addition of endogenous phospholipids and cholesterol from bile and phospholipids from shed intestinal cells (Carlier et al. 1991). It seems very unlikely, however, that these sources of crude fat alone should have led to an almost doubling of its content in the SI (Fig. 2). Published data on bile FA composition (in ruminants) indicate that both n-3 and n-6 PUFA content in bile is much lower (<12%) than in the stomach and SI contents determined here (Miller and Rice 1967; Garton 1969). Therefore, any substantial addition of bile FA would have led to a much stronger change of FA composition between stomach and SI than was actually observed here, with FA composition showing a linear change from stomach to caecum (Fig. 4a).

**Fatty acid absorption**

The significant decrease of PUFA content in the SI compared to the stomach, and the observed simultaneous increase of SFA (Fig. 4a), indicates a preferential absorption of PUFA. Partly, this result can be attributed to the lipophilic chemical properties of these FA. The permeation of intestinal enterocyte membranes increases with chain length (Carlier et al. 1991), which is higher in dietary PUFA (C18) than in SFA (C14-18, mean chain-length 16.2 in stomach contents of hares studied here). Further, there is some evidence that absorption of PUFA is enhanced by a facilitated diffusion mechanism and may involve a specific long-chain FA binding protein (FAPB, reviewed in Carlier et al. 1991). Preferential absorption of PUFA may contribute to the relatively high proportions of PUFA in the tissues of European hares (Valencak et al. 2003) and other hindgut fermenters (Leiber et al. 2008).

Following absorption of a large fraction of PUFA in the SI, there was a further decline of PUFA proportions in the caecum. At least part of this reduction in the amount of PUFA (which reached similar proportions in caecum contents, CT and colon contents) was almost certainly due to...
biohydrogenation by microorganisms. This fate of PUFA, i.e. the removal of their double bonds by addition of hydrogen atoms, is well known—and even more pronounced—in ruminants (e.g. Doreau and Ferlay 1993; Hristov et al. 2005), but has also been documented in hindgut fermenters. In rabbits for instance, hydrogenation results in a similar predominance of SFA in caecotrophes as observed in the present study (Leiber et al. 2008). Microbial fermentation in the caecum is also supported by our finding that odd chain-length FA (C15:0; C17:0) were virtually absent in stomach and SI contents of hares, but reached substantial proportions in the caecum, CT and colon content (Table 1). As these FA occur only in trace amounts in higher plants, but can be produced by bacteria (Vlaemink et al. 2006), it seems clear that they originated from bacterial metabolism in the hindgut.

Differentiating between the two major PUFA (Fig. 4b) gave no clear evidence for a preferential intake or absorption of either linoleic acid or \( \alpha \)-linolenic acid. The predominance of \( \alpha \)-linolenic acid in stomach contents reflects that of typical forages of herbivores (e.g. Palmquist and Jenkins 1980; Kuntz et al. 2006; Leiber et al. 2008; Meluchova et al. 2008). Originally, we had hypothesized that European hares may generally show a preferential intake of linoleic acid. This is because in cell membranes of skeletal muscle, heart and liver of this species the ratio of linoleic to \( \alpha \)-linolenic acid (and their longer-chained n-6 derivates) is actually reversed compared to typical pastures and stomach contents. This is also true for other mammalian herbivores, but the proportion of linoleic acid and its n-6 derivates is even higher in European hares than in other mammals of comparable body size (Valencak and Ruf 2007; Ruf et al. 2006). Consequently, our present data indicate that the FA composition of tissue membranes must be governed by mechanisms that control the formation and incorporation of membrane phospholipids rather than by differential FA uptake. Still, as outlined below, this does not exclude an important physiological function of dietary selection for certain FA.

Fatty acid classes: season and reproduction

GI-tract contents showed a clear seasonal pattern of FA composition (Fig. 5). During summer, PUFA content significantly increased at the expense of SFA, and linoleic and \( \alpha \)-linolenic acid peaked at different times of the year. Thus, it seems that the FA composition of hare GI-tract contents closely follows the seasonal pattern of natural forage, as principally the same seasonal shifts have been observed in mixed plant samples of natural pastures in both alpine habitats (Ruf and Arnold 2008) and in a nearby western Slovakian habitat that is climatically and botanically very similar to our hare study site (Meluchova et al. 2008). The spring peak of \( \alpha \)-linolenic acid is almost certainly due to the fact that this FA is primarily produced during photosynthesis (Gurr et al. 1969; Kenyon and Stanier 1970) which is at its maximum during the spring growth phase of pastures. Later in the year (August/September), there is an increase in the proportion of linoleic acid, which is enriched in inflorescences and seeds (Ruf and Arnold 2008; Meluchova et al. 2008).

Whereas GI-tract contents seemed to largely reflect the FA composition of natural forage, there was one interesting exception: the significantly increased content of linoleic acid in GI-tract contents of lactating females. Since FA compositions in the GI-tract were statistically adjusted for season, this effect cannot be attributed to the time of lactation coinciding with elevated linoleic acid availability. Theoretically, higher proportions of linoleic acid could be due to reduced absorption of this FA. However, there is neither evidence for significantly altered overall assimilation efficiency in lactating females (Valencak and Ruf 2009) nor any reason to assume that lactating hares should down-regulate the absorption of this rarer of the two essential PUFA. Consequently, the most parsimonious explanation for this finding is a genuine dietary preference of lactating females for plants or plant-parts rich in linoleic acid. This conclusion can be reached without any need for the analyses of plant FA composition because both lactating and non-lactating females were shot at randomly distributed locations at the study site indicating that average available food composition was virtually identical for both groups. The apparent ability of European hares to preferentially select plants with increased linoleic acid content is not surprising. Firstly, it has been shown that different plant species and parts show a large variation in their FA composition. For instance, linoleic acid content in dietary plants of herbivores can range from 3 to 274 mg g\(^{-1}\) (Frank et al. 1998) or, as determined in another study, from 8 to 239 nmol mg\(^{-1}\) (Hill and Florant 1999). Secondly, there is ample evidence from both field and laboratory studies that animals do in fact select food on the basis of their FA content (e.g. Frank 1994; Frank et al. 1998; Hiebert et al. 2000, 2003; Bairlein 2002; McWilliams et al. 2004; Ruf and Arnold 2008).

As mentioned before, there also is recent evidence from a hare breeding colony that supplementing the diet with oils rich in linoleic acid increases both the number of young born and weaned compared to isocaloric \( \alpha \)-linolenic acid-enriched and—even more so—compared to MUFA enriched diet (Tufarelli et al. 2010). Further, young of females supplied with linoleic acid-rich diet grow significantly faster between birth and weaning compared to those of females on an isocaloric SFA diet, in particular when litter size is large (i.e. \( \geq 3\); Hauhart 2002). The pathways by which PUFA affect reproductive performance are not fully
understood, but may involve altered membrane properties, effects on reproductive hormones such as steroids and prostaglandins, as well as effects on gene regulation (Mattos et al. 2000; Bordoni et al. 2006; Wathes et al. 2007). However, irrespective of the mechanisms involved it seems clear that, given its beneficial effect on reproductive performance, diet selection for linoleic acid-rich forage may well increase the fitness of lactating females.

Conclusion

This study gives some indication for preferential uptake of plant parts rich in fat and provides good evidence for enhanced PUFA absorption in free living European hares. Our findings also point to selective uptake of a specific PUFA, namely linoleic acid, by females during lactation. All of these results from our field study fit to conclusions from previous, laboratory studies, that dietary fat and PUFA composition play an important role in particular for reproduction in the European hare (Hackländer et al. 2002; Valencak and Ruf 2009; Hauhart 2002; Tufarelli et al. 2010). It is also clear, however, that insights from data on GI tract contents alone are limited and that further experimental studies are needed to clarify both the ability of European hares to discriminate food items and to assess the impact of certain dietary plants on the physiology of this species in more detail.

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