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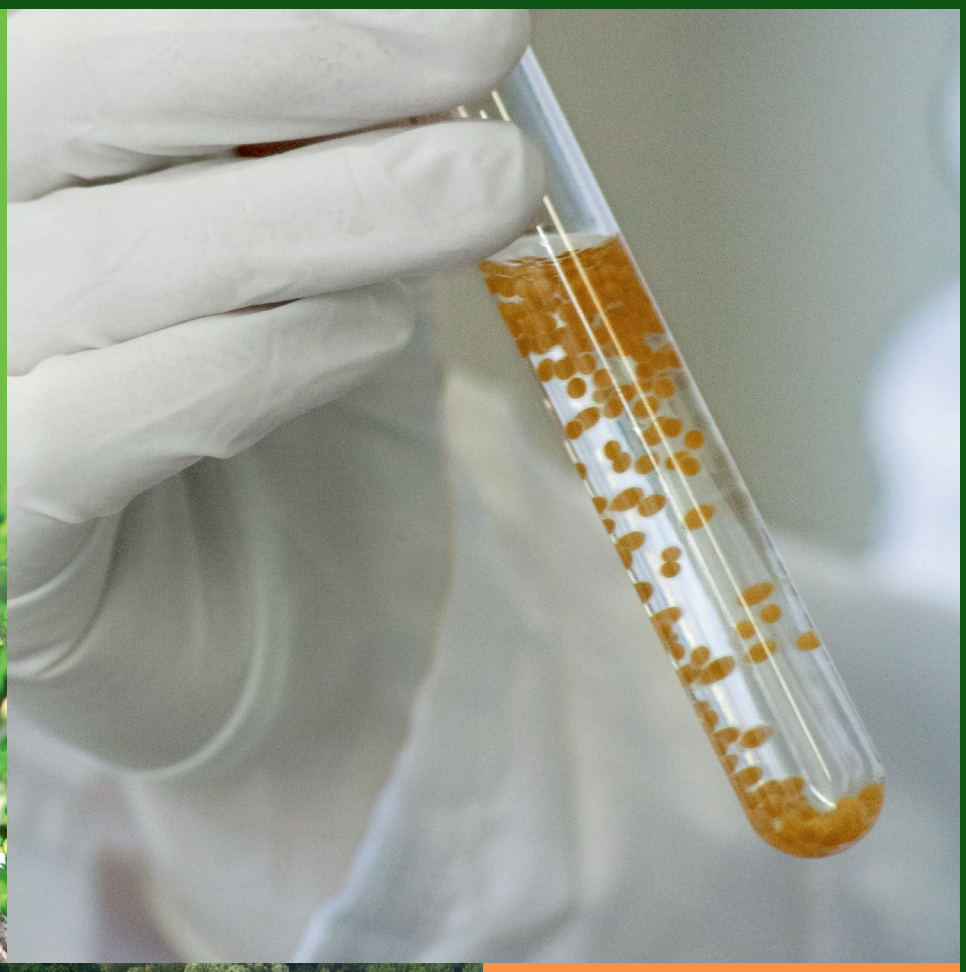

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Inhalt
Content

How wind can shape genetic variation and adaptability in metapopulations of wind-pollinated forest trees through its effect on pollen dispersal

Elizabeth M. Gillet*

Abstract

In wind-pollinated tree species, wind conditions during pollination are a primary determinant of mating. The oft patchy distribution of trees over spatially heterogeneous environments suggests a metapopulation approach to the study of wind effects on pollen contributions to seeds. A mathematical model of distance-dependent pollen dispersal among five populations on a transect yields distributions of pollen sources, and thus their potentially adaptive genetic characteristics, in the seeds of each population. For scenarios of symmetric and directional dispersal over variable mean dispersal distance, structural characteristics of the seeds are quantified by effective number of pollen sources within populations and compositional differentiation of pollen contributions among populations. These measures avoid the problems of the fixation index F_{ST}, which confounds diversity and difference. Effects of wind depend on location. The population with the highest effective number of pollen sources in its seeds need not be the most representative nor even central. Symmetric dispersal over long distances can be statistically indistinguishable from random dispersal, but directional dispersal is not. Consequences for adaptation and practical implications for forest management and seed harvesting are discussed.

Keywords: *Pollen dispersal, wind-pollination, forest tree, adaptation, metapopulation model, diversity, effective number, compositional differentiation*

Zusammenfassung

Wie Wind genetische Variation und Anpassungsfähigkeit in Metapopulationen windbestäubter Waldbäume durch Auswirkung auf die Pollenverbreitung beeinflussen kann

Bei windbestäubten Baumarten sind Windverhältnisse eine wesentliche Determinante von Paarungen. Die oft fragmentierte Verteilung von Bäumen über räumlich heterogene Umweltbedingungen legt einen Metapopulationsansatz nahe, um die Auswirkungen des Windes auf die Pollenbeiträge zu Samen zu untersuchen. Ein mathematisches Modell der entfernungsabhängigen Pollenverbreitung zwischen fünf Populationen an einem Transekt ergibt die Häufigkeitsverteilung von Pollenquellen mit ihrer potentiell adaptiven genetischen Information in den Samen jeder Population. Für Szenarien von symmetrischer bzw. gerichteter Verbreitung über variable mittlere Distanz werden strukturelle Eigenschaften der Samen durch eine effektive Anzahl von Pollenquellen in Populationen und der kompositionellen Differenzierung zwischen Populationen quantifiziert. Diese Maße sind frei von der Problematik des Fixierungindexes F_{ST}, welcher Vielfalt mit Differenz vermischt. Wind wirkt sich ortsabhängig aus. Die Population mit der höchsten effektiven Anzahl von Pollenquellen muss weder die repräsentativste sein noch im Zentrum liegen. Symmetrische Verbreitung über weite Distanzen kann von zufälliger Verbreitung statistisch ununterscheidbar sein, gerichtete Verbreitung jedoch nicht. Konsequenzen für Anpassung und praktische Implikationen für die Waldbewirtschaftung und die Saatgutgewinnung werden diskutiert.

Schüsselwörter: *Pollenverbreitung, Windbestäubung, Waldbäume, Anpassung, Metapopulationsmodell, Diversität, effektive Anzahl, kompositionelle Differenzierung*

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1 Introduction

In wind-pollinated plants, meteorological conditions during pollination are primary determinants of mating, in that they influence how many pollen from any one plant are available to fertilize the ovules of any other plant. Depending on wind direction, horizontal wind velocity, and turbulence but also on temperature and humidity (including washout by rain) after pollen emission (Prentice, 1985; Niklas, 1985; Jackson and Lyford, 1999; Helbig et al., 2004), wind-borne pollen can settle near source or be carried long distances. Tree pollen has been shown to be transported over distances of tens to hundreds of kilometers without losing viability (Williams, 2010; Buschbom et al., 2011; Kremer et al., 2012).

In spatially heterogeneous environments, the genetic variants transferred with the migrating pollen may therefore encounter different environmental conditions than those at its source. The adaptive genetic information (i.e., alleles at gene loci involved in adaptation) that it contributes to the seeds of a tree in a different environment may be detrimental or perhaps beneficial for survival of the offspring that descend from it. Between pollination seasons, variation in wind velocity and direction creates differences in the sources of pollen and thus the adaptive genetic information represented in each year's seeds. Wind thus shapes the spatial and the temporal distribution of adaptive genetic information in seeds, giving it considerable influence on the adaptability of forests to environmental change. This is especially true in temperate latitudes, where most tree species are wind-pollinated.

Since conspecific trees often occur in environmentally more or less uniform patches, and since these patches may give rise to the formation of subpopulations, mating can be characterized as the pattern of effective pollen dispersal from (sub)population to (sub)population. This calls for adaptive interpretation at the level of a metapopulation, which is defined as a set of partially isolated populations connected by gene flow (Levins, 1969). Populations of any size can form a metapopulation, even single trees. Extending metapopulation analysis beyond the basic consideration of colonization/extinction of habitats, the focus here is on the relative contributions of pollen from different sources to the seeds produced by each population and by the metapopulation as a whole. This metapopulation concept is reminiscent of that frequently conceived in community ecology as a set of local communities of potentially interacting species (here pollen of different sources) that are linked by species dispersal (here pollen dispersal) (see Liebold et al., 2004).

Each population in a metapopulation harbors a unique genetic structure (alleles, genotypes) at each gene locus that is involved in adaptation. Its alleles at each such locus contribute via pollen dispersal to the seeds of other populations, just as other populations contribute their alleles via pollen dispersal to the seeds of this population. Populations may or may not share alleles at a locus, so that pollen dispersal may change the frequency of the constituent alleles or even introduce new alleles. Since it is practically impossible to trace the dispersal of alleles at all of the presumably large number of adaptive gene loci among the populations, it is meaningful in metapopulation analysis to label each pollen with its source population as a synopsis of the complex adaptive genetic characteristics that have allowed the trees in this population to survive to maturity. Labelling of pollen by source population specifies a genetic trait within the set of all pollen produced in the metapopulation, where the trait state stands for the specific genetic structure of the source population.

The adaptive potential in a population's seeds is the range of environments in which a sufficient number of offspring can survive to support a viable population (Gregorius, 1977; Bergmann et al., 1990). This is primarily determined by the spectrum of environments in the source populations of the pollen contributions in interaction with the local environment of the population's ovule contributions. In changing environments, a large adaptive potential might be a safeguard for survival (adaptive reserve), but under constant environmental conditions, a high proportion of pollen from environments that differ from a population's local environment endangers survival (genetic load, migration load). Whether a sufficient number of offspring will survive to maintain the population depends on the distribution of environments around it, the degree of differences among the environments, and the direction and distance of pollen dispersal, all of which may vary over space and time.

In population genetics, the paradigm model of mating is panmixis. The panmixis hypothesis rests on three assumptions: (a) the same trait is viewed in both gametic sexes, (b) the frequency distribution of trait states is the same in both sexes (sexual symmetry), and (c) gametes fuse at random. If the trait state is the genotype at a diploid gene locus, panmixis yields Hardy-Weinberg proportions in the offspring. In the metapopulation framework of mating among populations, panmixis amounts to (a) consideration of the source population as a trait (state) for pollen as well as for ovules, (b) assumption that the distribution of source populations among the pollen produced within the metapopulation is the same as among the ovules, and (c) stochastic independence of pollen and ovule sources in the zygotes of the metapopulation. Panmixis is thus realized on the metapopulation level only if the relative contribution of each population through pollen equals its relative contribution through ovules to all zygotes, and in the absence of zygotic selection, to all seeds formed in the metapopulation. Even though it is frequently addressed as random dispersal, in a more strict sense this situation refers to equal mating success for pollen sources within populations. True random dispersal would, however, require special mechanisms of distance-independent pollen dispersal that are physically difficult to construe. It may, however, be possible that certain patterns of pollen dispersal approximate random dispersal so closely that statistical testing based on limited samples of the seeds cannot discriminate between them (Spasojevic et al., 2014). This may explain the frequent failure to detect deviation from the hypothesis of panmixis using real sampling data. One question is whether long distance dispersal can realize true random dispersal. Model characteristics that allow for such situations will be given special attention in order to allow for realistic interpretation of these observations.

The vagaries of wind defy general analytical treatment of mating for wind-pollination. By simulating wind conditions in models, possible effects of wind on the pattern of pollen dispersal among populations, and ultimately on adaptive potentials, can be demonstrated. To this end, a probabilistic model is presented that accepts wind direction and mean dispersal distance as the input variables that shape the dispersal of pollen beyond their source populations to other populations. By applying the model to scenarios of populations lined along a transect, a setting that is representative of a wide range of real situations (e.g. roadside, stream, lakeshore, valley), pollen dispersal need be considered in only one dimension. This makes it easier to separate the effects of wind direction and pollen dispersal distance from other factors that play a role in two-dimensional systems. Effects of wind direction and wind intensity are demonstrated for scenarios of symmetric (non-directional) and asymmetric (directional) pollen dispersal paired with increasing dispersal distance along the transect. Random mating on the metapopulation level provides a reference scenario. Under simple assumptions on gamete production and fertilization, the output of the model is the pollen source distribution in the seeds produced within each population and in the metapopulation as a whole.

The outcome of pollen dispersal is commonly analyzed in terms of amounts of gene flow among populations and the potential implication of preferential matings within or between these populations, as referenced above. Numerous experimental studies apply paternity analysis to the seeds of single mother trees in order to estimate the relative contributions of pollen donors (backward migration) or the distribution of pollen dispersal distances, including wind direction (Smouse et al., 2001; Austerlitz et al., 2004; Burczyk et al., 2004; Vranckx et al., 2014). In a wider evolutionary context, what ultimately counts are the effects of the mating system on providing potentials for adaptation, including general limits set by the available genetic variation.

The present paper therefore focuses on outlining the structural effects of different modes of wind-mediated pollen dispersal on the genetic variation within populations on a transect. It is shown that quantification of the distribution of pollen sources among populations by a different set of measures than those commonly used helps us understand how wind affects pollen source variation of populations along a transect. These measures set quantitative limits to the adaptive potential of populations when the metapopulation is spread over heterogeneous environments, showing how wind can shape adaptability to different types of environmental variation, whether static, gradual, or unstructured (chaotic).

Basically, the diversity, or effective number, of pollen sources in the seeds of a population represents the spectrum of pollen sources within the metapopulation. The compositional differentiation among populations reflects differences, or specializations, in the distribution of pollen sources in seeds between populations. The degree to which differentiation is absent corresponds to the homogeneity of the populations, allowing comparison with results for metacommunity models showing that dispersal has a homogenizing effect on species composition (Loreau et al., 2003; Mouquet and Loreau, 2003; Münkemüller et al., 2011). The commonly used fixation index F_{ST} or G_{ST} (Wright, 1978; Nei, 1973) is included to show how it confounds the effects of variation (polymorphism) within populations with differences between populations, making it less useful for prediction of adaptive potential.

2 Methods

2.1 The model

Consider $n = 5$ (sub)populations P_1, ..., P_5 lined along a transect far from other trees of the same species. The populations are of equal length $L = 100$ by some arbitrary measure (e.g. meter, decameter) and separated by treeless gaps of the same length to simulate fragmentation. The reason for division of the transect into equal stretches of populations and gaps is its suitability for analysis of the effects of partial isolation and pollen loss on the one hand and connectivity between populations via pollen dispersal on the other hand on within-population diversity. All five populations together are regarded as a metapopulation connected by pollen dispersal between populations. Seeds are heavy and do not disperse.

Model inputs are wind direction and mean dispersal distance (a composite of horizontal wind speed, turbulence, and humidity) during a single pollination season. Pollen produced within a population is carried by wind rightwards along the transect (e.g. towards populations of higher number) with probability p_r or leftwards with probability $p_l = 1 - p_r$.

Dispersal distance is governed by a dispersal kernel, i.e., the probability density function (pdf) of the distance $x \geq 0$ that pollen travels in either direction before settling. Equal dispersal distance in both directions is a simplification that may be realized, for example, by daily changes in convection up and down mountains or valleys.

A number of different dispersal kernels have been proposed and tested using data from paternity analysis on seeds or by studying the physical movement of small particles (Clark, 1998; Austerlitz et al., 2004; Katul et al., 2005; Kuparinen et al., 2007). Common to all is the property that the probability of reaching a certain distance is a decreasing function of this distance. This decrease is initially steep for small dispersal distance x and becomes more gradual as x increases, asymptotically approaching 0. These smooth functions neglect the possibility of occasional bulk dispersal of pollen from the same source to a spatially confined area (Lanner, 1966) and they may basically differ from dispersal kernels for non-wind-pollinated species, especially for insect pollination (see e.g. Gregorius et al., 2011). The frequent claim that they are limited to mean dispersal distances of not more than a few hundred meters may partially result from the infeasibility of identifying more distant pollen donors within the radially expanding pool of potential donors for a sufficient numbers of seeds.

The (negative) exponential distribution in its one-dimensional form

$$\theta_\gamma(x) = \frac{1}{\gamma} \exp\left(\frac{-x}{\gamma}\right)$$

is applied in this model, because it specifies the standard dispersal kernel against which other kernels are interpreted as deviations. The distance parameter γ in the same unit of length as x equals the mean (or expected) dispersal distance and defines the rate of decrease as -1/γ. The pdf θ_γ is unique in that it is the only distribution with a constant rate of decrease over distance (memorylessness).

Given the direction parameters p_r and p_l = 1 - p_r and the kernel θ_γ with its mean distance parameter, and assuming that pollen production is uniformly distributed along the length of each population, the probability $m_{i,j}$ that pollen produced in P_i settles in P_j (forward migration rate) equals

where $q_{i,j} = |i - j| \cdot 2L$ (see Figure 1).

Since pollen can settle outside of the bounds of the populations, the proportion $1 - \sum_j m_{i,j}$ of the pollen produced by population P_i is lost, i.e., excluded from mating a priori. Dispersal is well-behaved in the sense that the amount of pollen distributed from one population to another decreases with the distance between them.

In order to model the relative contributions of the source populations through pollen to the seeds of a given population, additionally assume that all populations produce the same number of pollen and the same number of ovules. Then the post-dispersal frequency of pollen from source P_i in P_j equals $p_i(j) = m_{i,j} / (\sum_k m_{k,j})$ (backward migration rate). Assuming further that pollen production is sufficient to ensure the (non-selective) fertilization of all ovules (with high probability), the frequency of seeds produced by P_j whose pollen was contributed by source P_i also equals $p_i(j)$. The outcome of the model is the pollen source distribution

$$m_{i,j} = p_l \cdot \frac{\gamma}{L} \cdot \left(\exp\left(\frac{-q_{ij} + L}{\gamma}\right) - 2\exp\left(\frac{-q_{ij}}{\gamma}\right) + \exp\left(\frac{-q_{ij} - L}{\gamma}\right)\right), \; if \; j < i$$

$$m_{i,j} = \frac{\gamma}{L} \cdot \left(\exp\left(\frac{-q_{ij} + L}{\gamma}\right) - 2\exp\left(\frac{-q_{ij}}{\gamma}\right) + \exp\left(\frac{-q_{ij} - L}{\gamma}\right)\right), \; if \; j = i$$

$$m_{i,j} = p_r \cdot \frac{\gamma}{L} \cdot \left(\exp\left(\frac{-q_{ij} + L}{\gamma}\right) - 2\exp\left(\frac{-q_{ij}}{\gamma}\right) + \exp\left(\frac{-q_{ij} - L}{\gamma}\right)\right), \; if \; j > i$$

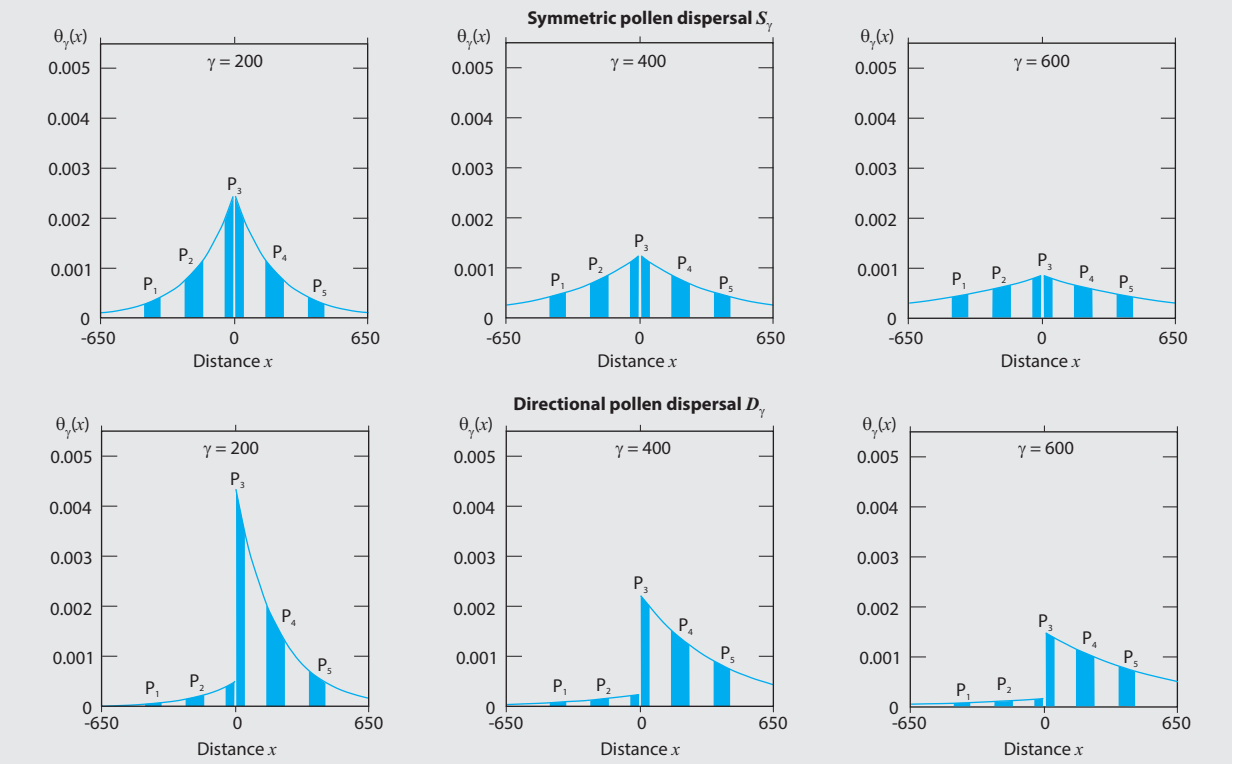

Figure 1

Dispersal of a single pollen: Probability density function $\theta_\gamma(x)$ for pollen that is produced at the center of P_3 to settle at a distance x in either direction under Scenarios S_γ and D_γ. The total area below the curve, including the two tails that extend to infinity, equals 1. The area of the shaded region below P_j equals the probability that such pollen settles in P_j. The area of a white region is proportional to the probability that the pollen settles in the gap between the two neighboring populations.

$(p_1(j), ..., p_5(j))$ in the seeds produced by each of the five populations .

The forward migration rates $m_{i,j}$ are invariant to changes in the unit of length or to a proportional change in γ and L that leaves the ratio γ/L constant (scale invariance). Increasing L but not γ would reduce the $m_{i,j}$, i.e., the dispersal among populations. By appropriate choice of the dispersal distance γ and population length L and the unit of length in which they are measured, the model can simulate metapopulations at any spatial scale for which the underlying dispersal kernel and the assumptions can be considered meaningful.

2.2 Scenarios

Symmetric dispersal S_γ Pollen is given equal chances $p_r = p_l = 0.5$ of dispersing in either direction along the transect over distances following the pdf θ_γ. For selected mean dispersal distances $\gamma \in \{200,400,600\}$, (forward) dispersal probabilities $m_{i,j}$ and the (backward) pollen source distributions $(p_1(j), ..., p_5(j))$ within the seeds produced by each of the five populations are listed in Table 1 and plotted as horizontal percent bar charts in Figure 2c. Interestingly, the symmetry of dispersal yields $m_{i,j} = p_j(i)$, that is, the probability that pollen of P_i disperses to P_j equals the probability that a seed of P_j has its pollen contribution from P_i.

Table 1

Pollen dispersal probabilities, pollen loss, and pollen source distributions in seeds for the random dispersal scenario R and the symmetric dispersal scenarios S_γ for $\gamma \in \{200,400,600\}$. The pollen dispersal probability (forward dispersal, left side) is shown as the standardized probability $m_{i,j} / \sum_k m_{i,k}$ that pollen produced in P_i (row head) and not lost will settle in P_j (column head) for $i,j = 1, ..., 5$ (see Figure 2). Loss equals the probability $1 - \sum_k m_{i,k}$ that it does not settle within the bounds of any population. Assuming equal pollen and equal ovule production over populations and non-selective fertilization of all ovules, the pollen source frequencies $p_j(i)$ (backward dispersal, right side) in the seeds of population of P_i (row head) equal $m_{i,j} / \sum_k m_{i,k}$ for each source population P_j (column head). Note the equality of forward and backward dispersal.

	Probability of pollen dispersal from P_i to P_j					Loss	Frequency of pollen from P_j in seeds of P_i				
R	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.200	0.200	0.200	0.200	0.200	0.000	0.200	0.200	0.200	0.200	0.200
2	0.200	0.200	0.200	0.200	0.200	0.000	0.200	0.200	0.200	0.200	0.200
3	0.200	0.200	0.200	0.200	0.200	0.000	0.200	0.200	0.200	0.200	0.200
4	0.200	0.200	0.200	0.200	0.200	0.000	0.200	0.200	0.200	0.200	0.200
5	0.200	0.200	0.200	0.200	0.200	0.000	0.200	0.200	0.200	0.200	0.200
S_{200}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.594	0.262	0.096	0.035	0.013	0.641	0.594	0.262	0.096	0.035	0.013
2	0.210	0.476	0.210	0.077	0.028	0.552	0.210	0.476	0.210	0.077	0.028
3	0.073	0.200	0.454	0.200	0.073	0.530	0.073	0.200	0.454	0.200	0.073
4	0.028	0.077	0.210	0.476	0.210	0.552	0.028	0.077	0.210	0.476	0.210
5	0.013	0.035	0.096	0.262	0.594	0.641	0.013	0.035	0.096	0.262	0.594
S_{400}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.408	0.270	0.163	0.099	0.060	0.717	0.408	0.270	0.163	0.099	0.060
2	0.223	0.337	0.223	0.135	0.082	0.658	0.223	0.337	0.223	0.135	0.082
3	0.128	0.212	0.320	0.212	0.128	0.640	0.128	0.212	0.320	0.212	0.128
4	0.082	0.135	0.223	0.337	0.223	0.658	0.082	0.135	0.223	0.337	0.223
5	0.060	0.099	0.163	0.270	0.408	0.717	0.060	0.099	0.163	0.270	0.408
S_{600}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.337	0.255	0.183	0.131	0.094	0.766	0.337	0.255	0.183	0.131	0.094
2	0.220	0.290	0.220	0.158	0.113	0.728	0.220	0.290	0.220	0.158	0.113
3	0.151	0.210	0.278	0.210	0.151	0.716	0.151	0.210	0.278	0.210	0.151
4	0.113	0.158	0.220	0.290	0.220	0.728	0.113	0.158	0.220	0.290	0.220
5	0.094	0.131	0.183	0.255	0.337	0.766	0.094	0.131	0.183	0.255	0.337

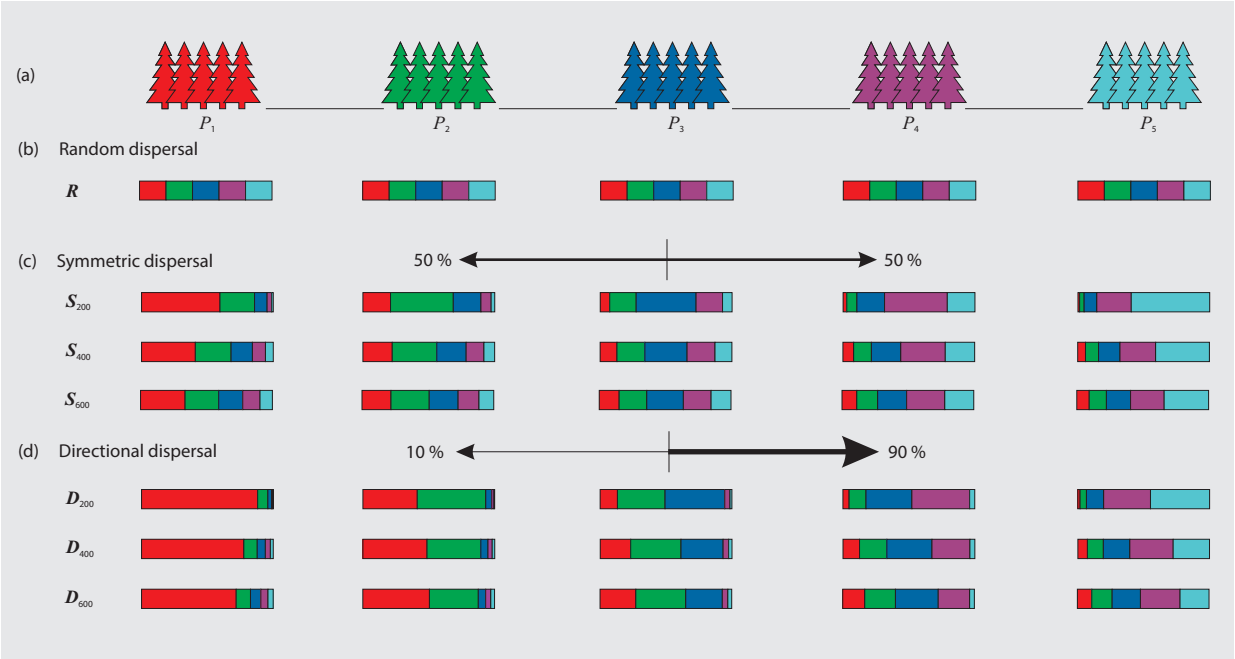

Figure 2
Distribution of pollen sources in seeds: (a) Diagram of the transect showing five populations of equal length $L = 100$ separated by gaps of the same length. (b to d) For each scenario and each population P_i ($j = 1, ..., 5$), the colored horizontal histogram of total length 1 shows the relative contribution $m_{j,i}$ of each of the five populations P_j as pollen sources to the seeds of P_i (e.g. the length of the red segment is proportional to the relative frequency of seeds with pollen from the "red" population P_1). (b) Random dispersal scenario R. (c) Symmetric dispersal scenario S_γ (50 % leftwards: 50 % rightwards) for mean dispersal distances $\gamma \in \{200,400,600\}$. (d) Directional dispersal scenario D_γ (10 % leftwards: 90 % rightwards) for $\gamma \in \{200,400,600\}$.

Table 2
Pollen dispersal probabilities, pollen loss, and pollen source distributions in seeds for the directional dispersal scenarios D_γ for $\gamma \in \{200,400,600\}$ (see Table 1 for explanation). Note the inequality of forward and backward dispersal.

	Probability of pollen dispersal from P_i to P_j					Loss	Frequency of pollen from P_j in seeds of P_i				
D_{200}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.448	0.355	0.131	0.048	0.018	0.524	0.880	0.078	0.028	0.010	0.004
2	0.039	0.438	0.348	0.128	0.047	0.514	0.412	0.519	0.046	0.017	0.006
3	0.015	0.040	0.453	0.360	0.132	0.530	0.132	0.360	0.453	0.040	0.015
4	0.006	0.017	0.046	0.519	0.412	0.590	0.047	0.128	0.348	0.438	0.039
5	0.004	0.010	0.028	0.078	0.880	0.758	0.018	0.048	0.131	0.355	0.448
D_{400}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.276	0.329	0.200	0.121	0.073	0.583	0.775	0.102	0.062	0.038	0.023
2	0.038	0.287	0.342	0.207	0.126	0.599	0.486	0.408	0.054	0.033	0.020
3	0.026	0.042	0.320	0.381	0.231	0.640	0.231	0.381	0.320	0.042	0.026
4	0.020	0.033	0.054	0.408	0.486	0.718	0.126	0.207	0.342	0.287	0.038
5	0.023	0.038	0.062	0.102	0.775	0.851	0.073	0.121	0.200	0.329	0.276
D_{600}	$j = 1$	2	3	4	5		$j = 1$	2	3	4	5
$i = 1$	0.220	0.300	0.215	0.154	0.110	0.641	0.717	0.109	0.078	0.055	0.040
2	0.036	0.238	0.325	0.233	0.167	0.669	0.505	0.370	0.056	0.040	0.029
3	0.030	0.042	0.277	0.379	0.271	0.716	0.271	0.379	0.277	0.042	0.030
4	0.029	0.040	0.056	0.370	0.505	0.787	0.167	0.233	0.325	0.238	0.036
5	0.040	0.055	0.078	0.109	0.717	0.890	0.110	0.154	0.215	0.300	0.220

Directional dispersal D_γ: Prevailing winds in the rightward direction (i.e., towards populations with higher numbers) are modelled by giving pollen the probability $p_r = 0.9$ of dispersing rightwards and $p_l = 0.1$ of dispersing leftwards. The dispersal distance in either direction follows θ_γ. Table 2 shows that $m_{i,j}$ and $p_j(i)$ are no longer equal. Figure 2d shows the horizontal percent bar charts of ($p_1(j)$, ..., $p_5(j)$).

Random dispersal *R*: In spatially less explicit population genetic models, pollination is assumed to occur at random, contributing equal proportions of pollen from all pollen sources to the seeds of all populations, i.e., $p_i(j) = \frac{1}{5}$ for all populations P_i and P_j. Random dispersal is included here as a reference scenario, since it is still a baseline hypothesis against which other models are tested based on actual data.

2.3 Measures of variation and their meaning

Structural characteristics of the distribution of pollen sources in the seeds produced within the populations of a metapopulation are quantified using the following measures (see classification of Gregorius and Gillet, 2015). Each seed is characterized by its "population" (i.e., the population of its seed parent), and its pollen source (i.e., the population of its pollen parent) or "type".

2.3.1 Effective number of pollen sources (types)

The adaptedness of the seeds produced by a population to a constant environment and its adaptive potential to changing environments are dependent on the spectrum of adaptive variation that it is contributed through pollen by the populations. The amount of pollen source variation (i.e., pollen source diversity) within the seeds of a population is measured here as the effective number of populations that contributed pollen according to Rényi diversity $v(j) = (\Sigma_i p_i(j)^2)^{-1}$ of order 2 (or Hill number 2D, where $p_i(j)$ is the frequency of pollen from P_i in the seeds of P_j. $v(j)$ ranges from its minimum of 1 for monomorphism (i.e., fixation of the seeds to a single pollen source) to its maximum of five for equal contributions $p_i(j) = \frac{1}{5}$ of all five source populations P_i. The Rényi-diversity of order 2 equals its own effective number of pollen sources and also the effective number by the Simpson diversity $(1 - \Sigma_i p_i(j)^2)$ (Gregorius, 1991).

The effective number of pollen sources in the seeds of the metapopulation, i.e., the aggregate of the seeds produced by the five populations, is expressed by the Rényi diversity $v_T = (\Sigma_i p^2)^{-1}$ (T stands for total), where the frequency p_i of pollen of source P_i in the metapopulation's seeds equals $\Sigma_j c_j p_i(j)$ and c_j is the relative frequency of seeds carrying pollen of P_j. Here $c_j = \frac{1}{5}$ holds due to the equality of the numbers of ovules and their complete fertilization in each population.

2.3.2 Compositional differentiation of pollen sources

Wind-dispersal of pollen creates differences between populations in the distribution of pollen sources in their seed production and thus in the spectrum of adaptive variants. Compositional differentiation measures the tendency for seeds produced by different populations to have pollen contributions from different sources or, inversely, for seeds with pollen contributions from the same source population to have been produced in the same population (Gregorius, 2010; Gregorius, 2014; Gregorius and Gillet, 2015). Differentiation is complete if the pollen sources among the seeds produced by the different populations are disjoint, and it is absent if all populations have the same distribution of pollen sources in their seeds (Gregorius and Roberds, 1986). In its "pure" form, compositional differentiation should be independent of levels of diversity (i.e., effective number) within the populations (Gregorius and Gillet, 2015). A measure that considers only the frequency differences between the pollen sources in the seeds, and not their diversities, is the complementary compositional differentiation

$$\delta_{SD} = \sum_{j=1}^{n} c_j \delta_{SD}(j)$$

(Gregorius and Roberds, 1986), where

$$\delta_{SD}(j) = \frac{1}{2} \sum_{i=1}^{n} | p_i(j) - \bar{p}_i(j) |$$

is the absolute difference between the pollen source distribution ($p_1(j), \ldots, p_n(j)$) in the seeds of population P_j and the pollen source distribution ($\bar{p}_1(j), \ldots, \bar{p}_n(j)$) in this population's complement, i.e., the aggregate of the seeds produced by all other populations. The frequency of pollen from P_i in the complement of the seeds of P_j equals $\bar{p}_i(j) = \Sigma_{k,k \neq j} c_k p_i(k) / (1 - c_j)$. Thus δ_{SD} equals the mean difference between the pollen source distributions in the seeds of each population and in their complements. The range of δ_{SD} stretches from its minimum of 0 for identical pollen distributions in all populations to its maximum of 1 when no two populations share pollen from the same population. In other words, $\delta_{SD}(j)$ measures the uniqueness of the seed production of P_j, and $1 - \delta_{SD}(j)$ measures its representation for the others. δ_{SD} measures the mean uniqueness or disjunction of the seeds produced among the populations and $1 - \delta_{SD}$ the homogeneity.

2.3.3 Fixation index F_{ST} or G_{ST}

Because of its wide application in the analysis of (sub)population structure and as an overview of results that are obtainable for indices of fixation versus indices of compositional differentiation, a brief account of this index is provided.

The general term "differentiation" as it is commonly used in the literature is ambiguous. It is often interpreted to mean the difference in the distributions of types (here, pollen sources) among populations, but the most commonly used measures of "differentiation" actually quantify the degree of monomorphism of types within populations. This family of measures comprises various standardizations of the increase in Simpson diversity $H_T = 1 - \Sigma_i p_i^2$ of types in the metapopulation compared to the mean $H_S = 1 - \Sigma_i \Sigma_j c_j p_i(j)^2$ of the Simpson diversities within the populations. The oldest member of this family measures this increase relative to H_T as

$$F_{ST} = G_{ST} = \frac{H_T - H_S}{H_T}$$

(Wright, 1978; Nei, 1973). Its minimum $F_{ST} = 0$ is assumed when the pollen source distributions in the seeds of all populations are identical, which suggests that it might measure "difference", but at the other extreme, its maximum $F_{ST} = 1$ is reached only when the seeds of all populations are monomorphic for pollen from only one source (though not from the same source in all populations, in which case F_{ST} is undefined). As a consequence, F_{ST} is maximal even if all but one of a large number of populations are fixed for the same type, making them identical, and only the remaining population is fixed for a second type. As pointed out by Gregorius and Roberds (1986), this contradicts the common interpretation of F_{ST} as a measure of the "difference" among populations. This was recognized by Sewall Wright, who introduced F_{ST} in the context of his Island Model of population genetics as a *fixation index*, that is, a measure of "differentiation within the total array in the sense of the extent to which the process of fixation has gone towards completion" (Wright, 1978, p. 82). F_{ST} is "thus not a measure of degree of differentiation in the sense implied in the extreme case by absence of any common allele", i.e., it does not measure compositional differentiation. Moreover, Wright's interpretation as a fixation index assumes fulfillment of the highly idealistic assumptions of the Island Model. As opposed to δ_{SD}, F_{ST} depends heavily on the diversities within populations, decreasing rapidly towards 0 as the degree of polymorphism increases within populations (Hedrick, 1999; Gregorius et al., 2007).

The fact that F_{ST} approaches zero for high polymorphism within populations despite high differentiation between them as well as for low differentiation marks a salient point in interpretation of this index, and this will be given special emphasis in the present paper. Beyond this, other standardizations of $(H_T - H_S)$ may not solve this problem. G'_{ST} (Hedrick, 2005), for example, assumes its maximum for complete fixation, for complete compositional differentiation, or both, meaning that it does not discriminate between tendencies towards monomorphism and disjunction (Gregorius et al., 2007). Jost's D is maximal only for complete disjunction, but its intermediate values are sensitive to the within-population diversity (Jost, 2008; Gillet, 2013).

2.3.4 Comparison of compositional differentiation and fixation

Both F_{ST} and δ_{SD} assume their minimum of 0 when the types are identically distributed in all populations, independently of the number of types in relation to the number of

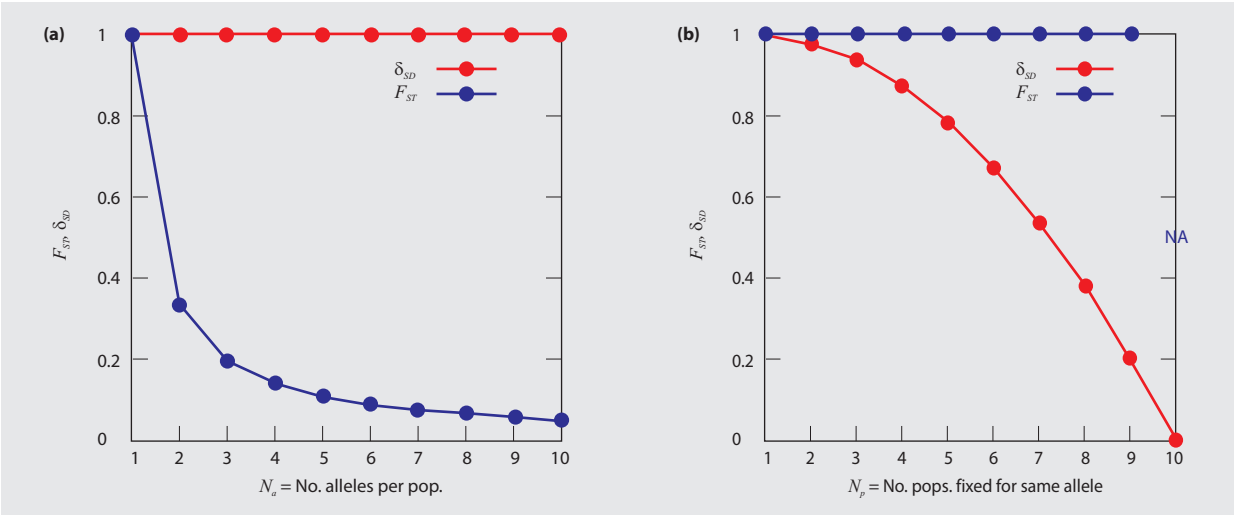

Figure 3
Examples of deviant characterizations of structural variation in metapopulations by two measures of variation among populations, the compositional differentiation δ_{SD} (red) and the fixation index F_{ST} (blue). (a) Two populations each possess N_a equally frequent alleles that are not shared between populations. For $N_a = 1$, both measures equal their maximum of 1. As the populations become more polymorphic with rising diversity $v_2 = N_a$, F_{ST} approaches its minimum of 0, while the non-sharing of alleles leaves the diversity-independent δ_{SD} at its maximum. (b) Ten populations are fixed for one allele, N_p of the populations for the same allele, the others for different alleles. For $N_p = 1$, both measures are maximal. As N_p increases, the decreasing difference of each population to its complement reduces δ_{SD} to 0 for $N_p = 10$, while the monomorphism of all populations leaves F_{ST} maximal for all N_p except 10, for which F_{ST} is undefined ("NA").

populations. Complete fixation (F_{ST} = 1) and complete compositional differentiation δ_{SD} = 1, in contrast, occur simultaneously only when all populations are fixed for a different type, implying that the number of populations equals the number of types (here, pollen sources). Here it happens in the absence of dispersal (γ = 0), where all seeds contain local pollen only.

In general, F_{ST} and δ_{SD} show quite different behavior, demonstrating that fixation (commonly called "differentiation") and compositional differentiation are very different concepts. At the extremes, fixation cannot be complete if there are more types than populations, and compositional differentiation cannot be complete if there are more populations than types. If, for example, each population is polymorphic for N equally frequent alleles that are not shared between populations, the diversity-dependence of F_{ST} pushes it towards 0 for increasing polymorphism, while δ_{SD} remains at its maximum of 1 (Figure 3a). On the other hand, if all populations are monomorphic and if an increasing number of populations are fixed for one particular allele, the decreasing disjunction between populations pushes δ_{SD} from 1 towards 0, while F_{ST} remains maximal but is undefined if all are fixed for the same allele (Figure 3b). For more rigorous treatment of the connection between fixation (or "apportionment") and compositional differentiation, see the papers of Gregorius et al., 2007; Gregorius, 2010; 2014; 2016.

2.3.5 Adaptive potentials and migration load

Adaptedness to a constant environment: A high proportion $p_j(j)$ of local pollen ensures adaptedness in the current environment, since offspring containing local pollen contributions have a better chance of surviving than offspring containing pollen from most other environments. Pollen contributions from the other populations may confer poorer adaptation in a population's environment, reducing the number of offspring that can survive. The potential migration load as the genetic load contributed via pollen dispersal to the offspring of P_i (i.e., the seed parent is in P_j) is measured as the proportion

$$p_{imm}(j) = 1 - p_j(j)$$

of immigrant pollen. For high potential migration load, the effective number of sources of immigrant pollen becomes important. A small effective number of sources bears the danger of flooding the populations with poorly adapted pollen from a few sources, while a higher effective number signifies a larger number of adaptive variants and thus a higher capacity for adaptation.

Adaptability to environmental change: In a changed environment, offspring descending from local pollen may have poor survival, while offspring containing pollen that immigrated from a population well adapted to the new environment would survive. The proportion $p_{imm}(j)$ of immigrant pollen is now a measure of the adaptive potential in the offspring. A high proportion is necessary for adaptability, though it is not sufficient to guarantee that pollen from adapted populations will be among them. Nevertheless, a large effective number

$$v_{imm}(j) = \sum_{i,i\neq j} \left(\frac{p_i(j)}{\sum_{k,k\neq j} p_k(j)}\right)^2$$

of sources of immigrant pollen increases the chances that some of the pollen will come from populations that are adapted to the new environment, giving offspring the ability to survive. The adaptive potential of the offspring of P_i in the face of environmental change is expressed as a combination of $p_{imm}(j)$ and $v_{imm}(j)$.

3 Results

Application of the model to the scenarios yields the following characterizations of the pollen source distributions along the transect, both in the seeds of the populations and in the aggregate seeds of the metapopulation. Since Scenario R is the idealized situation to which the other scenarios are compared, its characterization is presented first.

3.1 Random dispersal R

Under the hypothesis of random dispersal, pollen from each population has equal probability of settling in each of the five populations, i.e., $m_{ij} = \frac{1}{5}$ for all i,j. Under the further assumptions of the model, i.e., equal pollen production and non-selective ovule fertilization, the frequency $p_i(j)$ of every pollen source P_i in the seeds produced by each population P_j equals $\frac{1}{5}$. The pollen source distributions are illustrated by the five evenly striped horizontal percent bar charts in Figure 2b. Metapopulation structure is characterized for random dispersal as follows:

- *Effective number:* The equal proportions of all pollen sources in the seeds of each population yield equal proportions among the seeds produced by the metapopulation, so that the effective number of pollen sources is the actual number of populations ($v_T = v(j) = 5$ for all P_j).
- *Compositional differentiation:* Compositional differentiation is absent ($\delta_{SD} = 0$), yielding complete homogeneity of the populations for the distribution of pollen sources in their seeds. The seeds of every population are completely representative of the seeds of the metapopulation ($\delta_{SD}(j) = 0$).
- *Fixation index:* F_{ST} equals 0 due to the complete homogeneity of seeds among populations.
- *Adaptive potential:* The proportion $p_{imm}(j) = \frac{4}{5}$ of immigrant pollen in each population is large, among which each of the other four populations is equally represented ($v_{imm}(j) = 4$).

3.2 Symmetric dispersal S_γ

The pollen source distributions among the seeds of the populations are shown for selected mean dispersal distances

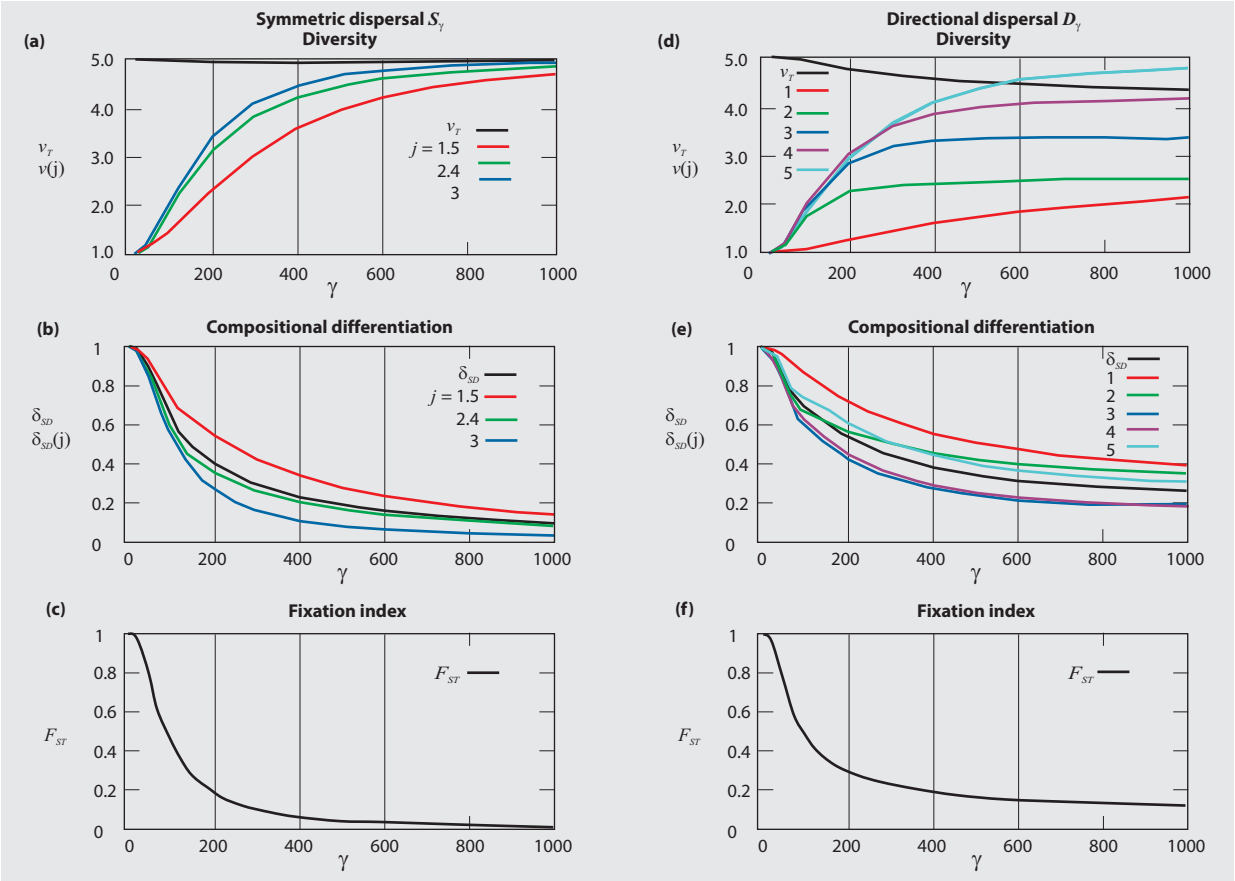

Figure 4

Measures of variation of the pollen source distributions in the seeds for scenarios S_γ and D_γ for mean dispersal distance γ up to 1000, i.e., 10 times the length L of the populations and the gaps. Dotted lines mark the scenarios in Tables 1 and 2; the numbers in the legends refer to the populations P_j.

Table 3

Variation of pollen sources in the seeds of the populations and the metapopulation for the scenarios in Tables 1 and 2.

Scenario	Effective number of pollen sources in seeds						Pollen loss
	$v(1)$	$v(2)$	$v(3)$	$v(4)$	$v(5)$	v_T	
R	5.000	5.000	5.000	5.000	5.000	5.000	0.000
S_{200}	2.317	3.118	3.375	3.118	2.317	4.935	0.583
S_{400}	3.584	4.202	4.446	4.202	3.584	4.949	0.678
S_{600}	4.201	4.582	4.738	4.582	4.201	4.967	0.741
D_{200}	1.280	2.263	2.823	3.000	2.884	4.771	0.583
D_{400}	1.620	2.458	3.295	3.853	4.085	4.573	0.678
D_{600}	1.862	2.516	3.370	4.062	4.529	4.468	0.741
Scenario	**Compositional differentiation among populations pollen contributions to seeds of variation**						**Partitioning**
	$\delta_{SD}(1)$	$\delta_{SD}(2)$	$\delta_{SD}(3)$	$\delta_{SD}(4)$	$\delta_{SD}(5)$	δ_{SD}	F_{ST}
R	0.000	0.000	0.000	0.000	0.000	0.000	0.000
S_{200}	0.566	0.379	0.294	0.379	0.566	0.424	0.198
S_{400}	0.351	0.218	0.124	0.218	0.351	0.244	0.062
S_{600}	0.246	0.152	0.076	0.152	0.246	0.169	0.029
D_{200}	0.737	0.585	0.457	0.474	0.635	0.560	0.306
D_{400}	0.569	0.467	0.294	0.306	0.456	0.395	0.189
D_{600}	0.482	0.411	0.233	0.242	0.375	0.325	0.149

$\gamma \in \{200,400,600\}$ as bar charts in Figure 2c. Pollen source variation is characterized as follows (see Table 3 and Figures 2a to c and 4a):

- *Effective number:* Pollen source variation is unevenly distributed over the populations (Figure 2c). Within populations, the effective number of pollen sources in the seeds increases differentially from 1 (monomorphism) for $\gamma = 0$ towards the maximum of five with increasing distance of symmetric dispersal. The increase is steepest for short-distance dispersal and flattens out quickly as dispersal distance increases. The increase becomes faster when moving from the periphery to the center, giving the central population P_3 the highest effective number and both peripheral populations P_1 and P_5 the lowest. In the seeds of the metapopulation, the effective number v_T of pollen sources is only slightly lower than the maximum of five for all dispersal distances (Figure 4a).
- *Compositional differentiation:* Starting from complete differentiation ($\delta_{SD} = 1$) for $\gamma = 0$, pollen sources become more homogeneous as dispersal distance increases (Figure 4b, "snails" in Figure 5a). Differentiation seems to be approaching $\delta_{SD} = 0$ for longer dispersal distances ($\delta_{SD} = 0.053$ for $\gamma = 2000$). The pollen source distribution of the central population best represents the seeds of the metapopulation, i.e., $\delta_{SD}(3)$ is the smallest $\delta_{SD}(j)$ and is even lower than the average differentiation δ_{SD} among all populations.
- *Fixation index:* When there is no wind ($\gamma = 0$), all populations are initially monomorphic for pollen source, yielding $F_{ST} = 1$). As dispersal distance γ increases, F_{ST} approaches 0 (i.e., identical pollen source distributions in all populations) more rapidly than δ_{SD} (Figure 4c). The faster decrease of F_{ST} towards 0 is due to the rapid increase in diversity within the populations. The dependence of F_{ST} on within-population diversity implies that F_{ST} would start out being much smaller than 1 for $\gamma = 0$ if allelic variation within the pollen contributions to the seeds were taken into consideration instead of only the pollen source (see Figure 1).
- *Adaptive potential:* The proportion $p_{imm}(j)$ of pollen immigrating into the populations is initially absent for $\gamma = 0$ and increases toward $p_{imm}(j) = 0.8$, the value for random dispersal, as dispersal distance increases (Figure 6a). The peripheral populations have a slightly lower proportion of immigrant pollen than the others. The effective number $v_{imm}(j)$ of sources of immigrant pollen is undefined (non-existent) for $\gamma = 0$ and increases from an initial positive value of 1 for the peripheral populations P_1

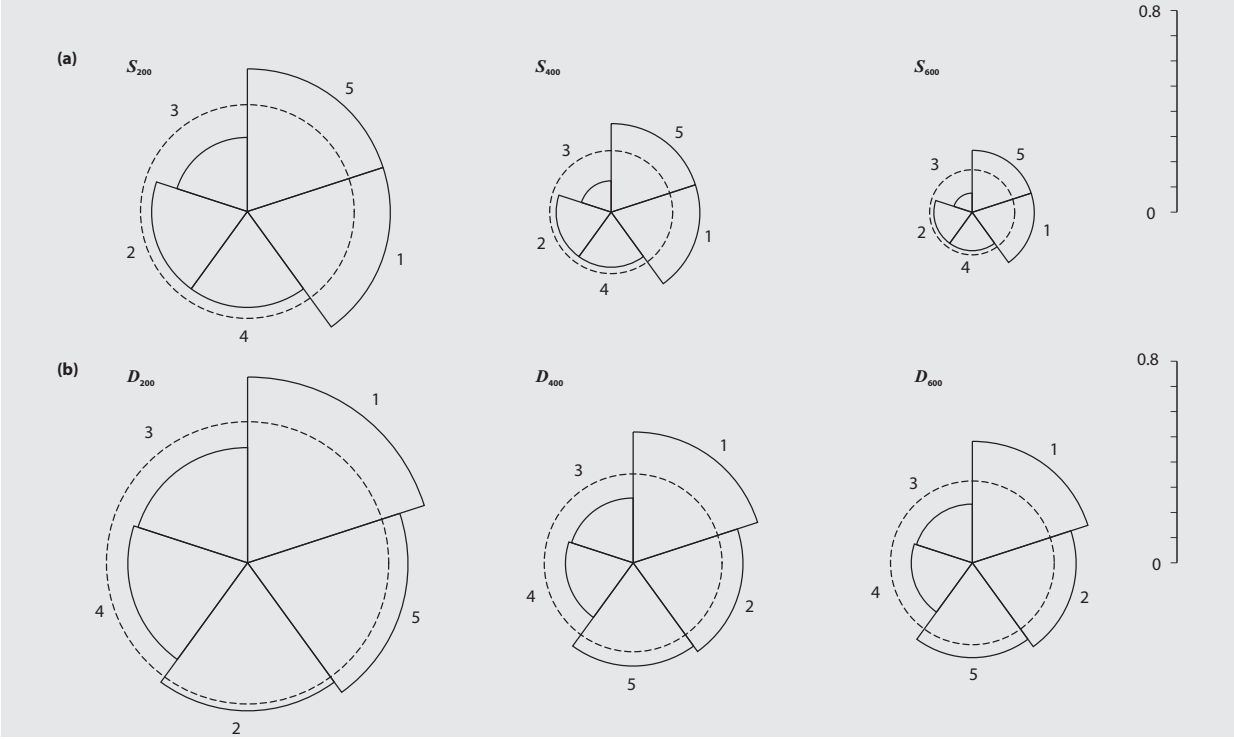

Figure 5

Snail diagrams of compositional differentiation δ_{SD} among the pollen source distributions of the seeds for three symmetric and three directional scenarios in Table 1 and 2 (after Gregorius and Roberds, 1986). The radius of the dotted circle is proportional to δ_{SD}. Population P_j is represented as a sector of radius proportional to $\delta_{SD}(j)$ and central angle $c_j = 2\pi/5$ marked by the number j. Populations are arranged in ascending order, starting with the population of smallest difference $\delta_{SD}(j)$ from its complement placed to the left of the top vertical axis and proceeding counterclockwise to the population of largest $\delta_{SD}(j)$. The smaller the snail, the more homogeneous are the pollen source distributions among populations. The population with the lowest $\delta_{SD}(j)$ has the most representative pollen source distribution, that with the highest $\delta_{SD}(j)$ has the most unique distribution. Since $\delta_{SD}(j) = 0$ holds for the random dispersal scenario R, its snail is a single point (not shown).

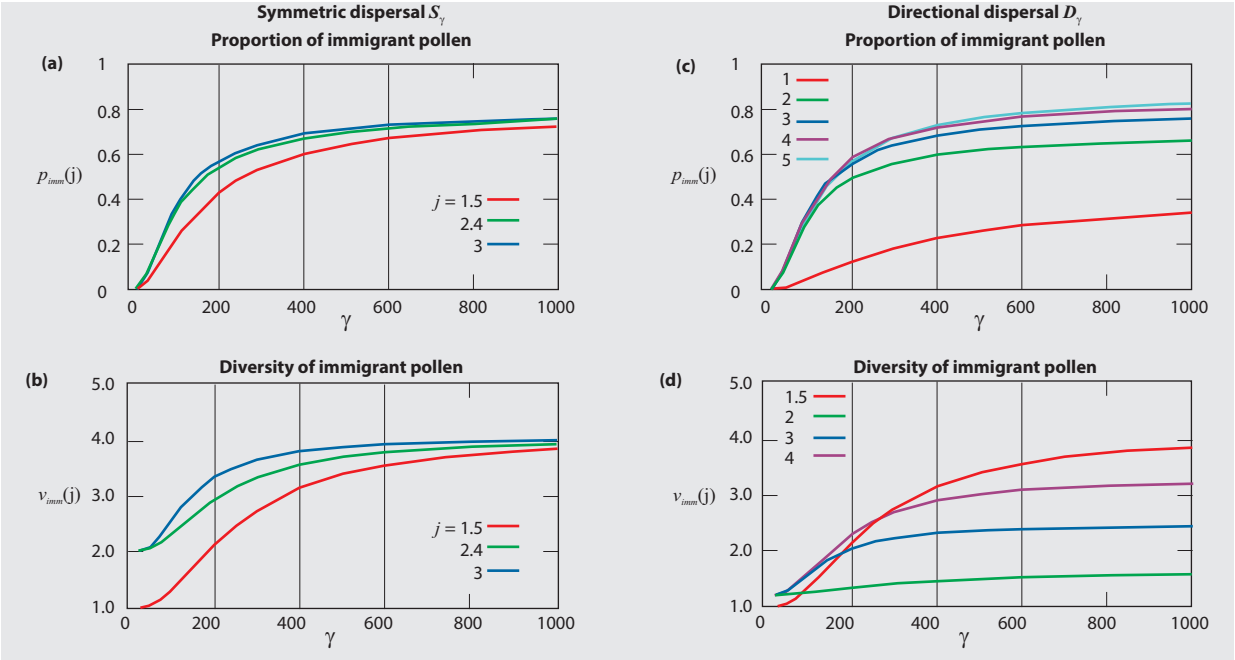

Figure 6

Adaptive potential: Proportion of immigrant pollen $p_{imm}(j)$ and its diversity $v_{imm}(j)$ in the seeds of each population P_j for scenarios S_γ and D_γ and mean dispersal distance γ up to 1000. The numbers in the legends refer to the populations P_j.

and P_5 and from 2 for the three interior populations as dispersal distance increases (Figure 6b). It remains highest in the central population and lowest in the peripheral populations. As dispersal distance increases, the effective number of immigrant pollen sources approaches 4 for all populations, as for random dispersal. The seeds of the peripheral populations P_1 and P_5 have the lowest potential migration load, since the proportion of immigrant pollen is smaller. The effective number of sources of immigrant pollen is also lower than for the interior populations. Both the migration load and the effective number of sources of immigrant pollen increase as dispersal distance increases.

3.3 Directional dispersal D_γ

The nine-fold chance (9:1) of dispersing rightwards, or downwind, along the transect yields a high level of asymmetry for pollen source distributions around the central population, as shown for $\gamma \in \{200,400,600\}$ in Figure 4d. Pollen source variation is characterized as follows (see Table 3 and Figures 2d to f and 4b):

- *Effective number:* As for symmetric dispersal, the pollen source variation is unevenly distributed over the populations (Figure 2d). The effective number v_j of pollen sources within populations increases in prevailing wind direction from its lowest level in the leftmost, or upwind, population P_1 to its highest level in the rightmost, or downwind, population P_5 (Figure 4d). Only the downwind population's effective number approaches the maximum of 5, and even exceeds the effective number v_T of pollen sources in the metapopulation. In the metapopulation, the effective number v_T of pollen sources is lower than under symmetric dispersal and actually decreases with increasing dispersal distance (Figure 4d), deviating from the value of five for random dispersal.
- *Compositional differentiation:* Among the populations, the distributions of pollen sources in the seeds initially become more homogeneous as dispersal distance increases, but their compositional differentiation levels off well above 0 even for long-distance dispersal (δ_{SD} = 0.234 for γ = 2000), which is over 4 times that for symmetric dispersal (Figure 4e, "snails" in Figure 5b). The pollen source distribution in the seeds of each population is less representative of the metapopulation's seeds than for symmetric winds (smaller δ_{SD} (j)), and the most representative is now not only the central population but also its downwind neighbor.
- *Fixation index:* F_{ST} for pollen sources in the seeds decreases from its initial value of 1 (no wind for γ = 0) as dispersal distance γ increases. F_{ST} again falls faster than δ_{SD}, but as opposed to symmetric dispersal, F_{ST} remains well above the value 0 that corresponds to identical pollen source distributions. This rules out random mating, which is a special case of identical distributions. The reason is the persistence of differences in pollen source distributions among the populations, as is confirmed by the positive compositional differentiation δ_{SD} (Figure 4f).
- *Adaptive potential:* The proportion $p_{imm}(j)$ of pollen immigrating into the populations increases from its initial absence for γ = 0 as dispersal distance increases, but only the three populations farthest downwind tend toward the proportion of 0.8 for random dispersal

(Figure 6c). The upwind population has by far the lowest immigration for all positive dispersal distances, and its neighbor also remains well below the value for random dispersal. The effective number $v_{imm}(j)$ of sources of immigrant pollen is undefined for $\gamma = 0$, then increases from 1 as dispersal distance increases (Figure 6d). It does not exceed the corresponding number for symmetric dispersal for any population. It remains small for population P_2. Populations P_3 and P_4 have successively larger effective numbers that level out far below 4, the value for random dispersal. The two peripheral populations P_1 and P_5 have the same effective number as under symmetric dispersal, but here it is the highest among all populations. As dispersal distance increases, the adaptive potential of the seeds of all populations increases, since more pollen immigrates and the effective number of its sources increases. For distances over ca. 300, the downwind population P_5 has the highest adaptive potential, since it has the most immigrant pollen $p_{imm}(j)$ and the highest effective number of sources $v_{imm}(j)$. Though the upwind population P_1 has the same effective number as P_5, the proportion of immigrant pollen upon which the effective number is based is extremely small and thus of little import for adaptation. P_2 has more immigration than P_1 but a lower effective number of sources.

4 Discussion

4.1 Effects of wind on structural characteristics in seeds

Variation of the effective number of pollen sources in seeds along the transect: In comparison to random dispersal, where the seeds of every population and the metapopulation itself have the maximum effective number of pollen sources (here five), the scenarios demonstrate that wind conditions can have a pronounced effect on the effective number of pollen sources, i.e., diversity. For symmetric dispersal, the seeds of the metapopulation are a comprehensive adaptive reserve due to the near maximality of the effective number of pollen sources, but for directional dispersal the metapopulation becomes less diverse as dispersal distance increases (black curves in Figures 4a, d). Within populations, the pollen source diversity varies along the transect. For symmetric dispersal of all dispersal distances, the central population P_3 has the highest and the peripheral populations P_1 and P_5 the lowest diversity. For directional dispersal, in contrast, the population of highest diversity moves from P_4 to the peripheral population P_5 as γ passes from 200 to 300, in contradiction to the central-peripheral hypothesis (Lira-Noriega and Manthey, 2014), while P_1 has very low diversity for all γ. For $\gamma \geq 600$, the diversity in the downwind population P_5 is even higher than in the metapopulation, making the seeds of this population a more comprehensive adaptive reserve than the entire seed production of the metapopulation.

Differences in the distribution of pollen sources in seeds along the transect: The partial connectedness among populations that is typical for metapopulations has an adaptational advantage over single unconnected populations ($\delta_{SD} = 1$) as well as over one big population ($\delta_{SD} = 0$) in that intermediate differentiation between the constituent populations is a factor of additional genetic variation. For populations on a transect, it would seem adaptationally advantageous for the central population to be the least differentiated, since its short distance to all of the other populations and best representation of the metapopulation's adaptive variants would allow it to replenish lost variants in the others. This is true for all symmetric wind conditions, where the central population P_3 is the least differentiated. It also holds for directional dispersal up to $\gamma = 800$, but it is perhaps surprising that for stronger winds the neighboring downwind population P_4 becomes the least differentiated. Even for γ as high as 2000, P_4 still maintains the least differentiation.

As opposed to directional dispersal, symmetric dispersal over longer dispersal distance can have a homogenizing effect on pollen source distributions in seeds, ultimately causing them to become statistically indistinguishable from random dispersal for samples of the sizes commonly encountered in experimental studies. To see this, consider two additional scenarios S_{1000} and D_{1000} of symmetric and directional dispersal, respectively, of large mean dispersal distance $\gamma = 1000$ and a sample of 100 seeds from each population that closely matches the pollen source distribution in the model (data not shown). Under the hypothesis of random dispersal, the pollen source distributions would be nearly identical, i.e., homogeneous, among samples. For S_{1000}, the likelihood-ratio test of homogeneity yields a non-significant P-value of 0.183, leading to acceptance of the hypothesis of homogeneity. For D_{1000}, in contrast, the test yields a highly significant P-value of 0.000, leading to rejection of homogeneity. Thus it may be difficult to detect deviation from random dispersal in seeds formed by symmetric long-distance dispersal, while the deviation from random dispersal should be obvious in seeds formed by directional dispersal. Yet, distinguishing between symmetric and directional dispersal as the causes for an observed significant deviation from random dispersal requires an analysis of the local differentiation patterns or explicit paternity analysis in order to avoid erroneous conclusions about mating.

Relationship between diversity and differentiation: The scenarios show that wind dispersal refutes the commonly held thought that the population with the highest diversity should be the most representative for the metapopulation, that is, the least differentiated. While it does hold for symmetric dispersal with respect to the central population P_3 for all dispersal distances (Figures 4a, b), it is not generally valid for directional dispersal (Figure 4d). For example, in the directional scenarios, P_5 has the highest pollen source diversity for $\gamma \geq 300$, but P_4 is the least differentiated for $\gamma \leq 800$ (Figures 4d, e).

4.2 Effects of wind on adaptability to environmental variation

Survival of a metapopulation and its member populations depends on the relationship between the amount and the

effective number of sources of immigrant pollen and the type of environmental variation to which the populations are subjected. In the following, hypothetical implications of the simulations for responses of individual populations and the metapopulation as a whole for potential adaptive challenges are pointed out. Such challenges and their responses differ depending on the degree and kind of environmental variability, whether locally stable, locally gradual, or unstructured.

Stable environment: If the populations inhabit different but stable environments, short-distance dispersal maintains adaptedness of offspring by ensuring a high proportion of local pollen. As dispersal distance increases, increasing pollen immigration introduces maladapted pollen into the seeds as migration load that may reduce offspring survival. Symmetric dispersal gives all seeds nearly the same proportion of immigrant pollen, which becomes very large for longer dispersal distance, endangering survival. Directional dispersal gives all but the upwind population about the same amount of immigration as symmetric dispersal. Only the upwind population maintains a low migration load over all dispersal distances, ensuring its continued adaptedness for longer dispersal distances.

Gradual environmental change: If the transect follows an environmental gradient and if environmental change is gradual, environmental conditions may just shift in one direction along the transect. In such a situation, a neighboring population may have already experienced the environmental conditions that are now facing a population. The more pollen this neighbor contributes to the population's seeds, carrying with it adaptive genetic information that was beneficial in the neighbor's old environment, the better would be the chances of survival in the changed environment. Symmetric dispersal contributes more pollen from adjacent populations than from more distant ones (Figure 4c). The three interior populations have two direct neighbors and thus good chances of surviving gradual environmental change in either direction, while the one peripheral population whose single direct neighbor has not experienced the new environment is vulnerable. Thus the offspring of the three interior populations and one of the peripheral populations should be able to survive under symmetric dispersal. For directional dispersal, the upwind neighbor contributes considerably more pollen than for symmetric dispersal (Figure 4d). For $\gamma \geq 400$, even more pollen is contributed by the upwind neighbor than by the local population itself (Table 2, Scenarios D_{400} and D_{600}). Thus directional dispersal benefits survival for all but the upwind population if environmental change follows the prevailing wind direction. If change proceeds against the prevailing wind direction, all populations are endangered.

Environmental change by disturbance: Environmental disturbances are sudden, often local, and temporary. Populations that are hit by a disturbance may have reduced survival, causing them to contribute little pollen to other populations. In addition, immigrant pollen finds few ovules to fertilize in the disturbed populations. Disturbances thus reduce the degree of connectedness among populations and, due to the decrease in the amount of pollen from the disturbed populations, the effective number of pollen sources in the metapopulation's seed production. A population's recovery from disturbance may depend on the immigration of pollen from other populations that had received the disturbed population's local adaptive genetic variants in previous generations.

In a highly connected and thus nearly undifferentiated metapopulation that arises from symmetric, longer-distance dispersal, all populations accumulate adaptive variants from the entire metapopulation in undisturbed times. In the presence of pronounced environmental differences among the populations, this high diversity would present a large migration load within the populations as long as they are undisturbed. Nevertheless, their ability to provide disturbed populations with these populations' own locally adapted variants through pollen after cessation of the disturbance could ensure persistence of the metapopulation. In a more loosely connected and thus well-differentiated metapopulation that arises from symmetric dispersal over intermediate distances, populations accumulate the adaptive genetic variants of the nearby populations and can return them after the disturbance. In this case, the effective number of pollen sources is smaller, but so is the migration load. Thus symmetric dispersal of intermediate distance is better than longer-distance symmetric dispersal when metapopulations are subject to disturbance. In contrast, prevailing directional winds spread the locally adapted variants to populations farther downwind and prevent these variants from returning when the disturbance ends.

In summary, short-distance dispersal or an upwind location maintain initial adaptedness in a constant environment. In a gradually changing environmental gradient, prospective adaptive potential depends on the direction of environmental change: the three interior populations and one of the peripheral populations have the highest adaptive potential for symmetric dispersal, while directional dispersal either ensures survival of all but the upwind population or endangers the survival of all populations. For the recovery of populations subject to disturbance, symmetric dispersal of intermediate distance is advantageous.

4.3 Practical implications

The model findings have practical implications for forest management, especially in the face of climate change, and in particular for interpretation of the results of common garden trials. The planting of trees originating from a wide range of different environments in each of a limited number of environments is used to predict which provenance would be best adapted to which environment for the purpose of reforestation. The genetic variation needed for adaptation to a changing environment is often judged based on phenotypic variability shown in the trial. For example, based on data from such trials, Yeaman and Jarvis (2006) found that variation in growth response is correlated with environmental heterogeneity in the region surrounding the population of origin in lodgepole pine (*Pinus contorta* Doug. ex Loud). It would be interesting to see whether consideration of wind

direction during pollination in the measure of environmental heterogeneity, in addition to dispersal distance, would affect this result. In another study, Kapeller et al. (2016) detected higher variability for growth parameters for trees originating from higher alpine elevations in Norway spruce (*Picea abies* [L.] Karst.). Perhaps the greater genetic diversity at higher elevations is due to the transport of pollen from lower elevations by the morning updrafts on mountainsides ("valley winds"), during the time of day when pollen shed is heaviest. Directional winds may be a natural form of "assisted gene flow", which is increasingly being discussed as a management measure to augment the dispersal of pollen or seeds from populations that are already under the same environmental conditions that the recipient population is expected to experience (Aitken and Bemmels, 2016).

The results also have implications for seed harvesting in natural stands. The common objective is to obtain a representative sample of the genetic variants in the stand by harvesting seeds from a sufficient number of trees that are preferably spread over the interior of the stand. The expectation is that pollen contributions to the seeds are a random sample from the entire stand. The model results contradict this expectation by demonstrating that the genetic diversity in the seeds of a tree depends on its location relative to the prevailing wind direction within the stand. This is confirmed, for example, by findings that the amount of genetic diversity contained in seeds from single trees increases from the upwind edge of a stand of European beech (*Fagus sylvatica* L.) towards the downwind edge (Gillet and Ziehe, 2012).

4.4 Outlook

There are several ways to extend this relatively simplistic model to allow for more variability. One is to consider additional forms or families of probability density functions (Austerlitz et al., 2004). The exponential function θ_γ applied here is only one member of the two-parameter exponential power family with distance parameter γ and shape parameter $c = 1$ (Clark, 1998; Hardy, 2009). In some studies, fat-tailed kernels with their increased probability of long-distance dispersal ($c < 1$) have been found to fit well with data (Clark, 1998; Austerlitz et al., 2004; Katul et al., 2005; Robledo-Arnuncio and Gil, 2005; Goto et al., 2006; Piotti et al., 2012). Their effects on metapopulation structure should be quantitatively stronger but not qualitatively different than the effects demonstrated here. Another extension would be to drop the assumption that all ovules are fertilized. By limiting the ratio of pollen-to-ovule production within the populations, the effect of fertilization probabilities less than 1 could be examined, as proposed by Gregorius (1983). Populations that receive less pollen would produce fewer seeds, skewing the distribution not only of pollen sources and but also ovule sources in the seed production of the metapopulation. Thirdly, the model framework can be applied to examine the meaningfulness of new and emerging measures of variation that describe different kinds of effective number and untangle the long-standing confusion between differentiation and apportionment (Gregorius, 2010, 2014, 2016; Gregorius and Gillet, 2015) in the framework of pollen dispersal and adaptation. Ultimately, the subsequent spatial distribution of seeds and its implications could be added.

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Glossary

Seeds of a population:
The set of all seeds formed by fertilization of the population's ovules during the pollination season.
Seeds of the metapopulation:
Aggregate of the seeds produced by all populations $P_1 - P_5$
Complement of population P_j:
Aggregate of the seeds produced by all populations except P_j
Pollen source distribution of population P_j:
Vector of relative frequencies $p_i(j)$ of seeds generated through fertilization of its ovules by pollen from the source populations P_i
Effective number of pollen sources in population P_j:
Rényi-diversity $v(j)$ of the pollen source distribution in the seeds of P_j
Representation of population P_j:
Difference $\delta_{SD}(j)$ between the distribution of pollen sources in its own seeds and in the complement, equaling 1 for complete disjunction, 0 for complete identity
Homogeneity among populations:
Degree of similarity $1 - \delta_{SD}$, i.e., of absence of compositional differentiation, among the pollen source distributions in the seeds of the populations, equaling 1 for identity of all distributions, 0 for complete disjunction
Proportion of local pollen for population P_j:
Relative frequency $p_j(j)$ of seeds fertilized by its own pollen
Proportion of immigrant pollen for population:
Relative frequency $p_{imm}(j) = \Sigma_{k,k \neq j}(1 - p_k(j))$ of seeds fertilized by pollen from all other populations, presenting potential migration load under constant environmental conditions and adaptive potential under changing environmental conditions.
Effective number of immigrant pollen sources in population P_j:
Diversity $v_{imm}(j)$ of the pollen contributions of all other populations, equaling 4 for equal representation of all pollen sources, 1 for monomorphism
Central population:
Population located at the center of the transect (P_3)
Peripheral population:
Population at the edge of the transect (P_1, P_5) as opposed to interior (P_2, P_3, P_4)
Prevailing wind direction:
For directional winds, direction in which the majority of pollen disperses, i.e., rightwards towards the higher-numbered populations
Upwind/downwind population:
Population located at the upwind/downwind end of the transect with respect to the prevailing wind direction (P_1 resp. P_5)

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Regenerative Kraftstoffkonzepte für Plug-In-Hybrid-Fahrzeuge

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Zusammenfassung

Bei Plug-In-Hybrid-Fahrzeugen erlangt aufgrund möglicher längerer Verweilzeiten des Kraftstoffs im Tank die Alterungsproblematik eine größere Relevanz. Dies ist insbesondere bei biodieselhaltigen Kraftstoffen der Fall, die eine kürzere Lagerstabilität im Gegensatz zu fossilen Kraftstoffen aufweisen. Um dennoch Biodiesel als Kraftstoffkomponente langfristig einsetzen zu können, wurden verschiedene Fettalkohole und auch Tributylcitrat (TBC) als neuartige, stabilisierende Kraftstoffkomponenten getestet. Dabei wurde darauf geachtet, dass die neuen Kraftstoffformulierungen die Dieselkraftstoffnorm DIN EN 590 einhalten und somit als Drop-in-Kraftstoffe in den bestehenden Kraftstoffmarkt integriert werden können.

Durch die Beimischung ausgewählter Fettalkohole und TBC können Alterungsprodukte des Biodiesels in sehr unpolaren Kraftstoffen wie HVO (Hydrotreated Vegetable Oil) in Lösung gehalten werden. Durch den Einsatz dieser Komponenten konnte ein Multikomponentenblend (REG50) definiert werden, der bei einem hohen regenerativen Anteil von bis zu 50 Vol.-% die Dieselkraftstoffnorm DIN EN 590 erfüllt und mit den enthaltenen Lösungsvermittleranteilen auch den gestiegenen Anforderungen von Plug-In-Hybrid-Fahrzeugen genügt. REG50 erwies sich bezüglich der Kraftstoff- und Emissionsparameter als unproblematisch beziehungsweise durch sehr gute Teilergebnisse als besonders geeignet.

Schlüsselwörter: *Plug-In-Hybrid, Kraftstoffalterung, Biodiesel*

Abstract

Regenerative fuel concepts for plug-in hybrid vehicles

There is an increasing relevance of aging problems in plug-in hybrid vehicles, because in those cars, the fuels will remain in the tank for a longer time. In particular biodiesel-containing fuels, which have a shorter storage stability, will form oligomers. To be able to use biodiesel as a fuel component further on, different fatty alcohols and tributyl citrate (TBC) were tested as novel, stabilized fuel components. It was ensured that the new fuel formulations comply with the diesel fuel standard DIN EN 590 and thus can be integrated in the existing fuels market as a drop-in fuel. Aging products of biodiesel can be kept in solution by the addition of selected fatty alcohols and TBC. With these components, a multi-component blend (REG50) was defined, which has a high proportion of renewable energy (up to 50 % by volume) and fulfills the diesel fuel standard DIN EN 590. The employed solubilizers enable the fuel to meet the advanced requirements of plug-in hybrid vehicles. In respect of emissions and fuel parameters REG50 proved to be unproblematic. The emissions of REG50 showed equal or better results compared to fossil diesel fuel.

Keywords: *plug-in hybrid, fuel aging , biodiesel*

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1 Motivation

Plug-In-Hybrid-Fahrzeuge vereinigen die Vorteile von Elektrofahrzeugen und konventionellen Verbrennerfahrzeugen. Sie besitzen sowohl einen elektrischen Antrieb als auch einen Verbrennungsmotor. Der Verbrennungsmotor wird nur gestartet, wenn die leistungsstarke Batterie auf längeren Fahrten nicht genügend Energie liefern kann. Bei Kurzstreckenfahrten reicht die Energie der Batterie jedoch aus und sie kann nach der Beendigung der Fahrt wieder am Stromnetz aufgeladen werden. Bei einem Fahrprofil mit hauptsächlich Kurzstrecken wird der Verbrennungsmotor selten benötigt. Es ist daher anzunehmen, dass sich durch verlängerte Standzeiten des Kraftstoffs im Tank die Alterungsproblematik mit dem Risiko der Bildung von Präzipitaten verstärkt. Dies ist insbesondere bei biodieselhaltigen Kraftstoffen der Fall, die eine kürzere Lagerstabilität im Gegensatz zu fossilen Kraftstoffen aufweisen. Um dennoch Biodiesel als Kraftstoffkomponente langfristig einsetzen zu können, wurde zur Vermeidung von solchen Ausfallprodukten nach geeigneten regenerativen Kraftstoffkomponenten gesucht.

2 Präzipitatbildung und Lösungsvermittler

Als negative Eigenschaft von Biodiesel (Fettsäuremethylester, FAME) kann die Neigung zur Präzipitatbildung in Dieselkraftstoffblends gesehen werden (Schaper et al., 2014; Fang und McCormick, 2006). Hierzu trägt jedoch auch die Dieselkraftstoffqualität bei. Diese Präzipitatbildung kommt dadurch zu Stande, dass bei der Kraftstoffalterung vor allem mehrfach ungesättigte Fettsäuremethylester initialisiert durch Autoxidation zu polaren Oligomeren reagieren. Dabei nimmt der Grad der Oligomerisierung und damit die Polarität mit fortschreitender Alterung weiter zu. Eine Löslichkeit der Oligomere ist im polaren FAME-Reinkraftstoff gegeben. Wenn der gealterte FAME-Kraftstoff aber als Beimischungskomponente zu aktuellen Dieselkraftstoffen hinzugegeben wird, fallen die Oligomere aufgrund der geringeren Polarität des resultierenden Kraftstoffs aus.

Ein Ansatz zur langfristigen Lösung dieser Problematik muss über den Einsatz von Oxidationsstabilisatoren hinausgehen. Während auf diese Weise die Oligomerbildung nur zeitlich begrenzt verhindert wird, ermöglichen Lösungsvermittler permanent die Löslichkeit von bereits gebildeten Oligomeren im Blend. Eigene Studien ergaben, dass bestimmte Fettalkohole als Lösungsvermittler ein großes Potenzial besitzen. Neben anderen getesteten 1-Alkoholen sind 1-Hexanol (HexOH), 1-Heptanol (HeptOH) und 1-Octanol (OctOH), die über die Fischer-Tropsch-Synthese zumindest begrenzt zugänglich sind (Anderson, 1980), hinsichtlich der DIN EN 590-Kriterien, insbesondere der Siedelage und des Flammpunkts, gut geeignet. Der Einsatz der einwertigen C_5-Fuselalkohole ist in Bezug auf das Flammpunktkriterium und einer damit verbundenen möglichen Dampfdruckproblematik noch Gegenstand aktueller Forschung. Bei Verwendung von 1-Dodecanol ist bereits die Kältestabilität deutlich gefährdet.

Ebenfalls wurde Tributylcitrat (TBC - $C_{18}H_{32}O_7$) (Schaper et al., 2014), das aufgrund von drei Ester-Gruppen und der Hydroxygruppe zur Steigerung der Polarität beitragen kann, hinsichtlich einer möglichen Lösungsvermittlereffizienz für alterungsbedingte Abbauprodukte und der generellen Eignung als Kraftstoffkomponente untersucht.

3 Material und Methoden

3.1 Untersuchungen zur Lösungsvermittlereffizienz

Die Polarität sowie ihre Verteilung im Molekül ist für die Effizienz von Lösungsvermittlern für Alterungsprodukte des Biodiesels ein entscheidender Faktor. Zur Überprüfung der Lösungsvermittlereffizienz für Alterungsprodukte wurden daher die ausgewählten Alkohole und TBC mit einer Berechnung der räumlichen Struktur und der Oberflächenladungsdichte mit dem Cosmo-Therm Programm (conductor-like screening model, COSMO-Logic GmbH) untersucht. Damit kann abgeschätzt werden, wie stark die Lösungsvermittler mit polaren Gruppen von Oligomeren wechselwirken.

Des Weiteren wurde zur quantitativen Erfassung der Effizienz der Lösungsvermittler ein gravimetrischer Ansatz gewählt (Munack et al., 2013). Hierfür wurde über 40 Stunden ein Luftstrom durch auf 110 °C erhitzten RME (Rapsölmethylester) geleitet und somit der RME künstlich gealtert. Dieser gealterte Kraftstoff wird im Folgenden mit RMEalt bezeichnet. 2 ml des künstlich gealterten Biodiesels (RMEalt) wurden unter Rühren 8 ml einer Mischung aus Dieselkraftstoff und fünf bzw. zehn Prozent Lösungsvermittler langsam zugegeben, bis ein Anteil von 20 Vol.-% RMEalt erreicht war. Dieser Anteil wurde gewählt, da hier mit einer maximalen Präzipitatbildung zu rechnen ist (Fang und McCormick, 2006). Nach weiteren zwei Wochen wurde die überstehende Lösung dekantiert und das Gefäß mit den verbleibenden Präzipitaten mit geringen Mengen an n-Hexan gespült. Nach anschließendem Abblasen von Lösemittelresten mit synthetischer Luft wurde die Masse des kompakten Bodensatzes durch Differenzwägung bestimmt.

3.2 Versuchsmotor

Für motorische Messungen der ausgewählten Kraftstoffe stand ein Nutzfahrzeugmotor des Typs OM 904 LA der Daimler AG mit einem auf Vanadiumoxid basierenden SCR-Katalysator (Selective Catalytic Reduction) zur Verfügung, der die nach Euro IV geltenden Grenzwerte erreicht (Tabelle 1). Zur Reduzierung der Stickoxide wurde ein SCR-Katalysator eingesetzt. Der Motor wurde gemäß der Richtlinie 2005/55/EG im europäischen transienten Zyklus (ETC) betrieben.

Tabelle 1
Technische Daten des Prüfmotors OM 904 LA

Zylinderhub	130 mm
Zylinderbohrung	102 mm
Zylinderanzahl	4
Hubvolumen	4250 cm^3
Nenndrehzahl	2200/min
Nennleistung	130 kW
Maximales Drehmoment	675 Nm bei 1200 bis 1600/min
Abgasnachbehandlung	SCR-Katalysator (Vanadiumoxid)
Abgasnorm	Euro IV

3.3 Emissionsanalytik

Die gesetzlich reglementierten Abgasbestandteile Kohlenmonoxid (CO), Gesamt-Kohlenwasserstoffe (HC) und Stickoxide (NO_x) wurden durch herkömmliche Gasanalysatoren bestimmt. Die Probenahme der Partikelmasse erfolgte mit einem Abgasteilstromverdünnungstunnel, der nach ISO 16183:2002 ausgelegt war. Die Partikelmasse wurde nach dem Verdünnungstunnel auf zwei PTFE-beschichteten Glasfaserfiltern abgeschieden und gravimetrisch bestimmt. Dazu wurden die Filter jeweils vor der Wägung über 24 Stunden bei 22 ± 3 °C und 45 ± 8 % relativer Luftfeuchte konditioniert. Zusätzlich erfolgte eine Bestimmung von polyzyklischen aromatischen Kohlenwasserstoffen (PAK, Tabelle 2) sowie des mutagenen Potenzials mittels Ames-Test. Die Auswahl der untersuchten PAK erfolgte entsprechend der Festlegung der amerikanischen Umweltbehörde EPA (EPA, 1984).

Tabelle 2
Liste der aus dem Abgas bestimmten PAK

Name	Anzahl der Ringe	Verwendete Abkürzung
Naphthalin	2	Nap
Acenaphthen	3	Ace
Fluoren	3	Flu
Phenanthren	3	Phe
Anthracen	3	Ant
Fluoranthen	4	Fla
Pyren	4	Pyr
Benz[a]anthracen	4	BaA
Chrysen	4	Chr
Benzo[b]fluoranthen	5	BbFla
Benzo[k]fluoranthen	5	BkFla
Benzo[a]pyren	5	BaPyr
Dibenz[a,h]anthracen	5	DBAnt
Benzo[ghi]perylen	6	BPer
Indeno[1,2,3-cd]pyren	6	IPyr

Die PAK sind aufgrund ihrer geringen Flüchtigkeit in der Regel im Abgas an Rußpartikel adsorbiert. Die Probenahmeeinrichtung für die Bestimmung der PAK war in Anlehnung an die VDI-Richtlinie 3872, Blatt 1, aufgebaut. Die Sammlung der Partikel erfolgte auf PTFE-beschichteten Glasfaserfiltern aus dem unverdünnten Rohabgas. Die Konditionierung der Filter wurde analog zur Partikelmasse durchgeführt. Zusätzlich wurden Komponenten aus der Gasphase in einem auf -15 °C gekühlten Kühlsystem abgeschieden. Das resultierende Kondensat und das Partikulat wurden getrennt mit einer HPLC (high performance liquid chromatography) mit Fluoreszenzdetektor untersucht.

3.4 Mutagenitätstests

Der Ames-Test (Ames et al., 1973 und 1975) ist das weltweit am häufigsten eingesetzte In-vitro-Testverfahren zur Untersuchung der Mutagenität komplexer Gemische, wie zum Beispiel Verbrennungsprodukten. Er ist seit 1997 von der OECD als Guideline 471 „Bacterial Reverse Mutation Test" anerkannt (OECD, 1997). Die direkte Mutagenität von Dieselrußpartikeln wird substituierten PAK, insbesondere den Nitro-PAK zugeschrieben, während die indirekte Mutagenität eher durch unsubstituierte PAK hervorgerufen wird. Die Probenahme von Partikulat und Kondensat wurde analog zur PAK-Analytik durchgeführt. Die Tests erfolgten mit und ohne metabolische Aktivierung durch mikrosomale Monooxygenasen (S9-Fraktion) zur Bestimmung der direkten und indirekten Mutagenität.

4 Ergebnisse

TBC hat einen Siedepunkt von 325 °C (Alfa Aesar, 2016) und liegt damit im Bereich von Biodiesel. Da TBC auch ebenso eine erhöhte Polarität aufweist, ist davon auszugehen, dass bei TBC die gleichen Probleme wie bei Biodiesel auftreten. Das betrifft insbesondere die Anreicherung im Motoröl. Damit kann man den Schluss ziehen, dass TBC nur bis zu 5 Vol.-% einem Blend zugemischt werden sollte. Mit einer Dichte von 1,045 g/cm^3 bei 15 °C und einer kinematischen Viskosität von 12,5 mm^2/s bei 40 °C ergeben sich weitere Faktoren, die die maximale Zugabemenge einschränken würden. Es kann jedoch durch Zugabe von bereits wenigen Volumenprozenten die geringe Dichte der regenerativen Kraftstoffe HVO (Hydrotreated Vegetable Oil) sowie GtL und BtL (regenerative Fischer-Tropsch-Prozessführung mit Gas und Biomasse) kompensiert werden, sodass der maximale Anteil dieser Kraftstoffe in neuen Kraftstoffformulierungen deutlich erhöht werden kann. Die Eignung der ausgesuchten Fettalkohole ist hinsichtlich der Dichte und dem Siedepunkt unproblematisch.

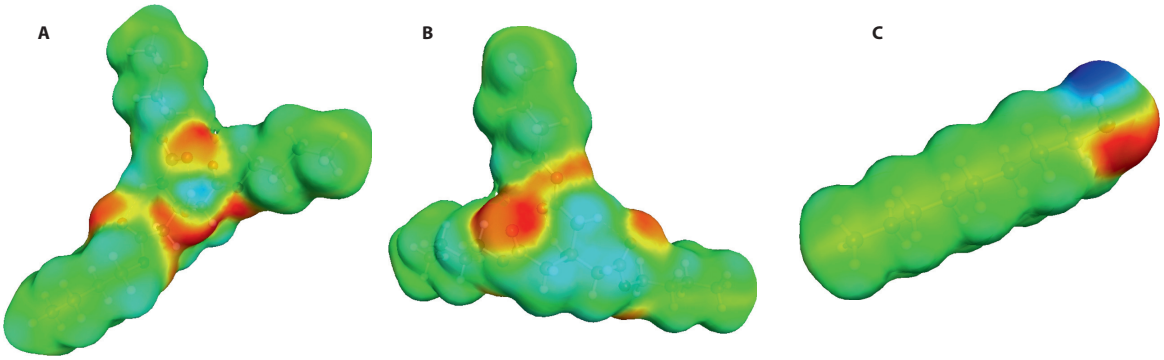

Abbildung 1
Oberflächenladung des TBCs aus unterschiedlichen Betrachtungswinkeln (A) und (B) und des 1-Octanols (C) (grün = unpolar; blau = polar (partial positiv); rot = polar (partial negativ) – Farbintensitäten symbolisieren die Ausprägung der einzelnen Bereiche)

4.1 Lösungsvermittlereffizienz von Fettalkoholen und TBC

Bei Betrachtung der räumlichen Struktur und der Oberflächenladungsdichte des TBC-Moleküls mit dem Cosmo-Therm Programm (COSMO-Logic GmbH), Abbildung 1, wird deutlich, dass die polaren Bereiche im Zentrum des Moleküls liegen und durch die unpolaren Butylgruppen abgeschirmt werden. Auch ist bei dem TBC nicht zu erkennen, dass auf einer Seite des Moleküls eine Teilladung dominiert. Dagegen ist 1-Octanol aufgrund der Kombination aus einer Hydroxygruppe und einem verhältnismäßig großen Alkyl-Rest als Lösungsvermittler unabhängig von bestehenden Unterschieden in der Molmasse besser geeignet. Der unpolare Teil ist ausreichend effektiv, um eine Löslichkeit in unpolaren Lösungsmitteln wie Dieselkraftstoff zu gewährleisten. Der polare Teil ist aber zusätzlich so stark ausgeprägt, dass ein großes Potenzial als Lösungsvermittler für Oligomere angenommen werden kann. Mögliche sterische Hinderungen können hier nicht auftreten.

Die Effizienz der Lösungsvermittlung ist in Abbildung 2 dargestellt. Da mit dem verwendeten Versuchsaufbau auch geringe Mengen von Kraftstoffresten, die nicht komplett entfernt werden können, im Bodensatz und an den Glasoberflächen verbleiben, sind die gemessenen Rückstandsmassen als Obergrenzen zu betrachten. Die Masse des Rückstands liegt gegenüber dem Messwert um ca. 5 bis 10 mg niedriger. Durch Zugabe von ungealtertem RME, der auch als Lösungsvermittler wirkt, und TBC, zeigt sich, dass mit steigender Lösungsvermittlermenge auch der Bodensatz verringert werden kann. Dabei zeigt TBC gegenüber RME eine deutlich höhere Wirksamkeit. Jedoch reicht die Wirksamkeit dieser beiden Lösungsvermittler nicht an die der getesteten Alkohole heran. Bei diesen zeigt sich, dass schon 5 Vol.-% ausreichen, um den gesamten Bodensatz zu lösen. Aufgrund der untereinander gleichbleibenden hohen Effizienz der eingesetzten Fettalkohole bietet es sich für eine Realisierung an, mit einem Gemisch von Alkoholen mit Beimischungen unter 5 Vol.-% zu arbeiten.

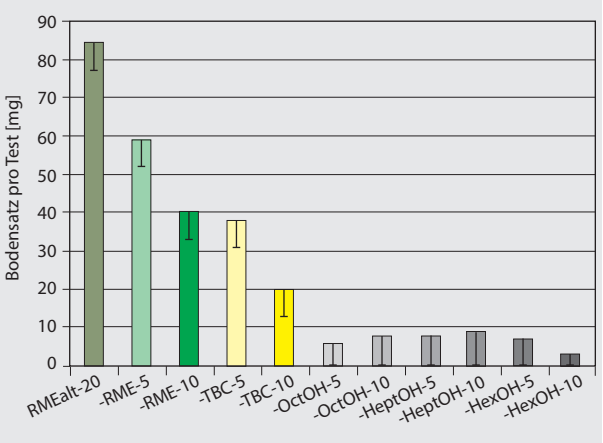

Abbildung 2
Gravimetrische Bestimmung des Bodensatzes ausgewählter Blends (10 ml); der Basiskraftstoff RMEalt-20 ist eine Mischung aus DK und 20 Vol.-% RMEalt (die Angaben bezeichnen den prozentualen Anteil des entsprechenden Lösungsvermittlers im Basiskraftstoff)

4.2 Emissionsanalytik

Ziel der Untersuchungen war es, einen Multikomponentenblend mit hohem regenerativem Anteil unter Berücksichtigung der Dieselkraftstoffnorm DIN EN 590 zu formulieren. Durch die Zugabe von 2 Vol.-% TBC kann der Anteil des regenerativen Kraftstoffs HVO auf 38 Vol.-% erhöht werden. Zusammen mit RME und des zumindest im Experimentierstadium befindlichen regenerativen Herstellungsweges entsprechender Fettalkohole ergibt sich ein potenziell möglicher regenerativer Anteil von 50 Vol.-%, sodass REG50 als Akronym für diesen Kraftstoff gewählt wurde. Der Kraftstoff ist dabei in seiner Zusammensetzung variabel. HVO kann durch entsprechende Alkangemische, wie sie alternativ über Fischer-Tropsch-Synthese zugänglich sind, ergänzt beziehungsweise ersetzt werden. Rapsölmethylester wurde als heimische Komponente gewählt. Andere FAME-Kraftstoffe können aber auch unter Berücksichtigung möglicher Einschränkungen in der Lagerstabilität, bedingt durch eine höhere Iodzahl, Verwendung finden. Auch der Fettalkoholanteil kann, da diese Gruppe zurzeit nur bedingt durch einen

Tabelle 3
Ausgewählte Multikomponentenblends basierend auf den vorangegangenen Entwicklungsstufen im Vergleich zu DK, HVO und RME

Kraftstoff	Zusammensetzung (Vol.-%)	Cetanzahl	Dichte bei 15 °C (kg/m³)
DK	CEC RF 06-03	53,2	0,834
HVO-26-RME-7	67 % DK, 26 % HVO, 7 % RME	57,6	0,824
HVO-26-RME-7-Oct-5	62 % DK, 26 % HVO, 7 % RME, 5 % 1-Octanol	56,6	0,823
HVO-26-RME-7-TBC-2	65 % DK, 26 % HVO, 7 % RME, 2 % TBC	57,1	0,828
REG50	50 % DK, 38 % HVO, 7 % RME, 3 % 1-Octanol, 2 % TBC	60,5	0,821
HVO	Hydrotreated vegetable oil	79,9	0,780
RME	Rapsölmethylester	54,0	0,883

regenerativen Prozess zugänglich ist, auf eine Mischung der 1-Alkohole von 1-Hexanol bis 1-Decanol verteilt und durch weitere geeignete Fettalkohole ergänzt werden. Zusätzlich zu REG50 wurden weitere Kraftstoffzusammensetzungen ausgewählt, um den Einfluss von 1-Octanol und TBC getrennt untersuchen zu können, Tabelle 3.

Bei Betrachtung der CO-Emissionen wird deutlich, dass Dieselkraftstoff den höchsten Wert aufweist, Abbildung 3. Schon mit dem Kraftstoff HVO-26-RME-7 kommt es aufgrund einer damit einhergehenden Cetanzahl-Steigerung zu einer deutlichen Absenkung der CO-Emissionen. Sowohl durch die Beimischung von 5 Vol.-% 1-Octanol als auch mit 2 Vol.-% TBC erfolgt eine weitere Absenkung. REG50 zeigt bei einer Erhöhung des HVO-Anteils auf 38 Vol.-%, vergleichbar mit HVO und RME, den niedrigsten CO-Wert. Es kann festgestellt werden, dass sowohl durch eine Cetanzahl-Steigerung mit

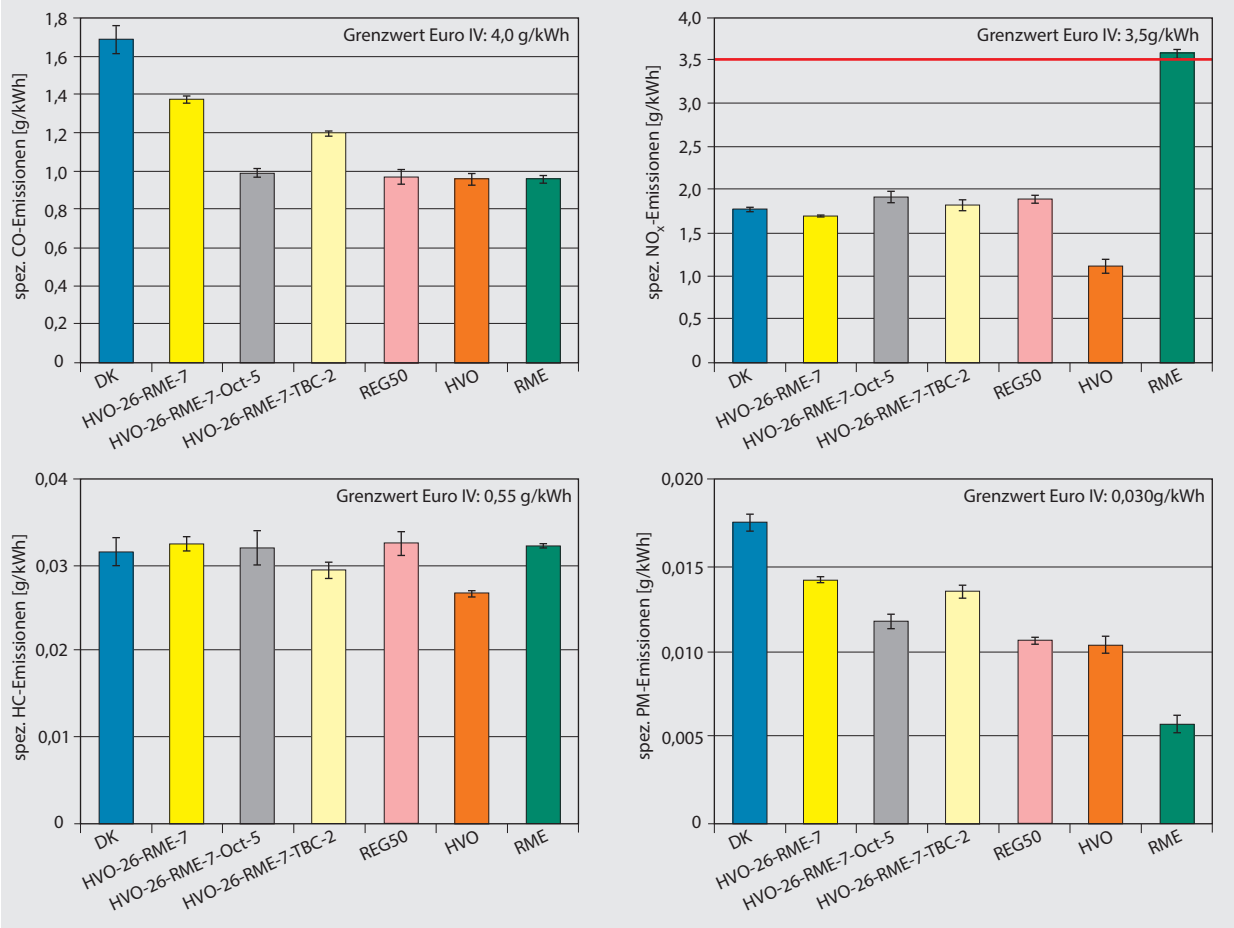

Abbildung 3
Spezifische CO-, NO_x-, HC- und PM-Emissionen mit Standardabweichung ausgewählter Multikomponentenblends im Vergleich zu DK, HVO und RME mit Angabe des Grenzwerts nach Euro IV-Abgasnorm

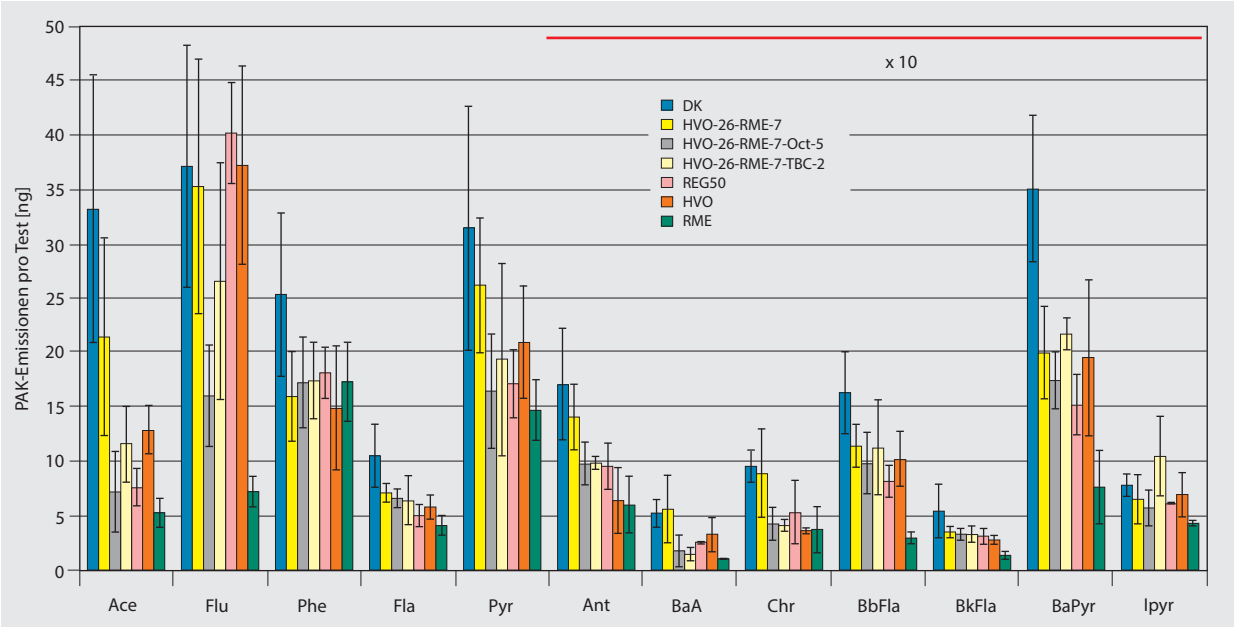

Abbildung 4
PAK-Emissionen (gesamt) mit Standardabweichung ausgewählter Multikomponentenblends im Vergleich zu DK, HVO und RME (Werte ab Anthracen sind mit dem Faktor 10 dargestellt)

HVO als auch mit 1-Octanol und TBC die CO-Emissionen gesenkt werden können.

Die NO_x-Emissionen sind beim Motorbetrieb mit RME gegenüber Dieselkraftstoff deutlich erhöht. Dieses Verhalten ist aus einer Vielzahl von Untersuchungen bekannt (Lapuerta et al., 2008). Bis auf HVO, das eine signifikante Absenkung des NO_x-Werts aufweist, liegen alle anderen Kraftstoffe bezüglich der NO_x-Emissionen im Bereich des Dieselkraftstoffs. Die drei Kraftstoffe mit 1-Octanol und TBC weisen dabei eine leichte Erhöhung gegenüber Dieselkraftstoff auf.

Aufgrund der Verwendung eines SCR-Katalysators mit integriertem Oxidationskatalysator liegen die HC-Emissionen auf einem allgemein sehr niedrigen Niveau (Shah et al., 2009) und weisen keine signifikanten Unterschiede auf.

Bei Betrachtung der PM-Emissionen zeigt sich ein ähnliches Verhalten wie bei den CO-Emissionen. Lediglich bei RME ist eine noch weitere Absenkung der Emissionen feststellbar. Auch hier scheint sich eine Kombination aus ausreichend hohen Cetanzahlen und einem Sauerstoffanteil des Kraftstoffs bei dem verwendeten Motor positiv auszuwirken.

Insgesamt liegen mit Ausnahme des NO_x-Werts für RME alle Werte unterhalb des jeweils geltenden Grenzwerts gemäß der Euro IV-Norm.

Bei den PAK-Emissionen, dargestellt als Summe aus Partikulat und Kondensat, ergibt sich durch den Einsatz des SCR-Katalysators ein generell niedriges Emissionsniveau, Abbildung 4. Es kann tendenziell eine Verbesserung der PAK-Emissionen bei den ausgewählten Multikomponentenblends und HVO gegenüber DK erkannt werden. Eine weitere Absenkung der PAK-Emissionen ist für RME bei wenigen Spezies statistisch abgesichert. Insgesamt wird dieses Verhalten nur in Bezug auf DK deutlich; gegenüber den anderen Kraftstoffen kann lediglich von einer tendenziellen Absenkung gesprochen werden. Es kann festgehalten werden, dass die ausgewählten Multikomponentenblends hinsichtlich der PAK-Emissionen unkritisch sind, da sie wie RME gegenüber DK eher eine tendenzielle Verbesserung des Emissionsniveaus bewirken. Dies wurde insbesondere bei der stark krebserregenden Substanz Benzo[a]pyren deutlich.

Auch bei der Mutagenität findet sich unter Berücksichtigung der Standardabweichungen ein sehr niedriges Werteniveau. Hierfür lässt sich wie bei den PAK-Emissionen der Einfluss des SCR-Katalysators verantwortlich machen. Der Stamm TA98 zeigt bei den Partikulaten sowohl mit als auch ohne Aktivierung durch S9 eine zwar statistisch abgesicherte, aber dennoch insgesamt unkritische Mutagenität,

Abbildung 5. Auch hier ergeben sich beim Dieselkraftstoff tendenziell höhere Mutagenitätsraten.

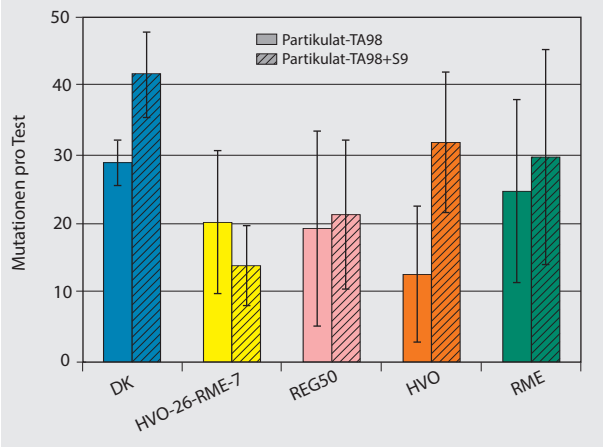

Abbildung 5
Mutationen (Partikulat) mit Standardabweichung im Stamm TA98 mit und ohne Aktivierung durch S9 am Beispiel ausgewählter Multikomponentenblends im Vergleich zu DK, HVO und RME

5 Zusammenfassung

Durch eine Polaritätssteigerung im Kraftstoff, bedingt durch die Beimischung ausgewählter Fettalkohole und TBC, können Alterungsprodukte des Biodiesels, wie sie verstärkt beim Plug-In-Hybrid aufgrund länger Standzeiten des Kraftstoffs im Tank vorkommen können, in Lösung gehalten werden. Die Kraftstoffparametrierung führte zu einer Definition eines vielversprechenden Multikomponentenblends (REG50), der bei einem hohen regenerativen Anteil von bis zu 50 Vol.-% die Dieselkraftstoffnorm DIN EN 590 erfüllt und mit den enthaltenen Lösungsvermittleranteilen auch den gestiegenen Anforderungen von Plug-In- Hybrid-Fahrzeugen genügt. REG50 erwies sich bezüglich der Kraftstoff- und Emissionsparameter als unproblematisch beziehungsweise durch sehr gute Teilergebnisse als besonders geeignet. Auf TBC kann bei geringeren Anteilen an gesättigten Alkanen auch vorerst verzichtet werden. Sollten aber gesättigte Alkane in Zukunft in Blends unter Beibehaltung der aktuellen Dieselkraftstoffnorm DIN EN 590 wesentlich stärker eingesetzt werden, ist diese Substanz als Kraftstoffkomponente der zweiten Generation eine vielversprechende Beimischungskomponente, da sie neben mittleren Lösungsvermittlereigenschaften für Alterungsbestandteile des Biodiesels auch als Dichtemodifikator wirken kann.

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Laying performance in hens of two breeds testing soybean meal or rapeseed meal plus peas as protein feed

Ingrid Halle*

Abstract

In hens' feed soybean extracted meal (SBM) was replaced by rapeseed (*Brassica napus*) meal (RSM) solvent extracted plus peas in order to investigate the effects on feed intake, laying performance and composition of eggs. A total of 460 laying hens (230 Lohmann Brown-LB/230 Lohmann Dual-LD) were used in the feeding study. The hens were allocated to four groups of 115 hens each. The hens were kept in pens (23 hens per pen) with five pens per group and fed for six laying months.

The daily feed intake, the number of eggs related to the trial's days (laying intensity) and egg weight of LB hens was significantly higher compared with LD hens. While no effect of substituting SBM through RSM/peas in the diet was seen on laying intensity of all hens, the egg weight was decreased and the feed to egg mass ratio increased. The daily egg mass production was lower in the LB RSM/peas group compared with LB SBM hens and not significantly different between two LD groups. Eggs of LD hens showed a significantly higher percentage of egg yolk and a reduced proportion of egg white. The replacement of SBM by RSM/peas reduced the final body weight of hens. After slaughtering of ten hens per group a statistically higher body weight and proportion of carcass and breast meat of LD hens compared with LB hens was found. The replacement of SBM by RSM plus peas reduced the yield of breast meat in the LD hens. The change of the protein source led in hens to reduced fat content.

Keywords: *Rapeseed meal, peas, hen, layer, dual layer, laying performance, egg composition*

Zusammenfassung

Leistungsentwicklung von Hennen zweier Rassen bei Prüfung von Sojaextraktionsschrot oder Rapsextraktionsschrot plus Erbsen als Proteinfuttermittel

Im Versuch an Legehybriden wurde ein vollständiger Austausch von Sojaextraktionsschrot (SBM) durch Rapsextraktionsschrot (RSM) plus Erbsen im Futter durchgeführt, um den Einfluss auf die Leistungsentwicklung und Eizusammensetzung zu prüfen. Für die Untersuchung wurden 460 Hennen (230 Lohmann Brown-LB/230 Lohmann Dual-LD) in 4 Gruppen mit 115 Hennen in je fünf Abteile aufgeteilt und über eine Versuchsdauer von sechs Legemonaten gehalten.

Die tägliche Futteraufnahme, die Anzahl an gelegten Eiern bezogen auf die Versuchstage (Legeintensität) und das Eigewicht der LB Hennen waren gesichert höher im Vergleich mit den LD Hennen. Während kein Einfluss des Austausches von SBM durch RSM+Erbsen auf die Legeintensität festzustellen war, wurde das Eigewicht gesichert reduziert und der Futterverzehr pro kg produzierter Eimasse stieg an. Die tägliche Eimasseproduktion war gesichert niedriger bei den Hennen der LB RSM/Erbsen Gruppe und nicht signifikant unterschiedlich zwischen den LD Gruppen. Die Eizusammensetzung der LD Hennen ergab einen gesichert höheren Prozentsatz an Eidotter und reduzierten Anteil an Eiklar. Der Austausch der Proteinquelle im Futter reduzierte die Lebendmasse aller Hennen am Ende des Versuches. Die Schlachtung von zehn Hennen pro Gruppe ergab ein gesichert höheres Lebendgewicht und einen höheren Schlachtkörper- und Brustfleischanteil der LD Hennen. Durch den Ausstauch von SBM gegen RSM/Erbsen wurden der Ertrag an Brustfleisch bei den LD Hennen und der Anteil an Abdominalfett bei allen Hennen reduziert.

Schlüsselwörter: *Rapsextraktionsschrot, Erbsen, Henne, Dual-Henne, Leistungsmerkmale, Eibestandteile*

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1 Introduction

Soybean meal (SBM) is the most common protein source in diets of laying hens. The majority of this soybean meal is imported to Germany from non-European countries and often derived from genetically modified varieties. Therefore, the interest has increased to find out alternative protein sources derived from non-genetically modified varieties, such as rapeseed and grain legumes. In Germany, in 2014, rapeseed was grown on 1.39 million ha contrasting with 41.7 thousands ha used for feed peas. As protein feed, 4 million tons of rapeseed meal (RSM) and 4.8 million tons of other oil seed meals, mainly imported soybean meal, were used (UFOP, 2015). Rapeseed feeds and pea feeds are listed in the "German Positive List of Feed Materials" (Normenkommission für Einzelfuttermittel, 2016).

The international relevance of the legumes is underlined by the 68th UN General Assembly declaring 2016 the International Year of Pulses: "The International Year of Pulses represents a unique opportunity to raise awareness on the potential of pulses in the agricultural development sector and provides an additional impulse to increase their production at global level. In addition, pulses can improve soil fertility and increase biodiversity. They reduce the risk of total crop failure in multiple cropping systems improving food security. Pulses are amenable to a diversity of food processing and can be easily stored when dried compared to vegetables and fruits. Finally, they can be sold and therefore generate income making food more accessible. Altogether, pulses reduce dependency on external inputs and improve sustainability of integrated crop - livestock - aquaculture production systems." (FAO, 2016).

In this study, two layer strains, Lohmann Brown hens and the new strain Lohmann Dual were included. In consequence of breeding of approximately 40 million commercially laying hybrids every year, the same number of male chickens is hatched. These male laying-type cockerels or spring chickens cannot be reared economically (Damme and Ristic, 2003; Schäublin et al., 2005; König et al., 2010; Halle et al., 2012; Halle et al., 2015). The routine culling of these day-old male chicks is more and more an ethical problem and to find alternative solutions is a great challenge. The dual-purpose chicken of the Lohmann Tierzucht Company produce eggs and meat. Accordingly, the hatched male chickens are fattened and the females reared to later laying hens. At the moment, by the strongly negative correlation between reproduction and growth the Dual hens do not achieve the high laying performances of the LB hens (Schmutz, 2013).

Objectives of this study were to study the effect of a total replacement of soybean meal by rapeseed meal plus peas in hens' feed on laying performance and egg quality parameters of two strains of hens.

2 Material and methods

A total of 460 laying hens (230 LB/230 LD) were used in this feeding study. The basal diet contained soybean meal. In the treatment diets, soybean meal feed was totally replaced by rapeseed meal and peas (Table 1).

Table 1
Study design (115 hens per group; duration of the trial – six laying month = 168 days)

	LB Hen		LD Hen	
Groups Ingredients (%)	1	2	3	4
Soybean meal	21.6	0	21.6	0
Rapeseed meal	0	12	0	12
Peas	0	35	0	35

Table 2
Ingredient composition and analyzed and calculated nutrients of the diet (g/kg)

Group	LB Hen		LD Hen	
Ingredients	1	2	3	4
Soybean meal	215.62	-	215.62	-
Rapeseed meal	-	120.00	-	120.00
Peas	-	350.00	-	350.00
L-lysine-HCL	0.19	-	0.19	-
DL-methionine	1.37	1.38	1.37	1.38
Corn	213.94	-	213.94	-
Wheat	400.00	346.10	400.00	346.10
Rapeseed oil	40.00	47.44	40.00	47.44
Premix[1)]	10.00	10.00	10.00	10.00
Calcium carbonate	77.60	76.34	77.60	76.34
Di-Calcium-phosphate	12.91	8.82	12.91	8.82
Sodium chloride	3.67	3.69	3.67	3.69
Grass meal	24.70	36.23	24.70	36.23
Dry matter [2)]	891.6	897.0	891.6	897.0
Crude protein [2)]	164.8	162.0	164.8	162.0
ME, MJ/kg [3)]	11.25	11.32	11.25	11.32
Lysine, g/kg [4)]	8.5	9.4	8.5	9.4
Methionine+Cystine, g/kg [4)]	7.1	6.9	7.1	6.9

[1)] Vitamin-mineral premix provided per kg of diet: Fe, 40 mg; Cu, 10 mg; Zn, 80 mg; Mn, 100 mg; Se, 0.25 mg; I, 1.2 mg; Co, 0.21 mg; vitamin A, 10000 IE, vitamin D_3, 2500 IE; vitamin E, 20 mg; vitamin K_3, 4 mg; thiamine, 2.5 mg; riboflavin, 7 mg; pyridoxine, 4 mg; vitamin B12, 20 µg; nicotinic acid, 40 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 25 µg; choline chloride, 400 mg

[2)] Analyzed values

[3)] Calculated values according to the equation WPSA(1985) AME_N (kJ/g) = 15.51 Crude protein + 34.31 Crude lipid + 16.69 Starch + 13.01 Sugar (Vogt, 1986)

[4)] Calculated values

The peas were originated from the variety "James". All diets contained a balanced concentration of 16 % crude protein, 0.85 to 0.9 % lysine, 0.7 % methionine plus cystine and 11.2 to 11.3 MJ ME/kg feed. (Table 2, 3). The hens were allocated to four groups of 115 hens each. The hens were kept in pens

(23 hens per pen) with five pens per group. The study commenced when the hens were 22 weeks old and continued until the 6th laying month (168 days). Each hen was offered the respective diet and water ad libitum. Number of eggs laid was recorded daily per pen and the feed consumption monthly. Each month the collected eggs were weighed four times within two weeks. In the 2nd, 4th and 6th laying month, all eggs laid over three consecutive days were collected per pen to measure the yolk weight, albumen weight and shell weight. The color of the egg yolk was read with the Roche-Fan. Body weight of hens was recorded at the start and at the end of the trial. Ten hens of each group (two per pen) were slaughtered at the end of the trial to determine carcass and organ weights. In the feed dry matter (DM), crude protein, ether extract, crude fiber and ash content were determined according to methods of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA, 2012). The glucosinolates of rapeseed were determined with the official method of the European Commission (1990); (Rothe et al., 2004) by HPLC (Dionex, Idstein, Germany).

Data were analyzed with a two-way ANOVA: yijk = μ + SCi + Dj + SCDij + eijk, with yijk = observation, μ = general mean, SCi = diet (soybean, rapeseed + peas), Dj = origin of hens (Lohmann Brown, Lohmann Dual), interaction SCDij and eijk = error term (random). Multiple comparisons of means were carried out using the Student-Newman-Keuls Test ($P \leq 0.05$). The statistical analyzes were performed by the SAS, 2002 to 2012 software package (Version 9.4).

3 Results

The peas and the rapeseed extracted meal (RSM) are characterized by their high content of protein (24 %, 34 %; Table 3). The RSM had a higher content of fiber (13.4 %) compared with peas (5.2 %). Crude fiber and thus the content of metabolisable energy (ME) of RSM was only 8.6 MJ/kg compared to peas with 11.8 MJ/kg. The glucosinolates of the RSM amounted to 8.2 mmol/kg.

Table 3
Analyzed compounds and metabolizable energy (ME) calculation of peas and rapeseed meal

Nutrients, g/kg	Pea „James“	Rapeseed meal
Dry matter	875	894
Crude protein	236	340
Crude lipid	17	35
Crude fiber	52	134
Crude ash	30	66
Starch	414	60
Sugar	48	86
Lysine	17	19
Methionine	2	7
Cystine	3	8
Threonine	9	16
ME, MJ/kg [1)]	11.8	8.6
Glucosinolate. mmol/kg	-	8.2

[1)] Calculated values according to the equation WPSA; 1985, see Table 2

Table 4
Laying performance of hens (six laying month)

Treatment	Hen	Feed intake g/day	Laying intensity [1)] %	Egg weight g/egg	Egg mass production g/hen/day	Feed to egg mass ratio, kg/kg
LS means						
1 Soya	LB	127.6 a	92.0 a	63.2 a	58.2 a	2.2 c
2 Rapeseed/peas	LB	125.0 a	92.6 a	61.3 b	56.8 b	2.20 c
3 Soya	LD	110.2 c	82.1 b	58.4 c	47.7 c	2.31 b
4 Rapeseed/peas	LD	115.6 b	83.8 b	57.0 c	47.5 c	2.43 a
Standard error (SE)		7.2	5.9	2.8	2.7	0.14
ANOVA, P-value						
Diet		0.3	0.3	0.002	0.1	0.009
Soya		118.9	87.1	60.8 a	53.0	2.25 b
Rapeseed/peas		120.3	88.2	59.1 b	52.2	2.32 a
Hen		<0.001	<0.001	<0.001	<0.001	<0.001
LB		126.3 a	92.3 a	62.3 a	57.5 a	2.20 b
LD		112.9 b	83.0 b	57.7 b	47.6 b	2.37 a
Diet x Hen		0.003	0.6	0.6	0.2	0.02

a; b; c; – Means with different letters differ significantly

[1)] Number of eggs in the trial divided through trial's days

There were significant effects of protein source and origin of tested hens on the determined performance parameters (Table 4). The daily feed intake of LB hens was 125.0 to 127.6 g and significantly higher compared with LD hens (110.2 to 115.6 g). During the trial duration of 168 days the laying intensity of LB hens was 92.0 to 92.6 % and 10 % units higher than that of LD hens. While exchanging SBM through RSM/peas in the diet had no effect on laying intensity, the change of the protein source decreased weight of LB hens' eggs from 63.2 g to 61.3 g ($P < 0.01$). The egg weight of LD-hens and egg mass production per day was altogether lower than that of LB hens. The daily egg mass production was lower in the LB RSM/peas group (56.8 g/hen/day) compared with LB soya fed hens (58.2 g/hen/day). LB hens fed the RSM/peas diet did not need more feed per kg egg mass compared with the SBM diet (2.2 kg/kg). The hens of the LD peas/RSM groups had the highest feed to egg mass ratio of 2.4 kg/kg.

Table 5 summarizes egg color, egg weight and the weight percentages of yolk, egg white and shell besides the total egg weight recorded during the 2nd, 4th and 6th laying month. During the complete trial, eggs of LD hens showed a

Table 5
Egg quality

Treatment	Hen	Egg weight g/egg	Yolk %	Egg white %	Egg shell %	Colour of yolk Roche fan
2nd laying month (253 to 315 eggs/per group)						
LS means						
1 Soya	LB	62.4 a	25.3 c	62.6 b	12.1 c	12.8 b
2 Rapeseed/Peas	LB	60.5 b	24.7 d	63.0 a	12.2 c	12.8 b
3 Soya	LD	55.6 c	27.8 a	59.4 d	12.8 a	12.9 b
4 Rapeseed/Peas	LD	54.4 d	27.4 b	60.0 c	12.6 b	13.2 a
SE		4.1	2.0	2.3	1.0	0.7
ANOVA, P-value						
Diet		<0.001	<0.001	<0.001	0.5	<0.001
Hen		<0.001	<0.001	<0.001	<0.001	<0.001
Diet x Hen		0.2	0.6	0.6	0.02	<0.001
4th laying month (n = 256 to 288 eggs per group)						
LS means						
1 Soya	LB	64.6 a	26.2 c	62.0 a	11.9 b	12.9 ab
2 Rapeseed/Peas	LB	62.1 b	25.6 d	62.1 a	12.3 a	12.8 b
3 Soya	LD	60.2 c	29.7 a	58.2 c	12.1 a	12.8 b
4 Rapeseed/Peas	LD	58.7 d	29.2 b	58.5 b	12.3 a	13.0 a
SE		4.6	2.0	2.2	1.0	0.7
ANOVA , P-value						
Diet		<0.001	<0.001	<0.001	<0.001	0.1
Hen		<0.001	<0.001	0.04	0.04	0.4
Diet x Hen		0.06	0.9	0.4	0.05	0.005
6th laying month (n = 204 to 288 eggs per group)						
LS means						
1 Soya	LB	64.4 a	26.2 b	61.5 a	12.2 a	12.6 b
2 Rapeseed/Peas	LB	62.6 b	26.1 b	61.6 a	12.3 a	12.7 b
3 Soya	LD	62.8 b	30.2 a	57.5 b	12.2 a	13.0 a
4 Rapeseed/Peas	LD	61.4 c	30.1 a	57.7 b	12.1 a	12.9 a
SE		4.7	1.9	2.1	1.0	0.6
ANOVA, P-value						
Diet		<0.001	0.3	0.4	0.2	0.6
Hen		<0.001	<0.001	<0.001	0.9	<0.001
Diet x Hen		0.5	0.9	0.5	0.2	0.06

a; b; c; – Means with different letters differ significantly

significantly higher percentage of egg yolk and reduced part of egg white. In the 2nd laying month higher egg shell content was obtained in the LD hens; these differences however were lower in the later laying period. The differences in the yolk color were between 12.6 to 13.2.

The body weight of the hens was significantly different between LB and LD hens both at the start and the end of the trial (Table 6). The exchange of the protein source SEM through peas/RSM reduced the body weight of LB hens (39 g) and LD hens (59 g) ($P < 0.02$).

Table 6
Body weight and number of hens (n = 115 hens per group at the start of the trial)

Treatment	Hen	Body weight, start point g/hen	Number of hens, end of trial	Final body weight, g/hen
LS means,				
1 Soya	LB	1774 b	111	1911 c
2 Rapeseed/Peas	LB	1781 b	114	1872 c
3 Soya	LD	1880 a	114	2108 a
4 Rapeseed/Peas	LD	1853 a	114	2049 b
SE		183		217
ANOVA, P-value				
Diet		-	-	0.02
Hen		<0.001	-	<0.001
Diet x Hen		-	-	0.6
a; b; c; – Means with different letters differ significantly				

The mortality during the 6-month trial was 1.5 % without differences between the groups.

Slaughtering showed for LD hens a statistically higher body weight and a larger part of carcass (76.8 to 79.0 %), breast meat (10.6 to 11.8 %) and abdominal fat (3.0 to 3.4 %) compared with LB hens (71.4 to 72.9 %; 8.6 to 8.9 %; 2.1 to 3.1 %; Table 7). The exchange of SES through RSM/peas in the LD hen diet reduced the yield of breast meat ($P < 0.05$). LB hens showed a higher part of gizzard (1.84 %) and thyroid weight (0.17 to 0.21 g) than the LD hens (1.34 to 1.43 %, 0.14 g). The replacement of SBM by RSM plus peas led in both hen breeds to reduced fat content.

4 Discussion

Rapeseed and its oil are the basis for European biodiesel production besides their use for food and feed. Therefore, after extraction, rapeseed meal is increasingly available as protein and energy component for animal feed (UFOP, 2015). Whereas meals consisting "00-rapeseed" cause no problems in the feeding of ruminants, there is a maximum content of 10 % rapeseed meal and 5 % rapeseed cake in the feed for hen breeds laying white eggs (Spiekers and Südekum, 2004; Jeroch and Dänicke, 2013). For some brown-egg laying hen breeds, their inability to metabolise trimethylamin (TMA) originating from the bacterial degradation of rapeseed sinapine in the large intestine, results in "fishy taint" eggs (Butler and Fenwick ,1984). The Lohmann Tierzucht Company identified the gene which is responsible for the inability of some hens to metabolise TMA and hens carrying this gene were eliminated from breeding. Thus, Lohmann Tierzucht announced that all commercial laying hens of Lohmann and H&N origin hatching from January 2007 onwards are free of the mentioned genetic defect and may receive diets containing rapeseed products (Pottgüter, 2006). In 2014, the rapeseed monitoring published the analytical results of

Table 7
Slaughtering of hens at the end of the trial (n = ten hens per group, two per pen)

Treatment	Hen	Body weight, g	Carcass, %[1)]	Breast meat, g	Breast meat, %	Legs, g	Legs, %[1)]	Liver, %[1)]	Heart, %[1)]	Gizzard, %[1)]	Fat, %[1)]	Thyroid weight, g
LS means												
1 Soya	LB	1900 b	71.4 b	163.5 c	8.6 c	342.0 b	18.0	1.77	0.36	1.84 a	3.12 a	0.17 ab
2 Rapeseed/Peas	LB	1886 b	72.9 b	167.8 c	8.9 c	337.9 b	17.9	1.81	0.35	1.88 a	2.11 b	0.21 a
3 Soya	LD	2089 a	79.0 a	247.8 a	11.8 a	374.1 a	17.9	1.76	0.38	1.34 b	3.37 a	0.14 b
4 Rapeseed/Peas	LD	2071 a	76.8 a	220.5 b	10.6 b	358.3 ab	17.3	1.95	0.37	1.43 b	3.02 a	0.14 b
SE		72	4.8	24.4	1.1	23.6	1.1	0.2	0.04	0.3	0.9	0.04
ANOVA, P-value												
Diet		0.5	0.9	0.1	0.2	0.2	0.3	0.2	0.5	0.5	0.02	0.1
Hen		<0.001	<0.001	<0.001	<0.001	0.001	0.3	0.4	0.08	<0.001	0.04	0.001
Diet x Hen		0.9	0.5	0.05	0.04	0.4	0.5	0.4	0.9	0.8	0.2	0.3

a; b; c; – Means with different letters differ significantly

[1)] Relative to the final body weight

rapeseed samples (n = 65) (UFOP, 2015). Rapeseed meal then contained 8.8 (0.8 to 14.9) mmol glucosinolates/kg dry matter (89 %), 27 (4 to 46) g crude fat/kg, crude fiber 108 (93 to 123) g/kg, crude protein 342 (312 to 371) g/kg and 7.4 (6.8 to 8.1) MJ ME/kg. These values are well comparable with the rapeseed meal in the presented hen trial (Table 4).

The laying intensity of the LB hens showed in this trial, no dependence on the protein source in the diet. Replacement of 22 % SBM by 12 % RSM plus 35 % peas did not influence the daily feed intake nor number of laying eggs in the first six laying months. The egg weight was decreased by 3 % ($P < 0.05$) and the daily egg mass production by 2.5 % ($P = 0.1$) after totally changed protein source. The reduction of the egg weight after feeding RSM was described in the literature (Rodehutscord et al., 2012; Halle and Schöne, 2013). Studies of Roth-Maier and Kirchgessner (1995) and Jeroch et al. (1999) with laying hens and a diet with 15 % rapeseed showed no difference in the laying parameters (feed intake, laying intensity, egg mass production) compared with the control groups without rapeseed feeds. Richter et al. (1996) measured negative effects (feed intake, laying intensity, egg weight) due to feeding of only 5 % dietary rapeseed. After a combined chemical and hydrothermal treatment of the rapeseed or rapeseed cake to reduce antinutritive compounds a higher percentage of rapeseed (22.5 %) or rapeseed cake (30 %) could be fed without negative impact on the laying performance (feed intake, laying intensity, egg mass production) (Jeroch et al., 1995; 1999; 2008). Jeroch et al. (2008) conclude that the inclusion of the rapeseed feeds into compound feeds, i.e. their maximum level in the diets, is particularly determined by the glucosinolate content. Fixing maximum glucosinolate content in the laying hen feed is not possible through, as specific studies these difficulties are missing.

Legumes – peas, field beans and lupines are important protein feedstuffs. Studies of Igbasan and Guenter (1997), Richter (2004) and Halle (2005) with laying hens and diets with up to 40 % peas showed no differences in laying performance (feed intake, laying intensity, egg mass production) compared with control groups. Perez-Maldonado et al. (1999) concluded that 25 % peas per kg hen feed could be fed without negative impact on the laying performance. The influence of micronisation, dehulling, and enzyme supplementation on the nutritional value of peas in a diet with 60 % peas was studied with hens (Igbasan and Guenter, 1997). Only the laying performance of the hens with a diet containing 60 % peas after micronisation showed no negative effect compared with controls. Kraft et al. (2013) studied the replacement of SBM by a mixture of concentrated pea protein, RSM and sunflower extracted meal in the hen diet during a period of 52 weeks. A replacement of 50 % did not change laying performance of hens while a total replacement of SBM caused decreased laying performance and increased feed to egg mass ratio.

The LD breed combines good laying performance of hens with an acceptable meat gain of males. Dual hens laid up to 250 eggs in twelve laying months (wk. 20 to wk. 68) and achieved a highest peak production of 85 % at 29 weeks of age (Schmutz, 2013; Icken et. al., 2013). The mean laying performance of the LD hens in this six laying month's trial was 83 % and it was not affected by the diet. The feed to egg mass ratio of the RSM/peas hens was 2.4 and significantly higher compared with SEM hens (2.3) (Table 4). The results after slaughtering of LD hens showed that the feeding of RSM/peas as protein source in their feed decreased the body weight, the breast meat weight and the abdominal fat (Table 6, 7).

Heavier thyroids of the LB hens may results from higher laying performance based on higher metabolic activity and showed a tendency to increase thyroid mass after feeding RSM. This confirms the results obtained by Schöne et al. (2013) showing that the feeding of rapeseed cake led to increased thyroid mass in hens.

The trial's results allow the conclusion that a total replacement of soybean meal with rapeseed meal plus peas in feed of LB hens had no effect on laying intensity but reduces the egg weight and increased the feed to egg mass ratio of LD hens.

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Effects of niacin supplementation and different concentrate proportions on ruminal lipopolysaccharide concentration, immunological response and health of dairy cows

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Summary

High concentrate proportions used in diets of high-yielding cows may lead to subacute ruminal acidosis, compromise the ruminal mucosal barrier and force transfer of lipopolysaccharides (LPS) with an eventual systemic inflammatory response. Because niacin (NA) increases performance and might exert anti-inflammatory effects, the present study investigates the effects of 60 % vs. 30 % concentrate proportions with or without 24 g niacin/cow/day on ruminal LPS content and indicators of inflammatory response. The experiment was carried out with four experimental groups, 60+NA, 60-, 30+NA, 30- and lasted from calving to week of lactation (WoL) 36. Ruminal LPS concentration was generally increased after feeding 60 % concentrate diets and also modified by niacin, parity and WoL. Also total leukocytes, hematocrit, fibrinogen and aspartat-aminotransferase were influenced in an interactive manner, while glutamate dehydrogenase and gamma-glutamyl transferase activities were elevated due to the 60 % concentrate proportion. The stimulation index of peripheral blood mononuclear cells was subjected to a 2-way interactive effect of concentrate and niacin feeding, being highest in 30- groups (cows and heifers). In conclusion, long-term feeding of high-energy diets increases the LPS load of the rumen and compromises the liver.

Keywords: *concentrate-to-roughage ratio, cow, PBMC, blood count, liver enzymes*

Zusammenfassung

Effekte einer Niacinsupplementation bei verschiedenen Konzentratanteilen auf den ruminalen Lipopolysaccharidgehalt, die Immunantwort und die Gesundheit von Milchkühen

Die bei hochleistenden Milchkühen eingesetzten hohen Konzentratanteile können zur subakuten Pansenazidose und zur Pansenmukosaschädigung verbunden mit erhöhtem Lipopolysaccharid (LPS)-Transfer und einer Immunantwort führen. Da Niacin (NA) leistungssteigernde und anti-inflammatorische Effekte haben kann, wurde der Effekt von 60 % vs. 30 % Konzentratanteil mit oder ohne 24 g Niacin/Kuh/Tag auf den ruminalen LPS-Gehalt und Indikatoren einer Immunantwort untersucht. Für vier Versuchsgruppen, 60+NA, 60-, 30+NA, 30- dauerte der Versuch von der Kalbung bis zur 36. Laktationswoche (WoL). 60 % Konzentrat führte zu erhöhtem ruminalem LPS-Gehalt, der auch durch Niacin, Parität (primi-/pluripare Kuh) und WoL beeinflusst war. Gesamtleukozytenzahl, Hämatokrit, Fibrinogen und Aspartat–Aminotransferase unterlagen einem interaktiven Effekt, während Enzymaktivität von Glutamatdehydrogenase und Gamma-Glutamyltransferase auch durch den 60 % Konzentratanteil erhöht waren. Der Stimulationsindex der mononukleären Zellen des peripheren Blutes wurde durch eine 2-fach-Interaktion von Konzentrat*Niacin beeinflusst und war am höchsten in den 30-Gruppen (Kuh/Färse). Insgesamt führt die Langzeitfütterung eines hohen Konzentratanteils zu erhöhten LPS-Gehalten im Pansen, sowie zur Beeinträchtigung der Leber.

Schlüsselworte: *Konzentrat-Grundfutterverhältnis, Kuh, PBMC, Blutbild, Leberenzyme*

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1 Introduction

To meet the energy demand of high yielding cows feeding of energy-dense diets is required. Consequently, rapid fermentable high concentrate proportions are used in such rations. This strategy predisposes cows for diverse nutritional disorders such as subacute ruminal acidosis (SARA) associated with an altered rumen microbial population (Andersen et al. 1994a; Nocek, 1997; Ametaj et al., 2005; Emmanuel et al., 2008; Zebeli and Ametaj, 2009) and increases concentration of lipopolysaccharides (LPS) in the rumen fluid (Nocek, 1997; Andersen, 2003).

Under SARA conditions, Kleen et al. (2003) suggested a translocation of rumen endotoxins into the bloodstream as the rapidly fermentable carbohydrates induce hyperacidity and consecutive parakeratosis with the possible consequence of provoking a systemic inflammation. Feeding of diets with a high concentrate proportion was frequently followed by increases in the ruminal LPS concentration. Gozho et al. (2005) showed that a diet with 60 % concentrates comprising mainly of cereal grains resulted in a grain-induced SARA and increased free LPS concentration in the rumen. LPS is released by gram-negative bacteria in rumen fluid (Nagaraja et al., 1978a; Andersen et al., 1994a; Khafipour et al., 2009; Zebeli and Ametaj, 2009) or from bacterial cell lyses due to excessive activity of autolytic enzymes that facilitates growth during the rapid bacterial growth phase (Wells and Russell, 1996). Plaizier et al. (2012) reviewed several studies which showed that inducing SARA by a nutritional challenge based on feeding excessively high grain diets increases LPS in the rumen of cattle (Nagaraja et al., 1978b; Emmanuel et al., 2008; Khafipour et al., 2009).

Only few studies have detected LPS in peripheral blood during grain induced SARA (Gozho et al., 2007; Nagaraja and Lechtenberg, 2007; Plaizier et al., 2008), probably due to its rapid clearance. When hepatic and whole body clearance capacity is exceeded, LPS might induce an inflammatory response characterized by the release of multiple pro-inflammatory cytokines and acute phase proteins (APP) which finally results in an acute phase response.

Recently, niacin was shown to modulate the LPS-induced inflammatory response in mice (Zhou et al., 2014) as it was found to attenuate the production of pro-inflammatory cytokines and to exert anti-inflammatory effects mediated by its hydroxycarboxylic acid receptor HCA2.

Apart from major inflammation markers other indicators such as fibrinogen and white blood cells can be used as indicators of an acute phase response (Arthington et al., 1996; Horadagoda et al., 1999). Therefore, the objective of the present study was to investigate the effects of feeding diets with either high or low concentrate proportions (60 % vs. 30 %), both in the absence or presence of niacin (24 g/cow/day) on ruminal LPS content, hematological variables, plasma parameters and immunological functional parameters to characterize the impact of feeding energy-dense diets on health of dairy cows.

2 Material and methods

2.1 Animals, experimental design, feeding and sample collection

The experiment was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Braunschweig, Germany according to the German Animal Protection Act concerning the protection of experimental animals and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (33.9 42502-04/085/09). The current investigations complete the results of a comprehensive feeding experiment which was recently published (Rauls et al., 2015). A total of 64 German Holstein cows were assigned to four groups of different dietary treatments according to milk yield, number of lactation and body weight. A high concentrate level of 60 % (in total ratio on dry matter basis) and a low concentrate level of 30 % were tested with the presence or absence of 24g niacin/cow/d and resulted in four experimental groups: 60+NA, 60-, 30+NA and 30- The niacin used was powdered non rumen protected NA with a content of at least 99.5 % NA (Lonza Ltd., Basel, Switzerland) which was included in the pelletized concentrate. The rations were formulated according to the recommendation of nutrient and energy supply of the Society of Nutrition Physiology (GfE, 2001). The cows had free access to water. The study started individually for each cow with the day of calving and was terminated in WoL 36. The composition of concentrate and roughage is shown in Table 1. The cows were housed in a free stall barn equipped with slatted floors and cubicles covered with rubber mattresses. The roughage was offered in self- feeding stations (type MJ/ RIC; Insentec B.V., Marknesse, The Netherlands), which were re-filled daily. Concentrate was offered by computerized concentrate feeding stations (type MJ, Insentec, B.V., Marknesse, The Netherlands). The cows were equipped with ear transponders to monitor the daily feed intake. The available amount of concentrate was adjusted twice weekly according to the individual roughage intake. In this way, the concentrate-to-roughage ratios were implemented in the different feeding groups while the feed was offered for ad libitum intake. A subgroup of 36 cows from a total of 64 animals (four pluriparous and five primiparous in each group) were studied more intensively: in WoL 5 and 16, blood samples were taken by jugular venipuncture into sterile heparinized monovettes for preparation of peripheral blood mononuclear cells (PBMC) and processed as described by Renner et al. (2011). To perform subsequent cell proliferation assays, the samples were stored at -80 °C. In WoL -3, 1, 3, 16, and 36 before or after calving, blood samples from the jugular vein were taken into lithium heparinized tubes for determination of clinical-chemical traits and citrate blood was taken to determine fibrinogen content. After centrifugation (Heraeus Varifuge® 3.OR Heraeus, Osterode, Germany, 2300 g, 15 °C, 15 min), plasma was stored at -20 °C until analysis. Furthermore, blood samples from a jugular vein were taken in WoL 2, 3, 4, 5, 10, and 36 (EDTA tubes) for the determination of

Table 1
Composition, nutrient and energy content of the concentrates and roughage (cf. Lohölter et al., 2013)

	Concentrate [1]				Roughage [2]
	Con 60+NA	Con 60 -	Con 30+NA	Con 30 -	
Components (%)					
Soybean meal	26.8	26.8	26	26	-
Wheat grain	50	50	50	50	-
Maize grain	20.8	20.8	20	20	-
Mineral premix [3] including supplemental niacin	2.4	-	4	-	-
Mineral premix [3]	-	2.4	-	4	-
Chemical composition (g/kg DM)					
Ash	49	51	62	63	58
Crude protein	224	219	219	217	106
Ether extract	30	30	30	30	34
Crude fibre	34	34	34	33	220
NDFom	156	185	130	128	440
ADFom	47	47	47	47	241
Niacin (g/kg DM)	1.76	-	3.52	-	-
Energy [4] (MJ NEL/kg DM)	8.3	8.3	8.2	8.2	6.4

[1] Con 60+NA, Con 60 -, Con 30+NA, Con 30 – used in groups 60+NA, 60 -, 30+NA, 30 -
[2] 60 % maize silage and 40 % grass silage on DM basis
[3] Per kg mineral feed: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu; 100mg I; 40 mg Se; 5 mg Co; 1 000 000 IU vitamin A; 100 000 IU vitamin D3; 1500mg vitamin E
[4] Calculation or roughage based on nutrient digestibilities measured with wethers (GfE, 1991). For the concentrates, tabulated values were used (DLG, 1997)

hematocrit and total and differential leukocyte counts which were evaluated immediately afterwards. Samples of rumen fluid were taken with an oral rumen tube and a hand vacuum pump from the ventral sac of the rumen in Wol -3, 1, 3, 16 and 36. After centrifugation (Beckmann J2-HS, Beckman Coulter Inc., Brea, CA, USA, 2400 g, 5 min), 2 ml of the supernatant were poured into Eppendorf-tubes and again separated by centrifugation (Hettich, Tuttlingen, Germany, 10 000 g, 15 min). The supernatant was percolated through a 0.22 µmol filter into Eppendorf-tubes and after heating for 30 minutes at 100 °C stored at -20 °C for subsequent LPS measurement.

3 Analyses

3.1 Feedstuffs

The composition of the feedstuffs (ash (ash), crude protein (CP), crude fibre (CF), ether extract (EE), neutral detergent fibre (NDFom) and acid detergent fibre (ADFom)) was determined according to the methods of the Association of German Agricultural Analysis and Research Centres (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten) VDLUFA (2012).

The niacin content in feedstuffs was determined microbiologically using *Lactobacillus plantarum* as an indicator strain (VDLUFA method Vol. III 13.9.1., HPLC-Method).

3.2 Ruminal lipopolysaccharides

The ruminal LPS-content was determined using the *Limulus amoebocyte* lysate assay (LAL QCL-1000, Lonza Group Ltd., Basel, Switzerland). Pretreated rumen samples were diluted using pyrogen-free water until their LPS concentrations were in a range of 0.1 to 1 endotoxin units (EU)/mL relative to the reference endotoxin (*Escherichia coli* O111:B4) and assayed as described by Gozho et al. (2005).

3.3 Hematological variables

Total leukocytes were counted using an improved Neubauer cell counting chamber and the evaluation of the differential leukocyte count after panoptic staining of the smear according to Pappenheim (Kraft and Dürr, 2005) was performed counting 200 cells. Hematocrit was determined after centrifugation with a hematocrit centrifuge. A 1 mL plasma sample of each cow was sent to the laboratory of the Clinic for Cattle of the University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. It was analyzed for activity of glutamate dehydrogenase (GLDH, EC 1.4.1.3), gamma-glutamyl transferase (GGT, EC 2.3.2.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) using a fully automatic apparatus (Cobas-Mira, Hoffmann-La Roche and Co. AG Diagnostika, Basel) and employing photometric standard procedures. Plasma fibrinogen was determined with gravimetric methods (Clauss method) directly after sampling.

PBMC viability and concanavalin A (ConA, Sigma- Aldrich, Steinheim, Germany, C 5275) stimulated proliferation were analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. PBMC were isolated from heparinized blood, diluted with phosphate buffered saline (PBS) at a ratio of 1:1, by density gradient centrifugation using Biocoll separation solution (Biochrome AG, Berlin, Germany). Isolation and subsequent proliferation assay were carried out as described by Renner et al. (2011). The results of the ex vivo tests were expressed as stimulation index (SI), defined as the ratio between the density of ConA-stimulated and ConA-non-stimulated cells.

3.4 Calculations

The energy balance was calculated as follows:

- The daily feed intake and live weight were condensed to weekly means before data analysis.
- The net energy requirements for maintenance (NEM) and lactation (NEL) were based on the equations published by the Society of Nutrition Physiology (GfE, 2001):
- Net energy maintenance (NEM) (MJ NEL/d) = 0.293 × $BW^{0.75}$
- Energy concentration of milk (MJ/kg) = 0.38 × milk fat (%) + 0.21 × milk protein (%) + 0.95
- NEL (MJ/d) = [Energy concentration of milk (MJ/kg) + 0.086] × milk yield (kg/d)

The energy balance was calculated as follows:

- Net energy balance (MJ NEL/d) = energy intake (MJ NEL/d) – [NEM (MJ NEL/d) + NEL (MJ NEL/d)]

3.5 Statistical analysis

Statistical analyses were performed using the software package SAS version 9.1 (SAS Institute Inc., 2004). All parameters were analyzed as repeated measures using the MIXED procedure. Dietary concentrate proportion, niacin supplementation, parity and WoL and the interactions between these factors were considered as fixed effects. Cows were treated as random effect. All results were presented as least squares means ± standard errors (SE). For all analyses, significance was declared when p-values were ≤ 0.05 and a tendency was noted when 0.05 < P < 0.10.

4 Results

4.1 Feed intake and energy balance

The results of the aimed concentrate-to-roughage proportions and performance parameters of the different feeding groups were already published in Rauls et al. (2015). The present results refer to the more intensively investigated subgroup of 36 animals out of the total of 64 cows. The concentrate-to-roughage proportions were nearly achieved: group 60+NA: 60 % to 40 %; group 60-: 57 % to 43 %; group 30+NA: 28 % to 72 %; group 30-: 30 % to 70 %. The consumed daily amounts of NA supplementation were slightly lower than the targeted amounts: group 60+NA obtained 21.1g ± 0.1 g/cow/day, group 30+NA 18.4 g ± 0.1 g/cow/day. The native amount of niacin in the unsupplemented groups was 2.0 g ± 1.1 g/cow/day in group 60- and 0.2 g ± 0.0 g/cow/day in group 30-. A 4-way interaction (C*NA*P*WoL = <0.001) was

Table 2

Effects of different concentrate proportion of the diet (30 vs. 60 % on a dry matter basis, **C)**, niacin supplementation (24 and 0 g/cow/day, **NA)**, parity (primi- vs. pluriparous cows, **P)** and week of lactation **(WoL)** on feed and energy intake, energy balance and ruminal lipopolysaccharide concentration (LS MEANS and standard error)

	group				p-value								
	60+NA[2]	60 -[3]	30+NA[4]	30 -[5]	C	NA	C*NA	P	WoL	C+P	NA*P	C*NA*P	C*NA* P*WoL
Intake	**(n = 9)**	**(n = 9)**	**(n = 9)**	**(n = 9)**									
DMI[1] (kg/d)	20.0 ± 0.2	18.3 ± 0.3	18.3 ± 0.3	19.8 ± 0.2	0.770	0.595	<0.001	<0.001	<0.001	0.427	0.001	0.002	<0.001
Energy intake (MJ NEL/d)	150.4 ± 1.8	136.3 ± 1.8	126.3 ± 1.8	137.3 ± 1.8	<0.001	0.334	<0.001	<0.001	<0.001	0.692	0.003	0.003	<0.001
Energy balance (MJ NEL/d)	15.7 ± 2.1	11.8 ± 2.0	14.7 ± 2.2	18.9 ± 2.0	0.148	0.943	0.054	<0.001	<0.001	0.002	0.111	0.072	<0.001
Ruminal Lipopolysaccharides													
LPS (EU/ml)	41457 ±3825	35486 ±4213	14975 ±4047	16570 ±3793	<0.001	0.584	0.345	0.959	<0.001	0.658	0.173	0.550	0.004

[1] Dry matter intake
[2] 60 % concentrate with 24g niacin/cow/day
[3] 60 % concentrate without niacin
[4] 30 % concentrate with 24g niacin/cow/day
[5] 30 % concentrate without niacin

detected for DMI, energy intake and energy balance (Table 2). In the beginning, the DMI of both groups receiving 60 % concentrate increased more rapidly than in the 30 % concentrate counterparts. Group 60+NA (cows and heifers) showed over the whole experiment a higher DMI than group 60-. This does not apply to the combination of low concentrate and supplemental niacin. Here, only the heifers developed temporarily a higher DMI while the cows from group 30+NA showed a lower DMI than group 30-. As energy intake is closely related to the DMI, it developed similarly. The energy intake increased in cows from both groups receiving 60 % concentrate proportion. The cows from group 60+NA had higher energy intake than group 60- over the entire trial. The energy intake of the 30 % concentrate groups was differently affected by parity, niacin, concentrate and WoL over the course of the trial.

Different energy intake and milk performance resulted in different energy balances. While the cows from group 60+NA almost reached a positive energy balance from WoL 5 on, the cows from group 60- remained in a nearly continuously negative energy balance until WoL 27. Heifers from all groups were in a positive energy balance.

4.2 Rumen fluid analysis/LPS

Ruminal LPS concentration was differently influenced by concentrate proportion, niacin supplementation, WoL and parity as indicated by the significant 4-way interaction (C*NA*P*WoL = 0.004, Table 2). LPS concentration was mainly higher when diets containing 60 % concentrate were fed compared to groups that received 30 % concentrate, except of heifers which developed different results in the beginning (WoL1 and 3, Figure 1). Niacin supplementation rather seemed to result in increased LPS and the heifers of 60+NA group appeared to respond more sensitively in early lactation (WoL 3), while heifers that received 30% concentrate showed the opposite with consistent lower LPS.

4.3 Hematological variables

A high variation between and within groups was visible for total leukocyte count (C*NA*P*WoL = 0.001, Table 3) during frequent sampling in the first lactation weeks. Afterwards, leukocyte number was similar between WoL10 and 35 being higher in cows and heifers receiving 60 % concentrate. The percentage of lymphocytes was influenced by concentrate proportion (p = 0.001) and niacin (p = 0.006) as it was lower in the groups receiving 60 % concentrate and higher in niacin supplemented groups. The percentage of banded neutrophil granulocytes was influenced by a C*NA interaction (p = 0.043) as it was higher in 60+ than in 30+ and 30- group (both p < 0.001).

The hematocrit (C*NA*P*WoL = 0.004) was until WoL 10 differently affected in cows and heifers, while 60 %

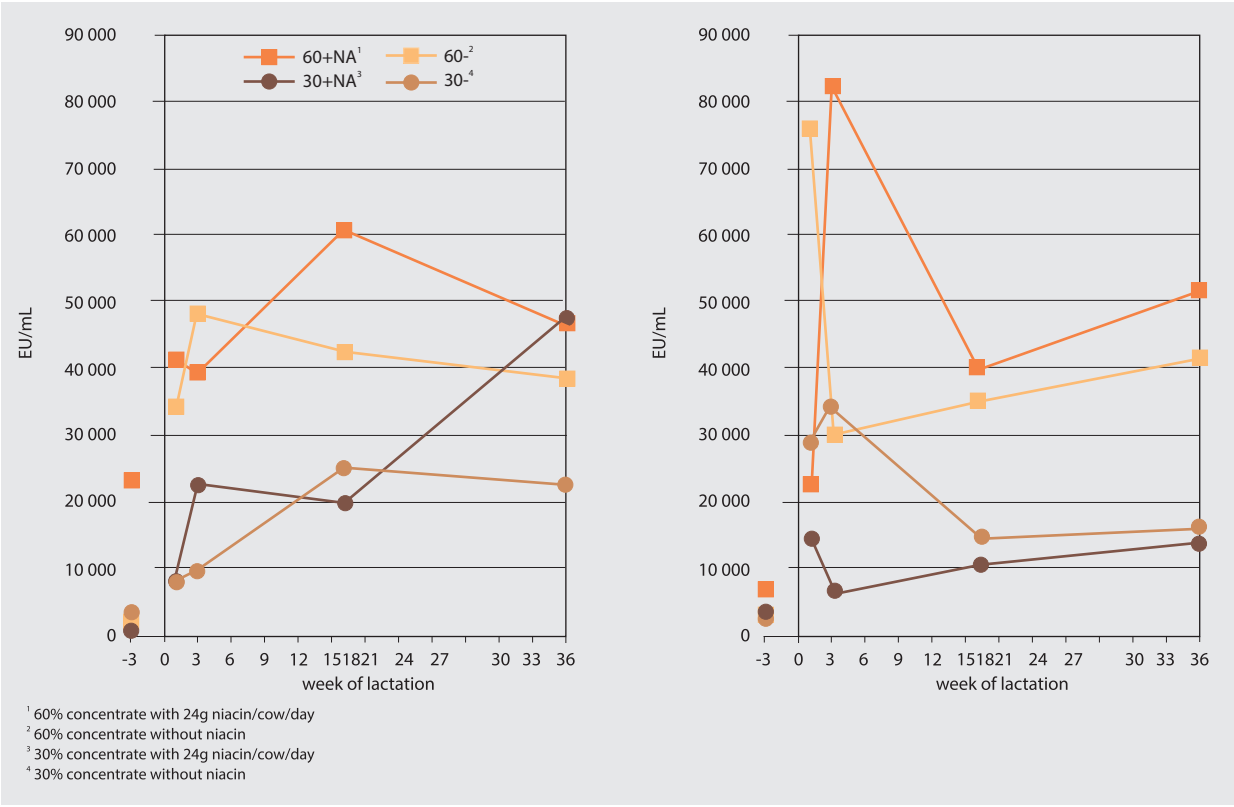

Figure 1
LPS concentration in rumen fluid of cows (left) and heifers (right) in week of lactation 1, 3, 16 and 36 in the different feeding groups.
The findings of the 3rd week a. p. were without niacin supplementation; Data points show LS means of n = 9.

Table 3

Effects of different concentrate proportion of the diet (30 vs. 60 % on a dry matter basis, **C**), niacin supplementation (24 and 0 g/cow/day, **NA**) parity (primi- vs. pluriparous cows, **P**) and week of lactation **(WoL)** on hematological variables and the SI for PBMC (LS MEANS and standard errors)

	group				p-value						
	60+NA[5]	60 -[6]	30+NA[7]	30 -[8]	C	NA	C*NA	P	WoL	C*P	NA*P	C*NA *P	C*NA* P*WoL
	(n = 9)	(n = 9)	(n = 9)	(n = 9)									
Hematolgical variables													
Total leukocytes (G/l)	7.14 ± 0.27	7.63 ± 0.24	6.78 ± 0.24	7.19 ± 0.22	0.104	0.067	0.868	0.079	0.018	0.001	0.077	0.235	0.001
Lymphocytes (%)	61.7 ± 2.1	53.8 ± 1.9	65.5 ± 1.8	62.7 ± 1.6	0.001	0.006	0.185	0.938	0.176	0.185	0.170	0.161	0.055
Monocytes (%)	0.7 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.136	0.054	0.226	0.968	<0.001	0.496	0.499	0.973	0.338
Segmented neutrophil granulocytes(%)	27.3 ± 1.8	31.8 ± 1.6	25.9 ± 1.5	28.0 ± 1.4	0.099	0.038	0.453	0.235	0.192	0.581	0.700	0.712	0.547
Banded neutrophil granulocytes (%)	9.4 ± 0.7	7.2 ± 0.6	5.5 ± 0.6	5.8 ± 0.5	<0.001	0.122	0.043	0.299	<0.001	0.274	0.691	0.623	0.240
Hematocrit (%)	30	31	28	29	0.001	0.151	0.690	0.050	0.134	0.676	0.091	0.282	0.004
Plasma Parameters													
GLDH[1] (U/l)	30.7 ± 4.6	21.4 ± 4.7	18.3 ± 4.8	14.5 ± 4.6	0.046	0.169	0.557	0.442	0.003	0.550	0.347	0.318	0.313
ASAT[2] (U/l)	92.1 ± 4.9	71.9 ± 4.9	71.7 ± 4.9	80.6 ± 5.0	0.239	0.260	0.005	0.473	0.004	0.669	0.581	0.668	0.022
GGT[3] (U/l)	31.4 ± 2.3	34.4 ± 2.3	27.0 ± 2.3	28.4 ± 2.3	0.027	0.347	0.728	0.228	<0.001	0.843	0.215	0.816	0.339
Fibrinogen (g/l)	5.7 ± 0.3	5.6 ± 0.3	5.2 ± 0.3	4.8 ± 0.3	0.031	0.381	0.668	0.188	0.001	0.482	0.417	0.563	0.037
Lymphocyte proliferation													
SI[4] for PBMC MTT assay	11.1 ± 1.5	7.8 ± 1.4	6.8 ± 1.2	12.8 ± 1.2	0.786	0.310	0.002	0.955	<0.001	0.224	0.237	0.402	0.265

[1] Glutamatdehydrogenase
[2] Aspartat-aminotransferase
[3] Gamma-glutamyltransferase
[4] Stimulation index
[5] 60 % concentrate with 24g niacin/cow/day
[6] 60 % concentrate without niacin
[7] 30 % concentrate with 24g niacin/cow/day
[8] 30 % concentrate without niacin

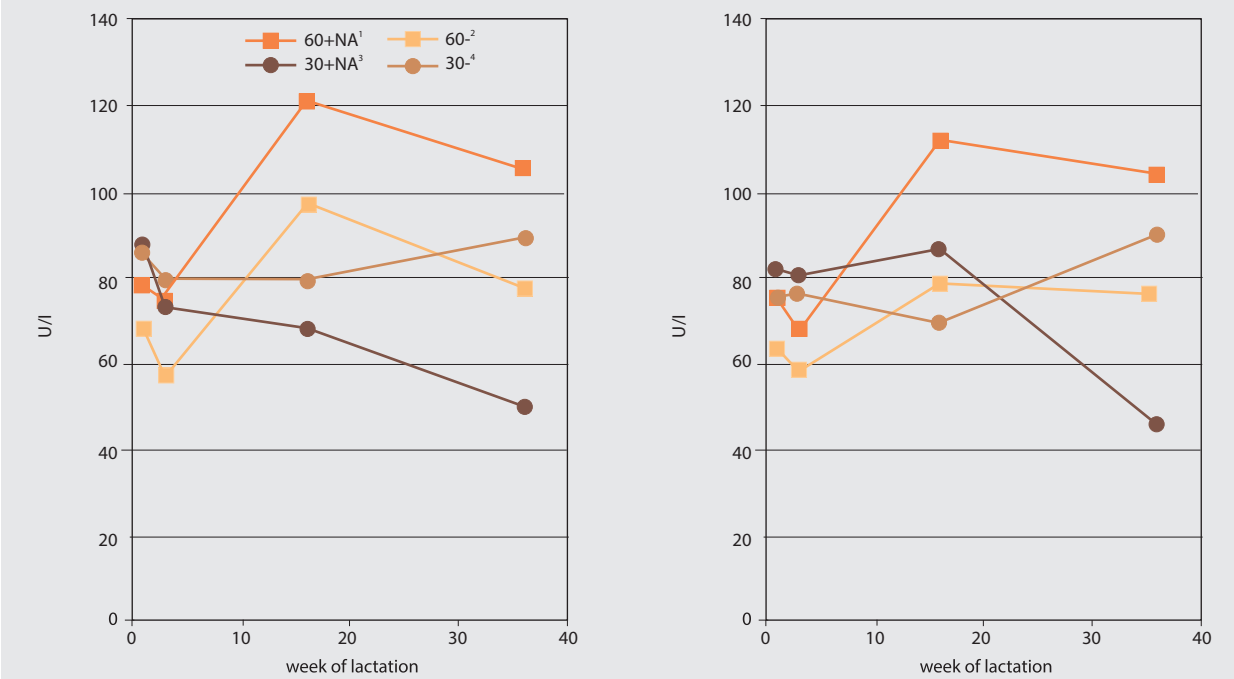

Figure 2
Plasma ASAT concentration of cows (left) and heifers (right) in WoL 1, 3, 16 and 36 in the different feeding groups.
Data points show LS means of n = 9.

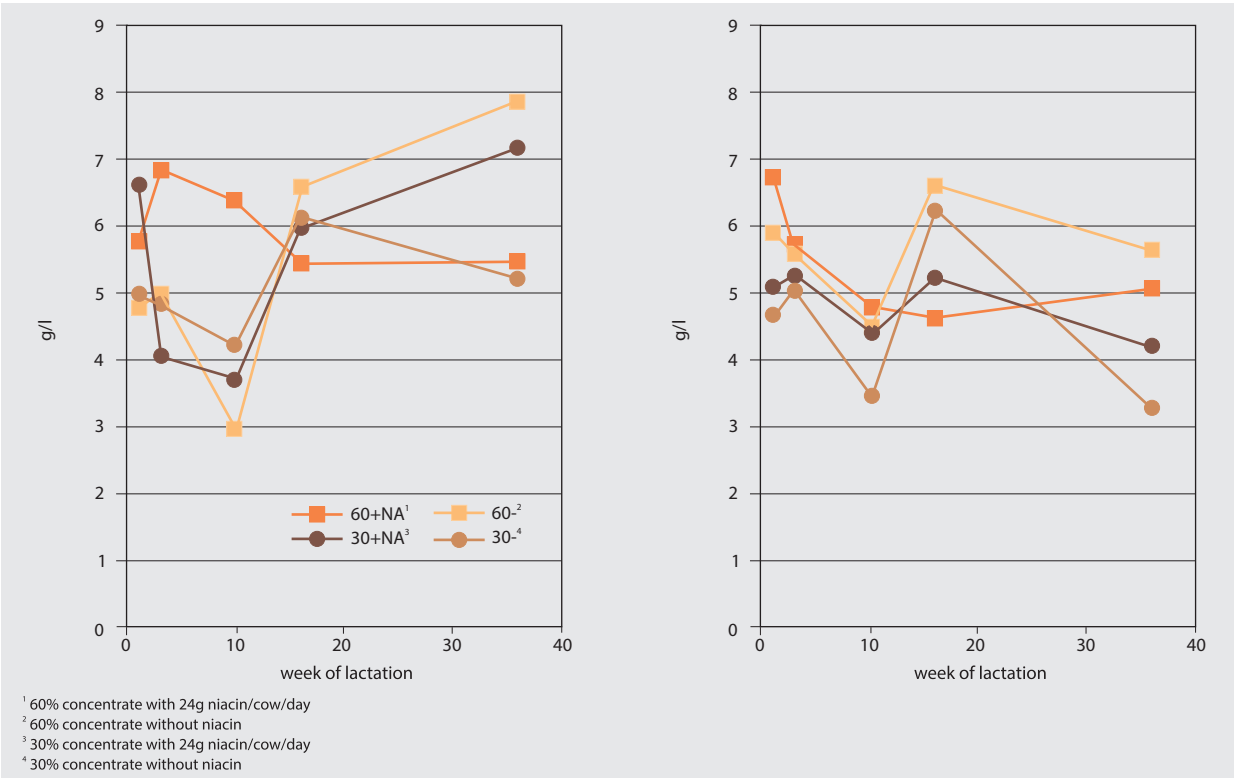

Figure 3
Plasma fibrinogen concentration of cows (left) and heifers (right) in week of lactation 1, 3, 10, 16 and 36 in the different feeding groups.
Data points show LS means of n = 9.

concentrate groups showed higher values over the course of the trial.

GLDH and GGT activity were higher due to high concentrate proportion, whereby serum ASAT activity (C*NA*P*WoL = 0.022, Table 3, Figure 2) was highest in group 60+NA (cows and heifers reacted similarly) while all other groups were over the course of time differently influenced by concentrate, niacin and parity.

Fibrinogen reacted similarly in all groups developing rather lower values in the first weeks with exception of cows from group 60+NA, which showed higher values. Between WoL 10 and 16, fibrinogen increased in all groups again with exception of group 60+NA (cows and heifers). To the end of the trial cows had rather increased, while heifers decreased fibrinogen (Figure 3).

A C*NA interaction influenced the SI ($p = 0.002$, Table 3) for the PBMC as stimulation ability was higher in 30- than in 30+ and 60- group ($p = 0.008$ and 0.048, respectively).

5 Discussion

In this study, the effects of different concentrate proportions on ruminal LPS concentrations were investigated. Zebeli et al. (2015) showed that rumen LPS mediates inflammatory responses while Zhou et al. (2014) found that niacin attenuates LPS-induced inflammatory responses in mouse alveolar macrophages in vitro. Therefore, the present study additionally investigated the impact of high dose niacin supplementation.

Feeding of the high concentrate diets (60 % of the total DMI) increased ruminal LPS concentrations which agrees with results reported by Gozho et al. (2006) who showed that a 60 % concentrate diet with gradual adaption was followed by a grain-induced SARA and an increase in free ruminal LPS. Also, Motoi et al. (1993); Andersen et al. (1994a); Gozho et al. (2007); Emmanuel et al. (2008); Khafipour et al. (2009) and Li et al. (2012) found an increased ruminal LPS concentration due to experimentally induced SARA. Additionally, Plaizier et al. (2012) found even greater increases in rumen LPS due to SARA challenges in studies that used diets consisting of a mixture of forages and grain just like the present study did. The authors explained these findings by the effects of concentrate feeding on microbial population in the rumen as it shifts towards gram-negative bacteria. As LPS is part of the outer membrane, it is released at bacterial cell death and lysis which might be increased in times of low ruminal pH and also during rapid growth phase of bacteria (Hurley, 1995; Wells and Russell, 1996). Also several other investigators showed that feeding cattle with high concentrate/low roughage diets is associated with major changes in the gastrointestinal microbiota in favor of gram-negative bacteria (Krause et al., 2003) resulting in a notably higher concentration of LPS in the rumen fluid (Gozho et al. 2007; Emmanuel et al., 2008; Zebeli and Ametaj, 2009). However, the mechanisms of release and removal or neutralization of endotoxins in the rumen fluid of dairy cows fed high concentrate proportion is still unclear. In the present study high concentrate/low roughage proportion in the diet might have modified rumen microflora also towards a shift to gram-negative bacteria and to an accumulation of short chain fatty acids (SCFA). The lack of fibre in the diet reduced time of chewing which is important for neutralizing the short chain fatty acids. Therefore, the rumen pH was probably lowered promoting bacterial cell death and lysis and therefore the release of LPS. Additionally, the rumen epithelium might have been damaged due to the depressed pH and therefore more LPS could have reached the blood circulation. In the present investigation the pH was only determined once (WoL -3, 1, 3, 16 and 36) via oral rumen tube as published by Rauls et al. (2015). Due to the way of sampling, the enhanced saliva production might have influenced the measured rumen pH. Therefore, the rumen pH values measured in the present trial may not be suitable to make a statement about the potential occurrence of SARA. Nevertheless, a SARA was reported to be induced by feeding 60 % concentrate in the diet (Gozho et al. 2005) as previously described. However, in the present study a negative correlation between pH and SCFA concentration (Rauls et al., 2015) was found ($r = -0.41$, $p = <0.0001$), caused by a negative correlation between pH and valeric acid ($r = -0.27$, $p = 0.02$). Nevertheless, since valeric acid is mostly in a very low concentration, the predictive value is rather low. Similarly, Allen (1997) postulated that rumen pH is mainly dependent on the concentration of total volatile fatty acids (VFA = SCFA) and on rumen buffering. A depressed rumen pH can essentially influence the release of LPS as mentioned above. But in contrast to that, Haubro and Jarlov (1990) and Andersen et al. (1994b) did not find increases in ruminal LPS concentrations due to induced SARA. Gozho et al. (2007) showed in three studies that feeding concentrate increases ruminal LPS and that the magnitude of the response depends on the level of concentrate in the diet and probably on how long such diets are fed before inducing SARA. Rapid growth of bacteria is associated with bacterial lysis due to excessive activity or autolytic enzymes during cell growth and division in the rapid growth phase (Wells and Russell, 1996). Andersen (2000) suggested that as much as 60 % of ruminal LPS is produced by rapidly growing gram-negative bacteria. During a SARA, the damaged rumen epithelium might lead to pathogen and/or LPS infiltration (Nordlund et al., 1995). When hepatic or body clearance is exceeded, LPS might induce inflammatory responses (Andersen, 2000). LPS content was not analyzed in peripheral blood samples. It is believed that free ruminal LPS that translocate into the portal vein can be cleared by the liver before reaching the peripheral blood circulation (Andersen, 2000). Also, Gozho et al. (2007) did not find detectable LPS in the peripheral circulation during a grain induced SARA. However, others detected very low LPS concentrations in the systemic circulation when compared to the ruminal ones. Zhang et al. (2013) detected free plasma LPS levels in a study investigating the influence of DMI and the effect of a *Saccharomyces cerevisiae* fermentation product on LPS-content. Therefore, it cannot be excluded that small amounts of LPS were present in systemic blood of the cows in the present experiment, especially of those fed the high-concentrate diets.

As we found no correlation between LPS and the SI for PBMC, fibrinogen or other hematological parameters and liver enzyme activity, results give no clear evidence for a connection between ruminal LPS and immune parameters of the present trial. However, fibrinogen which is considered only as weak APP in the bovine was elevated in cows fed the high-concentrate diets which contrasts to the findings of Pyörälä (2000).

Moreover, Gozho et al. (2007) attribute only an ancillary role to fibrinogen in SARA diagnostics under field conditions. Besides fibrinogen, which is synthesized by the liver, the high-concentrate feeding was associated with increases in GLDH and GGT activity in peripheral blood which might further hint at a LPS-associated involvement of the liver. Regarding the cellular components, the differential blood count was influenced by niacin supplementation. Niacin supplementation increased the proportions of lymphocytes and decreased that of the segmented neutrophil granulocytes. From a functional viewpoint it is interesting to note that stimulation ability of PBMC was differently influenced by niacin. In interpretation of these interactions between concentrate proportion in the diet and niacin supplementation it has to be considered that niacin acts both at the ruminal and the systemic level whereby direct effects from systemically absorbed niacin (Rauls et al., 2015), or indirect effects from an altered ruminal fermentative pattern and microbial protein synthesis (Niehoff et al., 2009) might affect the proliferative PBMC response. However, the nature of these interactions cannot be explained yet and requires further elucidation.

6 Conclusion

The aim of the present study was to investigate the influence of 60 % vs. 30 % concentrate in the diet of dairy cows with the presence or absence of 24 g/cow/day niacin supplementation. It was shown that ruminal LPS concentration, total leukocytes, hematocrit, ASAT and fibrinogen were subject to varying degrees of a 4-way interaction (C*NA*P*WoL). The SI of the PBMC was influenced by a C*NA interaction. Niacin supplementation increased lymphocyte percentages, while segmented neutrophil granulocytes decreased and there was also found a decreasing tendency for the total leukocytes and the monocytes. High concentrate proportion in the diet increased the GLDH and GGT.

As the mode of action of niacin is influenced by many different criteria like volume of niacin, parity, concentrate proportion, specific period, a large variety of diverse results has been generated. Therefore, further investigations should be conducted (only cows or heifers, shorter period) under more restricted experimental conditions to achieve more precise results.

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Effect of forage preservation method on fatty acid composition and oxidative stability of organic sheep milk

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Abstract

The aim of this study was to evaluate the effect of different forage preservation method in mountainous areas on the relationship between the fatty acid (FA) profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk. Twenty-four multiparous Turcana ewes were randomly assigned to 3 treatments: grazed grass (G), hay grass (H), and grass silage (GS). Indoor ewes were offered *ad libitum* grass hay or grass silage. All animals received 300 g DM grain mix (triticale and barley,1:1) per day. Conservation of grass by drying compared with ensiling resulted in lower forage 18:2n-6, 18:3n-3 and fat-soluble vitamin concentrations. Milk from ewes grazed, has a considerably higher concentration of n-3 FA and n-6 FA but and a higher content of nutritionally beneficial *trans*-fatty acids (e.g. CLA; conjugated linoleic acid, VA; vaccenic acid) than milk from ewes fed hay or grass silage. In spite of lower content, milk fat 18:2n-6 and 18:3n-3 content was higher ($P < 0.05$) for hay than for silage diets (2.34 *vs*. 1.64 and 2.11 *vs*. 1.70 % of total FA, for hay, and silages, respectively). Forage conservation method had no clear effects on milk VA or CLA content. Compared with silage, hay diets resulted in milk containing lower ($P < 0.001$) α-tocopherol and retinol concentrations, but had no effect on γ-tocopherol. There was no clear association between increased levels of the α-tocopherol and retinol in milk and a lower risk of lipid oxidation (lower concentration of malondialdehyde). There was a marked effect of composition of the lipids on the oxidative stability of milk, where high concentrations of PUFA (polyunsaturated FA), especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation.

Keywords: *roughage, fodder preservation, functional fatty acids, CLA, fat-soluble antioxidants, organic sheep milk*

Zusammenfassung

Die Wirkung von Raufutter-Konservierungsmethoden auf die Fettsäure-Zusammensetzung und Oxidationsstabilität der Milch von Schafen im Ökolandbau

Ziel der Untersuchung war die Bewertung der Einflüsse unterschiedlich konservierter Raufuttermittel, die ökologisch produziert wurden, auf das Fettsäurenmuster, den Gehalt an fettlöslichen Antioxidantien und der oxidativen Stabilitat in Schafmilch. 24 multipare Milchschafe der Rasse Turcana wurden in drei Interventionsgruppen randomisiert mit Weidegras (G), Heu (H) oder Grassilage (GS) gleicher Herkunft (Bergwiesen) *ad lib.* gefüttert. Alle Schafe erhielten pro Tag zusätzlich 300 g TM geschrotetes Getreide. Im Vergleich zu dem Silagefutter enthielt das Heu niedrigere Konzentrationen an Linolsäure (18:2n-6), Linolensäure (18:3n-3) und fettlöslichen Vitaminen. Die Schafe, die mit frischem Weidegras gefüttert wurden, hatten signifikant höhere Konzentrationen an wertgebenden n-3 bzw. n-6 Fettsäuren als auch trans-Fettsäuren (z. B. CLAs: konjugierte Linolsäure; VA: Vaccensäure) im Vergleich zu den Konzentrationen in der Milch von Schafen, die mit Heu oder Silage gefüttert wurden. Der Gehalt an 18:2n-6 und 18:3n-3 Fettsäuren ($p \leq 0,05$) war in der Milch von Schafen, die mit Heu gefüttert wurden, höher als in der Milch von Schafen, die mit Silage gefüttert wurden (2,34 vs. 1,64 und 2,11 vs. 1,70 % totale Fettsäuren für Heu bzw. Silage). Die Futterkonservierungsmethode hatte keinen signifikanten Effekt auf den Milchgehalt an CLA bzw. VA. Es konnte keine signifikante Korrelation zwischen den höheren Konzentrationen an α-Tocopherol und Retinol und einem niedrigeren Risiko an Fett-Oxidation (z. B. niedrigere Malondialdehydekonzentrationen) festgestellt werden.

Schlüsselwörter: *Raufutter, Futterkonservierung, funktionale Fettsäuren, CLA, Anti-Oxidanten, Ökologische Schafmilch*

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1 Introduction

Organic sheep milk production systems, compared with conventional farms, produce milk with markedly low levels of milk fat SFA (saturated fatty acids) and high levels of FA (fatty acids) having healthy properties (also named functional fatty acids), especially vaccenic acid (VA; *t*11-C18:1), conjugated linoleic acid (CLA; in particular isomer *c*9,*t*11-C18:2), and omega-3 FA (ALA; α-linolenic acid, EPA; eicosapentanoic acid, DHA; docosahexaenoic acid). These effects are essentially due to grass feeding because of the specific environmental conditions of the mountains as well as of the particular high botanical diversity of grass species which could specifically affect the FA composition of milk fat (Collomb et al., 2008).

The organic sheep sector is dominated by three Member States: the United Kingdom, Italy and Spain, representing together 62.7 % of the entire EU organic herd (3.9 million heads). Sheep breeding into organic farming system has a strong growing tendency in Romania in the future. The sheep livestock farmed organically in 2010 was 18883 heads and in 2012 the reached to 51722 heads (Eurostat, 2015).

Forages, even though containing a relatively low level of lipids, are the cheapest and often the major source of beneficial unsaturated fatty acids in ruminant diets.

Sheep farmed in mountainous areas are fed mainly on pastures, with little or none concentrates supplementation of concentrate during summer for the remainder of the year on hay from mountain grass and/or grass silage and low amounts of concentrate. Fresh forages are an important natural source of fatty acids and vitamins in ewes diets. Hay or silage from specific plant species of mountain areas could also affect the FA composition of milk fat (Shingfield et al., 2005; Collomb et al., 2008). Addis et al. (2005) showed that the percentages of CLA, VA and n-3 FA in milk fat seem to be strongly linked to the linolenic acid content of forages.

The disadvantage of milk enriched with functional fatty acids is the possibility to suffer oxidation due to its high content of double-bonded molecules, which are prone to oxidation onset (Puppel et al., 2012). The delicate balance between anti- and oxidative processes in milk is influenced by factors such as ruminant nutrition, degree of fatty acid unsaturation, content of transition metal ions, and content of antioxidants such as tocopherols and carotenoids (Havemose et al., 2006).

Lipophilic antioxidants (vitamin A and E) are naturally occurring compounds that can prevent some of the processes involved in the development of cancer (protecting DNA from oxidative damage) and cardiovascular disease (inhibiting oxidative damage to LDL-low density lipoprotein) (Schönfeldt and Holden, 2009). One of the most important indicators of oxidation process in milk is malondialdehyde (MDA), which is formed during peroxidation of PUFA by the action of reactive oxygen species. Mutagenic and carcinogenic properties of reactive oxygen species have been confirmed by Klaunig et al. (2010). Storage at low temperatures efficiently preserves saturated fats but is not sufficient to protect polyunsaturated fats against oxidation (Dervishi et al., 2012).

Vitamin A can be found in ewe's milk, in contrast to that of cows, only as retinol because dietary β-carotene is converted entirely into this form (Revilla et al., 2016), thus explaining differences in colour between bovine and sheep dairy products. Vitamin E is found in milk in three forms, α-, β-, and γ-tocopherols but α-tocopherol is the most abundant form (Revilla et al., 2014).

While some studies have failed to show an antioxidative capacity of α-tocopherol in milk (Havemose et al., 2006), others conclude that α-tocopherol is one of the most important antioxidants in cow's milk. Puppel et al. (2012) have demonstrated the protective role of vitamin E in relation to the introduction of PUFA supplementation, and thus demonstrated that tocopherol protects against growth of MDA in milk.

The aim of this study was to evaluate the effect of different forage preservation methods in mountainous areas on the relationship between the fatty acid profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk.

2 Materials and methods

2.1 Experimental design and treatments

The study was conducted at the University of Oradea (Romania) over a 8-week period (May to July, 2015). The first three weeks were used as a covariate period (week 1) and for adaptation to dietary treatments (weeks 2 and 3).

This study was undertaken in organic dairy sheep farm (a family farm) that has met the criterions stipulated by the Council Regulations of the EEC, 1991 (No. 2092/1991) and EU, 1990 (No. 1804/1999); Rahmann, 2013) on standards of organic animal husbandry. The organic farm status was checked with the existence of a valid certificate. The farm was located in the mountain area of western Carpathian Mountains (north-western Romania, 46^{0}40'N, 22^{0}29'W, 1240 m above sea level, total annual rainfall 778 mm; mean annual temperature 9.3 °C).

Sheep breeding into organic farming system in Romania is based on native breeds, which are well adapted to mountain and submontain regions with large areas of pastures, and prevalent breed is Turcana (over 50 % of the sheep in Romania belong to this breed). Milk production of the breed is generally low, with ewes producing yields of 40 to 60 kg of milked milk, with the average fat content ranging between 7 to 8 % and 6 % proteins.

Twenty-four multiparous (lactation number 3 to 4) Turcana ewes, were balanced for average body weight (45.7 ± 1.71 kg) and milk yield and were randomly assigned to three treatments: grazed grass (G), hay grass (HG), and grass silage (GS).

An area of pasture of 3.6 ha, situated on soil type argiluviosol, were subdivided into 3 equal parts who were randomly assigned to one of three ways to use: grazing, getting the

grass hay and getting grass silages. The most common species in the pasture in terms of occurrence were ca. 37.4 % *Festuca rubra*, 14.8 % *Dactylis glomerata*, 9.1 % *Phleum pratense*, 8.4 % *Poa pratensis*, 5.2 % *Agrostis capillaris*, 18.3 % legumes (mainly *Trifolium repens*) and other grasses. Botanical composition was determined on forage samples taken randomly by quadratic frame (0.25 × 0.25 m^2) by manual separation of plant species. Botanical composition was calculated by dividing individual species weight by the total weight collected (wet basis).

The pasture was harvested at the early flowering stage. Hay and silages were prepared simultaneously in May: grass used to prepare silages was cut and field-wilted for 6 h, and baled in bags while grass used to prepare hay was left to dry for 4 day, before baling. All the experimental animals were grazing sward before the feeding experiment.

The ewes were managed in experimental conditions exclusively. Outdoor grazing ewes had free access on pasture (12 to 14 h/day) and they were housed overnight. Rotational fenced grazing was practiced, pasture having three identical in size areas. Indoor ewes were offered ad libitum grass hay or grass silage and they had free access in outdoor paddock. All animals received 300 g DM grain mix (triticale and barley, into equal proportions) per day, divided into equal halves and given during the morning and evening milkings. All animals had non-restricted access to water and salt blocks. All forages (pastures, hay, silage and concentrates) were organically produced on farm. Ewes were milked twice per day, by hand, to a sheltered area.

The animals were managed according to the Animal Welfare and Good Clinical Practice (European Commission, 2010) and to those laid down by the local Bioethics Committee (Rahmann et al., 2016).

2.2 Sampling and chemical analyses

Forage samples were collected twice weekly and combined into one sample per week. All the forage samples collected were freeze-dried and ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA). Samples were analysed for dry matter (DM) (ISO 6496, 1999), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest et al., 1991) on a Fibersac analyser (Ankom Technology, Fairport, NY) and nitrogen (N) (Kjeldahl technique: Vapodest Distillation Systems – Gerhardt; AOAC International, 1995).

Samples of forage (n = 5) collected for determine the FA profile were stored immediately at -20 °C, and later lyophilized and kept until analysis.

The milk samples were collected once a week for a period of five weeks. Thus, five samples for analysis were obtained from each ewe (40 milk samples for each group and 120 total samples, respectively). Each sample (200 mL milk) was divided into two parts, one for fatty acid determination and the other one for analysis fat-soluble antioxidant content and MDA (malondialdehyde). Milk samples were kept in 100 mL containers and were protected from light, refrigerated immediately and then brought directly to the laboratory and was stored at -20 °C.

To determine the concentration of FAs in the diets, fatty acid methyl esters (FAME) were prepared by the one-step extraction-methylation method of Sukhija and Palmquist (1988). In order to determine the composition of FAs in milk, the fat was extracted according to the international standard, ISO 14156 / IDF 172, 2001. FAME were prepared according to the method proposed by Christie (1982) and Chouinard et al. (1999) and were determined by gas chromatography using a Varian GC 3600 equipped with FID and a fused silica capillary column (SP 2560 Supelco), 100 m × 0.25 mm i.d., film thickness 0.20 µm. Helium was used as the carrier gas at a flow rate of 1 mL/min. The split ratio was 1 : 100. The oven temperature was programmed at 90 °C and held for 1.50 min, then increased to 210 °C at a rate of 9 °C/min, held at this temperature for 25 min, then increased to 230 °C at 15 °C/min, and held for 7 min. The temperatures of the injector and the detector were set at 270 °C. The FA identification was based on external standards, and calculation of the distribution (in weight percentage) was based on the area of each FA ester corrected for the response factors for the individual FAs. Internal standards were used to determine the percentage of recovery. The CLA isomer reported is *c*9,*t*11-CLA and *t*10,*c*12-CLA. The percentage of each fatty acid was calculated by dividing the area under the FA peak by the sum of the areas under total reported FA peaks.

Determination of vitamins, was made by HPLC (High-Performance Liquid-Chromatography), the extracts obtained from milk samples. Extraction of fat soluble vitamins has been made with a mixture of diethyl ether and petroleum ether (1:1), in aliquots of 20 ml. The ether phase, after separation, was saponified with methanolic KOH solution (10 %), after which vitamins have been extracted in hexane, washed with water in a separator funnel and evaporated to dryness. For the determination of retinol was conducted in a first stage, curve standard using total-trans-retinol solutions (10 to 80 mg/ml). Standard solutions (Sigma) and samples were injected into a system Parkin-Elmer LC-295, equipped with Alltech C18 column (length 15 cm, 4.6 mm internal diameter and particle size 3µm). The mobile phase consisted of acetonitrile/methanol (85:15), with the addition of 2-propanol (30 %) after 8 min.

The analysis of carotenoids in the forages, ethanol: methanol:tetrahydrofuran (75:20:5) was used as a HPLC buffer. The flow rate was at 1 ml/min and the injection volume was 100 µl. The wave length for detection was 450 nm. External standards of β-carotene were used in order to quantify the carotenoids.

Dosage tocopherol was made using the same analysis conducted extract retinol. After evaporation of the hexane phase was added to methanol, after which separation of the tocopherol was by HPLC using the column with characteristics described above. The mobile phase for the different tocopherols analysed was acetonitrile:methanol (85:5)/isopropanol 90/10. Tocopherol analysis in feed samples was performed in the same way as in the milk samples. Instead of 2 ml of milk, 0.4 g of feed was taken to start the analysis. Detection wavelengths for α- and γ-tocopherol were set at 290 nm and 296 nm respectively. By injecting known

amounts of α-tocopherol (Sigma-Aldrich) and γ-tocopherol (Sigma-Aldrich) standard containing internal standard Tocol, realized a standard curve. Quantitative analysis of α- and γ-tocopherol in the samples was performed by HPLC Millennium software using peak area measurement and standard curve.

Malondialdehyde (MDA) is the main product of oxidation of PUFAs, it is widely used as a marker of lipid peroxidation in food and biofluid (Andrei et al., 2008). Thiobarbituric acid (TBA) reacts with MDA to form a pink compound in an acid medium, which was measured photometrically at 532 nm using a spectrophotometer UV-VIS Jenway 6315. The standard curve to calculate the required concentration of MDA was obtained by acid hydrolysis of 1,1,3,3-tetrametoxipropan (TMP) (Andrei et al., 2008).

2.3 Statistical analysis

Statistical analyses were carried out with SAS (SAS Institute Inc., 2005). Data for fatty acids profile and fat-antioxidants in milk were analysed using ANOVA model with factorial term for diet composition. Comparison among means was carried out using Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Pearson correlations between the parameters content was done with the XLSTAT Pearson Edition (Addinsoft SARL, 75018 Paris). Multivariate analysis was carried out with PAST (Paleolontological Statistical Software) (Hammer and Harper, 2005). There was used a multivariate sequence (PCA – Principal component analysis, MANOVA – Multivariate analysis of variance and HCA – hierarchical cluster analysis) in order to get the proper number of clusters with 95 % level of significance.

3 Results

The chemical composition, FA levels and fat-soluble antioxidants of the forages from diets is presented in Table 1. As expected, the fresh grass had a higher PUFA concentration, especially α-linolenic acid (C18:3n-3, ALA) (51.8 % of total FAME) and fat-soluble antioxidants, compared with the hay grass, which had a higher SFA content. The sums of total PUFA were 41.3 % and 17.1 % greater in the fresh pasture compared to hay and grass silage. Conservation of grass by drying compared with ensiling resulted in lower forage linoleic acid (C18:2n-6, LA), α-linolenic acid and fat-soluble vitamin (α-tocopherol and β-carotene) concentrations. The grain mix was good source of LA and MUFA.

Table 2 shows the results of fatty acids and fat-soluble antioxidants contents in milk in the three groups. The results clearly show a higher concentration of beneficial *trans*-fatty acids (e.g. CLA, VA), sum of n-3 FAs and fat-soluble

Table 1
Fatty acid composition and fat-soluble antioxidants content of experimental forages and concentrate supplement

	Forages			SEM	p-value	Concentrate
	Grass fresh	Grass hay	Grass silage			supplement
Dry matter (DM), g/kg	207	846	267	-	-	877
CP, g/kg DM	184	139	107	-	-	134
NDF, g/kg DM	461	526	486	-	-	178
ADF, g/kg DM	241	320	305	-	-	94
Fatty acids, (% of FAME)						
C12:0	0.46^{a}	0.61^{a}	0.11^{b}	0.06	< 0.05	0.04
C14:0	0.50^{b}	0.70^{b}	1.53^{a}	0.04	< 0.01	0.21
C16:0	16.71^{c}	26.18^{a}	22.43^{b}	0.63	< 0.01	17.43
C16:1	0.19	0.32	0.40	0.05	NS	0.64
C18:0	1.90^{c}	5.24^{a}	3.17^{b}	0.10	< 0.01	3.29
C18:1	3.71^{b}	6.97^{a}	3.32^{b}	0.14	< 0.001	24.69
C18:2 n-6 (LA)	19.40^{a}	16.18^{b}	18.43^{a}	0.21	< 0.01	41.20
C18:3 n-3 (ALA)	51.80^{a}	34.20^{c}	42.35^{b}	0.92	< 0.01	3.52
SFA	19.57^{c}	32.73^{a}	27.24^{b}	0.74	< 0.01	20.97
MUFA	3.89^{b}	7.69^{a}	3.74^{b}	0.09	< 0.01	25.33
PUFA	71.20^{a}	50.38^{c}	60.78^{b}	0.86	< 0.01	44.72
Fat-soluble antioxidants, mg/kg DM						
β-carotene	63.70^{a}	19.30^{c}	46.90^{b}	0.98	< 0.01	1.80
α-tocopherol	71.70^{a}	8.40^{c}	54.80^{b}	1.17	< 0.001	9.10
γ-tocopherol	15.80^{a}	5.20^{b}	8.90^{b}	0.52	< 0.01	12.30

CP – Crude Protein; NDF – Neutral Detergent Fibre; ADF – Acid Detergent Fibre.
FAME – fatty acid methyl esters; SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA – polyunsaturated acid.
^{a,b,c} – Values within rows with different superscript are significantly different ($P < 0.05$).

Table 2
Effects of feeding either grazing fresh, hay or grass silage on fatty acid composition, fat-soluble antioxidants and oxidative stability of milk

	Grass fresh (G)		Grass hay (H)		Grass silage (GS)	
	Mean	SEM	Mean	SEM	Mean	SEM
Fatty acid composition (% of FAME)						
C4:0 – C10:0	17.26^{b}	0.93	21.52^{a}	1.24	19.23^{a}	1.18
C12:0	2.07^{c}	0.41	2.81^{b}	0.50	3.54^{a}	0.55
C14:0	7.83^{b}	0.70	9.37^{a}	0.65	8.99^{a}	0.83
C16:0	18.12^{b}	1.34	22.68^{a}	1.52	22.44^{a}	1.39
C18:0	9.76	0.81	10.11	0.80	10.28	0.92
C18:1 n9t	0.59	0.04	0.47	0.04	0.52	0.04
C18:1 *trans*-11 (VA)	5.69^{a}	0.40	3.28^{b}	0.28	3.05^{b}	0.32
C18:1 n9c	24.51^{a}	1.72	20.07^{b}	1.59	23.46^{a}	1.64
C18:1 *cis*-11	0.64^{a}	0.18	0.41^{b}	0.21	0.40^{b}	0.20
C18:2 n6t	0.39	0.12	0.36	0.11	0.38	0.14
C18:2 n6c (LA)	2.87^{a}	0.39	1.76^{b}	0.27	1.02^{c}	0.20
CLA *cis*-9, *trans*-11 (RA)	3.06^{a}	0.35	1.81^{b}	0.32	1.82^{b}	0.31
CLA *trans*-10, *cis*-12	0.17^{a}	0.03	0.11^{b}	0.02	0.09^{b}	0.02
CLA-total	3.23^{a}	0.38	1.90^{b}	0.35	1.93^{b}	0.29
C18:3 n-3 (ALA)	2.17^{a}	0.51	1.63^{b}	0.47	1.15^{c}	0.44
C20:4 n-6	0.28	0.02	0.22	0.02	0.24	0.02
C20:5 n-3 (EPA)	0.41^{a}	0.06	0.20^{b}	0.04	0.21^{b}	0.04
C22:6 n-3 (DHA)	0.47^{a}	0.07	0.28^{b}	0.05	0.34^{b}	0.05
Unidentified FA	3.71	0.62	2.93	0.78	2.82	0.59
SFA	55.04^{c}	2.34	66.49^{a}	2.73	64.48^{b}	2.47
UFA	41.25^{a}	1.64	30.58^{c}	1.32	32.70^{b}	1.26
MUFA	31.43^{a}	2.86	24.23^{c}	2.59	27.43^{b}	2.71
PUFA	9.82^{a}	0.88	6.35^{b}	0.61	5.27^{c}	0.64
PUFA n-6	3.54^{a}	0.36	2.34^{b}	0.29	1.64^{c}	0.32
PUFA n-3	3.05^{a}	0.41	2.11^{b}	0.30	1.70^{c}	0.24
HFA[1]	28.02^{b}	1.73	34.86^{a}	2.11	34.97^{a}	1.97
PI[2]	7.60^{a}	0.82	5.38^{b}	0.67	3.70^{c}	0.41
Fat-soluble antioxidants (µg/100 g of milk)						
Retinol	93.46^{a}	3.16	48.81^{c}	2.04	71.16^{b}	2.27
α-tocopherol	187.39^{a}	14.11	89.34^{c}	9.72	122.60^{b}	10.34
γ-tocopherol	16.23	0.70	13.36	0.54	14.11	0.63
Oxidative stability of milk						
MDA – g/L of milk	3.31^{a}	0.18	3.04^{a}	0.13	2.67^{b}	0.13

1 HFA (hypercholesterolaemic fatty acids) [C12:0 + C14:0 + C16:0].
[2] PI (polyunsaturated index) = C18:2 n-6 + (C18:3 n-3 x 2).
FAME – fatty acid methyl esters; CLA – conjugated linoleic acids. SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA – polyunsaturated acid; FA – fatty acid; MDA – Malondialdehyde.
[a,b,c] – Means within the same row with different letters differ significantly according to Duncan's tests ($p < 0.05$).

antioxidants in milk produced from grazing animals, than ewes fed hay or silage grass. G ewes presented a significantly greater proportion of PUFA in milk than the H and GS group, which agrees with their higher proportion in grazed pasture compared to hay and grass silage (Table 1). Total PUFA n-6 milk fatty acids were greater in the G group than in the H and GS group due to the greater amount of linoleic acid in milk from G ewes than in milk from H and GS ones (Table 2).

In spite of lower intakes of LA and ALA, their contents were higher in the milk fat of ewes fed hay than in that of ewes fed grass silages. The forage conservation method had no clear effect on milk *trans*-18:1 acids or CLA contents.

Table 3
Pearson's correlation matrix between the bioactive compounds (fatty acids and fat-soluble antioxidants) and oxidative stability (MDA concentration) of milk.

	MDA	PUFA	n-3 FA	n-6 FA	CLA	Retinol	γ-tocopherol	α-tocopherol
MDA	**1**	**-0.5971**	**-0.6351**	**-0.5792**	**-0.5374**	0.1076	0.1389	0.1988
PUFA	**-0.5971**	**1**	**0.9462**	**0.9864**	**0.9585**	0.2036	0.3510	**0.5196**
n-3 FA	**-0.6351**	**0.9462**	**1**	**0.9165**	**0.8584**	0.1127	0.3790	0.4747
n-6 FA	**-0.5792**	**0.9864**	**0.9165**	**1**	**0.9155**	0.1425	0.3210	0.5068
CLA	**-0.5374**	**0.9585**	**0.8584**	**0.9155**	**1**	0.3429	0.3423	**0.5191**
Retinol	0.1076	0.2036	0.1127	0.1425	0.3429	**1**	0.3730	0.4970
γ-tocopherol	0.1389	0.3510	0.3790	0.3210	0.3423	0.3730	**1**	-0.0217
α-tocopherol	0.1988	**0.5196**	0.4747	0.5068	**0.5191**	**0.5970**	0.0217	**1**

Bold values significantly different, $p < 0.05$
MDA – malondialdehyde, PUFA – polyunsaturated acid; FA – fatty acid, CLA – conjugated linoleic acids.

Milk fat of ewes fed hay or grass silages than in that of ewes fed grass fresh has a higher HFA (hypercholesterolaemic FA: C12:0+C14:0+C16:0) proportion, whereas the polyunsaturated index is lower.

Concentrations of α-tocopherol and retinol were higher ($P < 0.05$) in milk from grazing fresh. Relative to silages, milk from hay diet contained lower ($P < 0.05$) amounts of α-tocopherol and retinol, but the concentrations of γ-tocopherol were independent of forage conservation method (Table 2).

The TBA test (thiobarbituric acid) provides information about the level of MDA, a secondary compound formed in lipid oxidation. The highest level of MDA was recorded in group G (3.31 g/l of milk). On the other hand, the lowest level was recorded in GS group, 2.67 g/l of milk. In the milk we found a negative association between the MDA and PUFA for all PUFA categories analysed (PUFA total, n-3 FA, n-6 FA and CLA) (Table 3). There was no clear association between increased levels of the α-tocopherol and retinol in milk and a lower risk of lipid oxidation (lower concentration of MDA, respectively). There was a marked effect of composition of the lipids on the oxidative stability of milk, where high concentrations of PUFA, especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation.

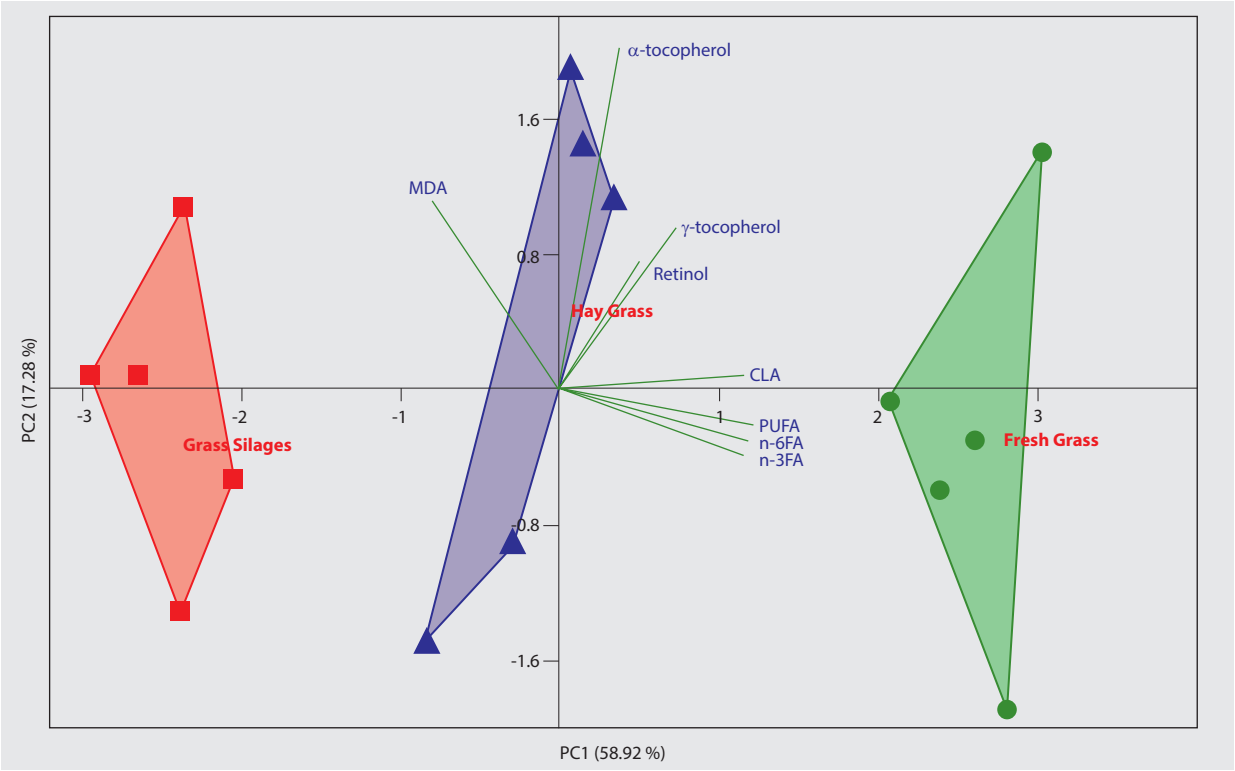

Figure 1
Relationship between healthy fatty acid, fat-soluble antioxidants concentration and oxidative stability of milk (component analysis biplot).

In order to present a complete evaluation of the effect of different forage preservation method in mountainous areas using the fatty acid profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk, a multivariate sequence was performed.

The multivariate sequence was intended to assess the sample clusters, and consists of: principal component analysis (PCA), multivariate analysis of variance (MANOVA) and hierarchical cluster analysis (HCA). Figure 1 shows the PCA biplot that contains both the scores (i.e. samples principal coordinates) and loadings (i.e. variables/parameters principal coordinates). Loadings are represented as vectors with origin as starting points. The vectors are correlated each other and with the principal axes (PC1 and PC2). From the PCA biplot (see Figure 1), it can be considered four variable groups. First group gathers: CLA, PUFA, n-6 FA and n-3 FA; the second group: retinol and γ-tocopherol; third group has only one variable α-tocopherol and the same the fourth group MDA. These groups are "shell" distributed over the three quadrants: first, second and fourth, and geometrically generates non-overlapping sample groups distributed along the first principal axis. The fresh grass sample group has highest abundance of first group of variables. The hay grass sample group is divided in two parts; one has highest content of α-tocopherol and second variable group, and the other part has average abundance of the first variable group. The grass silages sample group has the highest content of MDA and the lowest content of the first variable group.

4 Discussion

Forages are often the main source of fatty acids in the diet but the effects of conservation method on milk fatty acid composition and oxidative stability of milk are not well defined (Chilliard et al., 2001; Shingfield et al., 2005). Exposure to solar radiation and the duration of wilting affecting oxidative losses of unsaturated fatty acids and fat-soluble antioxidants (e.g. α-tocopherol and β-carotene) from cut grass. In the present experiment, hay contained lower amounts of all measured fatty acids, α-tocopherol and β-carotene than silages.

Fresh grass is a rich source of ALA and compared to hay or grass silage diets, results in increased milk fat concentrations of *c*9-C18:1, *t*11-C18:1, total n-3 FA and *c*9,*t*11-CLA, and decreased C4:0-C16:0 concentrations. Fresh grass resulted in higher concentration of *c*9-C18:1 in milk fat than hay and silage that may arise from higher uptake of *c*9-C18:1 from arterial plasma across the mammary glands (Halmemies et al., 2013). Feeding fresh grass decreased milk fat C4:0-C16:0 content relative to dried hay and grass silage primarily due lower de novo synthesis in the mammary glands, consistent with increases in the supply of preformed fatty acids inhibiting synthesis of C4:0-C16:0 de novo (La Terra et al., 2010; Shingfield et al., 2013).

The effects of fresh grass on increased the milk fat content of ALA, VA and CLA are related to the high content of ALA in green pasture, which is partly biohydrogenated into VA in the rumen and then secreted into milk and partially converted into *c*9,*t*11-CLA in the mammary tissue by the action of stearoyl-CoA desaturase (Nudda et al., 2014). Grazing pasture may enhance the growth of specific bacteria in the rumen, stimulating the production of CLA and/or blocking the final reduction of VA to stearic acid (C18:0), as well as increasing n-3 FA (Dervishi et al., 2012). Cabiddu et al. (2005) obtained a linear relationship between the total daily intake of ALA and the estimated daily amount of VA and *c*9,*t*11-CLA in sheep milk. In the present study, fresh pasture are richer in ALA (51.8 and 34.2 *vs*. 42.35 for fresh pasture and hay *vs*. grass silage, respectively), which can stimulate the CLA production.

Grass conservation through hay making or ensiling leads to decreases in ALA concentrations, with hay having lower LA and ALA concentrations than grass silage (16.18 *vs*. 18.43 and 34.2 *vs*. 42.35 % of FAME, respectively) (Table 1). Higher oxidative losses of LA and ALA in hay as compared with silage of Italian ryegrass (*Lolium multiflorum*) were reported by Dewhurst et al. (2006). Nevertheless, milk from hay diets they are richer in LA and ALA than milk from silage diets (Table 2), due to higher transfer efficiency from diet to milk with hay than with grass silage (Shingfield et al., 2005). Incubations in vitro show that the rate and extent of LA and ALA biohydrogenation are higher for ensiled than for dried grass (Boufaied et al., 2003). Earlier studies show the rate of C18:0 formation during incubations with mixed rumen bacteria in vitro to be higher for silage than for hay prepared from the same grass (Boufaied et al., 2003), which suggests that both lipolysis and biohydrogenation of forage lipids are lower when grass is dried rather than ensiled.

The forage conservation method had no clear effect on milk *t*11-C18:1 acids or CLA contents. This result was not expected as Collomb et al. (2008) reported a positive association between proportion of grass silage in the cows diet and content of *c*9,*t*11-CLA and *t*11-C18:1 in milk. Previously, forage conservation by drying compared with ensiling has been reported to decrease (Onetti et al., 2004) or have no effect (Nozière et al., 2006) on milk fat *c*9,*t*11-CLA content. The inconsistencies in milk fat responses between studies may be explained by the large differences in treatment effects on LA and ALA intake.

Compared to the hay diet the decrease in the concentration of SFA in milk fat from ewes fed grass silage may be due in part to the more important amounts of dietary PUFA provided by silage than by hay. These PUFA and/or their biohydrogenation products are potent inhibitors of mammary SFA synthesis by directly inhibiting acetyl-CoA carboxylase activity (Kalač and Samkova, 2010). Milk fat of ewes fed hay or grass silages than in that of ewes fed grass fresh has a higher HFA proportion, whereas the proportion of FA having healthy properties (e.g. VA, *c*9,*t*11-CLA and n-3 FA) is lower. Extensive lipolysis during forage preservation can be among the causes of these differences. The higher concentration of oleic acid (23.46 and 20.07 % of FAME) in milk fat from ewes fed grass silage compared to hay may be due to the fact that silage contains more PUFA than hay.

The effect of forage preservation method on α-tocopherol and β-carotene concentrations reflected more extensive

losses of these vitamins during the drying than during the ensiling of grass. Havemose et al. (2006) also report higher concentrations of α-tocopherol in silage than in hay (35 *vs*. 15 mg/kg DM) prepared from the same mixed timothy-alfalfa swards. There can be losses of up to 80 to 90 % of α-tocopherol and β-carotene in hay, but in well-fermented silages the losses are often less than 20 % (Havemose et al., 2006; Marino et al., 2012).

Fresh grass is rich in β-carotene and α-tocopherol and higher intake of α-tocopherol has been shown to result in a higher output of α-tocopherol in milk (Focant et al., 1998). In the present study, α-tocopherol and retinol in milk increased when ewes were feeding to pasture, which could be considered as a natural consequence of what was stated above. This increase, not found to be sufficient to prevent milk oxidation. Focant et al. (1998) showed improved oxidative stability of the milk when cows were fed supplements of α-tocopherol to the diet, and the concentration of α-tocopherol in the milk reached 2,670 µg/L. This indicates that concentrations of α-tocopherol in the milk (893 to 1873 µg/L) in this study needed to be higher to improve the oxidative stability.

In this studies we found a negative association between the MDA and PUFA for all PUFA categories analysed (PUFA total, n-3 FA, n-6 FA and CLA) (Table 3). These results are in agreement with previous studies in which lipid oxidation have been shown to be highly dependent on the content and composition of the PUFA in milk (Havemose et al., 2006; Juhlin et al., 2010). A higher degree of lipid oxidation was found in milk of ewes fed the grass fresh compared of hay or grass silages diet. The higher content of natural antioxidants (retinol and α-tocopherol) did not prevent oxidation and higher contents of n-3 FAs (3.05 %, 2.11 % and 1.7 % of total FAs in fresh grass, hay and grass silages, respectively) were thought to be the cause. The role of α-tocopherol and retinol on the oxidative stability of the milk appears to be less important than the variation in the fatty acid profile. It was observed that oilseed (rapeseed and linseed) included in grass silage-based cow diets could increase the concentration of n-6 FA and n-3 FA in milk fat and the content of α-tocopherol but the resistance to oxidation was reduced (Focant et al., 1998). The relationship between levels of α-tocopherol, retinol and lipid oxidation is not clear and there are also other antioxidants present in milk which in combination with various prooxidants may add to the complexity of PUFA oxidation (Juhlin et al., 2010).

5 Conclusions

The nature of forages consumed by ewes has a large effect on fatty acid composition and oxidative stability of milk. High intakes of PUFAs and natural antioxidants (lipid-soluble vitamins) from fresh pasture, result in higher concentrations of FA n-3, CLA and antioxidants in milk fat. Ensiling is superior to haymaking in preserving PUFA, α-tocopherol and β-carotene in forages. Milk from hay diet, had concentration higher FA n-3 and CLA but lower levels of antioxidants, compared with the silage diet. The high concentrations of PUFA, especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation from milk.

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