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The use of nest boxes to survey marginally distributed Fat dormouse *Glis glis* in Latvia

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Abstract

The use of nest boxes in different studies of arboreal mammals, such as dormouse, is a widely accepted method. We examined the use of this method to survey a marginal population of Fat dormouse (*Glis glis*) living in a mixed, oldgrowth broadleaf forest in Gauja National Park. A total of 104 nest boxes and 15 nest tubes designed for dormice were set up in five study sites: both in areas with potential dormice presence and where likelihood of presence was only theoretical. Nest boxes were readily used by *G. glis* in two study sites. During a four-year period we obtained preliminary results also on dynamics of nest box use, habitat preferences, relative abundance and reproduction of *G. glis* living in the most northern part of the range. Altogether 41 dormice including 35 juveniles were captured. *G. glis* showed clear preference to areas with oldgrowth oaks having wide canopies. In this habitat type the abundance of *G. glis* was estimated to be two individuals per ha or 20 individuals per 100 nest boxes. Nevertheless, the species proved to be rare in Gauja National Park, probably due to scatter and isolation of suitable habitats.

Key words: *Glis glis*, marginal population, nest boxes, relative abundance, Latvia.

Introduction

The Fat or Edible dormouse (*Glis glis*), similar to other dormice species, is an arboreal mammal that uses tree holes as nest sites both for daily rest and rearing of young (Storch 1978; Airapetyants 1983; Rossolimo et al. 2001). *G. glis* is known also to use nest boxes set up for cavity-nesting birds. Therefore, the use of nest boxes is a widely accepted method to investigate the distribution and different aspects of ecology of Fat dormouse (e.g., Schlund et al. 1997; Juškaitis 1999; Burgess et al. 2003; Juškaitis 2003; Kryštufek et al. 2003; Milazzo et al. 2003; Bako, Hecker 2006). Long-term studies using nest boxes have shown the effect of climatic changes on *G. glis* populations (Koppmann-Rumpf et al. 2003). The National Dormouse Monitoring Programme in the UK is based on the use of nest boxes (Morris 2003).

In Latvia nest boxes for birds have been widely used already for a long time since the 1940s both to study and to monitor cavity-nesting birds (Vilka 1999) as well as to increase the abundance of cavity-nesting birds in managed forests in order to reduce populations of forest insect pests (Mihelsons, Vilka 1974). Common dormouse (*Muscardinus avellanarius*) is sometimes observed in nest boxes for birds if they are placed in habitats

suitable for this dormouse species (Štrauss 1959; Štrausa 1999). Nest boxes for birds were used successfully to identify the presence of *M. avellanarius* in a study (LIFE project) area (Pētersons 2003). The presence of the Forest dormouse (*Dryomys nitedula*) in Latvia was discovered by checking nest boxes (Kasparsons 1970). According to unpublished data of the Atlas of Latvian Mammals several cases are known when the Garden Dormouse (*Eliomys quercinus*) has been recorded in nest boxes for birds.

However, previously there are no records of Fat dormice in nest boxes in Latvia. One of the authors (V. Pilāts) recorded signs (faeces and nest material) which were identified as possibly left by *G. glis* in bird nest boxes set up in the Gauja valley in the mid 1990s. During 2000 to 2002 a study using altogether 70 bird nest boxes of various design were carried out by two secondary school students (Žagars, Rozenfelds 2003) in Daugava valley at Skrīveri, in an agricultural research centre, another well-known *G. glis* site (Fig.1). Dormice were found only in a few boxes that were set up inside the pavilion for agricultural studies. *G. glis* was known to inhabit this area since 1977 (Tauriņš 1982). Although in several cases students found food remains in nest boxes set up in a nearby forest and identified them as left by *G. glis* these records are doubtful. Neither students nor the authors of this study during the initial stage of the studies had practical experience on *G. glis* identification according to activity signs. Therefore we can not exclude misidentification of nestbox occupants, as several mammal species can use nest boxes (Juškaitis 1999).

Latvia lies at the northern limit of the *G. glis* range (Storch 1978; Pilāts 2003). Therefore, it is possible that dormice of the northern populations might avoid bird nest boxes for several reasons.

This study was aimed to examine the usability of dormice nest boxes in Gauja NP as a tool for detecting *G. glis* presence and for monitoring.

Materials and methods

Study areas

The study was conducted at five sites in four different areas of Gauja National Park (Fig. 1). A brief description on Gauja National Park is given in an earlier publication (Pilāts 2003) and detailed descriptions can be found in a monograph on the National Park (Pilāts 2007a). Study sites were established in natural undisturbed mixed woods with oak (*Quercus robur*) and hazel (*Corylus avellana*) in the understorey. Such forests are considered as suitable habitats for *G. glis* in the northern part of its range (Rossolimo et al. 2001; Juškaitis 2003; Pilāts 2003).

Site A is situated on a steep southern slope of the Gauja valley at the former farmstead "Paslavas". The height of the slope varies from 10 to 40 m, and there are small outcrops of sandstone. The slope is covered mainly by 60- to 140-year old, mixed broadleaf forest with oak (some of them up to 250-year old), lime (*Tilia cordata*), birch (*Betula pendula*), aspen (*Populus tremula*), pine (*Pinus sylvestris*), spruce (*Picea abies*) and shadbush (*Amelanchier* sp. – an alien species). Dry meadow is situated between the slope and the River Gauja. A 100-year old birch and pine wood is located to the North at the slope's upper edge. In that direction at a distance of 1.3 km where a dormouse (probably juvenile) was caught in a mouse-trap in October, 1995 at another farmstead "Cepļi" (Pilāts 2003).

Site B is situated on a steep southern slope of Gauja valley at the farmsteads "Klintis" and "Gūdas". The height of the slope is up to 50 m. In its eastern part rather large (up to 10

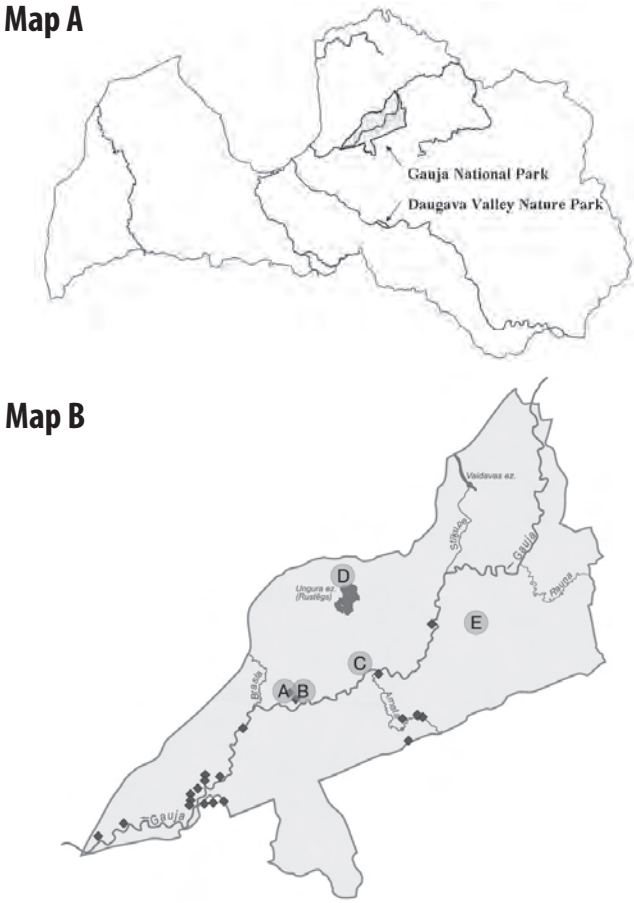

Fig. 1. Protected areas with Fat Dormouse in Latvia (Map A) and distribution of dormice records during 1937 - 2001 (♦) and study areas (A - E) during 2005 - 2008 (Map B).

m high) outcrops of sandstone appear on the slope. It is covered mainly by mixed 80- to 100-year old forest with lime, birch and aspen dominating and oak (some of them up to 250-year old), pine and spruce as minor species. A mixed 110- to 120-year old spruce-pinewood is situated between the slope and the River Gauja. An abandoned farmland is found to the North on the slope's upper edge. At that direction at a distance of 0.3 km a dormouse (probably juvenile) was caught by mouse-trap in late autumn, 1996 at farmstead "Klintis" (Pilāts 2003).

Site A and Site B are separated by a distance of 0.6 km. The side ravine with a small stream Draņķupīte, a tributary of the River Gauja, lies between the sites. The side ravine is covered by pine and birch woods of different age (mainly mature).

Site C is situated on a steep southern slope of Gauja valley at the farmstead "Ķūķi". The height of the slope is up to 40 m. Small outcrops of sandstone appear in lower parts on the bank of the River Gauja. The slope is covered by mixed, 130- to 150-year old forest where lime dominates and oak, birch, aspen and elm (*Ulmus glabra*) have minor proportion.

A farmyard is found on the slope's upper edge to the North. Historically the *G. glis* family has been found (Tauriņš 1982) on the opposite side of the River Gauja at a distance of approximately 1.3 km near the River Amata outflow.

Site D is situated on a flat area at a distance of 8.5 km from Gauja Valley at the former manor house "Ungurmuiža". The stand is 100- to 130-year old, almost solely oak. It is separated by a gravel road from a large park composed mainly of 200- to 300-year old oak.

Site E is situated on a steep southern slope of the Kazugrava valley at the farmstead "Mazceipi". The height of the slope is up to 40 m. The whole valley is situated in the area where dolomite is found on the slope almost everywhere. Important bat hibernation caves are situated nearby. The slope is overgrown mainly by mixed 60- to 110-year old forest with birch, aspen, oak, pine and spruce. The whole valley is surrounded by large crop fields and an abandoned meadow is situated in the valley bottom. Site E is located at a distance of 3 km from the Gauja valley. No *G. glis* records are known from the nearby sites D and E.

Methods

At all five study sites both wooden nest boxes and plastic nest tubes were used. Wooden nest boxes were composed of 25-mm thick planks, with internal dimensions 120 × 120 × 280 mm and with an entrance hole diameter of 40 mm. The design of the wooden nest boxes was similar to that introduced by Māris Čauns for small cavity-nesting birds in Latvia (for nest box design see Vilka 1999 and http://www.daba.gov.lv/public/files_uploaded/publikacijas/BRO_Putnu_burisi.pdf). The main feature of that nest box is the way how the box is fixed to the tree trunk: one side of the box is nailed to the trunk but the rest of the box is removable. This type of box is very good for inspecting the contents of the nestbox but it is not always easy to catch the dormouse found in the nestbox. In our study the entrance hole was in the side attached to the trunk, i.e. the nestbox entrance hole faced the tree trunk. The position of the nest box on the tree trunk was similar to that used for Common dormouse in Great Britain in 1982 (Morris et al. 1990) and is called a dormouse box.

Dormouse nest tubes, also designed in Great Britain (Morris, Temple 1998), are composed of two parts, a 300 mm long and 110 mm square plastic tube and a wooden tray.

Both wooden nest boxes and plastic nest tubes were positioned 3 to 4 m high on trees and arranged along transects throughout the wood. The distance between nest boxes and tubes varied from 15 - 20 m up to 60 - 70 m (Table 1).

In site A all nest boxes were arranged in two more or less parallel transects: one along the slope's lower border (and partly along the forest edge), the other along the slope's upper edge, mainly on a trees growing on the top. The height difference between both transects was approximately 20 m. The lower transect runs through broadleaf forest and the upper– mainly through the birch and pine stands at their border with broadleaf forest. This is the only site where indirect evidence (droppings and nest material) of *G. glis* presence were found in some bird nest boxes set up in the mid 1990s. At site A nest tubes were set up between nest boxes or in the same tree as the box.

In site B and C boxes were set up in the upper part of the slope, partly along its upper edge which was is also the forest edge. Also in site D the transect was established partly along the forest edge. In site E the transect was arranged on the slope.

In sites A and B nest boxes and nest tubes were checked irregularly: during May to July

Table 1. The number of nest boxes and tubes as well as the pattern of their setup (in brackets the increase of box number with additional 10 in site A in 2008 are shown)

Site	Number of nest boxes	tubes	Month and year of set up	Distance between boxes (m)	Placement	Coordinates
A	40 (50)	7	November 2004	15 - 45	in 2 lines	57°14'55.33" N 24°59'5.73" E
B	15	-	July 2006	20 - 70	in 1 line	57°15'1.49" N 25°0'7.84" E
C	13	2	July 2006	30 - 50	in 1 line	57°16'35.52" N 25°6'26.19" E
D	12	3	June 2006	20 - 30	in 1 line	57°21'41.74" N 25°5'34.01" E
E	14	3	August 2005	30 - 60	in 1 line	57°19'53.28" N 25°21'35.81" E
Total	94 (104)	15				

usually once in a month, during August to October in some years up to once in a week. In sites C, D and E they were checked mainly once a year. The total number of nest boxes and nest tubes monitored was 104 and 15 respectively (Table 1).

During autumn of 2005 to 2007 in sites A and B food items (mainly apples, plums, chestnuts, acorns and bred) were placed in some nest boxes and nest tubes to test if this might promote dormice to visit nest boxes and nest tubes.

All nest box occupants (mammals, birds and eusocial insects, as well as in most cases other invertebrates) and signs of their activity (mainly nests and droppings) were recorded. The point of reference for data processing was the use of each nest box during the dormouse activity season. If an animal or its activity was recorded at least once a season the nest box was regarded as used. Repeated records of the same kind in the same nest box were not summed. One reason for this was that nest boxes have different visitation times during the season. We consider that occurrence of use of each nest box during the whole season can better characterize the association of dormice with nest boxes. Juškaitis (2000) also suggested that the percentage of nest boxes occupied during the season reflects dormice abundance more accurately than the number of animals found during a single nest box visitation.

Since 2007 rat and mouse ear tags (AgnTho's AB, Sweden) were used to mark dormice individually. Altogether 28 dormice, of them 22 juveniles, were marked during a two-year period (of 41 individual including 35 juveniles captured during four years).

In site A the number of individuals marked in 2007 and recaptured during nest box checks in 2008 was assumed as the minimum number of dormice living in the particular area. As reference area (area trapped) for density calculations we used both the area `covered` by nest boxes (distinctive narrow belt of mixed broadleaf forest on a steep slope which is delimited from two longer sides by unsuitable habitats: pine/birch forest and meadow) and an additional boundary strip. We extended the belt of slope on both sides by 100 m taking in account that one of our dormouse males had moved between nest boxes at a distance of more than 100 m; in Germany the medium diameter of *G. glis* home

range was estimated to be 200 m (Gaisler et al. 1977) and in Poland species home range size varied from 0.55 ha to 7.0 ha (Ściński, Borowski 2008). The size of the whole reference area was 3 ha.

We estimated population abundance as the number of individuals per 100 nest boxes, as in the study of Kryštufek et al. (2003). This might be regarded as a derivative of another abundance index – the number of animals caught per 100 trap-nights which is a standard method for estimating rodent number and was used in a study of *G. glis* by Ivashkina (2006).

Results

Nestbox occupation

Up to present dormice have been recorded only in two (site A and B) of the five study areas. In a third study area (site C), a nest which might be made by *G. glis* was found in one nest box only once during the three year period. Neither dormice, nor their activity signs were recorded in sites D and E.

Besides the *G. glis*, the wooden nest boxes were occupied also by birds (*Parus major* and *Ficedula hypoleuca*), Yellow-necked mouse (*Apodemus flavicolis*), and eusocial insects (Vespidae) (Fig. 2).

Both the number of nest boxes used and number of *G. glis* found in boxes increased during the study period (Fig. 3). By the autumn 2008, the proportion of nest boxes visited by *G. glis* at least once during the four year period reached 55 %. Mainly the same nest boxes were visited by dormice both during one season and from year to year (Fig. 4). Some nest boxes most probably were visited by *G. glis* temporally – there either only droppings or only nest material e.g. leaves, but not well developed dens. In three cases we recorded two dormice older than one year sharing the same nest box.

Dormice were recorded in the nest boxes starting from late May until mid October. The older (reproducing) adults were recorded earlier and juveniles later in the season. At least a two week difference was observed between 2007 and 2008 in the date of the season's latest record of *G. glis* activity.

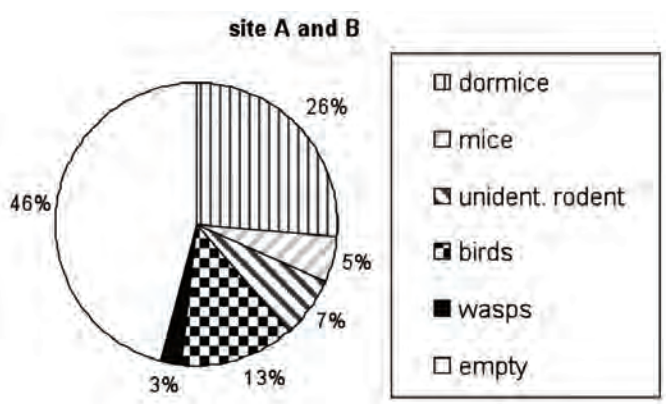

Fig. 2. Occupation rate of different nest-box inhabitants in 2008. Data from the two sites A and B were pooled in order to increase sample size.

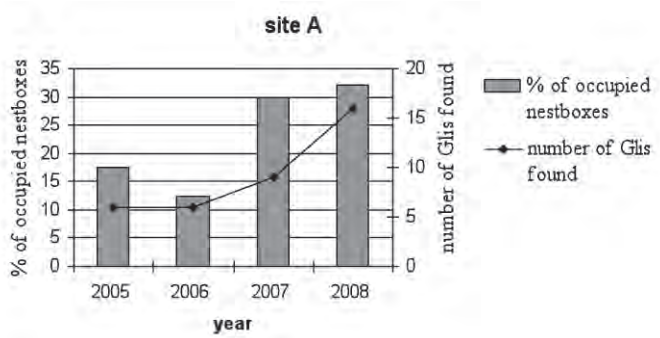

Fig. 3. Abundance dynamics of *Glis glis* according to nest boxes occupied and number of dormice found in nest boxes.

In site A a significant difference (chi-square = 8.29, p = 0.004) was found in nest box occupation rate when compared according to the topography and habitat where nest boxes were set up. During the study period 16 of the 23 nest boxes set up in broadleaf forest at the foot of slope were visited by *G. glis*, while three of 17 nest boxes were occupied at the upper edge of the valley slope (Fig. 4). Gestating females were recorded only in the lower transect of nest boxes.

Reproduction and abundance

Altogether 27 individuals of *G. glis* including one adult and five litters of three (one case), four (two cases) and five (two cases) juveniles were recorded in site A during the four year period (Fig. 5) and 14 individuals including two litters of four and six juveniles in site B during the two year period. In 2006 and 2007 we did not find any dormouse born in the previous year, in 2008 we recorded five in site A and three in site B. During late season 2007 we recorded also four juveniles (two per each site) born not in nest boxes. In 2006 and 2007 we did not find any dormouse born in the previous year, in 2008 five in site A and four in site B were recorded.

During 2008 we recorded 16 individuals (eight juveniles) of *G. glis* in site A (Fig. 5) and 10 individuals (four juveniles) in site B. Among them 12 were recaptured, i.e. dormice marked in the previous year. This means that the relative abundance of animals that survived the winter 2007/2008 was 18 individuals per 100 nest boxes, when data from both sites are pooled. For site A this is equivalent to two individuals per 1 ha at the beginning of season.

Discussion

Use of nest boxes and nest tubes

In the study plots A and B, Fat dormice not only occupied wooden nest boxes readily but in site A used them also for breeding as early as the first summer (Fig. 4, 5). Animal recapture data indicate that adult dormice, male and female, can use several nest boxes in turn, but they prefer to occupy one and the same nest box. For example, we recorded one adult male that in mid-season changed between two nest boxes located at a distance of 140 m. A similar nest box occupation pattern was demonstrated also in other G. glis

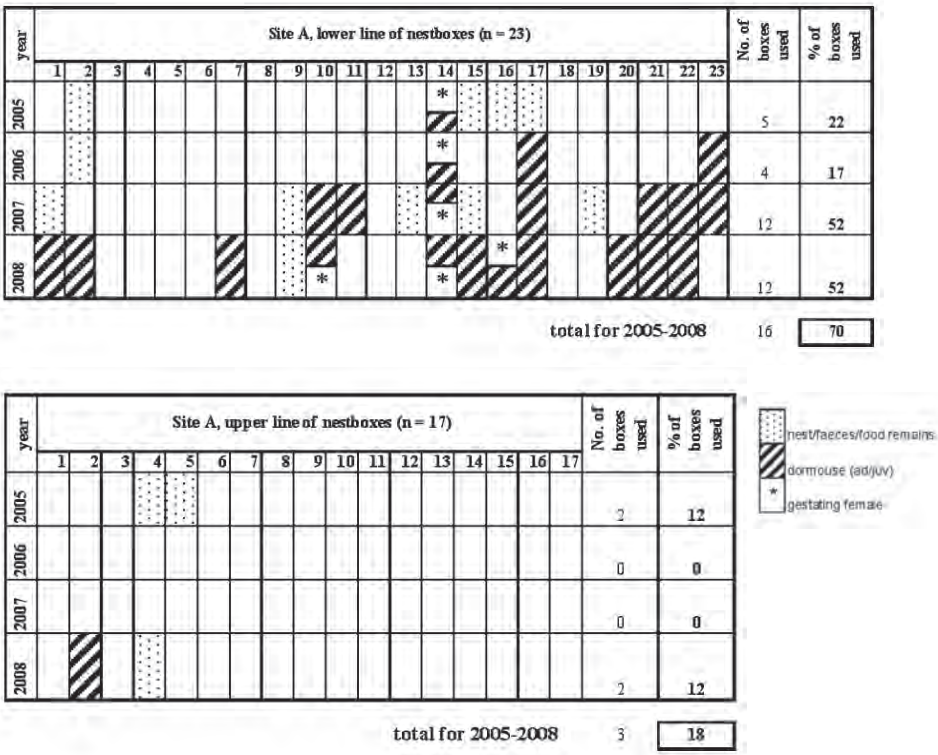

Fig. 4. Occupancy of nest boxes by *Glis glis* depending on nest box locality.

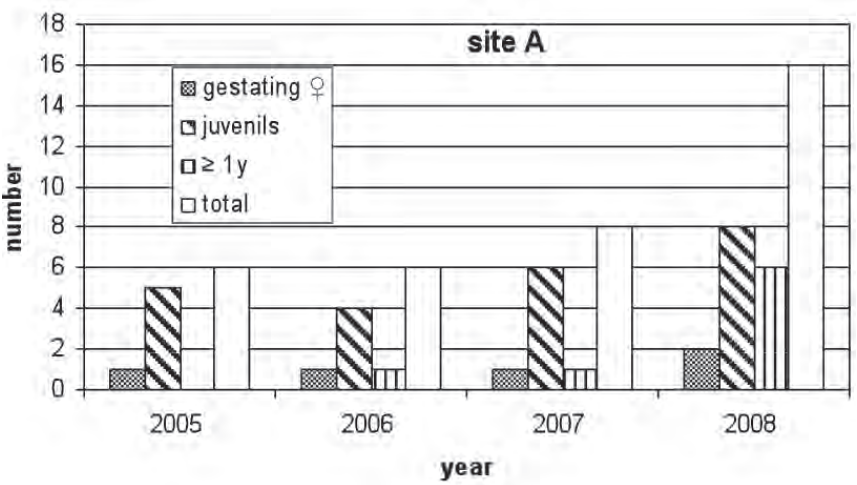

Fig. 5. Yearly number of *Glis glis* of different categories caught in site A.

studies (see Schlund et al. 1997; Rossolimo et al. 2001). Nest sharing by several, even up to six individuals have been reported (e.g. Airapetyants 1983) as a typical feature of Fat dormouse nestbox use, but we recorded only a few cases and up to two adults in one nest box. Similarly, we did not observe any competition/predation on bird nests in dormouse boxes, although elsewhere this is rather common (see Juškaitis 2006).

Although the greater part of nest boxes remained unvisited by *G. glis* during the study period, as in other dormouse studies (e.g. Morris et al. 1990; Schlund et al. 1997; Bako, Hecker 2006; Juškaitis 2008), nest boxes proved to be a good tool for dormouse monitoring, including marginal populations. However, plastic nest tubes were visited only in cases when food items were placed inside (Dzalba 2007). Avoidance of plastic nest tubes by *G. glis* was observed also in Hungary (Bako, Hecker 2006). We found that food items in wooden nest boxes did not promote their visitation, i.e. unvisited nest boxes kept this `status` also after food was put in. There was also no evidence that food in nest boxes would attract other dormice; i.e. animals which were not recorded yet in the area.

Different results on wooden nest box occupation were obtained in the study plot established in the Daugava valley. Nest boxes set up there in a forest evidently remained unoccupied as during 2000 to 2002 and later. In 2006 and 2008 the authors checked 20 nest boxes left there and found no evidence on dormouse presence. The reasons for *G. glis* absence in these nest boxes is most likely habitat suitability.

Distribution and dispersal of the Fat dormouse in Gauja National Park

This study proved the usefulness of dormouse nest boxes in survey of *G. glis* in the marginal population of Gauja National Park. The lack of dormice in many nest boxes can be considered as indicator for *G. glis* absence in an area if a sufficient number of nest boxes are established. Fifteen nest boxes set up in site B show that this number is sufficient to determine the presence of dormice. In sites D and E the Fat dormouse were probably absent even although suitable habitat was available. This indicates that the distribution of *G. glis* is restricted to the Gauja and Amata valleys (see also Pilāts 2003) despite suitable habitat availability in other parts of the National Park. Dormice absence in sites D and E might be explained by isolation of suitable habitats; they are too far one from other for dispersal of Fat dormice.

Our study is consistent with earlier records of *G. glis* dispersal pattern. In the autumn of 1995 and 1996 two individuals were trapped by mouse-traps in buildings of two farms situated near the study plots A and B (Pilāts 2003). The age of trapped animals is not known but according to the description given they probably were juveniles. The place of their birth is not known but obviously it was in the Gauja valley near sites A and B. There is no adequate habitat in between the Valley and both farms and in the other direction. The shortest distance between the valley and most distant farm is 1.3 km. Most probably both animals move within the forested areas as the Fat dormouse is considered as animal with pronounced arboreal lifestyle (Airapetyants 1983; Morris 2004) and avoids moving over open areas (Bieber 1995; Ivashkina 2006). Although *G. glis* can cross small treeless areas (at least up to 46 m; Bieber 1995) and disperse for even up to 2.8 km in a forested area (Rossolimo et al. 2001), this species is likely not able to colonize/recolonize the mosaic landscape in Gauja National Park. It should be also taken into account that formerly (before 100 - 300 years) the landscape in Gauja National Park was even more open, i.e. less forested (Pilāts 2007b) than today. The records of trapped individuals in farms confirms

another well-known habit of the *G. glis* – they readily enter buildings (Airapetyants 1983; Rossolimo et al. 2001; Morris 2004). However, cases are unique – *G. glis* was not seen in either farm previously nor afterwards. Fat dormouse in general is an unfamiliar species even for people living very close to *G. glis* habitats, both in the Gauja and Daugava valleys (see also Pilāts 2003). Thus these two records are an indirect indication on either low *G. glis* abundance or restricted distribution within the Gauja valley.

Habitat preferences

In the Gauja valley a significant and even surprising difference was observed in nest box occupation in association with topography and/or habitat quality (Fig. 4). The distance between the lower and upper transect (site A) is smaller than the distance between two nest boxes used by the one and same dormouse: 20 to 50 m and 140 m, respectively. Therefore, difference in topography is unreliable reason for the different nest box occupation rate, and most probably related to habitat quality. *G. glis* ignored not only nest boxes set up only some meters outside the broadleaf forest but even those situated in a particular habitat. It seems that the broadleaf forest in site A differs in quality for dormice, between the lower part and upper part of the slope. The lower part, mainly forest edge with more dense understorey and old oaks with big and broad branches (Dzalba 2007). This is the preferred type of trees for *G. glis* (Storch 1978; Juškaitis, Šiožinytė 2008). The upper part of the slope supports only a few thin oaks. Consequently, in site A dormice most likely inhabit a very narrow strip of forest where old oaks grow almost in a row. Also the study of Schlund et al. (1997) shows that Fat dormouse has clumped dispersion within one and the same habitat type.

Habitat quality might be reason for *G. glis* absence in nest boxes set up in the Daugava valley: there was no oak in the forest studied using nest boxes.

Additional studies are necessary to evaluate habitat quality within all our study sites to allow comparison with habitats in other *G. glis* localities including other parts of the species range.

Relative abundance

The abundance of *G. glis* varies from 0.6 to 50 individuals per ha (Rossolimo et al. 2001) depending of location within its range and habitats occupied. Our preliminary results suggest a density of two individuals per ha for site A, which is comparable with densities in other northern parts of the *G. glis* range, e.g., one to 11 individuals per ha in Poland (Jurczyszyn 1995), 2.3 to 6 individuals per ha in Germany (Schlund et al. 1997), 0.6 to 4.1 individuals per ha in England (Burgess et al. 2003).

However, density estimation may be biased for site A as the distribution of *G. glis* within the forest seems to be rather linear. Estimation of abundance by number of dormice per 100 nest boxes might be a better index. In 2008 the density of marked adults (animals older than one year) was 12 individuals per 100 nest boxes if we take in account both nestbox transects and even 20 individuals per 100 nest boxes when only nest boxes of the lower transect is considered. These density values are comparable even with densities estimated in southern parts of *G. glis* range, e.g. in Slovenia 23.5 and 25.8 individuals per 100 nest boxes in 1999 and 2000, respectively (Kryštufek et al. 2003).

We conclude that *G. glis* in the Gauja valley might be rather abundant very locally but is restricted to particular habitats and therefore is rare if a wider teritory is considered.

Dormice nest boxes proved to be useful tool for *G. glis* studies also in species in the northern part of its range but more data are necessary to verify most of our preliminary results and the ideas discussed above.

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Morphological variability and genetic diversity within Latvian and Swedish sweet cherry collections

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Abstract

Forty nine sweet cherry accessions at the Latvia State Institute of Fruit-Growing (Dobele) and ninety one at the Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences (SLU-Balsgård) were used for the analysis of morphological variability and genetic diversity by means of multivariate statistic analyses. Both applied statistical approaches (cluster and principal component analysis) showed adequate grouping of accessions according to morphological characterization. Multivariate analysis showed the most important traits for sweet cherry accession grouping were tree architecture and fruit traits, which should therefore be considered in further sweet cherry genetic resource characterization. Multiple parameter analysis of sweet cherry traits increased the value of phenotypical data and created the basis for common analysis of phenotypical and genetical data.

Key words: genetic diversity, germplasm, multivariate statistics, *Prunus avium*.

Introduction

Sweet cherry (*Prunus avium* L.) is one of the most important fruit crops both in Latvia and Sweden (Trajkovski, Andersson 1996; Ruisa 1998). Many varieties of sweet cherry are found in small gardens, and these have growing commercial importance. Therefore sweet cherry is important in breeding programs and plant genetic resource (PGR) activities in both countries. Since 1996, coordinated breeding and PGR activities were established for evaluation and characterization of *Prunus* genetic resources, e.g. for sweet cherry.

The Latvia State Institute of Fruit-Growing (LIFG) and the Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences (SLU-Balsgård) hold wide and valuable genetic resource collections of fruit crops, including sweet cherry (Trajkovski 1996; Rashal, Lacis 1999). The origin of the collections and their variability differ because they were formed by different strategies. The largest part of the collection in the LIFG are local sweet cherry accessions acquired by Pēteris Upītis, famous Latvian horticulturist (Blukmanis et al. 1997). This material includes both samples from expeditions (mostly wild growing trees and landraces), as well as hybrids from breeding programs. Unfortunately, detailed information on breeding and

collection sources is not available. It is only known that mainly local plant material was used for both selection of advanced varieties and for hybridization (Ruisa 1998). Varieties and advanced hybrids created by other Latvian breeders using old cultivars from the Western Europe as well as sweet cherry varieties developed in the former USSR (Belarus, Russia, and Ukraine) are also included in the LIFG collection.

The cherry germplasm collection at SLU-Balsgård is the result of a long term breeding programme. The Swedish germplasm has been built through collaboration with plant breeders from the entire northern temperate zone, and includes (i) local (Scandinavian) material, (ii) introduced advanced cultivars from Western Europe and North America and (iii) wide diversity of selections developed at SLU-Balsgård during the breeding process (Trajkovski 1996).

The importance of genetic diversity in breeding is obvious. Therefore the recognition and measurement of such diversity and its nature and magnitude are beneficial or even crucial to a breeding programme. The availability and informative value of plant germplasm are becoming more and more important for the future preservation and sustainable use of genetic resources. Evaluation and characterization as well as estimation of diversity have been performed for various sweet cherry collections (Christensen 1970; Christensen 1977; Hjalmarsson, Ortiz 2000; Rodrigues et al. 2008).

Modern developments in biology allow wide utilisation of molecular markers in PGR research (IPGRI 1997). Unfortunately, the available molecular markers mostly cover only a small part of a target genome. In general, the developed markers mostly are random, not associated with a particular trait. Investigations of germplasm should include also morphological and agronomical characterization of traits having breeding value. The main problem is detection of complex traits, like winterhardiness, which are difficult to detect by a single gene or random marker analysis. Therefore a combined approach is the most preferable for description of genetic resources (Dias et al. 2008; Smýkal et al. 2008).

Characterization of plant genetic resources (PGR) usually contains a wide range of data, on both qualitative and quantitative traits. Such data are generally large and multivariate with a considerable number of descriptors measured on each of many accessions. Analysis of diverse data is complicated, especially if it is necessary to evaluate not only single trait, but complex traits such as adaptivity, reproductivity or response to environmental or treatment conditions.

The most widely used multidimensional analysis methods in PGR characterization are Principal Component Analysis (PCA) and cluster analysis. The advantages of PCA in horticultural studies have been widely discussed (e.g., Broschat 1979; Iezzoni, Pritts 1991; Lacis, Rashal 2000; Lacis, Rashal 2001). PCA allows to evaluate multicollinear data and to determine the traits most suitable for classification (Iezzoni, Pritts 1991). Cluster analysis allows to analyze simultaneously both quantitative and qualitative traits, and each entry is treated as an individual entity of equal weight. The most appropriate approach for classification purposes is the group average clustering method (Peeters, Martinelli 1989). PCA and cluster analysis based on descriptive data on genetic resources can be useful in classifying accessions in a germplasm collection. This is the only possibility to classify accessions with unknown origin, as in case of the Latvian material.

The goal of this work was to characterize and evaluate Latvian and Swedish sweet cherry genetic resources using multivariate statistic analysis. Assessment of genetic diversity based on morphological data and determination of the variation patterns in

both sweet cherries genetic resources collections were performed. The best accessions for breeding and PGR maintenance were selected.

Materials and methods

Plant material

Morphological and agronomical characterization were performed on a representative sample of both collections: 49 (LIFG) and 91 (SLU-Balsgård) sweet cherry accessions.

Characterization and evaluation

Evaluation of sweet cherry accessions in LIFG and SLU-Balsgård was carried out according to the methods accepted in each institution based on morphology and phenology, fruit quality, disease resistance and hardiness data.

Survey at LIFG was made in July - August for three successive years using 26 leaf and fruit traits, phenological characteristics, measures of susceptibility to diseases and hardiness (Table 1). Each year 25 random samples were evaluated (five samples per tree with five trees per accession). Leaf and fruit samples were collected according to Yushev (1975; 1977). Initially data were recorded as results of direct measurements. the acquired data were processed by descriptive statistics to determine data structure and variability for each year separately. A scoring system was developed for measurement. Scores (10-point scale) were estimated for each year separately and average values were determined for each accession.

The collection at the SLU-Balsgård was evaluated using 19 traits (Table 2) for three successive years. The characterization descriptor lists were based on UPOV (UPOV 1995) and IPGRI (IPGRI 1985) lists, supplemented by local reference cultivars. The measurements were expressed as points, based on IPGRI/UPOV rules.

Statistical analysis

Evaluation and characterization of the data from the collections at LIFG and SLU-Balsgård were analysed separately due to different growing conditions and rootstocks. Statistical analysis was performed using average values from three-year measurements.

Principal Component Analysis (PCA) and cluster analysis was used to evaluate relationships among accessions. Cluster analysis was performed using the Group Average Method and the Squared Euclidean distance measurement. Data analysis was performed using the Multivariate Statistics modules of Statgraphics for Windows Version 3.3 (Users Guide 1994).

Results

Analysis of Latvian plant material

The collection at the LIFG was characterized mostly by accessions with early to medium flowering season. The Latvian accessions had low fruit quality (average size – 4.29 g, which fits to the small fruit size group), and a high proportion of fruits were yellow with colourless juice. However, they had high resistance to diseases (7.44 points of 10 on average) and also high winterhardiness (7.91 points of 10 on average).

PCA was conducted on all of 26 traits (Table 1). In total, 74.17 % of the observed

Table 1. Component weights in Principal Component Analysis using morphological traits of accessions from the Latvia State Institute of Fruit-Growing sweet cherry collection

	Principal Components							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Eigenvalues	5.1397	2.9088	2.5862	2.3735	2.1595	1.6033	1.3260	1.1873
Percent of variance	19.768	11.188	9.947	9.129	8.306	6.166	5.100	4.566
Factors / traits								
Fruit thickness	0.3271	–0.0059	–0.2695	0.0586	–0.1304	–0.0777	–0.1383	–0.1099
Fruit length	0.3751	0.0764	0.0819	0.0022	–0.0049	0.1011	–0.0096	0.0813
Fruit width	0.3796	0.1182	–0.0262	–0.0755	–0.1738	0.0046	0.0812	0.1142
Fruit weight	0.3932	0.0383	–0.1339	0.0750	–0.1271	–0.0212	0.0394	0.0114
Fruit skin colour	0.1166	0.2012	–0.1813	0.1850	0.0352	–0.3426	–0.2279	0.2054
Soluble solids	–0.1099	–0.0324	–0.3416	–0.1579	0.1038	–0.1960	0.2482	0.2797
Stone thickness	0.1481	–0.2939	–0.2489	0.2494	0.0603	–0.1164	–0.0366	–0.2753
Stone length	0.2642	–0.1424	0.2982	0.0357	0.1156	0.2593	0.1455	–0.0841
Stone weight	0.2464	–0.0767	–0.2491	0.1977	0.0656	0.1775	0.0882	–0.0477
Stone width	0.2989	–0.0619	–0.0268	–0.0283	–0.1759	0.0145	0.2970	0.1281
Length of fruit stalk	0.0636	0.0626	0.1169	0.2572	0.4061	–0.2725	0.0007	–0.0438
Yield	–0.0335	–0.0009	–0.1666	–0.2289	0.0397	0.2724	–0.2149	0.5036
Harvest maturity	–0.1829	0.0398	–0.1367	0.2537	0.0879	0.3012	–0.1485	0.0252
Leaf blade area	0.1753	0.2423	0.1849	–0.2457	0.2254	–0.1633	–0.0803	–0.0260
Leaf length	0.2305	–0.0662	0.0689	–0.3746	0.0937	–0.1166	–0.1180	–0.2536
Leaf blade width	0.0658	–0.0339	–0.1342	–0.3228	0.4158	–0.2034	–0.1135	–0.1281
Leaf vein angle (apex)	0.0249	0.4105	0.0132	0.2048	0.0191	–0.1068	0.1611	0.2009
Leaf vein angle (middle)	–0.0059	–0.2622	0.0904	0.4060	–0.0792	–0.2765	–0.0807	0.2361
Leaf vein angle (base)	0.0265	–0.4787	–0.0301	0.0179	–0.0678	0.0267	–0.3460	–0.0369
Leaf serration per cm	–0.0462	–0.1849	–0.0265	–0.1311	–0.2935	–0.3209	0.3500	0.1174
Petiole length	0.0830	–0.3697	0.2838	0.0433	0.1804	–0.1240	–0.0166	0.2623
Petiole thickness	0.0262	–0.1039	–0.1489	0.0456	0.4753	–0.0546	0.1480	0.2167
Gland number on the petiole	–0.0399	–0.2839	–0.2495	–0.3023	–0.0246	0.0331	0.1120	0.1375
Gland number on the basal leaf edge	0.0425	–0.1106	0.4967	–0.0522	–0.0709	–0.0881	0.0086	0.2274
Susceptibility to diseases	0.2071	–0.0107	0.0128	0.0356	0.1979	0.3629	–0.1236	0.2930
Winterhardiness	–0.0442	–0.1004	–0.0010	0.1066	0.2551	0.2091	0.5607	–0.1587

variability was explained by the first eight components (eigenvalues for each was larger than 1). The highest loadings on PC1 were shown by fruit traits (weight, length, width, thickness) and on PC2 by leaf traits (length, vein angle on leaf base and stone thickness). PC3 was associated with stone traits: length, weight, content of soluble solids. Other agronomicaly important traits were found on PC6 (disease resistance, harvest maturity), PC7 (winterhardiness) and PC8 (yield). Therefore grouping along principal components were performed to identify the best grouping variables and to select distinct accessions.

Thus, PCA identified the following accessions with distinct traits: Kompaktnaya and PU-14646 – small stone, the largest number of glands on leaf basal edge, according to the scores on the third and fourth Principal Components (PC3-PC4); PU-14684 – large leaves (PC3-PC4); Dogan's Gelbe Knorpelkirsche and PU-14419 – large yield (PC6-PC8); Leningradskaya Czornaya – very small leaves, low yield (PC3-PC8); PU-300 – early harvest maturity (PC2-PC6); Agris, PU-18619 and PU-20923 – very short fruit stalk (PC5-PC6); PU-14419 and AM 24-10-22 – very good winterhardiness (PC7-PC8). Cultivar Aija showed a distinct position in all combinations of PC2, represented by leaf shape measurements.

Cluster analysis formed four main clusters of accessions (Fig. 1). Finer grouping inside some of the main clusters was also found; therefore some were divided into sub-clusters to better describe the variability.

The variability of traits among clusters was relatively low; therefore most of the traits had little informativity in the cluster discrimination (Table 3). The highest variability found was for leaf blade area, fruit length, fruit width, fruit weight, fruit stalk length, winterhardiness, resistance to diseases, harvest maturity season and fruit yield.

Since there is no pedigree information for the most of accessions in the Latvian sweet cherry collection, it is not possible to make any cluster interpretation based on the origin.

Cluster 1 was characterized, in general, by accessions with large fruits, high resistance to diseases, and large leaves. This cluster could be divided into two sub-clusters. Accessions in the sub-cluster 1a had large leaves and fruits. The sub-cluster 1b was characterized by the largest fruits in the whole collection and a specific yellow vermilion fruit skin color not observed in other groups.

Cluster 2 was the largest group, represented by a diversity of accessions with average fruit size, late flowering and maturing season, and good fruit yield. Sub-cluster 2a was distinct due to a high content of soluble solids; sub-cluster 2b – wide leaf blade, high fruit yield; sub-cluster 2c – smal leaves, late flowering and harvest maturity season; high number of glands on petiole; 2d – short petiole, small fruit size, late maturing; 2e – the highest content of soluble solids among all clusters, the highest yield, small stone size. The sub-cluster 2f contained only one accession – cultivar Agris, which differed by having the smallest leaf blade size among all groups, late flowering and high yield.

Cluster 3 was characterised by a short, wide leaf blade with low serration per cm, long, thin petiole. Accessions of this cluster had the smallest fruit size of all accessions and low content of soluble solids.

Cluster 4 consisted only from one cultivar (Leningradskaya Czornaya), which was distinct because of very small leaf blade size.

Analysis of Swedish plant material

The collection at SLU was characterised by accessions with medium to strong tree vigour,

Table 2. Component weights in Principal Component Analysis using morphological traits of accessions from the Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences sweet cherry collection. * - average measurement of susceptibility to *Pseudomonas syringae*, *Monilia laxa* and *Monilia fructigena*

		Principal Components						
		1	2	3	4	5	6	7
Eigenvalues		2.8590	2.4014	1.9909	1.6272	1.4890	1.1848	1.0728
Percent of variance		15.047	12.639	10.478	8.564	7.837	6.236	5.646
Factors / traits	Tree vigour	–0.1743	0.4572	–0.0018	0.2744	0.0218	0.1500	0.1097
	Tree habit	0.1589	–0.4976	–0.0228	–0.1206	0.0018	–0.2979	0.1040
	Tree type	0.0253	0.1885	0.0628	–0.1557	–0.6214	–0.2104	–0.0955
	Density of the head	–0.0960	0.4802	0.1841	0.1559	–0.0925	–0.1740	0.1448
	Season of flowering	-0.0907	–0.0544	–0.4628	–0.2325	–0.1296	–0.1113	–0.0714
	Fruit shape	0.2902	0.1119	0.2457	–0.0649	0.4027	–0.2975	–0.0228
	Fruit size	–0.3094	–0.0567	–0.2606	0.2284	0.1486	–0.3403	0.2606
	Fruit firmness	–0.4176	0.0932	0.1921	–0.2944	–0.1544	–0.1465	–0.0789
	Fruit juiciness	0.4266	0.0175	–0.1646	0.3763	–0.0015	0.0859	0.0115
	Fruit separation from stalk	0.2498	0.1457	–0.1796	0.2573	–0.1136	–0.1123	–0.3773
	Fruit skin colour	0.3197	0.1933	–0.2398	–0.4172	–0.0007	–0.0257	0.0327
	Juice colour	0.2708	0.2769	–0.3278	–0.2518	–0.1636	0.0146	0.1864
	Susceptibility to fruit cracking	–0.2312	0.1027	–0.1573	–0.3258	0.3046	0.0540	0.1437
	Length of fruit stalk	0.2268	0.0495	0.1930	0.0031	–0.0598	–0.3025	0.6862
	Stone shape	0.0758	0.2474	0.1221	–0.1492	0.3636	–0.4354	–0.4261
	Stone relative size in comparison with fruit	0.1955	0.0425	0.3911	–0.2995	0.0608	0.4775	0.0585
	Susceptibility to diseases *	–0.0568	0.1958	–0.3580	–0.0342	0.3286	0.2075	0.1119

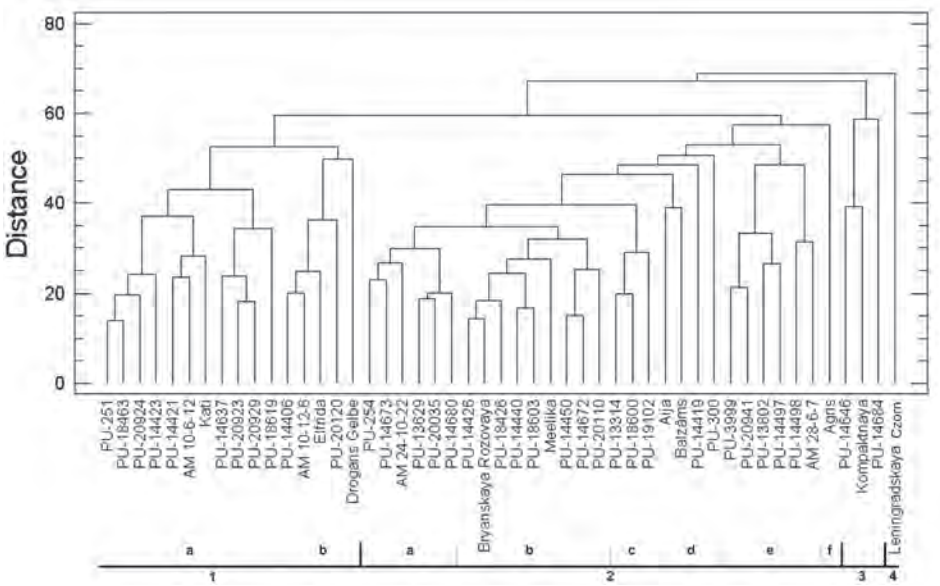

Fig. 1. Hierarchical analysis dendrogram obtained by Group Average Method (Squared Euclidean) using morphological traits of sweet cherry accessions of the Latvia State Institute of Fruit-Growing.

mostly semi-upright tree habit. The collection in general was characterized by medium to late season of flowering. Sweet cherry accessions in the Swedish collection had large fruits (with an average size of 6.36 points, which corresponds to about 7 g).

Swedish accessions in general had medium fruit firmness with good separation from a stalk. The collection at SLU contained mostly red and dark red colored fruits. Swedish sweet cherry genetic resources contained mostly accessions with intermediate susceptibility to diseases (4.30 points). Susceptibility to *Pseudomonas syringae* was an exception (5.57 points in average).

PCA analyses were performed on 19 measured traits (Table 2). Seven components with eigenvalues larger than 1.0 were extracted. They described 66.5 % of the variability of the original traits. The highest loadings on PC1 were by factors fruit size, shape, firmness and juiciness, on PC2 – tree traits. PC3 was associated with mostly season of flowering, fruit juice colour, and disease resistance. PC1 and PC3 were chosen to display accession variability pattern in the collection, as they represented the most important breeding criteria: fruit quality and disease resistance. Thise traits are used for selection of accessions with outstanding value in breeding.

PCA discriminated several distinct accessions based on particular traits. The following accessions were identified as distinct: Kaiser Franz and BPr 37239 – high fruit firmness, low tree vigour, low density of head, according to scores on the first and second Principal Components (PC1-PC2); Heinrich Riesen – high fruit firmness (PC1); BPr 36781 and 4570D – upright tree habit, low tree vigour (PC1); Regina – spur type of tree (PC5) in combination with all other PCs, large fruit size (PC1-PC6 and PC2-PC6), dark fruit skin colour, low susceptibility to fruit cracking (PC2-PC4); 13-116-76 – soft, juice fruits (PC1);

Table 3. Characterization of sweet cherry accession clusters in the Latvia State Institute of Fruit-Growing sweet cherry collection. * - cluster and sub-cluster numbers according results of cluster analysis presented in the Fig. 1

Trait / cluster	Average value per cluster and sub-cluster*												Total average	Variance
	1a	1b	1	2a	2b	2c	2d	2e	2f	2	3	4		
Fruit length	3.8	4.4	4.0	2.6	2.6	2.5	2.1	2.9	2.8	2.5	3.8	2.2	3.1	0.803
Fruit skin colour	1.0	3.4	1.8	1.0	1.7	1.6	1.0	2.0	1.0	1.4	1.0	1.0	1.5	0.131
Fruit thickness	4.8	5.6	5.1	3.8	4.1	4.1	3.1	4.4	3.3	3.9	2.4	3.2	4.1	1.291
Fruit weight	4.9	5.8	5.2	3.2	3.1	3.4	2.3	3.9	3.2	3.1	2.1	2.6	3.7	1.880
Fruit width	6.3	6.7	6.4	5.2	4.7	4.8	4.7	5.5	5.7	4.9	4.9	3.6	5.4	1.333
Gland number on the basal leaf edge	1.3	1.2	1.2	1.2	1.2	1.2	1.2	1.1	1.1	1.2	1.5	1.2	1.2	0.024
Gland number on the petiole	1.3	1.2	1.3	1.5	1.3	1.5	1.3	1.3	1.5	1.4	1.2	1.2	1.3	0.008
Harvest maturity	4.1	5.8	4.6	6.3	5.9	6.4	6.6	6.0	9.0	6.3	5.7	7.0	5.7	1.026
Leaf blade area	3.5	4.1	3.7	2.9	3.5	2.6	3.8	3.7	1.7	3.2	4.0	2.7	3.4	0.344
Leaf blade width	4.6	4.8	4.7	4.8	5.1	4.5	4.6	4.9	3.9	4.7	4.9	4.8	4.7	0.007
Leaf length	6.5	6.2	6.4	5.8	6.2	5.8	6.0	6.3	5.5	6.0	6.1	5.6	6.1	0.109
Leaf serration per cm	2.0	1.8	1.9	2.0	1.9	2.0	1.9	2.0	1.9	2.0	1.9	2.0	1.9	0.003
Leaf vein angle (apex)	4.8	5.9	5.2	4.3	4.6	4.8	5.7	4.9	4.9	4.8	4.8	5.5	4.9	0.114
Leaf vein angle (base)	5.4	4.9	5.2	5.4	5.7	6.0	4.8	4.6	6.1	5.5	5.5	5.2	5.4	0.029
Leaf vein angle (middle)	5.6	5.3	5.5	5.2	6.2	6.6	4.3	4.3	7.2	5.7	5.8	7.6	5.7	0.936
Length of fruit stalk	4.1	5.4	4.5	4.1	4.7	4.0	3.5	4.4	3.0	4.1	5.4	6.2	4.4	0.846
Petiole length	4.9	4.1	4.7	4.2	5.3	4.9	3.3	2.9	3.8	4.4	5.6	5.0	4.6	0.256
Petiole thickness	4.0	4.0	4.0	4.1	4.4	3.7	3.7	3.7	4.0	4.0	3.9	4.0	4.0	0.002
Soluble solids	5.0	5.4	5.2	6.5	5.8	6.2	6.2	6.5	3.6	6.1	4.0	5.9	5.6	0.900
Stone length	5.8	4.4	5.3	3.4	4.0	3.9	2.8	2.8	4.4	3.6	5.6	4.4	4.3	0.833
Stone thickness	5.1	4.0	4.7	4.2	5.4	5.0	2.8	3.7	3.1	4.4	1.9	6.4	4.4	3.511
Stone weight	4.6	4.2	4.5	3.9	4.0	3.9	2.3	3.7	3.4	3.6	2.9	3.1	3.9	0.488
Stone width	5.8	4.8	5.5	4.4	3.9	3.8	3.6	3.5	5.8	3.9	3.3	2.5	4.4	1.643
Susceptibility to diseases	7.8	9.0	8.2	5.8	7.7	7.5	6.5	6.2	10	7.0	8.0	5.0	7.4	2.084
Winterhardiness	8.0	7.7	7.9	8.6	7.8	8.0	8.0	4.2	5.5	7.7	7.6	9.0	7.9	0.409
Yield	6.5	6.8	6.6	7.2	7.6	7.1	7.4	8.0	8.0	7.4	7.0	2.5	7.0	5.174

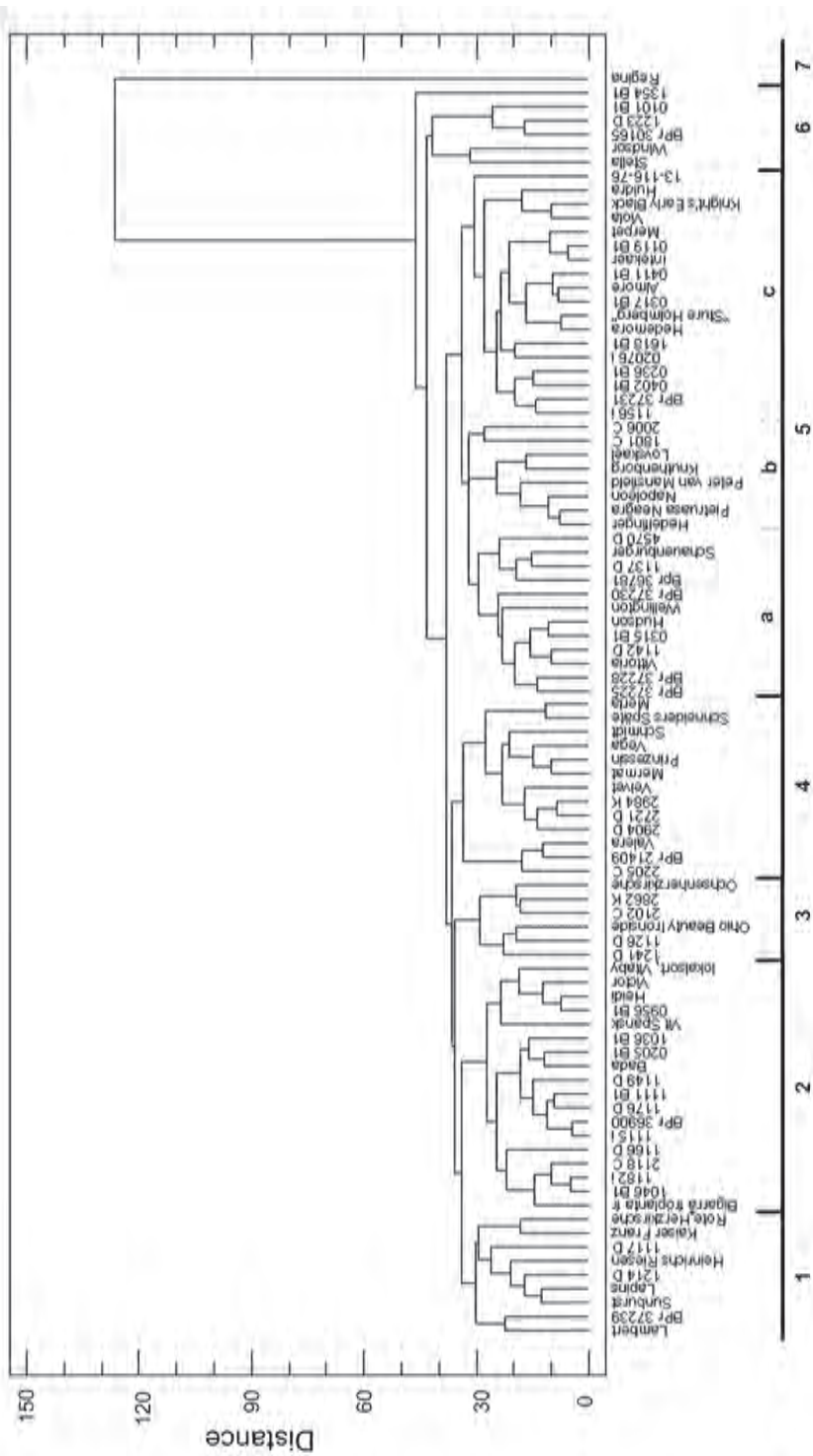

Fig. 2. Hierarchical analysis dendrogram obtained by Group Average Method (Squared Euclidean) by using morphological traits of the of sweet cherry accessions of the Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences sweet cherry accessions.

Table 4. Characterization of sweet cherry accession clusters in the Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences sweet cherry collection. * - cluster and sub-cluster numbers according results of cluster analysis represented in the Fig. 3. ** - average measurement of susceptibility to *Pseudomonas syringae*, *Monilia laxa* and *Monilia fructigena*

Trait / cluster	Average value per cluster and sub-cluster*										Total average	Variance
	1	2	3	4	5	5a	5b	5c	6	7		
Density of the head	5.7	4.3	6.7	4.8	4.5	4.0	3.3	5.3	5.3	7.0	5.09	1.352
Fruit firmness	6.8	4.8	5.3	5.0	4.0	5.2	3.8	3.3	6.0	7.0	5.11	1.507
Fruit juiciness	3.9	5.2	5.0	5.3	5.8	5.0	5.8	6.4	4.7	5.0	5.21	0.489
Fruit separation from stalk	4.6	6.3	6.3	6.2	6.5	6.0	6.8	6.8	6.7	7.0	6.32	0.472
Fruit shape	1.9	3.1	4.3	2.6	3.7	3.3	4.5	3.6	2.3	3.0	3.23	0.694
Fruit size	7.2	6.7	7.0	7.3	5.4	5.0	6.3	5.3	7.0	3.0	6.02	1.832
Fruit skin colour	4.1	3.3	5.5	5.2	5.7	5.3	6.6	5.6	6.0	6.0	5.34	0.950
Juice colour	3.2	1.2	4.7	6.3	7.0	6.2	6.1	7.9	6.8	9.0	5.84	5.157
Length of stalk	5.0	4.8	6.3	5.6	5.7	6.5	6.0	5.1	3.7	7.0	5.57	0.944
Season of flowering	5.7	5.7	5.7	7.9	5.9	6.2	6.8	5.4	6.0	7.0	6.22	0.604
Stone relative size in comparison with fruit	5.0	4.4	4.7	4.1	5.8	6.3	4.5	6.1	5.3	5.0	5.13	0.576
Stone shape	3.2	4.0	6.3	4.1	4.1	3.8	5.0	3.9	5.3	5.0	4.48	0.843
Susceptibility to diseases **	4.2	3.8	2.9	4.9	4.6	4.1	5.8	4.5	5.3	3.7	4.39	0.723
Susceptibility to fruit cracking	4.9	2.8	4.7	6.1	3.1	3.4	3.5	2.8	4.0	3.0	3.83	1.162
Tree habit	1.9	3.8	3.0	2.4	3.9	5.7	4.8	2.3	1.7	1.0	3.04	2.156
Tree type	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.10	0.100
Tree vigour	6.6	5.6	4.3	7.6	4.8	3.7	3.3	6.3	6.3	7.0	5.55	2.158

2862K and Ochsenherzkirsche – large fruit size (PC1-PC6, PC2-PC6); 1618 B1 – small fruit size (PC2-PC6, PC3-PC6); Ohio Beauty Ironside – dark juice colour, late flowering season, low susceptibility to diseases, specific fruit shape (PC3-PC5, PC3-PC6); Vit Spansk, 1354 B1 – short stalk, bad fruit separation from stalk (PC3-PC7).

Cluster analysis found seven clusters of different size (Fig. 2). Because a high number of accessions was found in cluster 5, it was divided in to three sub-clusters.

The highest variability among clusters was shown by traits such as tree vigour, density of head, tree habit, fruit size, fruit firmness, fruit skin colour, juice colour, and susceptibility to fruit cracking and susceptibility to diseases (Table 4). These traits had also the highest impact on grouping of accessions.

Accessions represented in Cluster 1 were characterized by early season of flowering, round shape of fruits, large to very large size of fruits, firm fruits with weak juiciness. The fruits were characterized by light fruit skin and juice colour, high susceptibility to cracking.

Accessions in cluster 2 were characterized by early flowering (approximately the same as cluster 1) and mostly round, medium fruit size. This cluster was associated with very light fruit skin and juice colour, very low susceptibility to fruit cracking and very short length of stalk.

Cluster 3 was distinguished by high density of head, and very low susceptibility to diseases. It was characterized by early flowering, good fruit separation from stalk, and more elongate fruit shape.

Cluster 4 was separated based on strong tree vigour, late flowering season and low stone relative size in comparison with fruit. The cluster was also characterised by large fruits, high susceptibility to cracking, and intermediate susceptibility to diseases. However, it was highly susceptible to *Monilia fructigena*.

Cluster 5 could, in general, be characterised by comparatively small soft, juicy fruits, small tree size and low density of head. This cluster showed quite distinguishable sub-division in smaller clusters with some specific traits. Sub-cluster 5a was distinguished by large stone relative size in comparison with fruit and long length of stalk. This sub-cluster was characterized by weak tree vigour, low density of head, tree habit mostly dropping and weeping. Sub-cluster 5b differed from sub-cluster 5a by weaker tree vigour, more elongated fruit shape, very soft fruits with dark juice colour and small stone relative size. This sub-cluster had also high susceptibility to diseases. Sub-cluster 5c differed from 5a and 5b by early flowering season, very soft and juicy fruits with very dark juice colour and large stone relative size. This sub-cluster showed very low susceptibility to *Blumeriella jaapii*.

Accessions of Cluster 6 had upright or semi upright tree habit, very low juiciness, very short fruit stalk and very high susceptibility to diseases. Fruits of accessions in this cluster had mostly elongated stone shape.

Cluster 7 was represented only by one accession – Regina. The main trait determining the distinct location of this cultivar was tree type: Regina was the only accession of the collection with a spur tree type. This cultivar was also characterized by strong tree vigour, high density of head, and high firmness of fruits. It also showed low susceptibility to diseases.

Discussion

Since both sweet cherry collections have different historical and geographical origin and, therefore genetic background, they showed clear differentiation of important breeding traits. The Latvian collection represents locally well adapted accessions, which showed comparatively high uniformity in traits suited to local environmental conditions: high disease resistance, winterhardiness, but comparatively low fruit quality. The Swedish collection includes mostly accessions which produce high quality fruits. Combining of both gene pools may favour development of locally well adapted, high quality cultivars. This may promote extension of harvesting season, since most of accessions in Latvian collection have early to medium harvest season, whereas Swedish accessions – medium to late. The detailed description of Latvian and Swedish sweet cherry genetic resources collections is valuable input for establishment of a targeted management and evaluation programme. It could serve also a base for further utilization of available genetic resources in breeding, because better plant material description allows to more easy identify interesting properties and make sure that the whole spectra of variation is preserved.

Characterization also provided data for further analyses of material, including multivariate statistical analyses (PCA and cluster analysis), which allowed to obtain a overall view of the existing plant material variability, based on the complete range of described traits. PCA showed the overall variability in collections and revealed traits with high impact on determination of similarity and relatedness. In this investigation it was not possible to make direct comparison of particular principal components between collections, since the used sets of traits differed. Therefore only general trend of trait groups were compared. From this point of view the most useful for both collections were fruit and tree architecture traits, which were associated with the first PC with a high percent of variability (Tables 1 and 2). As those traits are also important in breeding, more attention should be paid to them during further evaluation activities in sweet cherries collections. Fruit traits are especially important as they have high heritability (Hjalmarsson, Ortiz 2000). Leaf traits should be excluded from further evaluation due to low informativity and high dependence on environmental conditions (Lacis, Rashal 2000).

PCA based on morphological traits did not show good applicability in determining relationships between accessions in both sweet cherry collections, especially in the Swedish collection. This might be due to a common gene pool of accessions. A high degree of relatedness of accessions in this collection is confirmed by pedigree data (Lacis et al., 2008). Phenotypical similarity of breeding material is defined mostly by targeted breeding work towards valuable traits. However, PCA showed good usability in selection of distinct accessions based on a complete trait set for characterization, which is not possible to perform accurately by analysis of separate traits.

Cluster analysis was performed to classify accessions. The level of similarity discovered by cluster analysis was relatively high, especially for the Swedish collection. Nevertheless, cluster analysis discovered grouping of accessions within the collections. Although analysis was based only on phenotypical data, results could be supported by pedigree, which was available for the sweet cherry collection in Sweden (Lacis et al. 2008). There was some similarity of grouping based on morphological data on pedigree data. Many accessions or their parents in Group 1 and 2 had a cultivar Kaiser Franz as an ancestor. Kaiser Franz as a progenitor was ancestor of many accessions of Groups 5a and 6, but

not of Groups 5b and 5c, where the common ancestor of many accessions was cultivar Smidt. Accessions of Group 3 have no common ancestor. Several accessions in Group 4 have cultivar Windsor as an ancestor, while Windsor it self is located in Group 6. Cultivar Lambert, which is located in Group 1, is in the pedigree of several accessions from Group 6. Particular accessions showed distinctive separation from the other groups (Fig. 1 and 2), such as cultivar Regina, characterized by a spur tree type. Cultivars Leningradskaya Czornaya from the Latvian collection showed separation based on small leaf size. Cluster analysis is useful for grouping of accessions to obtain understanding of internal structure of germplasm collections (Peeters, Martinelli 1989). This was particularly informative for the Latvian sweet cherry collection, because of the lack of information on origin of most of accessions.

Comparison of sweet cherry accession groups by cluster analysis revealed the most important traits for grouping. For both collections tree architecture and fruit traits differed most between clusters (Table 3 and 4), which is in agreement also with the PCA analysis (Table 1 and 2). This should be taken into account in further characterization of sweet cherry genetic resources.

Combined application of PCA and cluster analysis of sweet cherry accession data revealed the most useful traits for further accession description and provided comprehensive information about the collection's genetic structure and internal relatedness of accessions, which was not possible by analysis of separate traits. Application of multivariate statistics increased the value of phenotypical data and created the basis for combined analysis with genetic data.

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Cloning and expression of a recombinant immunogenic truncated BBK32 protein of *Borrelia afzelii*

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Abstract

Borrelia burgdorferi, the Lyme disease-causing spirochete, is often found associated with host connective tissue, where it interacts with components of the extracellular matrix, including fibronectin. BBK32 is a surface-expressed lipoprotein with fibronectin-binding ability of B*orrelia burgdorferi*. A fragment of the *bbk32* gene of *Borrelia afzelii* strain ACAI encoding the N-terminus of the protein including the fibronectin-binding domain (designated BS4 in this study) was cloned end expressed in *Echerichia coli* under the control of arabinose promoter as six histidine-tagged protein. Expression for the target protein showed that BS4 was accumulated both in soluble and insoluble forms. The molecular weight of the recombinant protein was estimated by SDS-PAGE to be 35 kDa including the six histidine tag. The expressed protein was purified by Ni^{2+} affinity chromatography under denaturing conditions. The purified BS4 recombinant protein was evaluated as an antigen in the serology of Lyme disease. Western blot analysis of Lyme disease patient sera revealed that the recombinant truncated BBK32 protein has specific antigenic properties. The availability of recombinant immunogenic BBK32 protein provides a new opportunity for biochemical analysis of the protein, structure-function studies, examination of its role in microbial pathogenesis, and assessment of its diagnostic and vaccinogenic potential.

Key words: antigenicity, *Borrelia afzelii*, recombinant protein BBK32.

Introduction

Lyme disease (Lyme borreliosis; LB), the most common arthropod-borne disease in Europe and North America, is caused by spirochete *Borrelia burgdorferi*. It is a multisystem disorder characterized by dermatologic, cardiac, neurologic, and arthritic manifestations (Steere 2001). A total of 488 cases of LB were recorded in Latvia in the year 2008, with incidence 21.49 per 100000 population. Lyme disease is spread to humans and other mammals via bites of infected *Ixodes* ticks (Burgdorfer 1982). In Latvia, two tick species, *Ixodes ricinus* and *Ixodes persulcatus*, transmit the disease. Our recent studies indicated that on average 25 % of ticks in Latvia are infected with Lyme disease spirochetes. All of three main genospecies of *B. burgdorferi* that are pathogenic in humans are prevalent in Latvia: *B. burgdorferi* sensu stricto, *Borrelia afzelii* (the most prevalent species in Europe) and *Borrelia garinii* (Ranka 2004). Very recently, the fourth pathogenic borrelia species *Borrelia spielmanii* was delineated in Europe (Richter 2006). Significant antigenic variation

between and within each genospecies has been described (Wang 1999), and among different strains of *B. garinii* this antigenic variability is more pronounced (Wilske 1996). Several studies have shown differences in immunoblot reactivity patterns depending on the strain, serotype, or species used as the antigen (Zoeller 1991; Mathiesen 1996; Hauser 1997; for review see Wilske 2007). The existence of such heterogeneity challenge many approaches of LB research, especially the development of diagnostic tests and vaccine.

In spite of numerous investigations of the nature of Lyme disease, knowledge of the pathogenicity and the virulence factors involved is still insufficient. The ability of *B. burgdorferi* sensu lato to encounter and persistently infect diverse vertebrate hosts frequently requires complex environmental sensing mechanisms involving coordinated expression of proteins essential to overcome host defence, in particular the innate and adaptive immune responses. Nevertheless, there is little information on the function of such proteins. Binding to host extracellular matrix molecules is one of the common strategies that bacterial pathogens employ for adhesion and invasion of host tissues. *B. burgdorferi* is capable of binding to a variety of host extracellular matrix molecules (Cabello 2007). Moreover, the interaction with host ligands mediated by spirochetal surface adhesins has been hypothesized to be critical for the pathogenic strategy (Guo 1995).

Lipoprotein BBK32 was identified as a typical fibronectin-binding adhesin of *B. burgdorferi* (Probert 1998). Recently it was shown that BBK32 induces the formation of fibronectin aggregates (Prabhakaran 2009) and is required for the initiation of microvascular interactions between borrelia and the host (Norman 2008). The BBK32 protein was also identified as an antigen that elicits an antibody response in infected mice as well as in Lyme disease patients, and it is a potential agent for serological test and vaccine development (Suk 1995; Fikrig 1997; Brown 2005; Lahdenne 2006; Scogman 2008). Recent studies showed that inactivation of the BBK32 gene in infectious strains of *B. burgdorferi* reduces spirochetal binding to fibronectin, as well as its infectivity in mice (Seshu 2006). However the mutants were shown to have no apparent defect in tick vectors (Li 2006).

The goal of the present study was to prepare a recombinant antigen using gene encoding BBK32 protein fragment in *B. afzelii* in order to obtain a molecule useful for pathogenicity, immunogenicity and virulence studies of Lyme disease spirochetes.

Materials and methods

Organisms and growth conditions

Cloning and expression of recombinant DNA was performed in *Escherichia coli* strain TOP10 (Invitrogen). Cells were grown in small-scale (10 mL) or medium-scale (50 mL) Luria-Bertani broth [10 g of NaCl, 5 g of yeast extract (Difco), 10 g of tryptone (Difco) per liter] supplemented with ampicillin (50 μg mL^{-1}, selective LB media), with aeration at 37 °C on a rotary shaker.

PCR amplification of BBK32 *gene and sequence analysis*

A PCR-based approach was used to amplify and sequence the *BBK32* gene from the ACAI isolate of *Borrelia afzelii* genomic DNA (kindly donated by S. Bergstrom, Umeå), as described elsewhere (Heikkila 2002). The PCR-amplified full-length *bbk32* gene was sequenced by standard technique using an ABI Prism 3100 Genetic Analyzer (Perkin-

Elmer, USA). The clone gene was conceptually translated and compared with sequences in the GenBank database using the BLAST program. Protein sequence analysis was carried out by using Software from the ExPASy Proteomics Server (Swiss Institute of Bioinformatics, http://www.expasy.ch/).

Construction and cloning of recombinant plasmid

Insert DNA encoding full length *BBK32* gene and *BBK* gene fragment were amplified by PCR from *B. afzelii* ACAI DNA.

The coding region corresponding to BBK32 (Ala2 - Tyr352) was amplified with 5' ccccatgggaaaaattaaaagtaaatg 3' and 5' ggctgcagtcaatggtgatggtgatggtggtaccaaacaccattctt 3', where the restriction sites are underlined. The coding region corresponding to BBK32 fragment (Ala2 - Lys215, designated as BS4) was amplified with 5' ccccatgggaaaaattaaaagtaaatg 3' and 5' ggctgcagtcaatggtgatggtgatggtgtttaacaccctctagata 3', where the restriction sites are underlined. Cloning primers were designed to be complementary to the BBK32 sequence of *B. afzelii* strain 600 (AF472528). *Nco*I and *Pst*I sites were arranged in the forward and reverse primers, respectively, and six histidine coding DNA fragments were arranged in the reverse primer. Apmlification of the target gene was confirmed by agarose gel electrophoresis. Restriction endonuclease digestion, ligation, transformation, isolation of plasmid DNA and other standart recombinant techniques were performed as described by Maniatis et al. (1982). Briefly, the amplified fragment was treated with restriction endonucleases, purificated from agarose gel with a DNA extraction kit (Fermentas) following manufacturer's instructions, ligated into modified pBAD/Thio-TOPO vector (Invitrogen), and transformed into TOP10 *E. coli* cells. The cells were plated onto Luria-Bertani agar plates containing 50 μg mL^{-1} ampicillin. Positive recombinant clones were selected by colony PCR. The *E.coli* cells from each positive colony were inoculated into 2 mL of LB medium containing 50 μg mL^{-1} ampicillin and grown overnight. The cells were harvested by centrifugation and plasmid DNA was isolated. Double-digestion of plasmid DNA by *Nco*I and *Pst*I was used for the identification of positive clones followed by restriction analysis in agarose gel. The recombinant clones were confirmed by DNA sequencing.

Small-scale expression of recombinant protein

Optimal growth and expression conditions for the protein of interest were established with small-scale cultures. *E. coli* TOP10 cells were transformed with expression plasmids. LB media containing 50 μg mL^{-1} ampicillin was inoculated with bacterial colonies and incubated overnight at 37 °C. The next day, selective LB media was inoculated with 1 % volume of the overnight culture and grown for 2 to 3 h at 37 °C or until OD_{600} reached 0.6. A few mL of cells were collected as an uninduced control before induction. Protein expression was induced with arabinose at different concentrations (0.2 - 0.00002 %, final concentrations), and induction was carried out at 37 °C for 2 or 4 h. Absorbance was measured, and an aliquot of cells from each tube corresponding to 2 units of OD was removed. Aliquots were centrifuged in a microcentrifuge (~ 2400 rpm) for 5 min, supernatant was removed, and pellets were frozen at –70 °C.

Expression of recombinant protein was confirmed by SDS polyacrylamide (10 %) gel electrophoresis (SDS-PAGE, as described by Laemmli 1970) and Western blot analysis (as described by Towbin et al. 1979) using an Amersham Hybond-C Extra

nitrocellulose membrane (GE Healthcare). SDS-PAGE separated proteins were visualized by Coomassie brilliant blue R250 staining or by silver staining as described elsewhere (Walker 1996). The Western blot procedure used PentaHis Antibody (Qiagen) and anti-mouse immunoglobulin G peroxidase conjugate (Sigma-Aldrich). The bound antibodies were detected using chemiluminiscent ELC Western Blotting Detection Reagents (GE Healthcare) following manufacturer's instruction or visualized by incubating the membrane with 3,3`-diaminobenzidine (Walker 1996). A Spectra™ Multicolor Broad Range Protein Ladder and Prestained Protein Molecular Weight Marker (Fermentas) were used as protein molecular weight markers during SDS-PAGE and Western blot analysis.

Preparation of soluble and insoluble protein fractions

Preparation of soluble/insoluble protein fractions was performed as described by Maniatis et al. (1982). The obtained samples were analyzed by SDS-PAGE and Western blot analysis as described above.

Expression and purification of the recombinant antigen

Cultures were grown in 50 mL of LB broth at 37 °C with aeration to an absorbance 0.5 - 0.6 at 550 nm. Arabinose was added to a 0.02 % final concentration, and induction was carried out at 37 °C for 4 h. The cells were harvested from 50 mL of culture by centrifugation at 6000 *g* at 4 °C; pellets were frozen at –70 °C for future use. Purification of recombinant protein was done under denaturing conditions using a Ni-NTA Spin Kit (Qiagen) following manufacturer's instructions with some modifications. Briefly, the cell pellet was resuspended in 10 mL Qiagen Urea buffer B containing DNAse I (5 μg mL^{-1}) and sonicated on ice. The insoluble materials were removed by centrifugation at 10 000 g for 20 min. The clarified supernatant was loaded onto pre-equilibrated Ni NTA Spin columns (Qiagen, 600 μL per column). After centrifugation, columns were washed twice, and absorbed proteins were eluted with 2 × 200 μL of Qiagen elution buffer E (pH 4.5) containing 8M urea. The obtained samples were analyzed by SDS-PAGE and Western blot analysis as described above.

Clinical samples

Five serum samples from patients with confirmed Lyme borreliosis were obtained from the Infectology Centre of Latvia. Samples were used in Western blotting (immunoblotting) analysis to determine reactivity to recombinant ACAI BBK32 proteins.

Additionally, sera from two borrelia noninfected humans (without LB in history and commercial Lyme test negative sample) were also used in these immunoblotting experiments. All human sera were diluted 1:1000 in PBS-Tween for immunoblotting.

Immunoblot analysis with clinical samples

Immunoblot analysis for the reaction between recombinant antigens and serum samples from Lyme disease patients was performed as following. The recombinant antigens separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane. The membrane was cut onto slices, each slice contained a protein molecular weight marker (Fermentas) and recombinant antigen lines. Membrane slices were blocked by incubating in PBS containing 5 % skimmed milk and 0.1 % Tween 20 for 1 h at room temperature. After washing the membrane twice in PBST for 5 min, the membrane slice was incubated

with serum samples at 1:1000 dilution in PBS containing 0.1 % Tween 20 for 1 h at room temperature. After washing the membrane twice in PBST for 5 min, secondary probing was performed with protein A-peroxidase conjugate (Sigma) (1:1000 dilution in PBS containing 5 % skimmed milk and 0.1 % Tween 20) by incubating 1 h at room temperature. After washing twice in 0.1% Tween 20 PBS for 5 min, bands were detected with chemiluminiscent ELC Western Blotting Detection Reagents (GE Healthcare) following manufacturer's instruction or visualized by incubating the membrane with 3,3`-diaminobenzidine (Walker 1996).

Results

Sequence analysis

Sequence analysis of the amplified *bbk32* gene revealed 99 to 100 % homology with the *B. afzelii bbk32* genes from different isolates (*B. afzelii* strain 1082, A91, PKo, 600, 570, ACAI; data not shown). The deduced amino acid sequences of the BBK32 protein contained 352 residues. Secondary structure prediction analysis indicated that the *B. afzelii* ACAI BBK32 protein consisted mostly of alpha-helical regions with a few beta sheets. A signal peptide region (amino acids 1 - 20) and transmembrane region (amino acids 9 - 24) were also predicted. The greater part of the mature portion of the BBK32 protein was hydrophilic. The calculated molecular size of the mature BBK32 protein (without histidine tag) was 40.7 kDa. The obtained information was used for design of a truncated BBK32 variant. The sequence was selected from areas of predicted hydrophilicity including the N-terminal and mid-portion of the mature sequence (amino acids 2 - 215). The fibronectin-binding domain of BBK32 (amino acids 131 - 162; Probert 2001) was also included.

Cloning of the bbk32 *gene*

The DNA-coding region for the mature BBK32 (nucleotides 4 - 1059) and for the truncated BBK32 (nucleotides 4 - 645) of *B. afzelii* strain ACAI were produced by PCR and cloned into a modified bacterial expression vector pBAD/Thio-TOPO (Fig. 1).

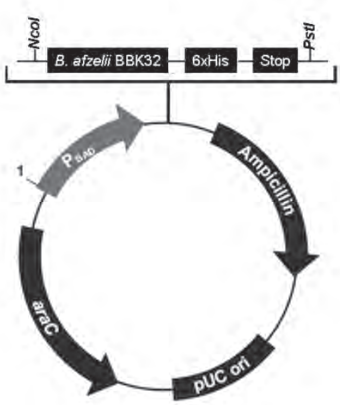

Fig. 1. Expression vector for *B. afzelii* BBK32. *B. afzelii* ACAI BBK32 coding sequence was inserted into modified pBAD/Thio-TOPO vector containing the ampicillin resistance marker. Expression of the recombinant protein was induced by arabinose.

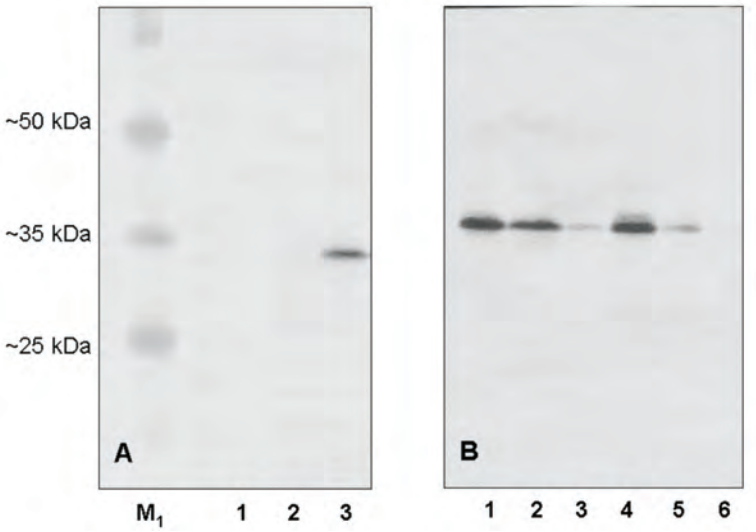

Fig. 2. Expression of recombinant protein BBK32 of *B. afzelii* in *E. coli*. Proteins from *E. coli* lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane and proteins of interest were detected by Western blotting using antibodies against His tag. Fragment of *bbk32* gene from *B. afzelii* ACAI was cloned into the modified pBAD/Thio-TOPO vector and expressed as a fusion protein with a six-histidine tag at its C-terminus (A, panel 3). The recombinant protein expression was not detected before the induction (A, panel 2). Expression of full-length recombinant *bbk32* gene was not detected (A, panel 1). Panel M_1 – protein molecular weight marker. The effect of different arabinose concentration (final concentration is shown) in the expression of recombinant truncated BBK32 protein at different expression time points (B). Panel 1 – 0.2 % Ara, 2 h; panel 2 – 0.002 % Ara, 2 h; panel 3 – 0.00002 % Ara, 2 h; panel 4 – 0.2 % Ara, 4 h; panel 5 – 0.002 % Ara, 4 h; panel 6 – 0.00002 % Ara, 4 h.

To verify the primary selection, each single colony was cultured and plasmid DNA was isolated. Plasmid DNA digestion by *Nco*I and *Pst*I restriction enzymes and DNA sequencing confirmed successful insertion of the *bbk32* gene and *bbk32* gene fragment into the modified pBAD/Thio-TOPO vector. Total length of the inserted DNA fragment with the six histidine tag was 1074 nucleotides encoding 357 amino acids and stop codon (designated BBK32ACAI), and 669 nucleotides encoding 222 amino acids and stop codon (designated BS4), respectively. The C-terminal histidine tag was used to affinity-purify the fusion protein by chelated Ni^{2+} resin. The obtained constructs were transformed into *E. coli* strain TOP10 for protein expression.

Expression of the recombinant antigen

The recombinant antigen candidates were expressed in *E. coli* strain TOP10, expression was proved by SDS-PAGE and western blot analysis with PentaHis antibodies (Qiagen) and Anti-mouse HRP antibodies (Sigma). The antigen BS4 was identified as a protein with molecular mass of approximately 35 kDa (Fig. 2). Bacterial expression was optimized by analysis of protein expression on different amounts of arabinose (Fig. 2B). *E. coli* containing recombinant vector expressed BS4 only after arabinose induction, but no significant increase of recombinant protein production was observed at different time

points (Fig. 2). Analysis of soluble and insoluble protein fractions showed that BS4 was expressed both in soluble and insoluble forms (data not shown).

Negligible amounts of antigen BBK32ACAI were detected in SDS-PAGE and western blot analysis, indicating that the expression of full-length BBK32 protein is problematic under the conditions used (Fig. 2).

Purification of recombinant antigen

To establish whether the recombinant truncated BBK32 protein could be purified via the C-terminal histidine tag, the cleared lysates were subjected to affinity chromatography on Ni-NTA Spin columns under denaturing conditions. The flow-through, wash and eluted fractions were collected and analyzed by SDS-PAGE and/or Western blotting (Fig. 3). The results showed that BS4 could be purified by this method; however, some modifications of procedure will be necessary in future. The results show that the capacity of used spin column was not enough to bind all of the expressed protein, because it was still detectable in significant amounts in the flow-through fraction. Western blot analysis showed that some extra bands of smaller size appeared in elution fractions in addition to the protein of interest indicating possible protein degradation during the purification procedure. A few other contaminants were copurified with the protein of interest under the conditions used, observed in the affinity purified fractions on SDS-PAGE (Fig. 3). Nevertheless, the quality of the obtained purified BS4 protein was good enough to perform further work.

Immunoreactivity of the recombinant antigens with patient serum

Five Lyme disease patient sera and two negative control human sera were used in immunoblott analysis with the recombinant BS4 antigen. In all reactions, the BS4

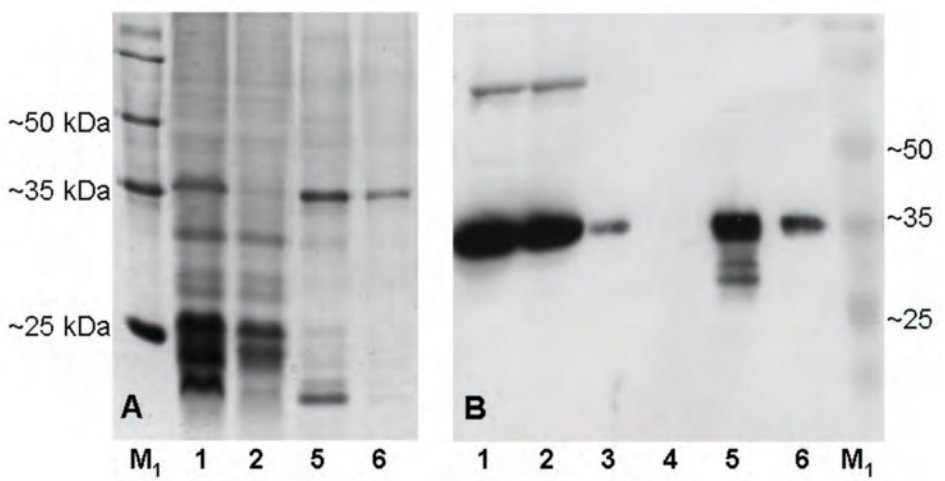

Fig. 3. Purification of recombinant truncated BBK32 protein. Protein of interest (BS4) was expressed in *E. coli* and purified by Ni^{2+} affinity chromatography. Proteins were separated by SDS-PAGE and detected by silver staining (A) or transferred to a nitrocellulose membrane for Western blotting with antibodies against His tag (B, chemiluminiscent detection). Panel 1 – *E. coli* crude cell lysate; panel 2 – *E. coli* cell lysate, flow-through; panel 3 – first wash fraction; panel 4 – second wash fraction; panel 5 – elution fraction 1; panel 6 – elution fraction 2; panel M_1 – protein molecular weight marker.

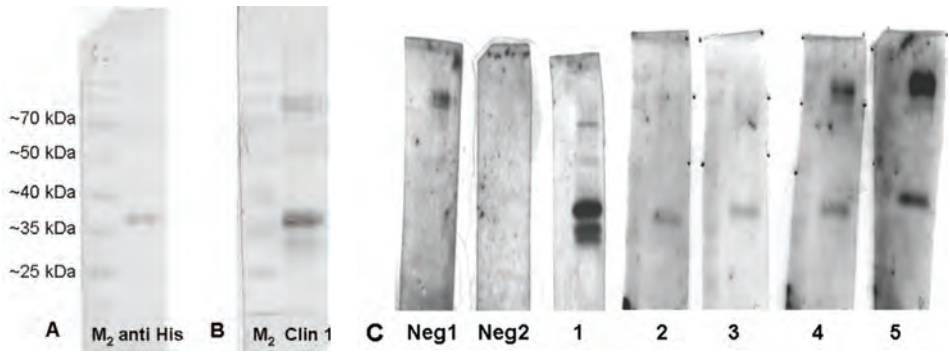

Fig. 4. Western Blot analysis of Lyme disease clinical samples with BBK32 protein. The truncated BBK32 protein of *B. afzelii* ACAI (BS4) was expressed in *E. coli* as a recombinant protein with six His tag. The protein was purified by Ni^{2+} affinity chromatography and the elution fraction 1 was used as an antigen in Western blot analysis. Probing with antibodies against His tag (A, detection by 3,3`-diaminobenzidine); probing with Lyme disease serum sample from patient No. 1 (B, detection by 3,3`-diaminobenzidine). Panel M_2 – protein molecular weight marker. Probing with different clinical samples (C, chemiluminiscent detection), Neg1 and Neg2 – Lyme disease negative serum samples; 1 - 5, Lyme disease positive serum samples.

elution fraction 1 was used. The results demonstrated that recombinant BS4 antigen had significant reactivity with Lyme disease patient sera (Fig. 4). Some differences in the immunoreactivities of the serum samples against BS4 protein were observed.

No positive reaction was observed between recombinant antigen and Lyme disease negative patients sera samples (Fig. 4).

Discussion

Since pathogenic mechanisms of Lyme borreliosis is still mostly unclear, detailed studies of the biological activity of borrelia specific proteins with antigenic nature are essential for further studies. A better understanding of the nature of predominant antigens of *Borrelia burgdorferi* will be useful in the attempt to detect factors involved in virulence and the study of pathogenicity, and also for serological differentiation of *Borrelia* species and for development of a vaccine. In search for these appropriate antigens with specificity and sensitivity, a large number of research papers have been published. These studies have helped to determine some spirochetal virulence factors (for review see Steere 2005), but Lyme disease pathogenesis is still poorly understood.

In this study we have cloned and expressed in *E. coli* the gene fragment of an immunogenic protein BBK32 from *B. afzelii* ACAI (designated BS4 in this study). Expression analysis showed that the maximal recombinant protein amount was produced after induction with 0.2 % arabinose (final concentration). However, the protein amount did not differ at time points 2 h and 4 h after induction (Fig. 2B). Analysis of soluble and insoluble protein fractions displayed that the protein of interest exists both in soluble and insoluble forms. In this study, to obtain maximal protein yields we purified BS4 under denaturing conditions. Western blot analysis of the obtained fractions showed that under these conditions certain amount of proteolysis occured shown by the appearance of some

new bands of proteins with smaller molecular weight that reacted with anti-His antibodies.

In this study we tested the antigenicity of truncated BBK32 protein of *B. afzelii* by using Lyme disease patient sera in Western blot analysis. We focused on *B. afzelii* proteins, because it is the most prevalent species of Lyme disease borrelias in Europe and also in Latvia (Steere 2001; Ranka 2004). We have shown that sera from Lyme disease patients recognize this recombinant protein. This finding is in accordance with the data of Lahdenne et al. (2003; 2006). However, these authors used full-length BBK32 protein and shorter mid-portion fragments of BBK32 (amino acid range 130-220) in ELISA studies. Our findings enhance the evidence that the BBK32 proteins are promising serodiagnostic antigens for the detection of LB. Variant BBK32 proteins may be used either in parallel or in combination in an immunoassay for LB to cover all the relevant borrelial species, whose prevalences differ regionally in Europe. In future studies, it will be interesting to identify possible antigenic epitopes of *B. burgdorferi* BBK32 proteins. There is some evidence that variant BBK32 proteins may have both individual and common antigenic epitopes and that epitope specificity varies in early and late LB (Lahdenne 2006).

In conclusion, we have been able to clone and express the N-terminal part of BBK32 protein of *B. afzelii*. This protein showed clear antigenic properties in Western blot analysis with Lyme disease patients' sera, which makes it useful for further studies of pathogenicity and for diagnostic applications of Lyme borreliosis.

Acknowledgements

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In vitro behaviour of nodal explants of *Portulaca grandiflora* under the influence of cytokinins

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Abstract

A method for the micropropagation of *Portulaca grandiflora* Hook is described to understand the *in vitro* behaviour of nodal explants in cytokinin-rich medium. Aseptic nodal explants were cultivated on Murashige and Skoog's medium containing different concentrations and combinations of 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin, KIN). The nodal explants of mature plant can be stimulated to undergo multiple shoot formation on the medium supplemented with BAP and KIN. Direct organogenesis without callus formation from the nodal explants was observed on medium supplemented with cytokinins. Using BAP (2 to 10 μM) alone had a significant effect on the formation of multiple shoots, which were stout and robust. Similar concentrations of KIN (2 to 10 μM) showed a weaker response. It was observed that when the optimum concentration of KIN (8 μM) was supplemented with low concentration of BAP (2 μM or 4 μM) there was a synergistic effect on the explant in terms of shoot regeneration. Single shoots were rooted on a half strength medium with 2 μM indole-3-butyric acid.

Key words: cytokinin, multiple shoots, nodal explants, *Portulaca grandiflora*, synergistic effect.

Introduction

Plants are a traditional source for many chemicals used as pharmaceuticals, biochemicals, fragrance, food colour and flavours (Leung 1980). Betalains are one such commonly used food additive. The interest of the food industry on this compound has grown after they were identified as antifungal (Kimler 1995), antioxidant (Escribano et al. 1998) and having positive health effects on humans (Tesoriere et al. 2004). Its wide range of pH stability also makes it an important natural colour in the juice industry.

The family Portulacaceae is characterized by the occurrence of betalains. *Portulaca grandiflora* Hook is a popular ornamental plant rich in betaxanthins, a subclass of betalains. Portulal, a root-promoting substance, has also been reported from this plant (Mitsuhansh et al. 1969) with a potential applicability in tissue culture.

An efficiently reproducible and rapid *in vitro* regeneration system is a prequisite for improving the conventional plant breeding programmes. Genetic transformation is one of the promising methods where specific traits can be added with minimal alteration of the target plant genome. Therefore, direct shoot morphogenesis from the primary tissue is more desirable than via an intermediate callus phase (Larkin, Scrowcroft 1981). In

this regard a method for the large scale propagation of *Portulaca grandiflora* needs to be developed. The aim of the present work was to study the influence of cytokinins on shoot formation from nodal explants of *P. grandiflora*, with the aim to obtain a fast and efficient regeneration.

Materials and methods

Murashige and Skoog (1962) culture medium supplemented with 3 % sucrose and the gelling agent (0.8 % agar; w/v) was used. Different concentrations of kinetin (KIN) (2 to 10 μM) and 6-benzylaminopurine (BAP) (2 to 10 μM) individually and in combination were added to this medium. The pH of the medium was adjusted to 5.8. The medium (20 mL) was dispensed in culture tubes which were closed with cotton bunks and further capped with paper. The culture vessels containing the media and instruments were autoclaved at 121 °C for 30 min.

Nodal explants were obtained from a healthy population of mature plants of *Portulaca grandiflora* growing in the botanical garden of the Maharaja Sayajirao University, Vadodara. The explants were kept in running water for one hour, washed with a plant detergent (Tepol) for 5 min, and again rinsed thoroughly with sterile distilled water four to five times. Explants were surface sterilized with 0.1 % (w/v) $HgCl_2$ for 3 min and rinsed with autoclaved double distilled water four to five times. The nodal explants were cut to appropriate size (2.5 to 3.5 cm) with three to four nodes and inoculated vertically on the culture medium. A single explant was placed in each culture tube. In total 15 replicates were used per treatment and the experiment was repeated twice. All cultures were maintained at 25 ± 2 °C under a 12 h photoperiod of irradiance provided with white florescent tube. The experiment was monitored for a period of 6 weeks and data for number of shoots per explant and number of leaves per explant was recorded. Standard error of the mean for each value was calculated.

For root induction, the shoots were harvested from culture tubes and transferred on half strength MS medium supplemented with 2 μM indole-3-butyric acid.

Results

Nodal explants of *P. grandiflora* were cultivated on MS medium supplemented with different concentrations and combinations of two cytokinins, BAP and KIN (2 to 10 μM). Withn three to four days after explanting the axillary bud proliferated and young leaves were observed. All the concentrations of individual BAP and KIN as well as their combinations showed enhanced shoot formation. The young multiple shoots formed were pale yellow, turning pink due to formation of betalain pigment. Axillary branching and multiplication of shoots occurred without a callus phase. Shoot tips showed necrosis after five to six days of cultivation. To avoid this effect, shoots were subcultured frequently on fresh medium of the same combination.

Of the two cytokinins BAP was found to be more effective in inducing multiple shoot formation as compared to KIN (Table 1, Fig. 1A and B). It was also observed that the number of shoots increased with an increase in concentration of BAP, while the number of shoots/explants decreased with increase of KIN concentration (Table 1).

New shoots initiated from each developing bud adjacent to the primary axillary shoot

Table 1. Effect of cytokinins kinetin (KIN) and 6-benzylaminopurine (BAP) on development of nodal explants of *Portulaca grandiflora* after four weeks in tissue culture

Cytokinin concentration (μM)		Number of shoots per explant ± SE	Number of leaves per explant ± SE
KIN	BAP		
-	-	0	0
2	-	2.8 ± 0.2	9.7 ± 1.0
4	-	2.2 ± 0.3	7.2 ± 0.9
8	-	2.5 ± 0.3	9.1 ± 1.1
10	-	2.7 ± 0.3	9.3 ± 1.1
-	2	2.7 ± 0.9	7.2 ± 1.8
-	4	3.2 ± 1.1	8.5 ± 3.0
-	8	3.5 ± 0.7	7.5 ± 1.0
-	10	3.7 ± 0.3	9.5 ± 1.0
2	2	2.8 ± 0.4	8.0 ± 0.6
2	4	2.5 ± 0.3	8.6 ± 0.4
2	8	2.4 ± 0.6	8.5 ± 1.2
2	10	3.1 ± 0.6	8.5 ± 1.4
4	2	3.3 ± 0.2	10.5 ± 1.0
4	4	3.1 ± 0.4	7.0 ± 1.2
4	8	3.6 ± 0.6	8.8 ± 1.6
4	10	3.6 ± 0.6	9.6 ± 0.8
8	2	4.0 ± 0.6	11.8 ± 2.8
8	4	3.8 ± 1.7	12.0 ± 1.9
8	8	3.2 ± 0.4	7.1 ± 0.7
8	10	3.0 ± 0.8	9.5 ± 2.2
10	2	2.7 ± 0.5	6.5 ± 1.0
10	4	3.7 ± 0.5	9.5 ± 1.3
10	8	2.7 ± 0.5	7.5 ± 1.8
10	10	2.0 ± 0.4	7.3 ± 0.8

within a week. All regenerated shoots were free from callus tissues at their proximal ends. For BAP alone the maximum effect was observed at 10 μM, while for KIN at 2 μM (Table 1). Similar results were observed for leaf formation. The number of leaves per explant increased with anincrease in BAP concentration with the maximum response at 10 μM. The number of leaves per explant decreased with increase in KIN concentration (Table 1).

When KIN was combined with BAP, it was found that KIN stimulated faster BAP-dependent shoot growth. When 2 μM KIN was used with increasing concentration of BAP (2 to 10 μM) the response was similar to that of KIN alone (Table 1). With 4 μM KIN and varying concentration of BAP (2 to 10 μM) there was an increase in the number of shoots per explant and the number of leaves per explant (Table 1). The maximum effect on shoot formation was achieved with 8 μM KIN and BAP (2 to 10 μM). When KIN was kept constant at 8 μM and concentration of BAP was increased gradually, it was observed

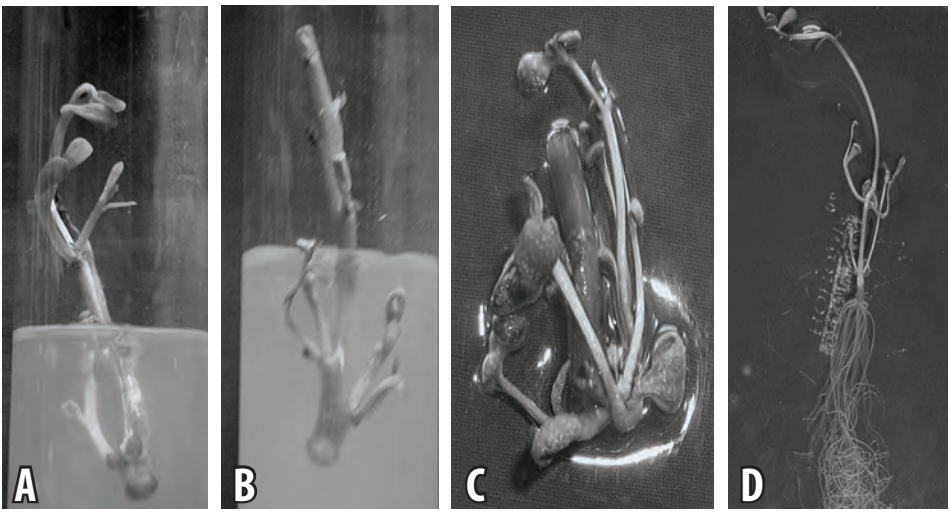

Fig. 1. Multiple shoot formation in *Portulaca grandiflora*. A, xillary bud proliferation in 2 μM kinetin. B, induction of multiple shoots in 2 μM 6-benzylaminopurine. C, synergistic effect of cytokinin (8 μM kinetin and 2 μM benzylaminopurine) D, rooting of the regenerated shoots in the presence of 2 μM indole-3-butyric acid.

that the frequency of multiple shoot formation and average number of leaves per explant decreased (Table 1). This suggests that at optimum concentration of KIN (8 μM) a low concentration of BAP (2 μM or 4 μM) is best suited for multiple shoot formation and for elongation of shoots. Further increase in KIN concentration (10 μM) lowered the number of shoots and a minimum was reached when the concentration of BAP also increased to 10 μM (Table 1). Thus the best response was obtained at 8 μM KIN plus 2 μM BAP (Fig. 1C) allowing to consider synergistic effect of the two cytokinins.

The regenerated axillary shoots were excised and transferred to ½ strength MS medium with sucrose (1 %) supplemented with 2 μM indole-3-butyric acid for induction of root formation. Root formation (Fig. 1D) was observed after 8 to 10 days. Initially roots were thin and slender. However, within three weeks they developed into slightly stout roots which were ready for hardening.

Discussion

In the present investigation cytokinins BAP and KIN were used for multiple shoot formation in *Portulaca grandiflora in vitro*. In general, bud break and development of shoots from stem node explants is a function of cytokinin activity (Sahoo, Chand 1998). In the present study axillary shoot buds were formed under the influence of both cytokinins. Further more the axillary branching and multiplication of shoots occurred without a callus phase. Similar findings have been reported in *Pterocarpus* on treatment with BAP and KIN (Anuradha, Pullaiah 1999).

Shoot tip necrosis was encountered in this experiment, which was augmented by subculturing on the same medium frequently. The use of cytokinins as plant growth regulators for cultures frequently is associated with the problem of shoot tip necrosis,

which can be solved by frequent subculture. Stem necrosis was also found in *Pterocarpus*, which was also reduced by subculture (Anuradha, Pullaiah 1999).

It was found that of the two individual cytokinins BAP was better than KIN in inducing multiple shoot formation. Cytokinins, especially BAP, are reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation (George 1993). Superiority of BAP in inducing multiple shoot formation has also been reported for a number of plants e.g. *Tridax procumbens* (Sahoo, Chand 1998), *Cypripedium flavum* (Yan 2006) and *Medicago truncatula* (Neves et al. 2001). For BAP alone, the best effect was obtained at 10 μM and a similar optimal concentration was also reported for *Ceropegia sahyadrica* (Nikam, Savant 2007) and *Andrographis paniculala* (Purkayastha et al. 2008).

KIN alone was not very efficient in inducing shoot bud multiplication in the present experiments. Low rate of multiplication in medium containing KIN has been observed in a number of plants e.a. *Bambusa balcooa* (Mudai, Borthakur 2009), *Ocimum gratissimum* (Gopi et al. 2006) and *Mentha arvensis* (Chishti et al. 2006). Thus, individually both cytokinins were found to induce multiple shoot formation, but the effect of BAP was more pronounced than that of KIN. Further it was noted that when the low concentration of KIN was supplemented with BAP, a synergistic influence on nodal cultures of *Portulaca grandiflora* was found in terms of number of shoots. A similar response is typical for a cytokinin combined with an auxin, which has been reported in a number of plants.

It was found in the present study that when the low KIN concentration was supplemented with BAP, both the number of shoots per explant and the number of leaves per explant increased. Optimum response was obtained at 8 μM KIN plus 2 μM BAP, supporting the synergistic effect of high concentration of KIN together with low concentration of BAP in *Portulaca grandiflora*. This type of effect has also been reported in many plants e.a. *Legenaria siceraria* (Shyamali, Kazumi 2007). Synergism of BAP and KIN was also reported in rootstock 1613C *Vitis solonis* × *V. labrusca* (Kumar et al. 2008).

The regenerated shoots were rooted in half strength MS medium with 1 % sucrose, supplemented with 2 μM IBA. Similarly, best rooting at half strength MS medium supplemented with IBA was obtained in *Vitex* (Balaraju et al. 2008), *Pappea capensis* (Mngomba et al. 2007), and *Bambusa balcooa* (Das, Pal 2005). Thus in the present investigation the development of nodal explants of *Portulaca grandiflora* in respect to multiple shoot formation *in vitro* was better in the presence of two cytokinins (BAP and KIN).

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Influence of climate on earlywood vessel formation of *Quercus robur* at its northern distribution range in central regions of Latvia

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Abstract

Annual variation in vessel cross-section area suggests that vessels as water transport tissues in plants can adjust size and numbers according to exogenous factors, such as climate. Earlywood vessels in ring-porous species such as oak *Quercus robur* are considered to contain climatic signals (climatic factors influence their formation). The aim of the study was to investigate the relationship between vessel cross-sectional area and climatic factors at its northern distribution range. Living oaks growing on dry forest sites in central parts of Latvia were cored. Cores were sanded and treated with chalk to expose vessels. Earlywood vessel cross-section areas were measured for each tree-ring. Relationship between climatic factors and vessel formation was examined using correlation analysis. Earlywood vessel formation depended on spring (March, April) and annual (previous year October to current year September) temperatures. Precipitation had low influence, which is not surprising considering location close to the northern limit of distribution, but precipitation was suggested as a non- limiting factor. Climatic factors of the current year were more important for vessel formation than previous year factors.

Key words: central Latvia; climatic signals; earlywood vessels; lumen area; *Quercus robur.*

Introduction

Many plants, including trees, can be used as bioindicators that respond to environmental changes over a long period of time. The past history of metabolic processes affected by the environment is reflected in wood structural features. Width, density and structure of the cells, tissues and organs, with their spatial relations and physiological states encode valuable annual information about climate, competition, predators, diseases, human impacts and radial growth of young and old trees grown on different soils (Wimmer 2002; García-González, Eckstein 2003; Schweingruber 2007).

Tree-ring width, earlywood and latewood width and density, and stable isotope composition in wood are the most widely used parameters in dendroscience. Genetic factors determine the basic structure of wood anatomy (Fritts 2001) but environmental factors such as climate during xylem development also can affect the anatomical characteristics of the woody cell, such as the size of tracheids in conifers and vessels in deciduous trees (Fonti et al. 2009).

Vessels are xylem elements involved in the transport of water (Rust et al. 2004; Thomas

et al. 2006; Steppe, Lemeur 2007). Earlywood vessels usually are generated before budbreak and controlled by the overall physiological vigor of the tree that is preconditioned by environmental stress and growth rates of previous growing season (Zahner 1968; Thomas et al. 2006). Vessels vary in size and in the type of openings (perforations) at their ends. Earlywood vessels in ring porous woods such as oak (*Quercus* spp.), elm (*Ulmus* spp.) and ash (*Fraxinus* spp.) are markedly wider than those in the latewood and tend to be barrel-shaped and as wide as they are long. There is variation in the size and arrangement of the pits that connect one vessel element to another, as well as vessel element to parenchyma (Schweingruber et. al. 2006). In oaks of the northern temperate zones earlywood vessels are only functional in the year of their formation (Thomas et al. 2006).

Interest in intra-annual anatomical features as indicators is steadily increasing in ecology, e.g., to determine water transport and effects of defoliation and drought stress on biomass. The oak species respond differently to defoliation and drought stress but even a small reduction in vessel diameter may result in a distinct decrease of water transport in the tree (Thomas et.al. 2006). In Mediterranean region it was suggested that in drought conditions only earlywood with large vessels is produced in Portuguese oak (*Quercus faginea* Lamk.) rather than latewood to compensate water loss by improving transport (Corcuera et al. 2004a). Holm oak (*Q. ilex* L.) (Corcuera et al. 2004a) and English oak (*Q. robur* L.) showed decrease in vessel size caused by drought but in sessile oak [*Q. petrea* (Mattuscha) Liebl.] repeated defoliation led to a significant increase in the vessel area (Zahner 1968; Corcuera et al. 2004b). This different anatomical response can be explained by the fact that sessile oak has a lower demand for water and nutrients and is less susceptible to drought stress than English oak (Gieger, Thomas 2002; Thomas et al. 2006). Although tension can strongly affect formation of vessels in tension wood, especially in young trees, vessel size variables still can be a useful tool in dendroecological studies (Heinrich, Gärtner 2008).

The use of time series of earlywood and latewood vessel features is becoming more popular also in dendroclimatology. Time series of vessels lumen area display little autocorrelation and show a greater stability through time compared to tree-ring width series, i.e., they provide more detailed information about seasonal climatic variability (García-González, Eckstein 2003).

Vessels such as the water-conducting elements (Rust et al. 2004; Steppe, Lemeur 2007) have been shown to reflect past rainfall regime in various ring-porous tree species, e.g., sweet chestnut (*Castanea sativa* Mill.) (Fonti, García-González 2004; García-González, Fonti 2006; García-González, Fonti 2008), beech (*Fagus sylvatica* L.) and oaks (Sass, Eckstein 1995; van der Werf et al. 2007). There have been a few studies on the influence of climate on the vessel size of ring-porous oaks in Switzerland (Eilmann et al. 2006; Fonti, García-González 2008; Fonti et al. 2009), at their northern distribution limit in Canada (St. George, Nielsen 2003; Tardif, Conciatori 2006) and in an oceanic climate (García-González, Eckstein 2003), and it was recommended to use vessels as a proxy for dendroclimatic reconstruction. Little is known about climate controls on vessel features in English oak and, considering climatic change, such studies would be particularly important at the northern distribution border.

The aim of the study was to assess the effect of climate on earlywood vessel formation in stem of English oak in dry habitats in Latvia and to evaluate of earlywood vessel cross-section area climatic signals as a potential proxy for climate.

Materials and methods

Study area

Five sampling plots were established in northwestern and central Latvia (Fig. 1), of which four were located in the eastern part of Kurland at Kandava, Sēme, Dzirciems and Tukums, and one in southern part of Vidzeme near Lobe. LKS-92 TM coordinates of sampling plats are shown in Table 1. All sites were located in dry habitats on clayey soils, covered with oak or mixed broadleaved managed forests. The relief in sampling plots was flat or with flat hills.

Sample collection, preparation and measurements

Samples were taken using a Pressler increment corer from living oaks at ~1.4 m height above tree base (breast height). Two cores were taken from each tree on opposite sides of the stem. Trees growing on relatively slight slopes were cored on sides of the stem perpendicular to the slope. Trees growing on steep slopes were not cored to avoid tension wood effect on vessel development. Samples were taken in autumn 2008.

In the laboratory samples (increment cores) were dried, glued into fixation planks and placed under pressure to dry. It was ensured that the direction of wood fiber and vessel cavity was perpendicular to surface of fixation plank. Fixed increment cores were gradually sanded (grain sizes from 80 to 400) using a vibration sander (Makita BO3700) until the radial plane of increment was uncovered. Dust from samples was removed with compressed air. Samples were rubbed with chalk to expose wood vessels and to increase their contrast. Quality checking was performed and only continuous cores with no rot present (breaking continuity) where all vessels were filled with chalk (in some samples vessels were filled with glue during fixation) were processed for scanning.

Chalked samples were scanned with 1200 dpi resolution and 24-bit colors using an

Fig. 1. Location of sampling plots in Latvia.

Table 1. Coordinates of sampling plots (LKS92 TM coordinate system)

Sampling plot	X coordinate	Y coordinate
Kandava	426640	6324050
Sēme	448460	6318850
Dzirciems	441840	6330950
Tukums	451180	6314740
Lobe	573940	6288760

Table 2. Numbers of cored trees, suitable samples and reference periods from sampling plots

Sampling plot	Number of trees cored	Number of suitable samples	Reference period (years)
Kandava	11	16	1906 - 2008
Sēme	10	15	1905 - 2008
Dzirciems	10	19	1900 - 2008
Tukums	10	15	1941 - 2008
Lobe	12	19	1900 - 2008
Total	53	84	

Epson GT-15000 scanner. Sample images were cut in sections along tree-ring borders and saved as separate tree-ring images for further analysis (wood vessels placed in central part of images). The youngest 100 to 110 tree-rings (where possible) were used.

Earlywood vessels were measured using the program WinCELL pro v.2007a (Regent Instruments). Tree-ring images were used as input data. Average lumen area in "H&W" mode of tree-ring earlywood vessels was measured using gray level pixel classification (values 220 - 235). Filters were used to avoid noise from chalk debris and sample surface defects. Batch function was applied to obtain data from tree-ring image groups (samples). Before starting batch function several randomly selected earlywood vessel images were manually checked to adjust measuring and filter parameters and to ensure measurement quality. Global data for each tree-ring image analysis was calculated for further analysis.

Data analysis

Mean yearly vessel lumen area (VLA) for individual trees was calculated, and used to estimate yearly mean VLA sites. To characterize influence of climatic factors on VLA correlation analysis was performed with DENDROCLIM 2002 program (Biondi, Waikul 2004). Climatic factors used were average monthly and seasonal (autumn, winter, spring, summer, and yearly – previous year October to current year September) temperature and precipitation sums of current and previous year on vessel formation.

Climatic data was obtained from the Latvian Environment, Geology and Meteorology Agency for the Riga meteorological station (missing data replaced by modeled). Annual mean VLA was compared with mean monthly temperature, monthly precipitation sum, and mean temperature and precipitation sum in season (i.e., from October of the preceding year to September of the growth year), as well as average temperature and precipitation

sums in autumn (September - November previous year), winter (December - February), spring (March - May) and summer (June - August).

Results

During the study 106 samples were taken from 53 living oaks. Number of cored trees, suitable samples and reference periods from sampling plots are shown in Table 2. In most cases rejection of suitable samples was caused by glue that filled the vessels or decay of parts of the samples. The Tukums plot had the shortest reference period due to relatively small tree age.

Mean vessel lumen areas for sampling plots are shown in Fig. 2. The formation of vessels among sampling places was rather similar, but there were some periods when quite large differences were visible (1900 - 1930, 1996 - 2008). Also a slight increase of VLA during the whole reference period could be suggested.

Significant correlations with climatic factors are shown in Table 3. In total 73 positive and four negative significant correlations were identified for 68 climatic factors. The highest absolute correlation value was 0.53 (with spring average temperature), lowest –0.16 (previous year March temperature). Significant monthly precipitation and temperatures correlation coefficients differed among the territories even in sampling plots from the same part of Latvia (Kandava, Sēme, Dzirciems and Tukums). Vessel lumen areas in all territories showed high correlation with winter, spring and yearly mean temperature. The highest correlation coefficients were observed with spring temperature, both seasonal and monthly. March, April and season mean temperatures overall showed highest correlation in most of sites.

Precipitation correlated with vessel formation in a few months, mostly positively. There were more correlations with current than with previous year climatic factors. Current year April precipitation showed a negative correlation only in one sampling plot, previous August precipitation showed three negative correlations. Overall only a few significant correlation coefficients were observed with current year summer precipitation data, correlation with previous year data was even less abundant.

Discussion

In the present study fluctuation and variability of VLA in English oak (Fig. 2) was similar to tree ring width fluctuations, suggesting that not only endogenous but also environmental factors had an influence on VLA formation (Fritts, Swetnam 1989; Wimmer 2002; Tardif, Conciatori 2006). The observed slight gradual increase of VLA during the reference period could be due to an ageing effect or changes in environmental factors (Fritts, Swetnam 1989; Schweingruber 1996). The most visible increasing trends of vessel formation occurred in periods of 1900 - 1930 and 1996 - 2008, which might be related with rising minimal temperatures under climate change (Walther et al. 2002), as previously observed in growth and establishment of Scots pine (*Pinus sylvestris*) (Brumelis et al. 2005).

Vessel formation more depended on current season climatic factors compared to previous season (Table 3). Influence of the previous season ending temperature on vessel formation in some regions might be related to local phenology of wood formation and microhabitat conditions (Menzel et al. 2001; Brüger et al. 2003). Although vessel formation

Table 3. Significant (a = 0.05) correlations (r) of mean vessel lumen area of *Quercus robur* with monthly and seasonal mean temperature and precipitation sum

		Current year					Previous year				
		Kandava	Sēme	Dzirciems	Lobe	Tukums	Kandava	Sēme	Dzirciems	Lobe	Tukums
Temperatures	Oct	0.29									
	Nov										
	Dec	0.23		0.34	0.21						
	Jan	0.27		0.23	0.30	0.29					
	Feb				0.30	0.27					
	Mar		0.24	0.23	0.25	0.49				0.16	
	Apr	0.26	0.32	0.42	0.40					0.27	
	May					0.30	0.28	0.24			0.26
	Jun		0.32			0.28		0.20		0.22	
	Jul										
	Aug		0.28		0.25			0.25	0.23	0.26	
Precipitation sum	Sep										
	Oct									0.25	
	Nov										
	Dec										
	Jan	0.25									
	Feb										
	Mar										
	Apr				-0.21						
	May	0.26	0.19								
	Jun	0.19					0.27				
	Jul										
	Aug							-0.17	-0.24	-0.29	
	Sep		0.17								
Temperatures	Autumn	0.24	0.30								
	Winter	0.27	0.22	0.35	0.37	0.28					
	Spring	0.24	0.35	0.34	0.34	0.53		0.27		0.26	0.28
	Summer		0.33		0.22			0.26		0.25	0.24
	Season	0.29	0.41	0.40	0.43	0.44		0.30		0.28	0.27
Precipitation sum	Autumn		0.20					0.22			
	Winter										
	Spring										
	Summer	0.20									
	Season	0.30					0.32				

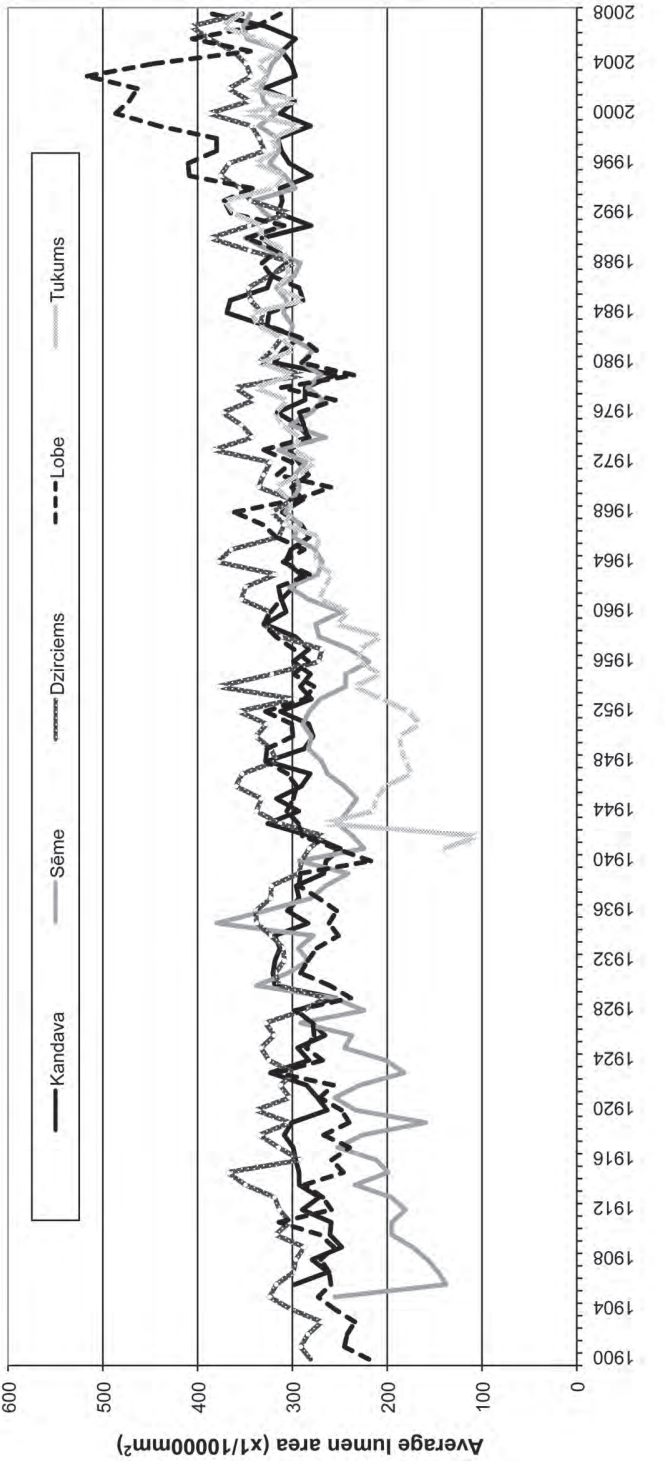

Fig. 2. Yearly fluctuation of vessel lumen area of *Quercus robur* at different sites.

depended on climate, site characteristics also had an influence; correlations with monthly temperature factors were not alike in all sites, even situated at short geographic distances from each other.

Correlation analysis showed that temperature has been a more important factor affecting vessel formation than precipitation. Current year winter and spring monthly mean temperatures (except February and May) had the most important role in vessel formation among temperature factors, showing the highest correlation coefficients and occurring in most sites. English oak is known to require a higher temperature in the beginning of the growth season (Jones 1959), and this is likely to be more evident close to its northern border of distribution area, i.e., in Latvia. Thus March-April mean temperature can be considered to be an important factor influencing vessel formation at the study sites, as they showed the highest correlation coefficients were detected. Early wood begins development in early spring or in some occasions even in end of winter before bud burst (Thomas et al. 2006; Fonti et al. 2009). Correlation with current season climatic factors after August most likely can be explained as coincidental, as early wood formation should have ended at that time (Jones 1959; Menzel et al. 2001). Correlation with previous year mean temperatures in May, June and August at several sites might be explained by nutrient reserve formation for next season affecting vessel formation (Zahner 1968; Larcher 2003; Tardif, Conciatori 2006; Thomas et al. 2006). VLA showed a lower dependence on previous than current season climatic factors; but still autocorrelation was suggested, but probably lower than observed in tree ring widths (Eckstein 1990; Fritts 2001; García-González, Eckstein 2003). Correlation with mean seasonal temperatures showed the summed effect of spring and winter temperatures on vessel formation.

Precipitation showed a minimal effect compared with temperature (significant correlation coefficients scattered between sites, low values) on vessel formation despite the fact that oaks were growing on dry forest habitats. This suggests non-limiting availability of water resources from groundwater and relatively low evaporation from clayey soil. Typically precipitation is a limiting factor for growth of oak in central Europe (Fonti et al. 2009). However, in Latvia a negative correlation with spring precipitation was observed only in one sampling plot [Lobe, (April, $r = -0.21$)]. Negative correlation with previous August precipitation (observed in three plots) might be related with water abundance in soil at the end of growing season causing stress and vessel size (area) reduction in the next season. Seasonal precipitation sums in general also had a low influence; correlation coefficients were relatively low and scattered among sites, suggesting precipitation as a non-limiting factor for vessel formation.

Climatic signals in vessels in Latvia compared with the Mediterranean region differed: importance of precipitation was much higher in Spain, and temperature was suggested to have secondary influence; showing different limiting factors. Still negative correlation with previous year August precipitation was observed (García-González, Eckstein 2003). Garcia-Gonzalez and Fonti (2006) indicated that vessel area of sweet chestnut showed negative correlation with spring and a positive relation (in some sites) with summer temperatures. Also they found a climate effect on vessel size (area) in different parts of tree-ring (early wood and latewood).

Climatic signals contained in vessel sizes can also be identified in central Latvia. Consequently, VLA can be applied for dendroclimatology. The climatic signals were stronger and more homogeneous compared to tree-ring climatic signals (Matisons, unpublished

results). Studies in Spain and Switzerland (with *Q. petrea*) suggest that VLA can be used in dendroclimatology and dendrochronology (Garcia-Gonzalez, Eckstein 2003; Fonti et al. 2009). Despite the extended time needed for material preparation and measurement, vessel size estimation is superior to tree-ring width for climatic reconstruction, as vessels provide more selective information (Wimmer 2002; Garcia-Gonzales, Eckstein 2003; Tardif, Conciatori 2006). VLA is well related to spring temperatures in the study sites (central parts of Latvia), but correlation with precipitation (as a non limiting factor) is poor. Tree-ring widths nevertheless are a good proxy to characterize ring-porous tree growth.

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Relationship between arterial pressure and pulse wave velocity using photoplethysmography during the post-exercise recovery period

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Abstract

Blood pressure is an important parameter for health assessment. Noninvasive continuous beat per beat blood pressure measurement is still a complicated and expensive procedure. There is a lack of agreement on the optimal blood pressure measurement technique. Potentially the most useful indirect parameter for blood pressure monitoring could be pulse wave velocity or the inverse – pulse transit time. The objectives of the study was to determine the relationship between blood pressure and pulse wave velocity under conditions when different cardiovascular parameters change independently but simultaneously with blood pressure and to clarify whether there is a difference in relationship between blood pressure and pulse wave velocity measured by two different methods in different vascular beds. We determined the relationship between arterial blood pressure (systolic, mean) and the pulse wave velocity from 20 healthy volunteers during a post-exercise recovery period after a load cycling test. A significant correlation ($p < 0.05$) and regression ($p < 0.001$) between arterial pressure and pulse wave velocity was obtained. Pulse wave velocity measurement measured in different vascular beds showed no impact on correlation of arterial blood pressure and pulse wave velocity. The average difference between measured and calculated arterial blood pressure values did not exceed 10 mm Hg.

Key words: non-invasive continuous blood pressure; photoplethysmography; pulse transit time; pulse wave velocity.

Introduction

In recent years cardiovascular disease has become a major reason for high mortality in western countries; therefore a non-invasive and continuous system for monitoring cardiovascular parameters would be of great interest to clinicists and sport medicine physicians. One of the most important parameter's for the assessment of cardiovascular system is arterial blood pressure (BP). Chronic high blood pressure may lead to cardiovascular diseases and consequently is a risk factor for death. Currently, ambulatory blood pressure monitoring is limited to simple measurement of systolic and diastolic blood pressure at intervals. A continuous measurement of BP might provide more useful

information about a person's health condition in clinics and at home. The most logical approach for continuous cardiovascular monitoring of a large population might be a simple, non-intrusive pressure measurement.

Despite many efforts in the past concerning non-intrusive methods for continuous blood pressure measurement, there is still a lack of agreement on the optimal measurement technique.

Potentially the most useful and convenient indirect parameter for continuos, non-intrusive blood pressure monitoring are either the pulse wave velocity (PWV) or the inverse – pulse transit time (PTT). In general the PTT refers to the time it takes a pulse wave to travel between two arterial sites. There are a number of different sophisticated pulse transit time measurement techniques such as the Ultrasound Doppler, arterial tonometry, and the so called "two point" PPG method (Smith et al. 1999; Kanda et al. 2000; Lykogeorgakis 2002). However, the simplest and most convenient method is to compute PTT as a temporal difference between the R wave in an electrocardiogram (ECG) and the beginning of the following pulse wave measured by photoplethysmography (Lutter et al. 2002; Kazanavicius et al. 2003). The reported disadvantage of this described method however is that it is not possible to obtain a "true" value of the pulse transit time, because of a varying pre-ejection time (Robin et al. 1999). Moreover, research by Pollak et al. (1983) has shown that the isometric contraction time is itself time varying and can also be influenced by the blood pressure and stroke volume. The theoretical framework that outlines the relationship between PTT and blood pressure is well-known by the Moens-Korteweg equation, which connects the pulse wave velocity with dimensions of the vessel and the distensibility of the vessel wall. An acute rise in blood pressure will cause the vascular tone to increase and hence the arterial wall will become stiffer, causing PTT to shorten. Equally, a fall in blood pressure will reduce vascular stiffness and consequently the PTT will become larger (McDonald 1974).

Several studies have confirmed the application of the PTT for pressure measurement (Naschitz et al. 2004; Ahlstrom et al. 2005; Kim et al. 2006; Sharwood-Smith et al. 2006) although others still argue and consider this method to be a weak surrogate-measure (Young et al. 1995; Payne et al. 2006). The relationship between pulse transit time and arterial pressure may vary due to numerous physiological factors.

The elastic properties of the arterial wall are a major determinant of the PTT-BP relationship. This relationship can be altered by active changes to vascular tone, via for example sympathetic activity or a particular duration of the cardiac cycle (Albaladejo et al. 2001; Lantelme et al. 2002). Previous studies have also indicated that various vasoactive substances (such as NO and PGI2) can also change the tone of smooth muscles, hence, modify the compliance of the arterial wall in conduit arteries, which in turn influences PWV.

It is important to resolve the contradictions of the PTT approach for non-intrusive pressure measurement, as mentioned in the literature above. Therefore in this present work we have addressed two major questions. First, to determine the relationship between blood pressure and pulse wave velocity under conditions when different cardiovascular parameters such as heart rate and the stiffness of arterial wall change independently, and simultaneously with blood pressure, as is the case during post-exercise recovery. Second, to clarify whether there is a difference in relationship between blood pressure changes and pulse wave velocity measured by two different methods in different vascular beds – by

radial artery sphygmography in conduit artery and by finger photoplethysmography in a diffuse vascular bed consisting of small arteries and arterioles.

Materials and methods

Subjects

The study was performed on 20 healthy volunteers (10 males and 10 females) aged from 19 to 21 years. Before the experimental procedure subjects completed a questionnaire and the findings were used during a health assessment. Only those individuals with no history of cardiovascular or other serious diseases were admitted to the study. Prior to the experiment all participants were informed about the procedure and informed consent was obtained. This study was approved by the local ethics committee. To calculate PWV, a tape measure was used to measure the distances between *fossa jugularis* and sensor points (index finger tip for PPG and radial artery line for SFG). The base P was obtained non-invasively. To alter the P, the subject performed a graded bicycle exercise test. Subjects cycled at three different stages: males – 2 min at 150 W, 2 min at 180 W and 3 min at 210 W; females – 2 min at 120 W, 2 min at 150 W and 3 min at 180 W.

The exercise and measurements were carried out in a well-ventilated room at temperature 23 to 25 °C.

We began data acquisition from one minute after cycling cessation and continued until the pressure returned to baseline. While measurements were recorded the subject's sat comfortably on the veloergometer seat. Measurement sensors were attached to the subject in the following manner: the cuff of P monitor on the right hand brachial artery at elbow, PPG on left hand index finger, SFG on the left hand radial artery. ECG was recorded from III limb lead.

Measurement technique and equipment

A previously tested and approved custom-made, one channel amplifier ECG-1 was used to record ECG's. To register SFG, a pressure transducer (PFSG-064) with a discrimination of 0.1 mm Hg was placed on the right arm on skin over the radial artery in the site where the maximal mechanical pulsation was palpated. The PPG signal was recorded from the right index finger using our custom-made one channel PPG device (University of Latvia ASI) with a wavelength of 940 nm.

All sensors (outputs) were connected to a 16-bit data acquisition card (SAMP-9, Umeå, Sweden), data sampled at 800 Hz and stored for further analyses.

PM was computed using an oscillometric method (Rithalia et al 2000). We developed a special custom-made set by attaching the cuff of a portable digital blood pressure monitor (UA-767 Plus, A&D Instruments LTD®) to a laboratory pressure transducer (BP1-B, World Precision Instruments Inc®). The transducer output was sampled, and PM computed via off-line analysis using Winzoom software package. The systolic blood pressure value was obtained from a digital blood pressure monitor as a single readout from LCD display.

All measurements (PPG, SFG, ECG and P) were continuously recorded during cuff deflation (approximately 25 s). The cuff was inflated again for 15 s and the recording was stopped until the next measurement. This therefore allowed the physiological signals to be registered in separate blocks, each lasting approximately 25 s. The number of blocks for a single subject depended upon the pressure recovery rate, thus the average number of

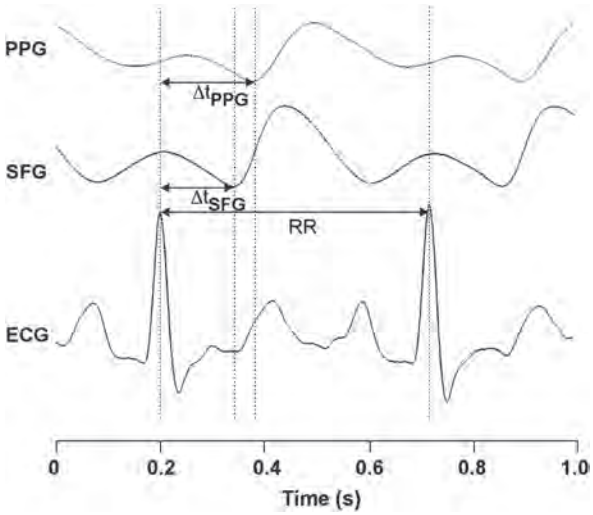

Fig. 1. ECG and simultaneously measured PPG and SFG pulse. Three different parameters were obtained for further research: the time interval between the ECG R wave and the start point of PPG pulse wave (Δt_{PPG}) and SFG pulse wave (Δt_{SFG}) and the time interval between the two ECG R waves (RR).

blocks (pressure readouts) varied individually from 10 to 14. The PPG and SFG pulse wave delay times (*Pulse Transit Time*) Δt_{SFG}, Δt_{PPG} (defined as the time interval between the ECG R wave and the start point of pulse wave) were computed by Winzoom using the method previously described by many authors (Drinnan et al. 2001; Lutter et al. 2002; Nitzan et al. 2002; Kazanavicius et al. 2003) (Fig. 1).

PPG and SFG Pulse wave velocities (PWV_{PPG} and PWV_{SFG}) were calculated using the Matlab (Math Works®) software package. To reduce the influence of the respiratory system we averaged 10-15 successive cardiac cycles (overlaps at least one respiration cycle), so that each block had a single RR, Δt_{SFG}, Δt_{PPG} value (Asmar et al. 1995).

Statistics

Statistical analysis of the data was performed on SigmaStat 2.0, SPSS 10.0 (SPSS®) and Matlab 5.3 (Math Works®) software packages.

Appropriate statistical methods were selected after the Kolmogorov-Smirnov normality test prior to other statistical procedures. The correlation (Pearson or Spearman) between PWV and PS as well as PWV and PM was calculated for both PPG and SFG signals. The same data was tested on different regression models to best fit the regression curve. We used a linear regression analysis (PWV as a dependent and P_S independent) to determine the coefficients of equation for calculation of arterial pressure from PWV measured by two different methods: PPG and SFG (PWV_{PPG} and PWV_{SFG}). The preceeding analyses were performed for single subjects ($12 < n < 16$) and between subjects ($n = 20$). To examine whether regression slopes of PWV_{PPG} and PWV_{SFG} are equal, we performed analyses of covariance (ANCOVA) for individual subjects (Neter, Wasserman 1974). To test whether PWV_{PPG} and PWV_{SFG} have the same correlation, we compared correlation coefficients (using a t-Test).

Results

Almost all measured parameters followed a normal distribution, and in most cases it was possible to apply parametric tests.

Fig. 2 shows pulse wave velocity (derived from pulse wave transit time), arterial blood pressure (systolic, mean) and duration of heart cycle (as R-R interval) recorded during the post-exercise recovery period. The pulse wave velocity measured by PPG (PWV_{PPG}) was significantly higher than the pulse wave velocity measured by SFG (PWV_{SFG}) ($p < 0.001$) (Fig. 3).

We observed a significant correlation between P_S vs PWV_{PPG} ($r = 0.856 \pm 0.109$; $p < 0.05$), P_S vs PWV_{SFG} ($r=0.850 \pm 0.091$; $p<0.05$), PM vs PWV_{PPG} ($r = 0.807 \pm 0.095$; $p < 0.05$) and P_M vs PWV_{SFG} ($r = 0.815 \pm 0.071$; $p < 0.05$), and there was no significant difference between correlation coefficients when we compared PWV_{PPG} with PWV_{SFG} (Fig. 4).

All data were significantly fitted to a linear regression model ($p < 0.001$), and slopes of the regression lines were significantly equal for P~PWV_{PPG} and P~PWV_{SFG} (PWV_{SFG} vs P_S and PWV_{PPG} vs P_S; PWV_{SFG} vs P_M and PWV_{PPG} vs PM) (Fig. 5).

Finally we used the linear regression equation

$$P = \frac{PWV - a}{b} \qquad (1)$$

with individual regression coefficients (a, b) and values of PWV (PWV_{PPG}, PWV_{SFG}) to compute P_S and P_M.

The precision of calculated P_S and P_M, was assessed by subtracting computed values from those measured. On average the difference did not exceed 10 mm Hg (error was plus or minus; Fig. 6).

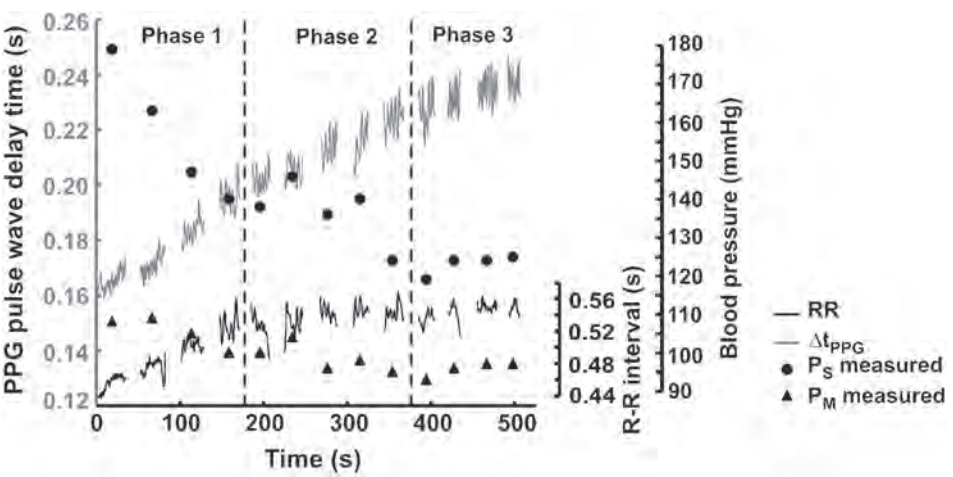

Fig. 2. Pulse wave delay time (Δt), systolic blood pressure (P_S), mean blood pressure (P_M) and duration of heart cycle (RR) – in the whole recovery period for one subject.

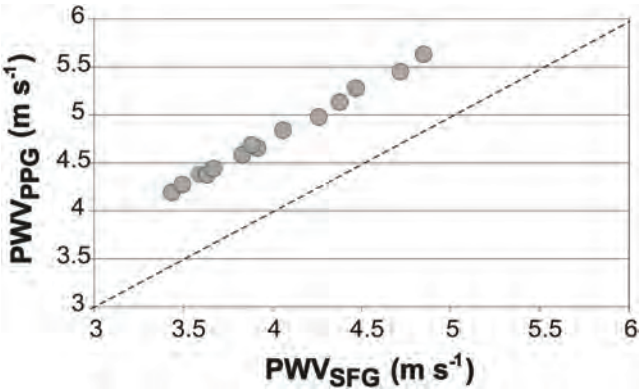

Fig. 3. Comparison of pulse wave velocity (PWV) measured by two different methods on different vascular beds – radial artery sphygmography (PWV_{SFG}) and finer photoplethysmography (PWV_{PPG}). The graph shows PWV data from one subject.

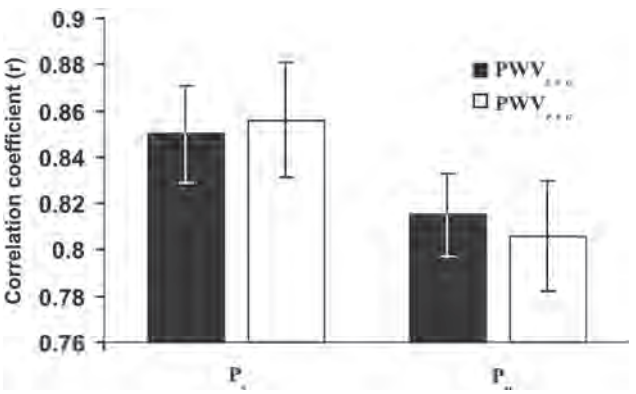

Fig. 4. Comparison of correlation coefficients (P_S vs PWV_{PPG} and PWV_{SFG}, PM vs PWV_{PPG} and PWV_{SFG}). Error bars show value of standart error.

Discussion

In this study we assessed the relationship between blood pressure and pulse wave velocity registered by PPG and SFG under conditions when different cardiovascular parameters such as heart rate and the stiffness of arterial wall change independently but simultaneously with blood pressure. As we predicted, there was a difference between PWV measured by PPG and PWV measured by SFG. The values of PWV, measured at different arterial sites, was not the same, likely due to other influencing factors, such as the reflection of pressure waves and different flow distributions within the arterial system. Differences in PWV measurement methods also should be taken into account. Both the SFG and the PPG records pulse waves, but they do so on the basis of different physical parameters – SFG measures oscillations of conduit artery diameter, while for PPG – blood volume oscillations are measured from a diffuse vascular bed.

We assume that in our case the most important factor that leads to the PWV_{PPG}

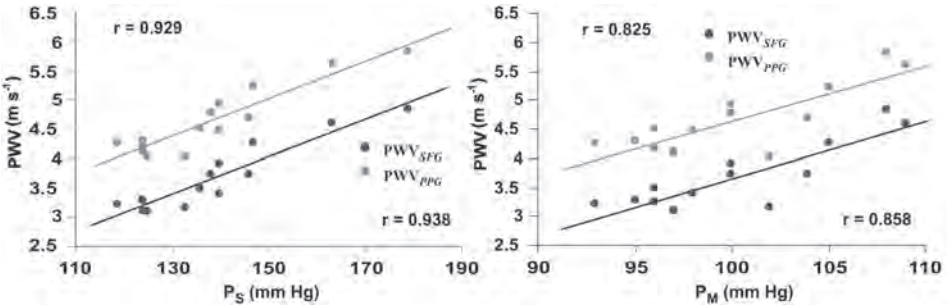

Fig. 5. Comparison of P (P_S in A, P_M in B) and PWV regression slopes when PWV was measured by different methods in different sites: sphygmography (PWV_{SFG}) and photoplethysmography (PWV_{PPG}). Upper correlation coefficient (r) for upper regression line, lower r for lower regression line. The graph shows typical data from on subject.

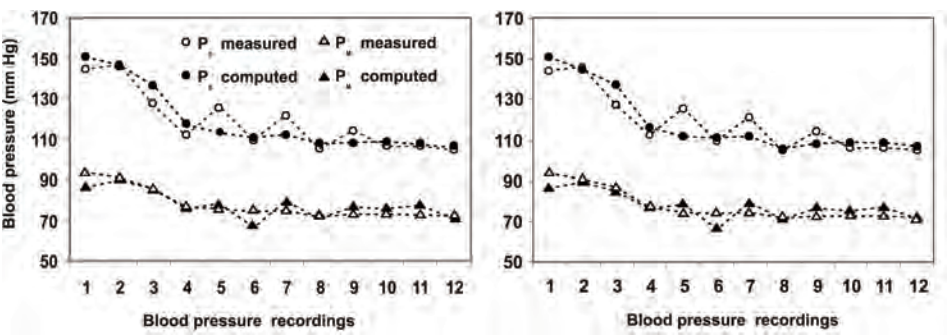

Fig. 6. Comparison of blood pressure values obtained by a conventional compression blood pressure measurement apparatus ($P_{S, M}$ measured) versus blood pressure values obtained based on the pulse wave velocity measurements ($P_{S, M}$ computed). A, PWV measured by SFG; B, PWV measured by PPG. The graph shows typical blood pressure data obtained during the recovery period after the cycling test.

recordings being higher than PWV_{SFG} is the difference in vascular beds, which is where a pulse wave travels and from which the signal is taken. Pulse waves travel from the heart to the radial artery line, but also from the heart to the fingertip. The common part of both paths (PPG and SFG) are large elastic type arteries and conduit arteries. However, 18 to 23 cm path for the PPG recording were only the small, muscular arteries and capillaries, which are located in the palm and fingers tissue. There is evidence that PWV in muscular arteries is higher than in elastic arteries (McDonald 1974; Harada et al. 2002). Thus, it seems reasonable that different vascular beds can have different influences on correlation of PWV~P.

Our study revealed that, contrary to results of other authors (Young et al. 1995, Payne et al. 2006) both PWV_{SFG}~P and PWV_{PPG}~P relationships are linear, PWV~P regression lines have the same slopes and finally the correlation coefficients for both P~PWV_{PPG} and P~PWV_{SFG} did not differ significantly.

As other authors (Barschdorff, Erig 1998; Kazanavicius et al. 2003; Lass et al. 2004; Kim et al. 2006) we also have confirmed in our study a close correlation between PWV

and arterial pressure.

Other authors confirm that PWV is not the only parameter that represents P changes, but others such as heart rate, elasticity of arterial wall and arterial vasomotions may also play a crucial role in blood pressure determination (Lu et al. 1992; Chen et al. 2000; Naka et al. 2003).

Previous studies (Mircoli et al. 1999; Drinnan et al. 2001; Lantelme et al. 2002) have proposed that the heart rate may change PWV by itself, thus there is a necessity to include this in P calculations. Others have shown that heart rate has no clear effect (Kingwell et al. 1997; Lass et al. 2004). Such controversial reports can possibly be explained by the involvement of various regulatory mechanisms that alter pressure in different ways. It is known that in many other studies, the experimental protocol was very similar to ours in that alterations of arterial pressure were induced by physical load. Recent studies showed that the application of a physical task usually leads to change of sympathetic tone, which in turn will increase heart rate (Mircoli et al. 1999). Moreover, regulatory hemodynamic mechanisms are not simple, because the sympathetic nervous system (sympathetic tone) may affect elastic-type and muscle-type arteries differently. Dynamics of the after-exercise recovery process itself may depend on various other factors such as age, type of nervous system and the individual properties of every subject whereby the physiological mechanisms involved can be very distinctive. For example, after-exercise hyperaemia itself may increase a linear velocity of the blood flow which consequently may change arterial tone by the mechanism of endothelium dependent flow mediated dilation (ref FMD). Recent research proved that flow mediated dilution has an remarkable effect on pulse wave velocity (Naka et al. 2006).

Our analyses of the recovery curves for 20 subjects showed that they involve different specific physiological mechanisms, and it was possible to split the whole process in to three consequent phases, based on changes in parameters (Fig. 2).

During the first phase (phase I) arterial pressure (P_M, P_S) and heart rate decrease rapidly and monotonously. During the second phase (phase II) the pressure continues to decrease but the heart rate remains fairly stable, because of its rapid recovery in the previous phase. During the final, third phase (phase III) the values of those three parameters do not change significantly, but the dynamics of pulse wave velocity still shows that relatively slower changes are still occurring, possibly due to normalisation of vascular tone.

We conclude that The PWV~P relationship which is unique for each individual, and that itremains stable during post-exercise restitution. Other factors that change along with P (heart rate and arterial tone) which might influence PWV did not show an impact on PWV~P relationship.

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Treatment of skin ulcers with adenylate deaminase, a glycoprotein from microscopic fungus *Penicillium lanoso-viride*

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Abstract

The effect of topical application of adenylate deaminase (AMPD), an immunomodulatory glycoprotein produced by the microscopic fungus *Penicillium lanoso-viride*, on the experimental skin ulcers was studied in rats. In total, 28 animals in four groups were used. Carbopol hydrogel containing AMPD in doses of both 3.0 and 0.3 U mL^{-1} accelerated healing of the ulcer ($p < 0.05$). Complete (100 %) epithelialisation on the 21st day was observed in a group of animals treated with AMPD 3.0 U mL^{-1} and partial (60 %) epithelialisation in the group treated with AMPD, 0.3 U mL^{-1}. No animal showed entired epithelialisation of skin ulcer treated with Carbopol and untreated control groups. The morphological results were supported by histological findings. Standard blood chemistry and complete blood counts parameters were within the normal range and without significant differences. Experimental results indicated that locally administered glycoprotein-adenylate deaminase hydrogel possesses ulcer healing activity.

Key words: adenylate deaminase, healing, microscopic fungi, *Penicillium lanoso-viride*, skin ulcer.

Introduction

Wound healing is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells. The healing has three phases – inflammation, tissue formation, and tissue remodeling, which overlap in time (Singer, Clark 1999).

At the cellular level, each phase is directed by the coordinated interaction of several types of cells, including inflammatory cells and native skin cells such as fibroblasts, keratinocytes and vascular endothelial cells. At the molecular level, cytokines regulate inflammatory cells during the early stages of wound healing, and throughout the process polypeptide growth factors play dominant roles in the regulation of the proliferation and differentiation of native skin cells and the synthesis of extracellular matrix (Martin 1997; Goldman 2004). It is known that various growth factors, cytokines, and chemokines function in the wound-healing process (Werner, Grose 2003). Platelets, neutrophils, macrophages, endothelium, fibroblasts and epithelium express and release epidermal

growth factor, transforming growth factors, platelet-derived growth factor, interleukins, proteases, matrix proteins and other compounds (Parenteau, Hardin-Young 2007). The regulation of the temporal and spatial expression of growth factors is of major significance for normal repair (Werner, Grose 2003).

Numerous experiments have been carried out to investigate the pharmacological potential of different growth factors and other mediators. Unfortunately, the overall experience with application of these agents to accelerate wound healing has been discouraging. This is not surprising, considering that wound repair is the result of a complex set of interactions among soluble cytokines, formed blood elements, extracellular matrix, and cells (Pierce, Mustoe 1995; Singer, Clark 1999; Bello, Phillips 2000). Nevertheless, intensive research in wound healing is continuing. Current research is leading to new therapies that can be divided into the following classes: growth factors, skin substitutes, extracellular matrix proteins, stem cell therapy, gene therapy, protease inhibitors, angiogenesis stimulants, nitric oxide-releasing agents, adenosine agonists, immunostimulants, vasoactive compounds and granulating agents (Petrova, Edmonds 2006).

Non-specific adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6; AMPD) is a glycoprotein (g_M 210 kD) produced by microscopic fungus *Penicillium lanoso-viride* during a particular phase of growth, i.e., conidiospore formation (Revelina et al. 1981). *In vivo* studies on experimental animals have demonstrated that purified AMPD has diverse immunomodulating properties that influence both cell-mediated and humoral immunity reactions. Injections of AMPD have been shown to augment the formation of antibody-producing cells in spleen, facilitate lymphocyte proliferation *in vitro*, activate mice peritoneal macrophages (Nikolajeva et al. 1999), stimulate natural killer cell and tumour adhesion, restrict growth of several tumours (Zak et al. 1986), enhance host resistance to infectious agents (Nikolajeva et al. 1996), and inhibit experimental autoimmune encephalomyelitis (Nikolajeva et al. 2000). On the basis of previous investigations on the influence of AMPD on the systemic immune response, we hypothesized that AMPD could contribute also the local immune processes in the skin.

The present investigations were designed to evaluate the influence of topically administered AMPD produced by microscopic fungus *Penicillium lanoso-viride* on healing of skin ulcer in rats.

Materials and methods

Animals and animal care

Male Wistar rats (123 to 169 g) were obtained from the laboratory of experimental animals of the Riga Stradins University. The rats were housed in a room with controlled environment (20 ± 2 °C, 55 ± 3 % relative humidity, ventilation and a 12 h alternating light-dark cycle). Standard pelleted complete diet and water were supplied *ad libitum*.

The study was carried out at the University of Latvia, Latvia. The protocol of study was reviewed and approved by the Animal Ethics Committee at the Food and Veterinary service Republic of Latvia.

Materials

AMPD that was sterile and free of endotoxin was prepared and purified in our laboratory

(Nikolajeva et al. 1996). One unit (I U) of enzyme activity was determined as the quantity of AMPD that deaminated 1.0 μmol of 5`-AMP to 5`-inosine monophosphate per min at 37 °C, pH 6.0. Specific activity of AMPD was 10 U per mg protein. Carbopol 940 was obtained from PharmaZell GmbH (Germany). All other chemicals were at pharmaceutical or analytical grade.

Dressings

The polymer-based bioadhesive hydrogel was generated from individual constituents, and finished composition contained 1 % Carbopol 940, 1.35 % triethanolamine, 0.9 % sterile NaCl and filter-sterilized AMPD used in two dosages 0.3 U mL^{-1} or 3.0 U mL^{-1}. The Carbopol gel without active components did not contain AMPD. Gels were prepared before the beginning of the experiment, and complete compositions were maintained in a refrigerator (4 ± 2 °C).

Measurement of healing activity

An experimental skin ulcer was developed under light ether anaesthesia by hypodermic injection of 0.5 mL 9 % acetic acid in the right foreleg and intraperitoneal injection of 6 % dextran in a dose of 300 mg kg^{-1}. Necrosis of the skin and a skin ulcer developed within three days.

The animals were divided into four groups of six to eight each. Animals of group 1 were treated with the Carbopol gel containing AMPD 3.0 U mL^{-1}. Animals of group 2 were treated with Carbopol gel containing AMPD 0.3 U mL^{-1}. The group 3 animals served as reference standard and treated with Carbopol gel without AMPD. The group 4 animals were left untreated and formed the control. The gels were topically applied to the wound bed once a day beginning with the 4th day following the initial wounding (hypodermic injection).

The following parameters were estinated: ulcer closure rate, time of epithelialisation and feeling of pain. The ulcer closure rate was assessed by planimetric measurement of the wound area. The measurements were taken in the beginning of treatment on the 4th day, and also on the 7th, 14th and 21st day. Ulcer closure (V) as a percentage was calculated as follows (Kirker et al. 2002): $V = (A_0 - A_t) / A_0 \times 100$, where A_0 is the original ulcer area and A_t is the area of ulcer on the day of measurement. The process of epithelialisation was evaluated by the coefficient of regeneration (R_{coef}) and regression index of regeneration (R_{reg}):

R_{coef} = the initial area of the ulcer (mm^2) / the area of the ulcer on the day of measurement (mm^2);

$R_{reg} = R_{coef}$ in the experimental group / R_{coef} in the control group.

The period of epithelialisation was calculated as the number of days required for dead tissue remnants to fall and elimination of residual raw ulcer. The feeling of pain was evaluated by light touching with a wood-pointer to the wounded region.

Blood tests

Blood tests were taken from sublingual vein in three to four animals from each group on the 14th and 21st day. The following biochemical and blood counts parameters were estimated – white blood cell count, red blood cell count, haemoglobin, hematocrit, platelet count, leukocyte subpopulation differential, alanine aminotransferase, aspartate

aminotransferase, albumin, alkaline phosphatase, cholesterol and total protein. The percentage of albumin, alpha 1, alpha 2, beta 1, beta 2 and gamma proteins was calculated by protein electrophoresis in PAAG. Blood tests were carried out with a Beckman Coulter (USA) and ILab-300 Plus (Instrumentation Laboratory, USA).

Pathomorphological and histological studies

Three to four animals from every group were sacrificed on the 14th and 21st day. Skin tissue samples (2 × 2-cm pieces) were collected while the animal was fully anesthetized. The skin was excised from the central regions of the ulcer. After tissue harvesting, animals were euthanatized. Samples were fixed in 10 % neutral buffered formalin, dehydrated and then embedded in paraffin. Tissue sections (2 to 4 μm in cross-section) were stained by routine procedures: hematoxylin and eosin for the evaluation of leukocyte infiltration, cellular debris and scar formation, and by van Gieson's connective tissue staining (Böck 1989). The main features were estimated by a scoring: – absent; + weak; ++ medium; +++ strong.

The weight of heart, kidney, liver, testes and epinephric glands was estimated, and their pathomorphological investigation was carried out..

Statistics

All values were expressed as the means ± standard deviations (S.D.). The differences between groups were analysed by one-way ANOVA test from summary data. Values of p less than 0.05 were considered to be significant. The experiment was conducted in duplicate for confirmation.

Results

Ulcer closure in rats treated with adenylate deaminase-containing gels

Hypodermic injection of solution of acetic acid resulted in the necrosis of the skin, and a skin ulcer developed in three days. Total open circular wound surface area reached its peak on the 4th day with an average of 219 ± 78 mm^2.

The rate of ulcer closure in both AMPD gels treated groups was significantly ($p < 0.05$) higher on the 14th and 21st day, as compared to that of the Carbopol and Control groups (Fig. 1). Moreover, the lowest dose of AMPD (0.3 U mL^{-1}) showed a significant increase ($p < 0.05$) in the ulcer closure rate already on the 7th day after three applications. There was a small decrease of the rate of closure at this dose in the subsequent week, while there was a significant increase at the higher dose of 3.0 U mL^{-1}. The rate of the closure decreased in both groups in the third week but there still remained differences in the closed area in comparison with the Carbopol and control groups. There was no significant difference ($p > 0.05$) in the rate of ulcer closure between Carbopol and control groups.

The estimated epithelialisation time corresponded to the closure rate. There was complete (100 %) epithelialisation on the 21st day in the group of animals treated with AMPD 3.0 U mL^{-1} and epithelialisation in 60 % of animals receiving AMPD 0.3 U mL^{-1} (Fig. 2). No animal showed complete epithelialisation of cutaneous ulcer in Carbopol and control groups.

The epithelialisation process evaluated by R_{coef} (Fig. 3) and R_{reg} (Fig. 4) confirmed that AMPD improves healing in a dose dependent manner. Both coefficients were higher in the group treated with AMPD 0.3 U mL^{-1} during first two weeks but the larger dose showed

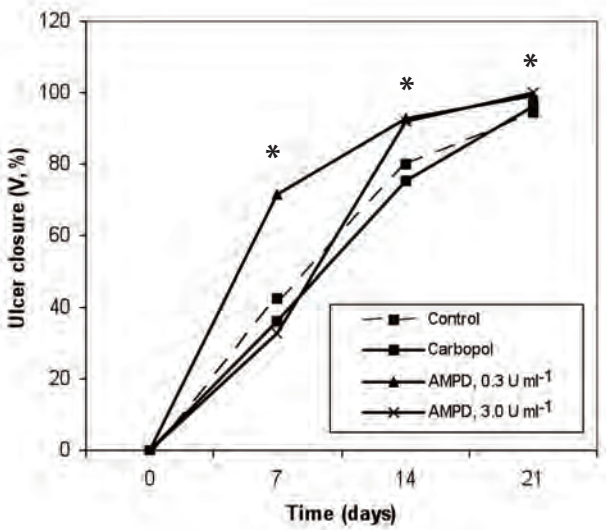

Fig. 1. Influence of AMPD on the ulcer closure (V). Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Percent of ulcer closure (V) was calculated as follows: $V = (A_0 - A_t) / A_0 \times 100$, where A_0 is the initial area of the ulcer and A_t is the area of ulcer on the day of measurement. Replication on days 7 and 14 was six to eight animals and on day 21 – three to four animals. *, significant difference ($p < 0.05$) when compared with the Carbopol and Control groups.

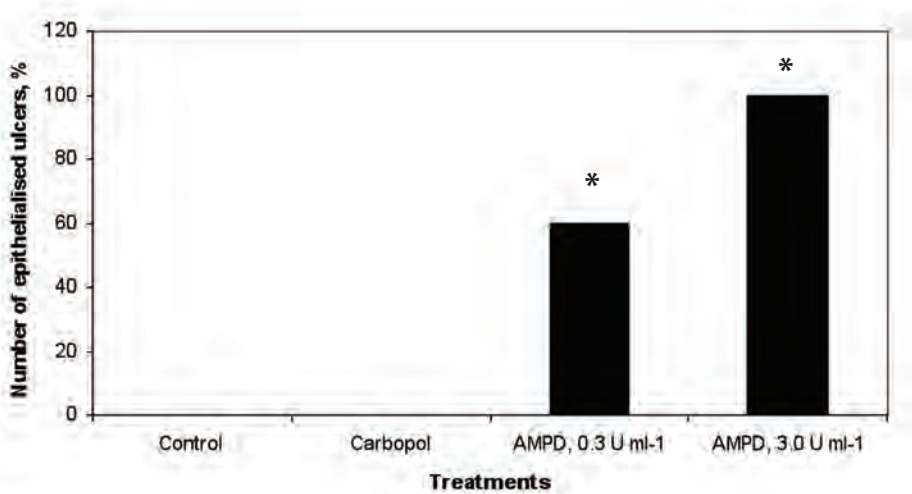

Fig. 2. Percentage of number of epithelialised ulcers at the end of experiment on the 21st day following wounding. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Replication was three to four animals in each group were. The experiment was conducted in duplicate. *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

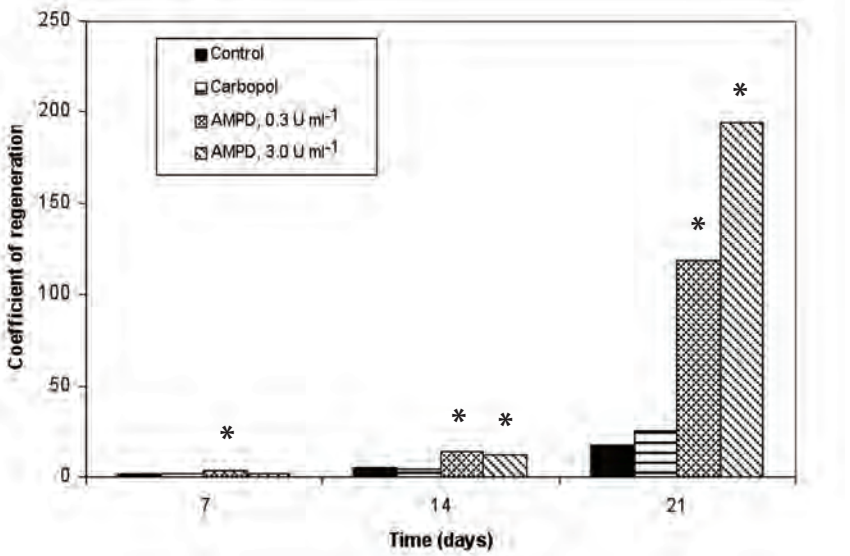

Fig. 3. Epithelialisation expressed as coefficient of regeneration (R_{coef}) in animal groups with different treatment of ulcers. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). R_{coef} was calculated by dividing the initial area of the ulcer (mm^2) by the area of the ulcer on the day of measurement (mm^2). *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

better results later in the third week of application.

Effects of adenylate deaminase on the parameters of blood tests and general health

To establish that no bias mediated via infection or inflammation occurred, standard blood chemistries and complete blood counts were carried out. All blood counts parameters measured (white blood cell count, red blood cell count, haemoglobin, hematocrit, platelet count, leukocyte subpopulation differential) were within the normal range (data not shown). Similarly, all blood chemistry parameters measured (alanine aminotransferase, aspartate aminotransferase, albumin, alkaline phosphatase, cholesterol, total protein) were within the normal range (data not shown). No significant differences ($p > 0.05$) between animal groups were found during the course of experiment.

All animals had a healthy course, with no clinical complications or deaths in either group. Additionally, all animals exhibited normal posture and appetite during the post-injury period. The increase of rat weight during the experiment showed a general well being of experimental animals, similar ($p > 0.05$) among treatment groups (data not shown).

Pathomorphological and histological findings

Ulcer healing differed in intensity and stages of re-epithelialisation, as well as development of granulation and connective tissue in the examined histological specimens.

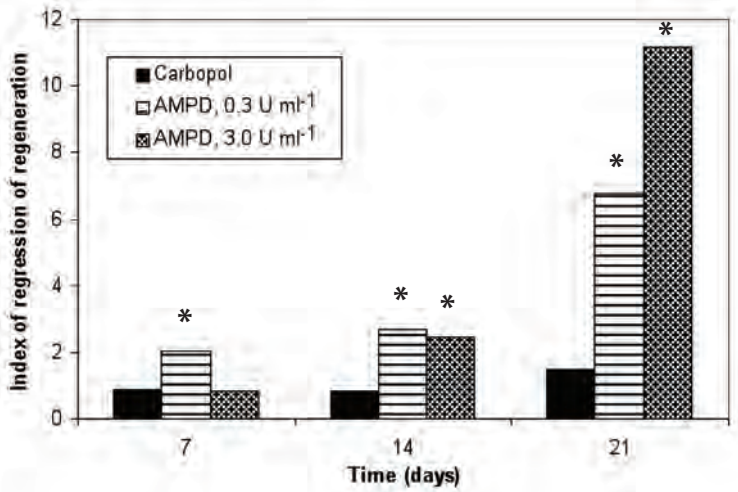

Fig. 4. Influence of AMPD and Carbopol on the epithelialisation of skin ulcers expressed as index of regression of regeneration (R_{reg}). Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). R_{reg} was calculated by dividing the coefficient of regression (R_{coef}) in the experimental group by R_{coef} of the control group (Fig. 3). *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

A strong infiltration of leukocytes was observed in the skin samples from the AMPD 0.3 U mL^{-1} and the Carbopol group on the 14th day, and a moderate infiltration in the AMPD 3.0 U mL^{-1} group (Table 1). There was also a significant amount of cellular debris: small amount in the group treated with AMPD 3.0 U mL^{-1}, moderate in the group of AMPD 0.3 U mL^{-1}, and strong in the Carbopol group. Histological estimation of epithelialisation confirmed planimetric measurement of the ulcer area. The dermis was covered with a fibrin clot what was infiltrated by inflammatory cells and fibroblasts. Connective tissue formation was evaluated as medium score in the AMPD 0.3 U mL^{-1} and Carbopol group, and with a strong score in the AMPD 3.0 U mL^{-1} group. There was a strong similarity between animals regarding features of the scars.

The amount of leukocytes and cellular debris in the region of ulcer decreased during the process of healing until the end of experiment on the 21st day, more or less in all treatment groups, possibly with exception of untreated control (Table 1). It was noted that epithelialisation was completed in the AMPD 3.0 U mL^{-1} group. Notable reduction of scar in comparison with the 14th day was observed only in the AMPD groups in particularly in the of the AMPD 3.0 U mL^{-1} group. The ulcer region revealed a structured type of dermis with ripening connective tissue, parallel to the surface arranged bundles of collagen and full-blooded capillaries, and did not visibly differ between groups.

Heart, kidney, liver, testes and epinephric glands, both on the 14th, and on the 21st day, showed normal appearance and structure without any shift from the norm including weight of organs (data not shown). There was no significant difference between groups of animals ($p > 0.05$).

Table 1. Effect of AMPD on the histological parameters of skin ulcer regions. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Skin tissue samples were excised from the central regions of the ulcer on the 14th and 21st day, processed and stained by hematoxylin and eosin for the evaluation of leukocyte infiltration, cellular debris and scar formation, and by van Gieson's connective tissue staining. The features were expressed by scoring: – absent; + weak; ++ medium; +++ strong. N – not determined

Parameter	Treatment			
	Control	Carbopol	AMPD (0.3 U mL^{-1})	AMPD (3.0 U mL^{-1})
14th day				
Number of animals	0	4	4	3
Epithelialisation	N	+	+++	++
Cellular debris	N	+++	++	+
Connective tissue	N	++	++	+++
Leukocytes	N	+++	+++	++
Scar	N	+++	+++	+++
21st day				
Number of animals	3	3	3	3
Epithelialisation	++	++	+++	+++
Cellular debris	+++	++	++	–
Connective tissue	+++	+++	+++	+++
Leukocytes	+++	++	–	+
Scar	+++	+++	++	+

Discussion

The two main objectives of pharmacology of wound healing are to determine the influence of various measures in wound management and to screen drugs that promote healing. Several materials have been used so far and are reported to affect healing differently. In the present study, we have shown that AMPD, a fungal glycoprotein enzyme, increased the healing rate of skin ulcers, compared with that of untreated ulcer or carrier hydrogel treatment. Although healthy young rats are known to heal skin wounds efficiently and there is likely little room for significant improvement, ulcer closure was significantly promoted in both AMPD hydrogel-treated groups (Fig. 1, 2). The morphological results were supported by histological findings (Table 1).

Acute wounds normally heal in a very orderly and efficient manner by a highly controlled repair process requiring numerous cell-signalling events (Diegelmann, Evans 2004). The initial response to a cutaneous wound induces powerful transcriptional activation of pro-inflammatory stimuli what may alert the host defence. Subsequently, and in the absence of infection, inflammation subsides and is replaced by angiogenesis and remodeling (Deonarine et al. 2007). In our experiments, a greater amount of inflammatory cells was observed in superficial and deep areas of the granulation tissue of the control

groups compared to the AMPD groups, both on the 14th and on the 21st day following wounding. Despite the absence of direct antibacterial activity (Nikolajeva et al. 1996), local application of AMPD to the ulcers shortened the time of neutrophil infiltration. It is possible that AMPD stimulated some or various cells of the local immune system in common with the systemic (i.v., i.m. or i.p.) administration of AMPD (Nikolajeva et al. 1996; 1999; 2000). However, we can not eliminate the possibility that AMPD has an impact also or solely on other cells of skin origin and the ulcer environment. Immunomodulation of the injury response may play a positive role not only with respect to the phase of inflammation, but also to the regeneration. Many of the growth factors present at a wound site can act as mitogens and/or as chemotactic factors for wound fibroblasts (Martin 1997). The involvement of the immune system in the response to tissue injury has raised the possibility that it might influence tissue, organ or appendage regeneration following injury (Godwin, Brockes 2006).

At present, our interests are focused on macrophages, as AMPD possesses stimulating effect on their activity (Nikolajeva et al. 1999). Also for example, chitosan, another well-known activator of macrophages, achieved recommendation for wound healing praxis (Mori et al. 2005). Moreover, most of the factors that regulate wound healing are glycoproteins (Gagneux, Varki 1999), and carbohydrates constitute about 20 - 25 % of the AMPD composition according to our previous study (Nikolajeva et al. 1996). Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response. Activated macrophages release various substances such as cytokines and reactive intermediates and then carry out nonspecific immune responses (Son et al. 2001). In contrast, some studies suggest that "macrophageless" mice are able to repair skin wounds with a similar time course as wild-type siblings, and that repair appears scar-free as in the embryo, which also heals wounds without raising an inflammatory response (Martin et al. 2003). The scientific underpinnings for healing are better understood than ever, although much remains to be discovered. Eventually, an improved understanding of cellular and subcellular physiology may lead to new or better forms of therapy (Baum, Arpey 2005).

Hydrogels have been used primarily in the pharmaceutical field as carriers for delivery of various drugs, peptides and proteins therefore Carbopol gel was chosen as a carrier for AMPD also in our experiments. Hydrogels offer good opportunities due to their inherent biocompatibility. They maintain an appropriate moist environment, which is considered to accelerate wound healing. Their hydrophilic, soft and rubbery nature ensures minimal tissue irritation and a low tendency of cells and proteins to adhere to the hydrogel surface (Chu et al. 1992; Van Tomme, Hennink 2007).

Another relevant question involves the enzymatic activity of AMPD, i.e., deamination of adenosine and their derivatives. Adenosine is a well-known important signaling molecule that is released, for example, under inflammatory conditions. It can show anti-inflammatory as well as pro-inflammatory activities, and the contribution of the specific adenosine receptor subtypes in various cells, tissues and organs is complex (Akkari et al. 2006). Certainly, native AMPD binds to their substrates and deaminates. However, according to our previous studies, AMPD affects the immune response mainly without expression of their enzymatic activity (Nikolajeva et al. 1999). Our future experiments are directed to determine the mechanisms involved in the wound healing activity of AMPD.

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Onset of breeding in Tawny Owl *Strix aluco* in eastern Latvia

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Abstract

Factors influencing onset of breeding in Tawny Owl in eastern Latvia were determined by examining time of egg laying in 344 nests during 1991 - 2008. Data on owls breeding in natural cavities and nest-boxes in six districts (Aizkraukle, Balvi, Gulbene, Ludza, Madona, Rēzekne) were used. Capture and ringing of females was performed at 132 nests and morphological parameters and age of the female were recorded. Data on small mammal abundance at the Teiču Nature Reserve (Madona District) and open-access information on weather conditions at the Zīlāni weather station (56° 31' N; 25° 55' E) were used for analyses. Onset of breeding between years varied substantially: the mean date of the laying of the first egg ranged between March 13 (2002) and April 14 (1996). Range of the recorded extremes is even greater: February 20 (2002) and April 30 (1998 and 2001). There was no trend of the timing of the laying of the first egg during the study period of 18 years. Female characteristics (age, weight, wing-length) had no effect on the onset of breeding. Also small mammal abundance had no effect neither in previous autumn, nor in summer of the breeding season. The only two factors having a statistically significant impact were mean air temperature in February (positive effect; $r^2 = 0.38$; $p = 0.008$) and March (positive effect; $r^2 = 0.55$; $p = 0.0005$), as well as depth of snow cover in February (negative effect; $r^2 = 0.53$; $p = 0.0009$) and March (negative effect; $r^2 = 0.51$; $p = 0.0013$). Mean air temperature and snow depth in January had no effect. We conclude that Tawny Owls benefits by warm springs with little snow cover. These factors may explain the range expansion of the species in the past and, probably, future.

Key words: breeding onset, Latvia, range expansion, *Strix aluco*, Tawny Owl.

Introduction

In general bird populations are regulated by available resources in a density-dependent manner (Lack 1954). However, density independent factors (e.g. weather) may have significant influence on density dependent populations (Newton 1998). The evidence for global warming now appears overwhelming (see review by Watkinson et al. 2004). Climatic factors are usually the limiting factors for species distribution, and thus it is predicted that climatic changes will most probably cause a shift in species distribution. Climate change has been attributed to cause changes in the geographic ranges of bird

populations in Europe already since the beginning of the 20th century (Kalela 1949). Recently, considerable shifts in species distribution ranges are predicted by complex analyses of climate change impact on habitats in Europe (Huntley et al. 2007).

Onset of breeding is critical for reproductive output of the individual. Too early broods may face increased predation and density-independent mortality by weather extremes, and too late broods may be subject to starvation, when the peak of the food source is over (Verboven, Visser 1998; Blūms et al. 2002; Blūms et al. 2005).

Tawny Owl is resident generalist predator, distributed throughout most of Europe (Hagemeijer, Blair 1997). It is a typical species of deciduous temperate forests of Europe (Mikkola 1983).

This species has expanded its range to the North relatively recently – e.g. the first observation of Tawny Owl in Finland was in 1875, when one individual was shot near Helsinki (Collin 1886). The species has also gradually increased in numbers from rare to common species in Estonia until 1960 (Kumari 1958). In Latvia, currently it is the most abundant owl species with a population estimate of 15000 - 20000 pairs and breeding density on average 27 pairs per 100 km^2 (Avotiņš 2000).

In this paper we discuss factors affecting onset of the breeding in Tawny Owls in the eastern part of Latvia and their potential impact on species expansion in the region in the past.

Materials and methods

Data on the laying date of the first egg during 1991 - 2008 were obtained from 344 nests in nine sample plots located in seven districts (Aizkraukle, Balvi, Gulbene, Ludza, Madona, Rēzekne and Ludza) of eastern Latvia (Fig. 1). The majority of the nests were in nest boxes, but in a few cases also in natural cavities. Nests were checked during late incubation stages indicated by behaviour of the females.

We estimated the laying date by back-calculating the date given the egg's incubation stage, measured by the water test method (Blūms 1990). The time period for a laying an egg is 48 hours and the incubation time for Tawny Owl is 30 days (Mikkola 1983). Incubation is started after the first (or sometimes second) egg has been laid (Mikkola

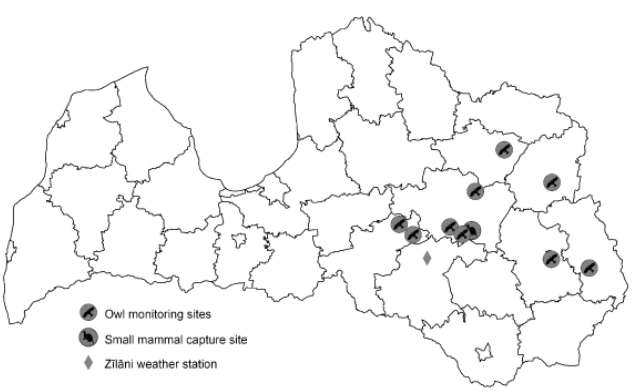

Fig. 1. Geographical location of owl study sites, small mammal trapping site and Zīlāni weather station

1983). In some cases, when the young were already hatched at the time of the first visit, we estimated the laying date also by the age of the nestlings. Young of the Tawny Owl are fledging by the age of 28 to 37 days (Mikkola 1983).

The mean date of the laying of the first egg was determined annually by calculating the arithmetic average of the Julian date of all first eggs laid in the respective year.

Capture and ringing of the breeding females were performed in 132 nests. Females were captured either by netting them at the opening of the nest box (cavity) or by trapping them in the nest box by attaching a swing door to the entrance. Capture was performed during late stages of incubation to avoid desertion of the nest. We recorded female morphological parameters (maximum wing length, tail length, weight) and age by moulting characters (Petty 1992).

Data on small mammal density at the Teiču Nature Reserve were used for analyses. The reserve is located in very close proximity of the Mētriena sample plot with some of the nest boxes placed inside the reserve. Small mammals were snap-trapped in autumn (September) and summer (June) in meadow and forest habitats (Pupila, Bergmanis 2006; A. Pupila, personal communication). Open access data on climate conditions at the Zīlāni meteorological station (56° 31' N; 25° 55' E) were used. Data were analyzed by Pearson product-moment correlation (Zar 1996).

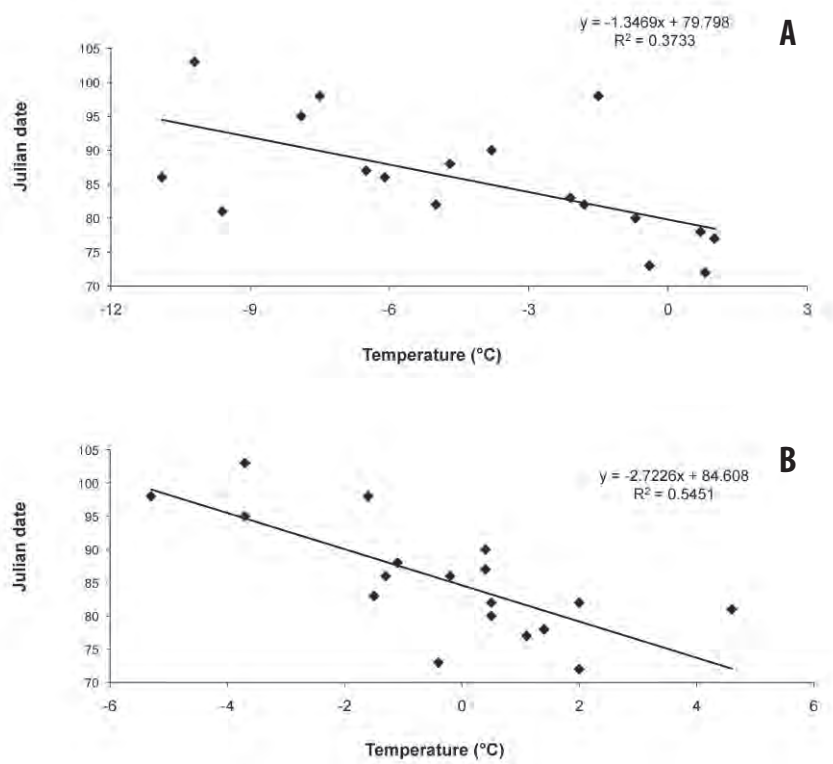

Fig. 2. Laying date of the Tawny Owl in Eastern Latvia explained by the mean air temperature in February (A) and March (B).

Results

The mean onset of breeding of the Tawny Owl in Eastern Latvia (Table 1) ranged between March 13 (2002) and April 14 (1996). Range of the recorded extremes was even greater: February 20 (2002) and April 30 (1998 and 2001). Onset of breeding was not shifted in either direction during the study period of 18 years ($r^2 = 0.007$; n.s.). Mean laying date in Eastern Latvia over the period of 1991 - 2008 was March 28.

Data showed that the laying date of the first egg had not been influenced by the female characteristics: age ($r^2 = 0.005$; n.s.; n = 129), weight ($r^2 = 0.062$; n.s.; n = 132), tail-length ($r^2 = 0.063$; n.s.; n = 112) and wing-length ($r^2 = 0.004$; n.s.; n = 115).

The annual variation in laying dates was not driven by the abundance of small mammals in the field in the previous October ($r^2 = 0.007$; p = 0.76), nor in June of the current breeding season ($r^2 = 0.05$; p = 0.36).

There were only two factors that had a statistically highly significant impact on the laying date of Tawny Owls in eastern Latvia: temperature and snow depth. Increased air temperature in February (Fig. 2A) and March (Fig. 2B) caused earlier onset of breeding, as expected. In contrast, increased snow depth in February (Fig. 3A) and March (Fig. 3B) delayed the onset of breeding. Mean air temperature and snow depth in January had no effect.

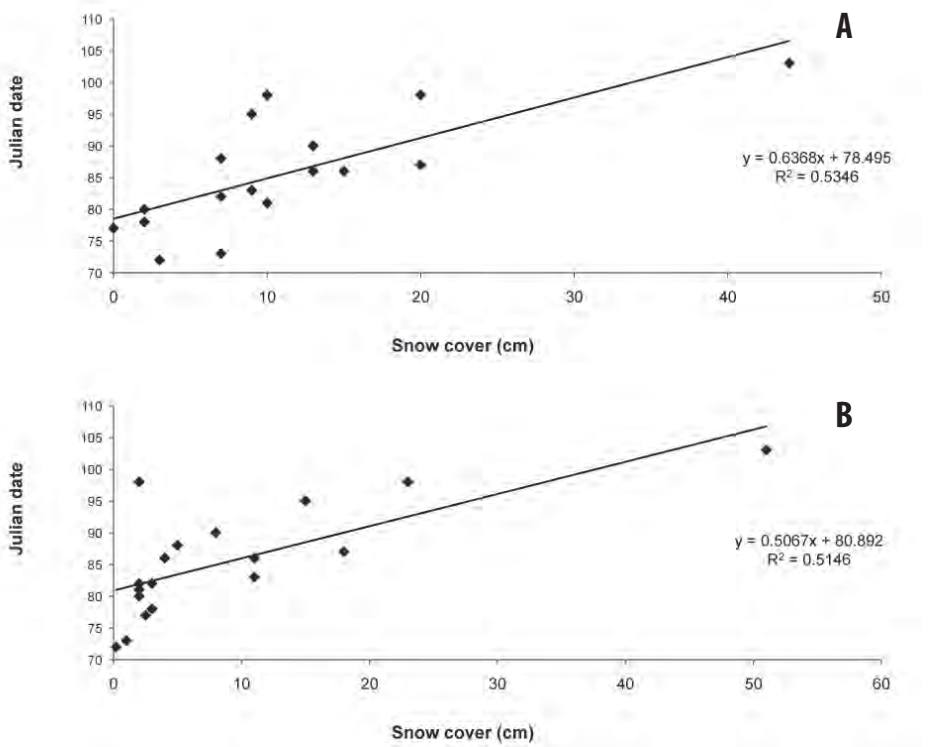

Fig. 3. Laying date of the Tawny Owl in Eastern Latvia explained by the depth of snow cover in February (A) and March (B).

Table 1. Laying dates of the first egg by Tawny Owl *Strix aluco* in eastern Latvia in 1991 - 2008

Year	Median laying date	Range	Nests studied (number)
1991	23 March	14 March - 2 April	11
1992	23 March	16 March - 30 April	12
1993	24 March	3 March - 8 April	24
1994	27 March	17 March - 6 April	15
1995	19 March	6 March - 31 March	22
1996	14 March	4 April - 26 April	23
1997	14 March	2 March - 27 March	17
1998	9 April	28 March - 30 April	12
1999	28 March	4 March - 2 April	20
2000	21 March	6 March - 2 April	15
2001	29 March	11 March - 30 April	18
2002	13 March	20 February - 24 March	29
2003	27 March	19 March - 4 April	19
2004	1 April	19 March - 4 April	25
2005	9 April	31 March - 27 April	19
2006	6 April	1 April - 15 April	15
2007	22 March	10 March - 30 March	22
2008	20 March	8 March - 3 April	26

Discussion

Onset of breeding of Tawny Owl in Eastern Latvia between years varies widely (Table 1). There is no trend to become earlier or later as described for other species e.g. onset of breeding in Pied Flycatcher is coming later in Finland (Laaksonen et al. 2006) and in Great Tit earlier in Germany (Winkel, Hudde 1997). It is probable that our data series are too short (18 years) to detect any considerable change in onset of breeding. As a resident species, Tawny Owl might respond quickly to changes in spring phenology, but the response to climate change can be expressed in other ways. The response to climate has been has been observed to differ in different populations of a single species (e.g. for Great Tit see Visser et al. 2003). However, a northwards range shift is the most common observed consequence of climate change for all taxa (Parmesan, Yohe 2003).

Climate change has been causing and is predicted to cause various ecological effects on avian species (Winkler et al. 2002) and ecosystems in general (McCarty 2001). The most serious concern raised is asynchrony between bird breeding time and the availability of food resources (Visser et al. 1998; Both, Visser 2001), particularly for long-distance migrants feeding mainly on insects (Sanz et al. 2003). However, food availability shifts might not be applicable to owl breeding time. The main food source of owl populations in Northern Europe is voles (Sundell et al. 2004). It is predicted that vole populations are much more numerous in autumn in comparison to spring, thus food availability in theory increases during the season. Early breeding in owls might provide other advantages unrelated to food (e.g. young have to acquire hunting skills before winter). However,

food has been documented as an important factor for other parameters of owl breeding (Brommer et al. 2004).

Tawny Owls breed also in cities, which can begin as early as in December (Petriņš 1986; O. Keišs, unpublished data). The factors determining the early onset of breeding in the urban environment remain unclear. Possible explanations are (i) availability of food in towns since rodents and small passerines accumulate in suburban areas in winter; this idea has been supported by Petriņš (1986); (ii) temperature – mean air temperature is higher in cities; (iii) light conditions – anthropogenic light sources in cities might mislead birds on onset of 'spring' by an altered photoperiod. Probably, the same factors are responsible for onset of breeding of this species also in the wild environment. Our observation that a deep snow cover delayed onset of breeding, might be indirectly related to the availability of food, as voles and mice are difficult to capture under snow. Thus breeding begins when snow disappears.

In other studies female age has been found to have an indirect effect on reproductive output by earlier breeding of older females (Blūms et al. 2002). Thus, we might expect older females to start to breed earlier. This seems reasonable if early breeding individuals gain more fitness than late breeders. In our study, however, female age had no effect on onset of breeding.

Female body mass in our study had only a slight (statistically insignificant) effect on the onset of breeding of Tawny Owl. Size-adjusted body mass adjusted to wing length in birds would have been better measure of female condition to consider for effect on onset of breeding.

We conclude that the Tawny Owl benefits from warm springs with little snow cover. These climatic factors might have an indirect effect on food availability, as snow limits access to rodent prey and thus may delay onset of breeding. Global warming thus might have driven the gradual increase from rare to common species status in Estonia until 1960 (Kumari 1958; Leibak et al. 1994). The further colonisation forecast for the late 21st century distribution includes mainland Europe up to the Arctic Ocean (Huntley et al. 2007).

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Method for identification of avian species by eggshell microstructure: preliminary study

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Abstract

It is widely known that birds and their eggs can be identified by their outer characteristics – shape, coloration, spots etc. These features can not be used when only a single piece of eggshell is available (e.g. remains at nests). The aim of this preliminary study was to determine whether microscopy methods can be used for avian species identification by eggshells. Stereo microscope, transmission and reflection light microscope and scanning electron microscope were used to describe eggshell microstructure, empasizing differences among species. Differences in eggshell structure of some Passerine bird species were obrerved in the eggshell mammillary layer by transmission light microscope. However the size of eggshell mammillae was overlapping, and more studies are necessary to discover species-specific structures in avian eggshell.

Key words: avian eggs, eggshell microstructure, identification of species, mammillae.

Introduction

Some research has been conducted on eggshell microstructure in to obtain a method for simple identification of differences among avian species, when only a small piece of eggshell is available. This would allow to identify species of eggs in museum collections, since it does not require destruction of the egg, and also to identify small remains of eggshells in nests (especially cavities), when species identification by other methods is not possible.

Microstructure of the eggshell has been studied with a focus on poultry and these studies are important for commercial purposes in order to improve eggshell strength (Dawkins et al. 2004; Peebles, McDaniel 2004). Most of the knowledge about eggs and eggshells originate from the studies of domesticated birds, but research on wild bird eggs were started only recently (Gosler et al. 2004; Massaro, Davis 2005). However, A.L. Romanoff and A.J. Romanoff (1949) described differences in eggshell microstructure of various species groups. Microstructure as well as pore differences of eggshells of ratite birds were found not to be related to the certain species (Board 1982; Board, Sparks 1991). Traditional identification methods of birds and their eggs are based on morphological

analysis (Svenson et al. 1999; Harrison 1975) and there is a lack of avian species identification methods based on only pieces of cracked eggshell.

Formation of an avian egg occurs in female reproductive organs (Romanoff, Romanoff 1949) whereas an eggshell itself is developed in uterus and has several layers (Fig. 1). The growth of the inorganic part of the eggshell initiates from the inner part consisting of organic shell membranes with mammillary cores (Romanoff, Romanoff 1949; Board 1982; Peebles, McDaniel 2004). The first conic layer in the eggshell mineralization stage is formed by growing mammillary knobs (mammillae or cones). When these knobs conjugate, the formation of a palisade layer begins. Mammillary cores are randomly placed on the shell membrane and therefore pores are formed on the eggshell (Romanoff, Romanoff 1949).

The aim of this preliminary study was to determine whether microscopy methods can be used in avian species identification based on eggshells.

Materials and methods

Eggshell samples

Eggshells of closely unrelated avian species for comparison of eggshell microscopy structures included: goose *Anser* sp. (Anseriformes; n = 1), dubbling duck *Anas* sp. (Anseriformes; n = 1), Great Tit *Parus major* (Passeriformes; n = 2), House Sparrow *Passer domesticus* (Passeriformes; n = 2), Chiffchaff *Phylloscopus collybita* (Passeriformes; n = 1), Starling *Sturnus vulgaris* (Passeriformes; n = 2) and Skylark *Alauda arvensis*

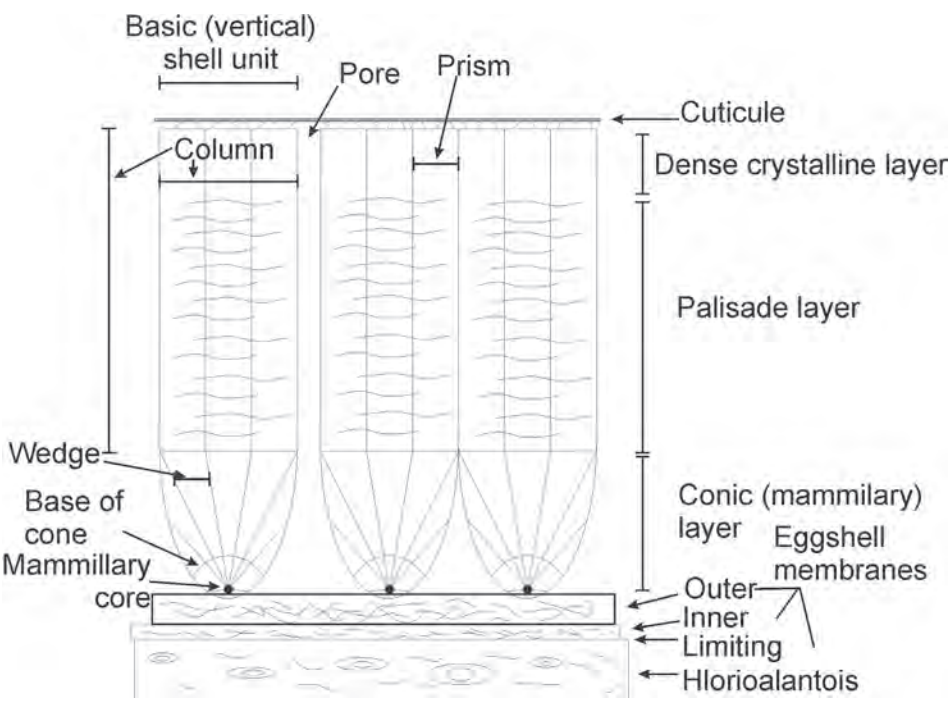

Fig. 1. Structure and elements of the avian eggshell in radial view (Mikhailov 1997; Peebles, McDaniel 2004; Lammie et al. 2005).

(Passeriformes; n = 1). All samples were kindly provided by the Museum of Zoology of the University of Latvia.

Eggshells for analysis of eggshell inner structure were taken from some Passerine bird (Passeriformes) species: Sedge Warbler *Acrocephalus schoenobaenus* (n = 5), Aquatic Warbler *Acrocephalus paludicola* (n = 3), Chiffchaff *Phylloscopus collybita* (n = 3), House Sparrow *Passer domesticus* (n = 5), Tree Sparrow *Passer montanus* (n = 3) and Linnet *Acanthis cannabina* (n = 4). Most of the samples came from collections in the Museum of Zoology (University of Latvia; 11) and Museum of Natural History of Latvia (10). Two of three samples of Aquatic Warbler were collected in Belarus (wild) and Germany (captive population).

Sample preparation and microscopy

Five different microscopes were used in the examination of structure of eggshells: (1) stereo microscope, (2) reflection light microscope, (3) transmission light microscope, (4) fluorescent light microscope and (5) scanning electron microscope.

Stereo microscope. Complete fragments (non ground) of eggshells were examined at magnification of 10 × 0.63 to 10 × 3.

Reflection, transmission and fluorescent light microscope. Leica microscopes were used with magnification of 100, 200 and 400 times. Specimens were prepared in two ways: (1) placing a ground eggshell with water a drop on a glass slide and covering it with cover-slip and (2) placing a non ground eggshell fragment on a glass slide. A digital camera *Canon Powershot S60* (resolution 4 Mpix) was used to take images of the specimens.

Scanning electron microscope (SEM). TM-10000 (*The Hitachi Tabletop Microscope*) was used at magnification of 300 and 1000 times for examination of non ground fragments of eggshells.

The main criteria was detailed investigation were the visibility of as many as possible of eggshell structures in order to compare and determine differences among species.

Analysis of the inner structure of eggshells was performed on a transmission light microscope *Leica DM2000* at magnification 200 times. Samples were prepared using non ground fragments of eggshells. A small fragment of the eggshell (at least 1 × 1 mm) was taken.

The shell membrane that covers the conic layer of the eggshell canblock the view of the inner part of the eggshell. The connection between the membrane and conic layer is very tight (Romanoff, Romanoff 1949), and thus removal of membrane from the conic layer by mechanical methods is impossible. Removal of organic membrane requires chemical methods. Use of acids in this case is not recommended, since calcite reacts with various acids (Brown et al. 2006). Therefore we used 5 % NaOH to remove the membranes (Peebles, McDaniel 2004). Samples were incubated in micro-tubes with 5 % NaOH placed in boiling water bath for 10 to 20 min.

The inner surface of the eggshell was examined. For further analyses images of specimens were taken with a digital camera *Canon Powershot S70* (resolution 7.1 Mpix). Dimensions of mammillae were measured with software *Scion Image for Windows*. Two images of each sample were measured. Minimum (L_{min}) and maximum (L_{max}) size of each mammilla were measured (Fig. 2). Mean size [(L_{min} + L_{max}) / 2] and difference between minimum and maximum size of a mammilla were calculated. For each specimen 33 to 92 measurements of mammillae (59 on average) were made. The number of measurements

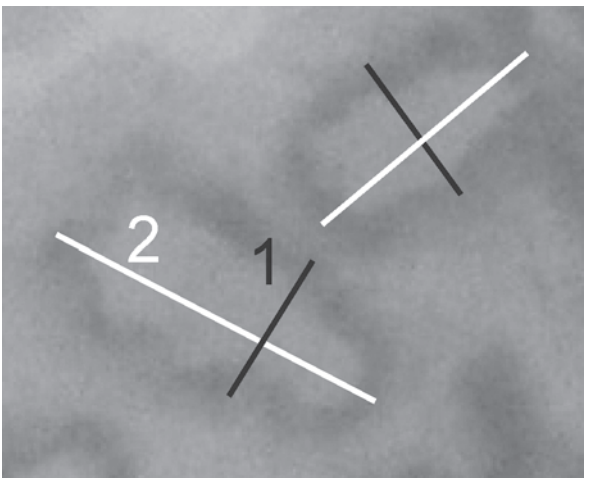

Fig. 2. Measurement of (1) minimum (L_{min}) and (2) maximum (L_{max}) size of eggshell mammilla using a transmission light microscope image at magnification 200 times.

made per specimen depended on quality and size of the shell fragment and quality of the picture. Differences in mammillae dimensions were tested by Scheffe and LSD (Fishers' Least Significant Difference Method) methods using *SPSS for Windows* software (Sokal, Rohlf 1995).

Results and discussion

Comparison of microscopy analysis of eggshell structures

Stereo microscope. No apparent differences were observed among eggshell fragments of various bird species in examination by stereo microscope. Some intra-specific variation was found in spot patterns and structure of the eggshell surface. It was possible to distinguish the inner and outer surface of the eggshell, which is important for identification of the eggshell position when preparing specimens for light microscope.

Reflecting and transmission light microscope. First, a ground eggshell fragment in a drop of water was examined. With reflecting light it was impossible to identify structures that could indicate inter-specific differences. However, observations were obstructed by glare from the cover-slip produced from the reflecting light. Under transmission light it was possible to identify potential variable structures in the ground eggshell substance (e.g. crystallised formations), but identification was impossible. The advantage of this method was the very small amount of eggshell required for the specimen. However, it was impossible to grind eggshells uniformly; hence the consistency of the eggshell was indicated rather than species-specific features. This method needs more exploration, since samples can be obtained with minimal damage to eggs.

Secondly, complete (non ground) eggshell fragments were examined. Differences between inner and outer surfaces of the eggshell were clearly distinguished. The outer surface of the eggshell was considerably glossier than the inner surface. This can be explained by the outer part of the eggshell, palisade layer and cuticle that makes the surface smooth. The inner surface of the eggshell consists of eggshell membranes and the conic

layer, which produces irregular structures. Comparing transmittion and reflection light microscopy, a better view was obtained with transmitted light. Reflected light produced a glare that made all structures less distinguishable. Specimens of complete eggshell fragments can be preserved and reused. Thus the procedure can be repeated, if necessary.

Comparing the inner and outer surface of the eggshell among various species, more differences were observed for the inner surface. However, it is known that the outer surface of the eggshell is sometimes covered by cuticle and shell accessory materials that can vary among species (Board 1982; Board, Sparks 1991; Peebles, McDaniel 2004). Evidently, shell accessory materials on the eggshell outer surface are too small to be studied under a light microscope or they are not regularly distributed on eggs. Differences among various species could be observed on the inner surface and are expressed at the conic layer of the eggshell. Eggshell membranes may occur on the conic layer, but differences among these membranes of various eggshells were not observed. This can be explained by the organic origin of the membranes. the conic layer is formed by inorganic material (calcite), and hence it may remain intact for a long time. In this study basal caps of the conic layer were found to be the varying structures of the inner surface – mammillae vary in dimensions, shape, quantity and, probably other parameters. Therefore this feature can be explored further to identify species specific differences of avian eggshell.

Fluorescent light microscope. Some structures of complete eggshell fragments under fluorescent light could be better observed in comparison with transmission light, which might be explained by the luminescent characteristics of calcite (Brown et al. 2006). under fluorescent light, it is possible to clearly separate calcite crystals from other structures in the field of view. However, it was difficult to make qualitative photographs with reflecting light, which limit the possibility to use this method.

Scanning electron microscope. There were similar results found with SEM as shown for transmitting light microscope – there were no clearly visible differences in the outer surface, but it was possible to identify variable structures on the inner surface: mammillae. The advantage of the electron microscope is the high quality pictures that can be produced. SEM has been used for eggshell studies (e.g. Mikhailov 1997), and the acquired images of the conic layer are promisingin the search for differences among species. SEM has been used to measure thickness of the eggshell (Romanoff, Romanoff 1949; Ar et al. 1979; Solomon 1997; Pantheleux 1999; Lammie et al. 2005; Mikhailov 2004; Peebles, McDaniel 2004), detect changes of structure in different stages of incubation (Simons 1971; Hunton 1995; Hunton 2005), observe pore status (open, closed) and dimensions (Board 1982; Board, Sparks 1991). SEM is recommended for further study of the variation of eggshell among species and even individuals (e.g. differences of ecological factors depending on breeding site), nevertheless it is a more complicated method and can not be used out in the field research.

Analysis of eggshell inner structure

Mean mammillae size (minimum, maximum and mean values; Table 1) differed significantly (Scheffe and LSD, $p < 0.04$) between all six studied species (Fig. 3 A - C; Table 2) except between minimum size for Chiffchaff and Linnet by Scheffe (Fig. 3 A; Table 2). The means differed significantly by the LSD test between species of genus *Passer* – Tree Sparrow and House Sparrow, and between these and the other studied species (LSD, $p < 0.01$; Fig. 3 D; Table 2). Using the Scheffe test the significant differences between

Table 1. Sizes and calculated values (μm) of eggshell mammillae

Species	Minimal size	Maximal size	Mean value of sizes	Difference in size	n
Sedge Warbler *Acrocephalus schoenobaenus*	24.23	28.12	26.18	3.89	5
Aquatic Warbler *Acrocephalus paludicola*	26.52	30.27	28.39	3.74	3
Chiffchaff *Phylloscopus collybita*	17.61	20.62	19.11	3.01	3
House Sparrow *Passer domesticus*	31.68	36.74	34.21	5.05	5
Tree Sparrow *Passer montanus*	38.93	44.88	41.91	5.95	3
Linnet *Acanthis cannabina*	18.87	22.88	20.88	4.01	4

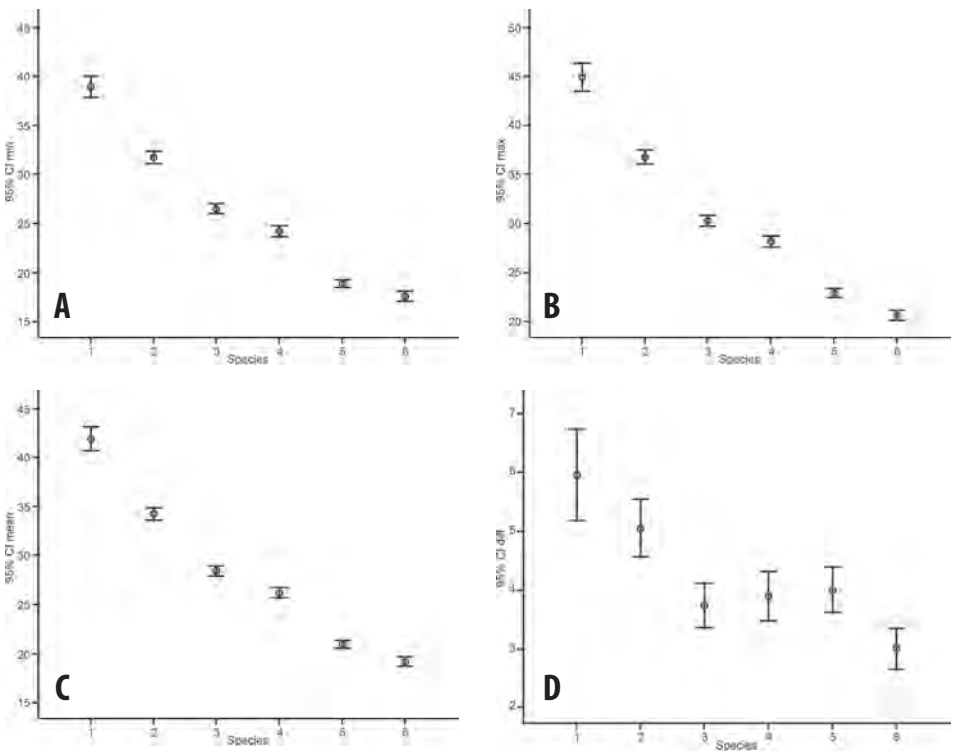

Fig. 3. Size of eggshell mammillae (μm) of the specimens (bars indicate confidence intervals of 95 %). A, maximal size of mammillae; B, minimal size of mammillae; C, mean size of mammillae; D, difference in size of mammillae. Species: 1, Tree Sparrow *Passer montanus*; 2, House Sparrow *Passer domesticus*; 3, Aquatic Warbler *Acrocephalus paludicola*; 4, Sedge Warbler *Acrocephalus schoenobaenus*; 5, Linnet *Acanthis cannabina*; 6, Chiffchaff *Phylloscopus collybita*.

Table 2. Significant differences in sizes of eggshell mammillae by analysis of eggshell inner structure.Data in parentheses are not significantly different

Species		Tree Sparrow *Passer montanus*		House Sparrow *Passer domesticus*		Aquatic Warbler *Acrocephalus paludicola*		Sedge Warbler *Acrocephalus schoenobaenus*		Linnet *Acanthis cannabina*	
		Scheffe	LSD	Scheffe	LSD	Scheffe	LSD	Scheffe	LSD	Scheffe	LSD
House Sparrow	Min.	<0.001	<0.001								
Passer domesticus	Max.	<0.001	<0.001								
	Mean	<0.001	<0.001								
	Diff.	(0.235)	0.009								
Aquatic Warbler	Min.	<0.001	<0.001	<0.001	<0.001						
Acrocephalus	Max.	<0.001	<0.001	<0.001	<0.001						
paludicola	Mean	<0.001	<0.001	<0.001	<0.001						
	Diff.	<0.001	<0.001	0.005	<0.001						
Sedge Warbler	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
Acrocephalus	Max.	<0.001	<0.001	<0.001	<0.001	0.012	<0.001				
schoenobaenus	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
	Diff.	<0.001	<0.001	0.004	0.001	(n.s.)	(n.s.)				
Linnet	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
Acanthis cannabina	Max.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
	Diff.	<0.001	<0.001	(0.052)	0.001	(n.s.)	(n.s.)	(n.s.)	(n.s.)		
Chiffchaff	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	(n.s.)	0.012
Phylloscopus	Max.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	<0.001
collybita	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.036	0.001
	Diff.	<0.001	<0.001	<0.001	<0.001	(n.s.)	(0.059)	(n.s.)	0.029	(n.s.)	0.01

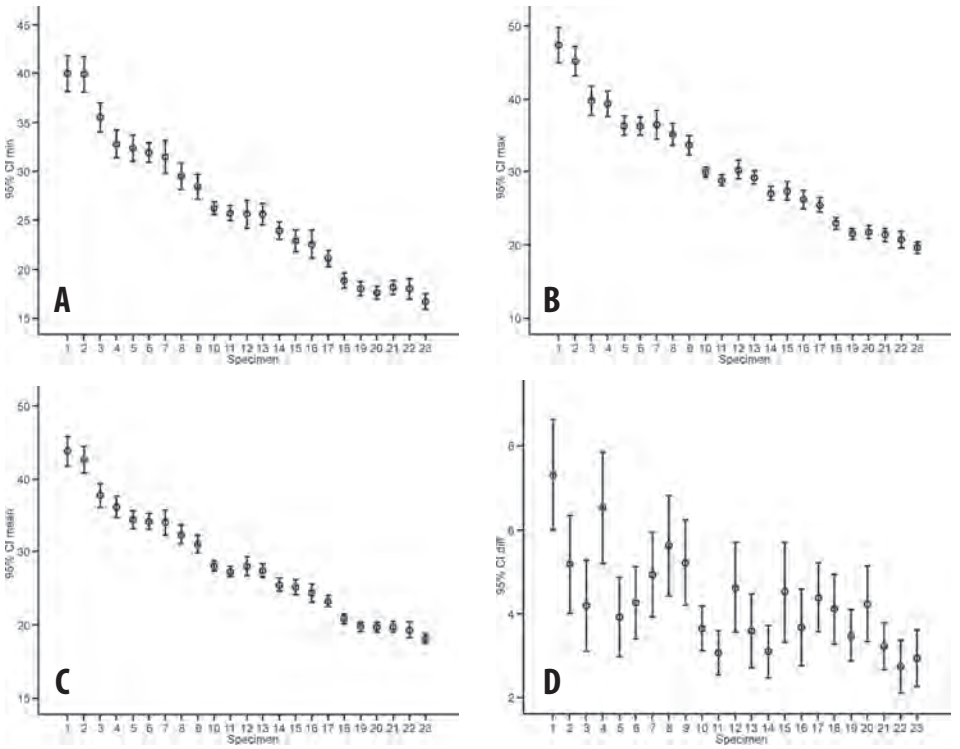

Fig. 4. Size of eggshell mammillae (μm) of the specimens (bars indicate confidence intervals of 95 %). A, maximal size of mammillae; B, minimal size of mammillae; C, mean size of mammillae; D, difference value of size of mammillae. Species: 1 to 3, Tree Sparrow *Passer montanus*; 4 to 8, House Sparrow *Passer domesticus*; 9 to 11, Aquatic Warbler *Acrocephalus paludicola*; 12 to 16, Sedge Warbler *Acrocephalus schoenobaenus*; 17 to 20, Linnet *Acanthis cannabina*; 21 to 23, Chiffchaff *Phylloscopus collybita*.

Tree Sparrow and House Sparrow disappears. Significant differences were observed also between Chiffchaff and Sedge Warbler (LSD, $p < 0.03$; Fig. 3 D; Table 1) and Chiffchaff and Linnet (LSD, $p = 0.01$; Fig. 3 D; Table 2).

The significant species differences largely remained also when any one egg of each species was considered, although in some cases we observed significant variation within one species e.g. Tree Sparrow (Scheffe and LSD, $p < 0.001$), House Sparrow (Scheffe and LSD, $p < 0.001$) and Aquatic Warbler (Scheffe, $p < 0.001$; Fig. 4). However, in some cases there were no differences between samples of different species e.g. we found no significant differences between Sedge and Aquatic Warbler as well as between Chiffchaff and Linnet (Fig. 4).

As significant differences were observed for species of one genus – Tree and House Sparrow this might suggest that phylogeny of the species is not related to size differences of eggshell mammillae. On the other hand, significant differences among samples of one species indicated that size of eggshell mammillae is not species specific. There might be other factors that affect the size of eggshell mammillae (nutrient availability for the

female, altitude of breeding site, incubation stage of the egg etc.). It is also likely that a larger sample size for each species might improve the differentiation among species. Some of the eggs at the Zoology Museum of University of Latvia and the Museum of Natural History of Latvia collections were collected in the 1920-ties and 1930-ties and might be misidentified (A. Petriņš, personal communication); thus collection of fresh and correctly identified eggs in the field is necessary for further study.

In the future it is important not only to analyze eggshell structure for species identification, but also eggshell structure in various environments, to exclude the possibility of ecological factors affecting phenological features.

For further studies it is important to explore not only species identification problems, but also eggshell structure in various environments, to exclude the possibility of these factors rather than species causing observed differences in eggshell structure. It can also reveal the intensity of environment and ecological factors affecting eggshell structure.

Conclusions

1. Differences observed in mammillae can be used for avian eggshell analysis.
2. Intra-specific differences of mammillae tends to be significant.
3. Mammillae (minimum and maximum, difference and mean) sizes could not be used for precise species identification and the differences were not species specific, but tendencies of differentiation in species that might be affected by other factors.

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The shape and dimensions of photoplethysmographic pulse waves: a measurement repeatability study

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Abstract

Photoplethysmography (PPG) is a non-invasive, simple and well-known physiological method for recording and monitoring blood volume pulse, but still there are many unknown issues concerning this method such as individual variability and repeatability of the PPG waveform. In our study we recorded two PPG signals (from the radial artery and from the finger) several times in resting conditions to evaluate the individual repeatability of PPG wave shape. Arbitrary amplitudes of incisura and maximum of dicrotic notch and time from foot to anacrotic maximum had the greatest repeatability among PPG wave shape parameters in both radial artery and finger. Maximum of the first derivative in the anacrotic phase in the PPG waveform showed the highest variability. A possible explanation for the existing individual variability of PPG parameters might be temporal and spatial summation of fluctuations of heart cycle length and changes of peripheral resistance in magistral artery and microcirculatory vessels.

Key words: blood volume pulse, photoplethysmography, pulse wave analysis, radial artery.

Introduction

The application of photoplethysmography has continued to expand and has become widely used for clinical research involving cardiovascular parameters (Allen 2007). The main advantages are it's non-invasiveness and reduced morbidity for subjects.

Extensive studies have been conducted on photoplethysmography to determine the ideal settings for measuring pulse wave velocity (Allen, Murray 2002; Alnaeb et al. 2007). In the last ten years much research has focused on the possibility on using a PPG signal to replace the pre-existing invasive procedure for cardiovascular parameter measurements. Many successful applications for PPG have been introduced: use of PPG in identifying flow mediated vasodilation and thus as a diagnostic for endothelium dysfunction (Donald et al. 2006); determination of arterial pressure wave shape and amplitude with high accuracy (Allen, Murray 2004), allowing virtual reconstruction of central arterial pressure wave (Munir et al. 2008) and calculation of "ankle-brachial" index; as an application for evaluating the mechanical properties of arterial walls using the finger volume pulse shape

analysis (Chowienczyk et al. 1999; Millasseau et al. 2002).

The output of an infrared PPG transducer is proportional to changes in the volume of red blood cells in the peripheral microvascular beds. In addition, fluctuation in RBC volume is associated with each heart beat, and thus can be associated with PPG transducer recordings.

There are two PPG operational configurations: transmission (transillumination) mode operation where the fingertip is placed between the source and detector, and reflection (adjacent) mode operation where the LED and detector are placed side-by-side (Crabtree et al. 2006). The detected optical radiation waveform comprises a pulsatile ("AC") component attributed to cardiac synchronous changes in the blood volume with each heart beat, and is superimposed on a slowly varying ("DC") baseline with various lower frequency components attributed to respiration, sympathetic nervous system activity and thermoregulation (Gonzalez et al. 2008).

The origins of the DC and AC components of the PPG signal are not fully understood, but it is generally accepted that they can provide valuable information about the cardiovascular system. The PPG signal (mentioned AC component) has a direct relationship to arterial pressure waves (Allen 2007). The PPG signal starts as a wave propagation from central arteries to the peripheral vasculature. In the periphery, the more resistant vessels will induce reflection of the wave, and finally the summation of the propagating and reflected waves will produce the PPG signal.

Detailed investigations in arterial pressure wave shape started in the early 1940s (Hamilton 1944). Similar shapes for the PPG and arterial pressure waves has been noted, thus it is reasonable to rely and use the previous arterial pressure pulse shape method for PPG wave shape analysis. Every individual has a unique characteristic shape for their photoplethysmography signal, but in previous research not enough attention has been focused on the existence of this variation, and on the reproducibility of PPG measurements when repeated at different times.

In our study we recorded two photoplethysmography signals: in reflection mode from the radial artery and in transmission mode from the fingertip, with the aim to evaluate the individual repeatability of PPG wave shape parameters recorded from finger and artery in rest conditions.

Materials and methods

Subjects

The study included 13 healthy adults (three men) with mean age ± standard deviation 21.3 ± 2.8 years. The main inclusion criterion was individuals with no cardiovascular diseases. This study was approved by the local ethics committee and each subject gave informed consent.

Study protocol

All measurements were recorded in a controlled and quiet environment at room temperature (17 ± 1 °C) after a 15 min resting period, during which personal information (name, age, gender) was obtained and the study protocol was explained to subjects. Then subjects were seated and systolic (SBP) and diastolic (DBP) arterial blood pressure as well as heart rate (HR) were measured immediately by pressure sensor application to the

subject in a systematic order thus ensuring possibility of occurrence of signal interactions. Measurements were performed continuously – beat per-beat during a period of 2 min while the subject was in a seated position with both arms bent in a 90 ± 5° angle and both forearms rested on an upholstered chair support frame. We allowed patients to keep their wrist and palm relaxed. All traces were recorded simultaneously for a period of 120 s, and the study protocol was repeated for each patient two to four times (with up to a 7-day resting period between measurements for each subject) to explore the variability and individual features of the PPG signal.

For measuring forearm blood flow (FBF) we used a venous strain-gauge plethysmograph (D.E. Hokanson Incorporated, Bellevue Washington USA). We placed the cuff on the upper left arm, the strain gauge was placed around the left forearm about 10 cm under the elbow and fixed with adhesive tape. We chose the shortest possible measuring interval (15 s), which allowed us to register BF data four times per minute. Continuous SBP, DBP and HR traces were obtained with a Finometer (Finometer model-2, FMS, Finapres Medical Systems B.V. Amsterdam, Netherlands); sensors were placed on the right arm. The PPG signal was recorded with an originally designed two-channel photoplethysmograph (LU ASI, Latvia) with two different sensors: reflecting probe sensor for the radial artery and transmission probe sensor for the finger. Diameter of the emitting area for reflecting probe was ~2 mm, radiant power ~10mW, peak wavelength ~940 nm, estimated mean penetration depth under the skin surface~2–3 mm. The analogue signals from PPG contact probes were digitized by an analogue-to-digital converter (16-bit accuracy, sampling rate 300 Hz) and transferred to the computer (Erts et al. 2005). We fixed the reflecting probe on the artery by taping and additionally bending it to the wrist. Skin temperature for each patient was constant during the measurements.

Fig. 1 shows the PRG parameters determined in this study: maximum of the first derivative in anacrotic phase of the PPG waveform (I_A), arbitrary amplitudes of anacrotic maximum (h_s), incisura minimum (h_i) and maximum of dicrotic notch (h_d), time intervals from foot to ancrotic maximum (t_s), incisura (t_i) and maximum of dicrotic notch (t_d) (Ratner 1993; Allen 2007). Amplitude parameters were measured in arbitrary units (a.u.).

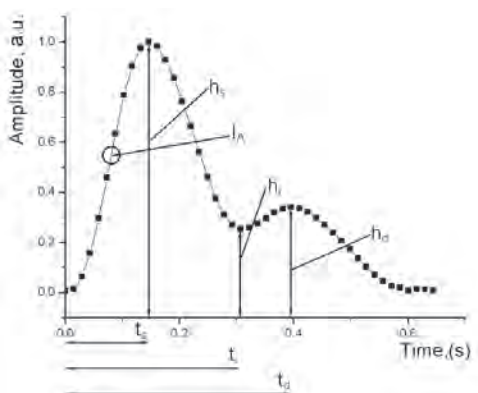

Fig. 1. Parameters of PPG signal studied in this work. I_A – maximum of the first derivative in anacrotic phase of the PPG waveform; h_s – arbitrary amplitude of anacrotic maximum; h_i – arbitrary incisura minimum; h_d – maximum of dicrotic notch; t_s – time interval from foot to ancrotic maximum; t_i – time interval from foot to incisura; t_d – time interval from foot to maximum of dicrotic notch.

Analysis

Offline analyses were performed for all channels (PPG, FBF and BP signals) and data processed by custom-made software developed for Matlab. PPG and pressure channels were filtered and a continuous FBF curve was interpolated from intermittent recordings by cubic spline function. Normalization within pulse wave was performed to compare PPG signals in different registrations. The synchronized traces were initially handled manually to select the unaltered (cut off movement artifacts) traces only, which were then exported to MS Excel and processed there, using the program SigmaStat for statistical analysis (t-test, one way repeated measure ANOVA).

Results

The rest period hemodynamic parameters DBP, SBP and HR, as expected, did not show statistically significant differences for each subject in the repetitions. The obtained parameters for the whole group (n = 13) were as follow: average systolic blood pressure 117.5 ± 8.9 mm Hg, diastolic blood pressure 75.5 ± 8.5 mm Hg, heart rate 66.4 ± 5.1 beats min^{-1}.

In our experiment regional circulation was only represented by the forearm blood flow, which showed slight variability; nine subjects of 13 showed no statistically significant differences between forearm blood flow in different repetitions.

According to variability of PPG parameters from the radial artery signal, the most stable PPG parameters were hi, hd and ts. The difference of these parameters within a subject was only ≤ 2 % (one out of 37 recordings differed significantly ($p < 0.05$).

A slightly larger variability was shown by h_d, t_i and t_d, which differed significantly ($p < 0.05$) in 7 - 18 % (three to seven out of 37 recordings). I_A was the only parameter that was variable within an individual subject and within the study group; the variability of this parameter was very high and significant differences ($p < 0.05$) between I_A values of individual subjects when repeated measurements were performed was found in more than 59 % (21 out of 37 recordings of subjects). Even within the same measurement the I_A parameter showed a very large fluctuation in values (mean ± SD; 0.84 ± 0.17). An example of a subject's both radial artery and digit hemodynamic and PPG parameters is shown in Fig. 2.

The most stable parameters in the PPG recorded from the finger were h_i and t_s, as in only 2 % (one out of 37 recordings) of cases they did not show significant differences ($p < 0.05$) between different recording times within a subject. Similar to the arterial PPG parameters, a larger variability was observed for h_d, t_i, t_d un I_A in the finger PPG signal. Altogether 11 to 30 % of recordings (four to 11 out of 37) showed a significant difference ($p < 0.05$).

Discussion

Previous research has confirmed that peripheral arterial PPG signal measurements can be used for diagnostic purposes (Allen 2007; Alnaeb 2007). The variation in heart rate allows us to understand individual characteristics of physiological regulatory mechanisms in the autonomic neurohormonal system.

Pulse wave velocity and multi-body site PPG measurements provide us with valuable

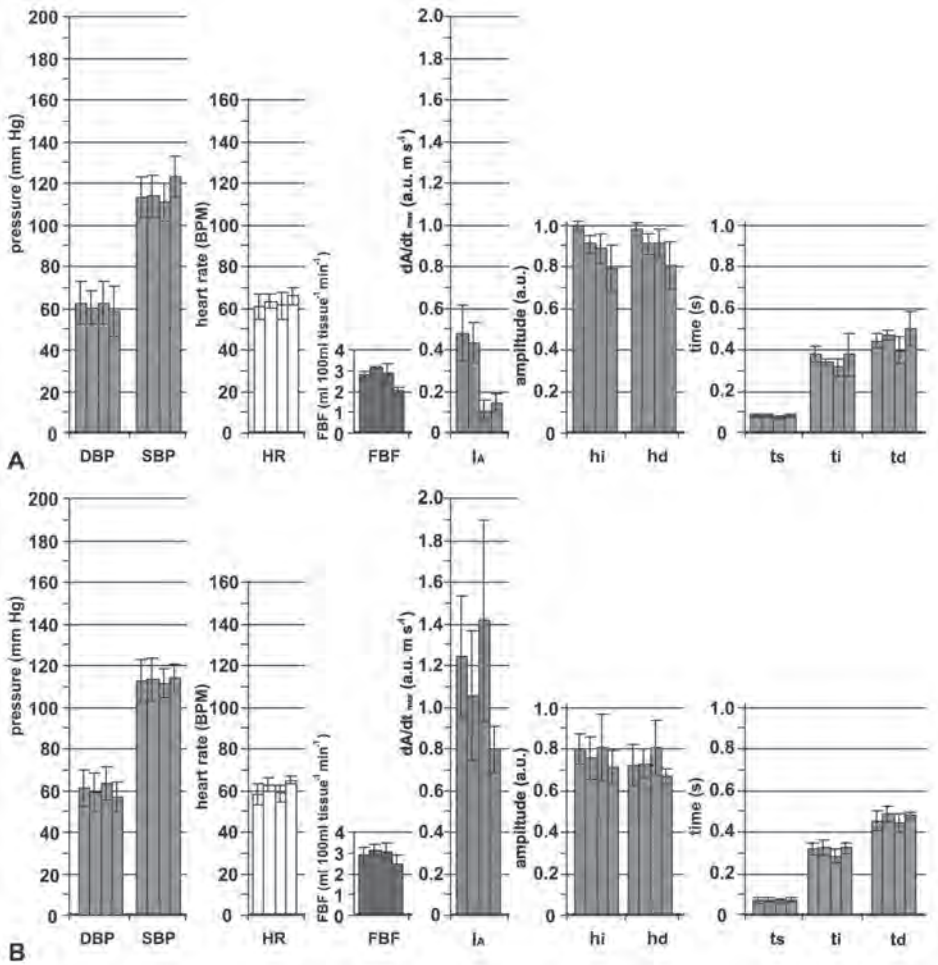

Fig. 2. A representative example for individual variability of one subject showing hemodynamic and PPG parameters. Recordings of four repetitions in two vascular beds (A - radial artery, B – finger). Data is shown as mean ± standard deviation; n = 43 cardiac cycles. SBP – systolic blood pressure (mm Hg); DBP – diastolic blood pressure (mm Hg); FBF – forearm blood flow (mL 100 mL^{-1} tissue min^{-1}); I_A – maximum of the first derivate in anacrotic phase of the PPG waveform (dA/dt_{max}); h_i – arbitrary incisura minimum (a.u.); h_d – maximum of dicrotic notch (a.u.); t_s – time interval from foot to ancrotic maximum (s); t_i – time interval from foot to incisura (s); t_d – time interval from foot to maximum of dicrotic notch (s); HR – heart rate (bpm).

information concerning the mechanical properties of arterial walls, and thus will enable us to diagnose peripheral arterial occlusive diseases. Photoplethysmography also makes it possible to evaluate flow-mediated vasodilatation in an artery, allowing the specific endothelial functional state to be determined. In addition photoplethysmography can be used as an alternative method for ankle brachial pressure index calculations, which is a crucial parameter in the diagnosis of peripheral artery disease (Khandanpour et al. 2009). Finger PPG may also provide us with the ability to obtain the parameters of the heart left

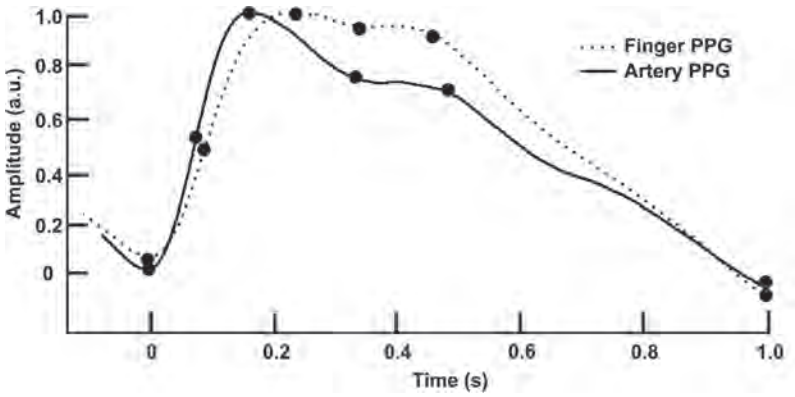

Fig. 3. Representative example of typical curve demonstrates shape differences for PPG_{fing} and PPG_{art}. Pulse waves were recorded simultaneously in the rest conditions. The shape parameter measuring points are marked with the dots.

ventricular volume and filling time, which has an important application in the case of heart arrythmia (Zheng et al. 2008).

The arterial pulse shape has been extensively studied by recording arterial wall diameter changes or by the arterial pressure wave. By use of applanation tonometry it is possible to record the arterial pulse shape signal, which is also practically identical to the invasively measured intraarterial pressure signal. Detailed analysis of the tonometry signal has proven that the shape analysis contains the relevant information (Kelly et al. 1989). In a specially designed experiment, pulse wave analysis using radial applanation tonometry demonstrated high levels of repeatability (Crilly et al. 2007).

Research into the PPG signal shape was first implemented by Hertzman. In 1937 he introduced two basic abbreviations: the anacrotic phase being the rising edge of the pulse, and the catacrotic phase being the falling edge of the pulse; the dicrotic notch is usually seen in the catacrotic phase (Allen 2007). It was recommended to measure the anacrotic time, which is the crest time from the rising edge of the pulse waveform, and to normalise this to the heart rate. Later research introduced more PPG wave shape parameters (I_A, h_i, h_d, t_s, t_i, t_d etc.) and it was recommended to normalise the values of these parameters to the heart rate and the amplitude of the pulse wave (Hamilton 1944; Kelly et al. 1989; Nitzan et al. 1998; Millasseau et al. 2000; Hayward et al. 2002; Millasseau et al. 2002; Gonzalez et al. 2008). In the finger pulse wave, special attention has been paid to the "notch" or point of inflection in pulse wave downslope, as it has been proven to be a sensitive index of nitrate bioavailability (Takazawa et al. 1998; Chowienczyk et al. 1999).

There are no previous studies that show the variation of individual photoplethysmography waves when repeated at different time intervals. More extensive research in this field has been carried out to determine the accuracy with which pulse transit times (PTTs) can be measured. Measurements of PTT between the ECG Q-wave and various peripheral sites was conducted in 10 normal subjects on 10 separate days. The day-to-day repeatability sigma (the square root of the within-subject mean square variance) of individual PPT measurements in a subject was 9 – 12 ms (Jago, Murray 1988). These results indicate individual repeatability of the PPG signal.

In our research, repeated measurements for each individual in the resting condition

showed almost the same arterial pressure and heart rate values (average variation in pressure was 5 %). In the resting condition, finger and artery plethysmogram curves for each subject in repeated measurements showed no significant differences for h_i and h_d. However, other PPG wave shape parameters (t_i and t_d) within a subject in different PPG_{fing} and PPG_{art} recordings might be similar or in some cases they might show a statistically significant difference.

The forearm blood flow was the only single hemodynamic parameter whose value varied throughout the study in different measurement periods. However, blood flow and PPG data analysis did not show any relationship between these two variables. We therefore propose another hypothesis for the explanation of this variability. From previous studies it is known that systemic and regional hemodynamic parameters are usually subjected to periodic fluctuation. PPG signal amplitude changes over time and has a low frequency of fluctuation, while the cardiac cycle length has a higher frequency and approximately corresponds to the respiratory rate (Nitzan et al. 1998; Avnon et al. 2004). Therefore individual variability of PPG parameters might be caused indirectly by temporal and spatial summation of fluctuations of heart cycle length and changes of peripheral resistance in magistral artery and in each microcirculatory vessels in the tissue region (Nilsson et al. 2003; Munir et al. 2008).

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Alteration of serum adhesion molecules and cutaneous endothelium-dependent vasodilatation in insulin resistant obese patients

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Abstract

Insulin resistance (IR) is associated with decreased endothelium-dependent vasodilatation. Adhesion molecules are regarded as endothelial dysfunction biomarkers, which are markedly up-regulated in obesity-linked diseases, including coronary artery disease (CAD) and type 2 diabetes mellitus (T2DM). The purpose of the study was to evaluate the relationships between IR, vascular cell adhesion molecule-1 (sVCAM-1), intercellular cell adhesion molecule-1 (sICAM-1), sE-selectin, and endothelium-dependent vasodilatation in metabolic syndrome (MetS) patients who were categorized as having T2DM, both T2DM and CAD, or neither. Obese MetS patients with dyslipidemia were classified into three groups: 34 patients with T2DM (D), 20 patients with T2DM and CAD (DC), and 26 patients with MetS alone (M). Eighteen healthy subjects were selected as controls (C). The study groups were matched for age and sex. IR was assessed by HOMA-IR method, and serum sVCAM-1, sICAM-1, and sE-selectin levels were measured by xMAP technology. Laser Doppler imaging with iontophoretic application of 1% acethylcholine (LDI-Ach) solution was used for the evaluation of cutaneous endothelium-dependent vasodilatation in the hand. Serum levels of sVCAM-1, sICAM-1, and sE-selectin were significantly higher in the group of DC patients compared with the levels in other groups ($p < 0.01$), except for sICAM-1 in the D group. Also, sVCAM-1, sICAM-1, and sE-selectin concentrations were significantly correlated with HOMA-IR indexes ($p < 0.0001$). Only D and DC patient groups demonstrated a significant and similar decline in LDI-Ach marker compared to the group of healthy subjects ($p < 0.001$). LDI-Ach values were significantly correlated with HOMA-IR indexes, sVCAM-1, sICAM-1, and sE-selectin levels ($p < 0.01$). Our findings show that obese MetS patients with T2DM have more higher serum levels of adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), simultaneously with both higher IR and lower endothelium-dependent vasodilatation than those with MetS alone, and the presence of

CAD in these patients is associated with greater changes in the endothelial dysfunction markers. IR was observed to be a closely related to endothelial dysfunction.

Key words: adhesion molecule, endothelium-dependent vasodilatation, insulin resistance, metabolic syndrome.

Introduction

Adhesion molecules are vascular inflammatory markers for endothelial dysfunction (Ponthieux et al. 2004). They mediate the binding of circulating leukocytes to endothelial cells and their subsequent migration into the blood vessel wall, which is an important step in the initiation of atherosclerotic lesions. Focal expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) has been demonstrated in human atherosclerotic plaques (Davies et al. 1993). The systemic inflammatory markers C-reactive protein (CRP), oxidized low-density lipoprotein, IL-1, and NF-κB can initiate VCAM-1 and ICAM-1 expression (van der Meer et al. 2002). The levels of soluble adhesion molecules like sICAM-1 and sVCAM-1 reflect the expression of membrane-bound adhesion molecules and vascular inflammation of the vessel wall (Gearing, Newman 1993). E-selectin binds neutrophils, monocytes, eosinophils, basophils, natural killer cells, and subsets of lymphocytes. This adhesion molecule is important in the initial steps of leukocyte extravasation into inflamed tissues (Wagers et al. 1996).

Adipose tissue is thought to be able to produce inflammatory markers such as IL-6 and TNF-α, which in turn are able to stimulate the expression of cellular adhesion molecules. Higher levels of sE-selectin in obesity have been described in both men (Hwang et al. 1997) and women (Ito et al. 2002), in relation with body mass index (BMI) (Weyer et al. 2002). Conflicting results exist for the relation of sVCAM-1 and sICAM-1 levels with obesity. Some reports have shown an association between both sVCAM-1 and sICAM-1 on the one hand, and BMI on the other, while others have not. When obesity is accompanied by type-2 diabetes mellitus (T2DM), levels of sE-selectin are associated with measures of obesity (Schram, Stehouwer 2005).

Studies on the association of endothelial cell adhesion molecules with insulin resistance (IR) in non-diabetic individuals showed that the concentrations of sVCAM-1, sICAM1 and sE-selectin were significantly elevated in IR individuals (Chen et al. 1999; Hak et al. 2001), although not all studies demonstrated this for sVCAM-1 (Weyer et al. 2002). Some studies showed no positive relationship between high levels of adhesion molecules and coronary artery disease (CAD) risk, but most have demonstrated this (Blake, Ridker 2002). Concentrations of soluble adhesion molecules have been consistently shown to be increased in diabetic patients and in subjects with IR. They correlate with various cardiovascular risk factors such as smoking, hypertension, low high-density lipoprotein (HDL)-cholesterol and hypercholesterolemia (Blankenberg et al. 2001).

Metabolic syndrome (MetS) is considered to be a state of chronic inflammation closely associated with endothelial dysfunction causing an increased incidence of ischemic cardiovascular events and high mortality. Low-grade inflammation is observed in patients with increased plasma levels of sVCAM-1, sICAM-1 and sE-selectin (Gonzalez, Selwyn 2003), which are correlated with inflammatory markers e.g., CRP, TNF-α, IL-6, and IL-1 (Ruotsalainen et al. 2008).

The purpose of the study was to evaluate the relationships between IR, sVCAM-1, sICAM-1, sE-selectin, and cutaneous endothelium-dependent vasodilatation in MetS patients who were categorized as having T2DM, both T2DM and CAD, or neither.

Materials and methods

Subjects

Obese MetS patients with dyslipidemia, who were recruited in the study, were classified into three groups: 34 patients with T2DM (D); 20 patients with T2DM and CAD (DC), and 26 patients with MetS alone (M). Eighteen healthy subjects were selected as controls (C). The study groups were matched for age and sex.

MetS was diagnosed according to the International Diabetes Foundation criteria with specific reference to the European population (Alberti et al. 2005). Patients were not included if their systolic blood pressure was ≥ 160 mm Hg or diastolic ≥ 95 mm Hg and if they were treated with antihypertensive drugs other than angiotensin-converting enzyme inhibitors. Diabetes was defined as a reported history of diabetes and treatment with antidiabetic drugs. Duration of T2DM was 8 ± 5 years and glycated hemoglobin HbA1c was less than 7.5 %. The diabetics did not undergo insulin therapy and lacked pronounced diabetic complications.

The diagnosis of CAD was substantiated by coronary angiography. Digital coronary angiography was performed by means of a GE Medical System X-ray digital angiography system. Results of coronary angiography were accepted as positive if stenosis ≥ 50 % of at least one of the three main epicardial branches of coronary arteries was detected. Patients with acute coronary syndrome and those who had evidence of peripheral vascular disease or cerebral ischemia were not included. Other exclusion factors were acute inflammatory condition or chronic inflammatory states such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis, inflammatory bowel disease, surgery and trauma within the preceding 30 days, and other diseases known to be associated with significant changes of cytokines. Malignancy, alcoholism and smoking were also exclusion criteria. We did not include patients who were using COX-2 inhibitors, nonsteroidal antiinflammatory agents or corticosteroids, or had used them within the preceding 30 days.

All subjects gave their informed consent to the protocol, which was approved by the local Medical Ethics Committee of the University of Latvia for Biomedical Research.

Biochemical measurements

Blood samples (5 mL) for the determination of cytokines were collected after a 12-h fast and allowed to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation at 4 °C for 20 min at 1600 × g. All specimens were immediately aliquoted, frozen, and stored at –80 °C. sVCAM-1, sICAM-1, and sE-selectin concentrations were measured by xMAP multiplex immunobead assay technology (Luminex200 analyzer, Luminex Corp., Austin, TX) (Kofoed et al. 2006). We used homeostasis model assessment (HOMA-IR) to quantify IR (fasting glucose × fasting insulin / 22.5) (Matthews et al. 1995). HOMA-IR values have been shown to correlate well with values obtained using the "gold standard" clamp technique (Bonora et al. 2000). Fasting concentrations of lipids, insulin, and glucose were analyzed by standard methods.

Blood flow measurements

Measurement of cutaneous endothelium-dependent vasodilatation was performed by Laser Doppler imaging (LDI; moorLDI2, Moor Instruments Ltd., UK) in conjunction with iontophoretic application of 1 % acethylcholine (LDI-Ach) solution on the dorsum of the hand (Turner et al. 2008).

Statistical analysis

After testing the normality of data distribution, statistical differences between the four groups were assessed by one-way ANOVA using Fisher's multiple comparison test. Data were recorded as the means ± SD and two-tailed values of $p < 0.05$ were considered to be significant. Correlation analyses were performed using one-factor linear regression analysis. All analyses were performed using STATISTICA 6.0 software (StatSoft Inc, USA).

Results

All patient groups demonstrated significantly higher HOMA-IR values than the group of healthy controls. The value of HOMA-IR in the diabetic group was higher than that in the group of patients with MetS alone (D 5.77 ± 3.06 vs M 3.87 ± 1.86, $p < 0.05$), but did not differ from the group of patients with both T2DM and CAD (Fig. 1).

Serum levels of sVCAM-1, sICAM-1, and sE-selectin in patients with T2DM and CAD ($p < 0.01$) were significantly higher than those in other groups ($p < 0.01$), except for sICAM-1 in the group of diabetics (Fig. 2 and 3). Also, sVCAM-1, sICAM-1, and sE-selectin concentrations were significantly correlated with HOMA-IR indexes ($p < 0.0001$)

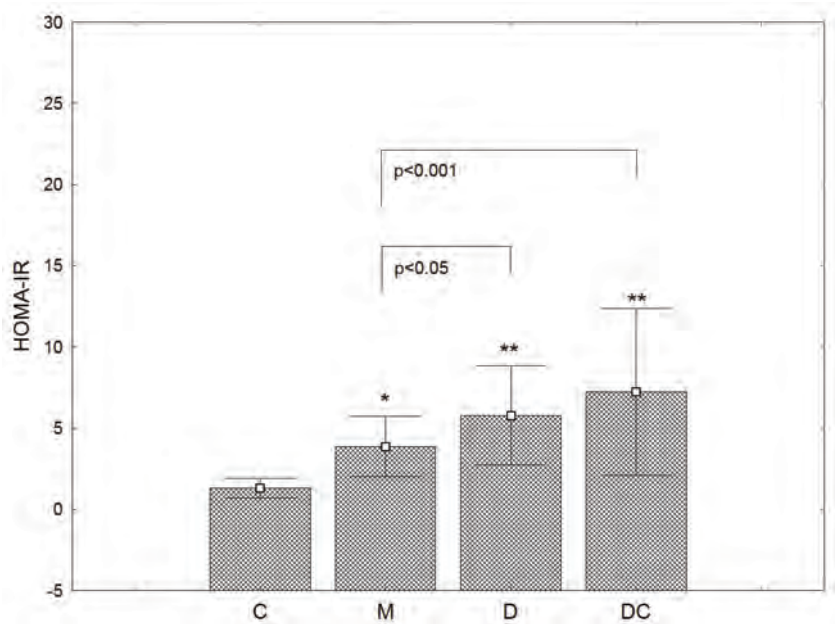

Fig. 1. Insulin resistance (HOMA-IR) in healthy subjects (C), obese metabolic syndrome patients (M), MetS patients with type 2 diabetes mellitus (D), and MetS patients with diabetes and coronary artery disease (DC). Data are expressed as mean ±SD. *, $p < 0.01$ vs. healthy subjects.

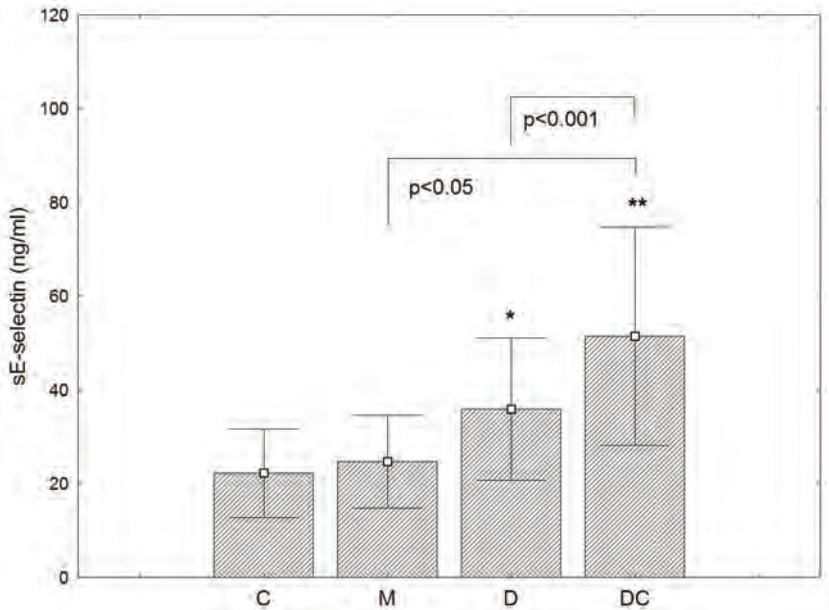

Fig. 2. Soluble E-selectin (sE-selectin) in healthy subjects (C), obese metabolic syndrome patients (M), MetS patients with type 2 diabetes mellitus (D), and MetS patients with diabetes and coronary artery disease (DC). Data are expressed as mean ±SD. *, $p < 0.01$ vs. healthy subjects.

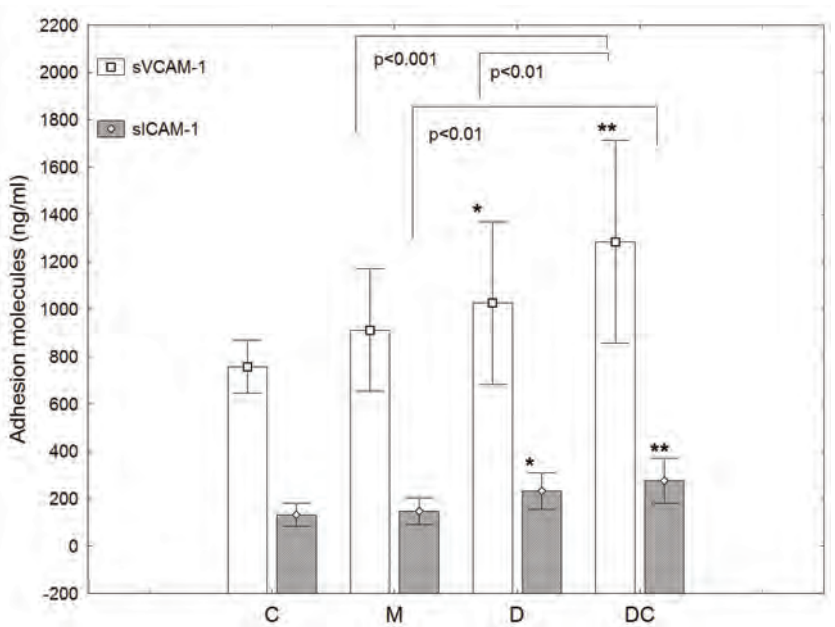

Fig. 3. Soluble vascular cell adhesion molecule-1 (sVCAM-1) and intercellular cell adhesion molecule-1 (sICAM-1) in healthy subjects (C), obese metabolic syndrome patients (M), MetS patients with type 2 diabetes mellitus (D), and MetS patients with diabetes and coronary artery disease (DC). Data are expressed as mean ±SD. *, $p < 0.01$ vs. healthy subjects.

Table 1. Correlations of adhesion molecules (sVCAM-1, sICAM-1 and E-selectin) with markers of insulin resistance (HOMA-IR) and acethylcholine induced endothelium-dependent vasodilatation (LDI-Ach) in the total study clinical material. * $p < 0.01$; ** $p < 0.0001$

Adhesion molecules	HOMA-IR	LDI-Ach
sVCAM-1	r = 0.48**	r = –0.36*
sICAM-1	r = 0.59**	r = –0.51**
E-selectin	r = 0.60**	r = –0.55**

(Table 1).

A significant and similar decline in endothelium dependent vasodilatation (LDI-Ach) was observed only for patients with T2DM and those who were diagnosed with both T2DM and CAD ($p < 0.001$; Fig. 4). LDI-Ach values were significantly correlated with HOMA-IR indexes, sVCAM-1, sICAM-1, and sE-selectin concentrations ($p < 0.01$; Fig. 5 and Table 1).

Discussion

The results of this study indicate that obese MetS patients with T2DM, independently of CAD, have higher serum levels of sICAM-1, sVCAM-1, and sE-selectin than those with MetS alone, whilethese patients had higher insulin resistance (HOMA-IR) and lower microvascular (cutaneous) endothelium-dependent vasodilatation (LDI-Ach). The

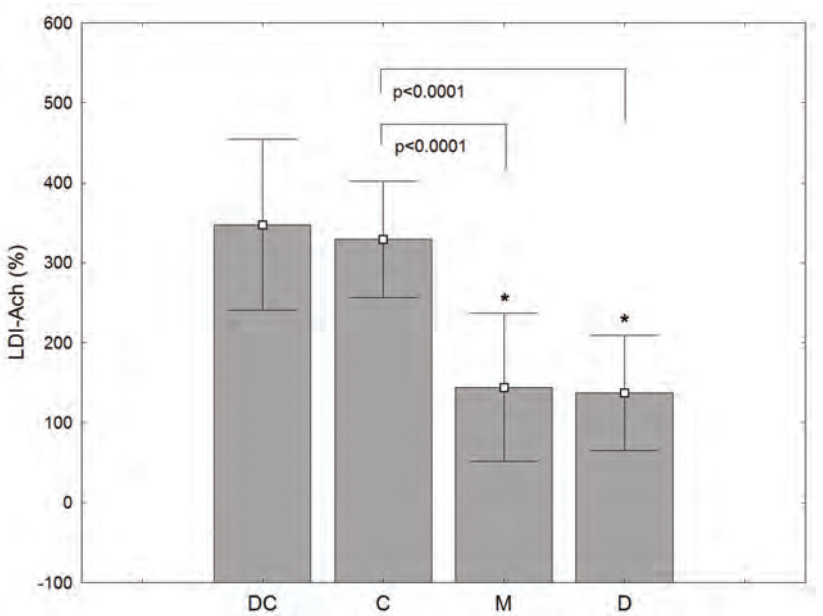

Fig. 4. Acethylcholine-induced endothelium-dependent vasodilatation (LDI-Ach) in healthy subjects (C), obese metabolic syndrome patients (M), MetS patients with type 2 diabetes mellitus (D), and MetS patients with diabetes and coronary artery disease (DC). Data are expressed as mean ± SD. *, $p < 0.01$ vs. healthy subjects.

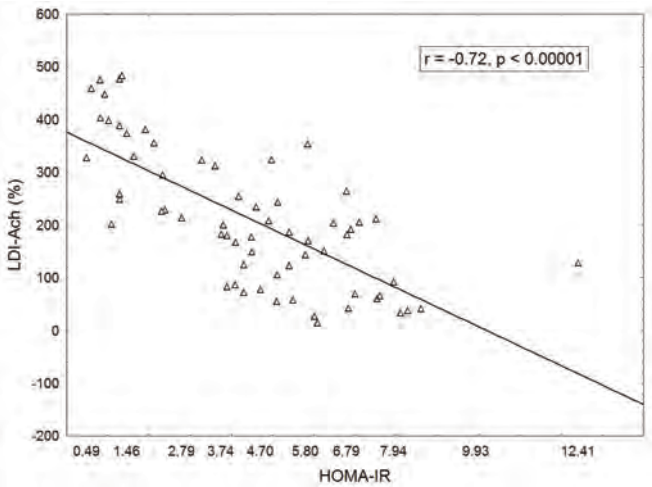

Fig. 5. Correlation between insulin resistance (HOMA-IR) and acethylcholine-induced endothelium-dependent vasodilatation (LDI-Ach) in the total study clinical material.

results also show that the presence of CAD in MetS patients with T2DM is associated with significant elevation of sICAM-1 concentrations and HOMA-IR values and with impairment of endothelium-dependent vasodilatation. Our findings suggest that in obese patients MetS can be characterized by elevated HOMA-IR. The study have confirmed correlations between HOMA-IR and serum levels of sICAM-1, and sVCAM-1, and additionaly demonstrated correlation between HOMA-IR, sE-selectin, and LDI-Ach and also between sVCAM-1, sE-selectin, and LDI-Ach (Fig. 5 and Table 1).

In recent years, much attention has been paid to the potential value of soluble adhesion molecules as biomarkers for CAD risk (Blankenberg et al. 2001). Cellular adhesion molecules may be important in atherosclerosis, inasmuch as they facilitate the immigration of leukocytes into the vessel wall (van der Meer et al. 2002). However, reports on adhesion molecules and atherosclerosis are not consistent. Several studies have reported association of sICAM-1 (Hwang et al. 1997), sVCAM-1 (Peter et al. 1997) or both sCAMs (Blankenberg et al. 2001) with measures of atherosclerosis. Our findings support the notion (Kressel et al. 2009) that adhesion molecules are excellent markers of CAD risk, especially in MetS and IR patients. Nevertheless clinical data on these soluble CAM forms are still limited and more research is necessary to elucidate the role of adhesion molecules as important risk markers in atherosclerosis and CAD.

The association of adhesion molecules with MetS can be explained by close interaction with proinflammatory cytokines which are known to be increased in MetS patients (such as IL-1ß, TNF-α, and IL-6; Rutter et al. 2005). An association between obesity and the expression of cellular adhesion molecules has also been demonstrated. This suggests that obesity is involved in the development of endothelial dysfunction. Studies on the effects of weight loss suggest that endothelial dysfunction caused by obesity is reversible. Mechanisms that may explain these relationships include increased stress to the cardiovascular system in overweight patients, increased production of inflammatory markers by adipocytes, or metabolic stimulus such as the effect of insulin on the endothelium (Schram, Stehouwer

2005).

Cellular adhesion molecules may play an important role in the development of the MetS and T2DM, as well as in their cardiovascular complications. All three cellular adhesion molecules, sVCAM-1, sICAM-1 and sE-selectin, have been investigated with the aim to improve our knowledge on endothelial function. The endothelium is thought to be the major source of the soluble forms of these molecules. Strong evidence exists that increased levels of the adhesion molecules reflect an alteration of endothelial function, which may have pathophysiological consequences. There is a growing body of evidence showing an association between cellular adhesion molecules and development of T2DM and its cardiovascular complications (Schram, Stehouwer 2005).

Our findings show that obese MetS patients with T2DM have higher serum levels of adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), simultaneously with both higher insulin resistance and lower endothelium-dependent vasodilatation than those who have neither T2DM nor CAD. The presence of CAD in these patients is associated with greater change in the endothelial dysfunction markers. Also, insulin resistance has a close relationship to endothelial dysfunction.

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Genetic diversity in milk proteins among goats bred in Lithuania

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Abstract

The aim of the study was to determine the main plymorphisms in AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin genes in goats bred in Lithuania and compare these among breeds. The study was based on determination of single polymorphisms among individuals tested by PCR-RFLP and A-PCR methods. Allele, genotype and haplotype frequencies were calculated and the Hardy-Weinberg equilibrium test was performed. Lithuanian native, Zaanen, Czech White and German White breeds were found to be polymorphic for AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin genes. The AlfaS1-casein gene B allele (0.981) and BB genotype (0.83) as well as Beta-lactoglobulin C allele (0.913) and CC genotype (0.87) had the highest frequency in Lithuanian native breed. Eight different casein locus haplotypes were found in the studied breeds. Haplotypes B/A/AB and B/B/AB were the most common among the Lithuanian goat population. B/B/C and E/A/C haplotypes were found solely in two breeds – Lithuanian Native and Zaanen. The haplotype E/B/C may be unique, as it was found only in one the Zaanen breed.

Key words: allele, genotype, goat, haplotype, milk protein genes, PCR-RFLP.

Introduction

It is widely known that animal productivity and other agricultural traits, such as milk quantity, composition, fat and protein quantity and chemical composition, suitability for processing and production of individual milk, milk discharge velocity during the milking process, succulence, speed of growth, etc., are inherited from generation to generation. However, their formation and functional features are determined by genes. The advances in molecule genetic technologies, especially DNA markers, over the last few decades have had an enormous influence on the compilation of gene maps, which has allowed to identify genes that control the changing part of multigenic features.

The detection of milk protein genetic variants gave rise to the analysis of milk protein polymorphisms in many animal species, such as cattle, sheep and goat. In the first stage of analysis cattle milk proteins were in the focus of attention; the aim was to indicate the relationship between milk protein variants, milk productivity and technological features. Cow Kapa-casein A and B variants were shown to improve milk productivity and milk quality. Kapa-casein BB genotype cow milk has more fat, protein and caseins (Ng-Kwai Hang 1998; Di Stacio et al 2000; Miceikienė et al 2005) and has better industrial features

– shorter coagulation time with aided by rennet performance, better consistence for curd formation and higher cheese output (Schaar 1985; Marziali et al. 1986).

The main focus of goat milk analysis was onthe AlfaS1-casein. „Strong" (A, B, C) alleles, „medium" (E), „low" (F, D) and „zero" alleles were identified in the AlfaS1-casein, associated with the high (3.6 g L^{-1}), medium (1.6 g L^{-1}), low (0.6 g L^{-1}) and zero AlfaS1-casein quantity in milk (Jordana et al. 1991; Feligini et al. 2005). Much research has been recently conducted on the AlfaS1-casein polymorphic relationship with the goat milk productivity, physico-chemical and technological features. However, the number of studies on goat AlfaS2-casein and Beta-lactoglobulin has been relatively low, perhaps due to low polymorphism (Folch et al 1994; Lagonigro et al 2001). However, goat Kapa-casein polymorphism was demonstrated using protein electrophoresis (Di Luccia et al. 1990), chromatography (Law et al. 1993) and capillary electrophoresis (Recio et al. 1997) and gene variants have been identified by molecular methods in different goat breeds (Caroli et al. 2001; Yahyaoui et al. 2001; Angiolillo et al. 2002; Feligini et al. 2002; Miceikienė et al 2007).

The aim of this study was to determine the main plymorphisms in AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin genes in goats bred in Lithuania and compare these among breeds.

Materials and methods

Samples for identification of milk protein genetic polymorphism were taken from unrelated individuals from Lithuanian Native (30), Zaanen (74), Czech White (29) and German White (18) goat breeds. DNA was extracted from hair roots.

AlfaS1-casein gene polymorphism was investigated by A-PCR method (Feligini et al. 2005). Primer sequences were following: forward BE 5'-CAA-CCT-CAA-ATT-GAA-GGC-ACT-3'; forward E 5'-CAA–CCT-CAA-ATT-GAA-GGC-ACT-3'; reverse R 5'-CAA-GCT-CTT-AGG-ACA-ATT-TCA-CTT-3'.

AlfaS2-casein gene polymorphisms were identified by PCR-RFLP (Cosenza et al. 1998). Primer sequences used: forward 5'-GCCATT-CAT-CCC-AGA-AAG-3' and reverse 5'-CTC-TTC-ATT-TGC-GTT-CCT-TA-3'. The digestion of PCR product was performed using endonuclease MseI (MBI Fermentas, Lithuania).

Kapa-casein gene polymorphisms were identified by PCR-RFLP (Yahyaoui et al. 2001). Primer sequences used were: forward 5'-TGT-GCT-GAG-TAG-GTA-TCC-TAG-TTA-TGG-3' and reverse 3'-GAT-TCC-TCT-GTA-GTT-TCT-CCT-GTT-GCG-5'. The digestion of PCR product was performed using endonucleases *Alw44*I (MBI Fermentas, Lithuania) and *Bse*NI (MBI Fermentas, Lithuania).

The beta-lactoglobulin gene polymorphism was assessed by PCR-RFLP (Yahyaoui et al. 2000). Primer sequences were following: forward 5'GTCACT TTCCCGTCCTGGGG-3 and reverse 5'GGCCTTTCATGGTCTGGGTGACG-3'. The digestion of PCR product was performed using endonuclease SmaI (MBI Fermentas, Lithuania).

The PCR reactions were carried out using the GeneAmp PCR System 2700 (AppliedBiosystem). The reaction products were analyzed by electrophoresis on 3 % agarose gel. EtBr was added to gels to visualize the analysis results under UV light in the Heliorab system.

Goat AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin allele,

genotype, haplotype frequencies in each breed and in all investigated group, expected and observed heterozygosities per locus and population, deviations from Hardy-Weinberg equilibrium were calculated using the R statistical package (http://www.r-project.org/).

Results and discussion

B and E alleles were identified in Lithuanian goat breed milk protein AlfaS1-casein. B „strong" allele, which conditions a larger quantity of AlfaS1-casein, was found very frequently (0.643 - 0.891) in the goats of all analyzed Lithuanian breeds, and was most frequent (0.89) in one Lithuanian goat breed (Table 1). This allele ranges among Italian goat breeds from a frequency of 0.32 to 0.52 (Feligini et al. 2002). In Hungarian milking goat breeds, B allele was identified along with the A allele and was found in 61 % of the goat population studied (Veress et al. 2004). Goat selection prefers „strong" alleles, which provide high output of making cheese out of such milk. „Low" effect or zero alleles are also important; they are not useful for milk manufacture industry, but are demanded by goat milk consumers, since this milk is suitable for allergic people. E allele had the highest frequent range (0.36) in the Lithuanian Zaanen goat population and the lowest frequency in the Lithuanian native goat breed (0.12; Table 1). These data are consistent with the frequencies of E allele in Zaanen goat (41 %) in France (Grosclaude et al. 1987) and Zaanen goat (46 %) in Italy (Ramunno et al. 2001). In the Spanish Canaria goat breed, the E allele was found at a 0.20 frequency range (Jordana et al. 1991). The Hungarian milking goat breed possessed this allele only in 8 % of all animals, in the Polish white goat breed – 5 % (Krolikowska et al. 2002). Therefore, the „strong" A and B alleles were most frequent in goat breeds of the Mediterranian region as well as in Lithuanian goat breeds; whereas, „medium"(E) and „low"(F) alleles were more common in French, Italian, Swiss and Spanish goat breeds (Jordana et al. 1996; Enne et al. 1997; Grosclaude et al. 1997).

In the local Lithuanian breed, AlfaS1-casein BB genotype was found at a high frequency,

Table 1. Allele frequencies of AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin genes in Lithuania goat breed

Milk protein allele types	Lithuanian Native	Zaanen	Czech White	German White
AlfaS1-casein				
B	0.891	0.643	0.875	0.778
E	0.109	0.357	0.125	0.222
AlfaS2-casein				
A	0.565	0.690	0.675	0.667
B	0.435	0.310	0.325	0.333
Kapa-casein				
A+B	0.826	0.857	0.925	1.000
C	0.174	0.143	0.075	0.000
Beta-lactoglobulin				
C	0.913	0.738	0.725	0.722
T	0.087	0.282	0.275	0.278

Table 2. Genotype frequencies of AlfaS1-casein, AlfaS2-casein, Kapa-casein and Beta-lactoglobulin genes in goats bred in Lithuania

Milk protein genotypes	Lithuanian Native	Zaanen	Czech White	German White
AlfaS1-casein				
BB	0.83	0.48	0.80	0.72
BE	0.13	0.33	0.15	0.11
EE	0.04	0.19	0.05	0.17
AlfaS2-casein				
AA	0.35	0.62	0.50	0.45
AB	0.43	0.14	0.35	0.45
BB	0.22	0.24	0.15	0.10
Kapa-casein				
A+B/A+B	0.74	0.80	0.85	1.00
A+B/C	0.17	0.10	0.15	0.00
CC	0.09	0.10	0.00	0.00
Beta-lactoglobulin				
CC	0.87	0.57	0.55	0.56
CT	0.09	0.33	0.35	0.34
TT	0.04	0.10	0.10	0.10

and the EE genotype at low frequent range (Table 2). In total, 30 % of Polish white goat breed possessed the BB genotype and 25 to 38 % of the Italian goat breed (Feligini et al. 2002). No BE and EE genotypes were found in the Italian Montefalkone breed (Belivacqua et al. 1999).

The analysis of distribution of the milk protein AlfaS2-casein gene variant showed that Lithuanian goat breeds possess the same milk protein AlfaS2-casein alleles as other European goat breeds – A and B. The A allele frequency in Lithuanian goat breeds varied from 0.56 in Lithuanian Native to 0.69 in the Zaanen breed with a mean of 66.3 % (Table

Table 3. Frequencies of casein haplotypes in goats bred in Lithuania. *, Haplotype was composed from AlfaS1-casein, AlfaS2-casein and Kapa-casein alleles

Haploytpe*	Lithuanian Native	Zaanen	Czech White	German White
B/A/AB	0.32	0.37	0.42	0.58
B/A/C	0.17	0.06	0.05	-
B/B/AB	0.24	0.17	0.32	0.26
B/B/C	0.13	0.06	-	-
E/A/AB	0.06	0.14	0.05	0.11
E/B/AB	0.04	0.08	0.16	0.05
E/A/C	0.04	0.06	-	-
E/B/C	-	0.06	-	-

Table 4. Observed and expected heterozigosity for four milk protein loci in Lithuanian goat breeds

	Investigated population	Lithuanian Native	Zaanen	Czech White	German White
Observed H	0.325	0.293	0.238	0.213	0.222
Expected H	0.341	0.313	0.397	0.297	0.298
χ2- test	1.852	1.226	0.015	0.428	0.184
(P-value)	(0.6035)	(0.2682)	(0.9014)	(0.5131)	(0.6684)

1). In the Italian Alpine goat breed the A allele was found at a 0.68 frequency (Cosenza et al.1998), in the French Alpine goat breed at a 0.85 frequency (Bouniol et al. 1994) and in the South Italian locals in 31 % of the animals (Ramunno et al. 2001). The recently found E allele was found in two Italian goat breeds of five (Lagonigro et al. 2001). It is possible that the E allele could be used as a breed marker to identify from what breed milk goat milk products are made. Thus it seems important to study this allele in Lithuanian goat breeds.

The AlfaS2-casein AA genotype was prevalent in Zaanen and Czech White breeds. In the Native Lithuanian breed, the AlfaS2-casein AB genotype was found at a high frequency, whereas the BB genotype at a low frequency. The frequency of AA genotype was 49 %, AB genotype – 35 %, and BB genotype – 16 % of tested goats (Table 2).

The Kapa-casein gene has been analyzed among Italian, Spanish, German, French and Hugarian goat breeds (Caroli et al. 2001; Sacchi et al. 2001; Yahyaoui et al. 2001; Feligni et al. 2002; Veress et al. 2004). Our results correspond with these provided by other scientists. The Kapa-casein C allele frequency varied among Lithuanian goat breeds from 0 in German White breed to 0.17 in Lithuanian Native, similarly, in Hungarian, Spanish and French goat breeds with a frequency of 0 to 0.15. The Kapa-casein A+B allele frequency in Lithuanian breeds varied from 0.83 to 1, whereas in Hungarian, Spanish and French goat breeds from 0.85 to 1 (Table 1). In the French Zaanen goat breed, the frequency of C allele was similar to the Lithuanian Zaanen breed, respectively 0.11 and 0.14 (Yahyaoui et al. 2001; Veress et al. 2004). The frequency of the A allele in Italian breeds varied from 0.44 to 0.67, while in German goat breeds this allele was found at a higher frequency range. A total of 90 % of the German Ionika goat breed possessed this allele, compared to 100 % in Togenburgo breed (Caroli et al. 2001). The A variant was found in all other breeds, but was not found among Italian Teramina and Montefalcone breeds. Excepting the E allele, which has a rather high frequency in the Montefalcone breed (0.41), the frequency range

Table 5. Observed and expected heterozigosity in Kapa-casein milk protein loci in Lithuanian goat breeds

	Investigated population	Lithuanian Native	Zaanen	Czech White	German White
Observed H	0.183	0.522	0.143	0.000	0.000
Expected H	0.258	0.423	0.278	0.180	0.000
χ2- test	7.0	1.24	4.95	20.0	
(P-value)	(0.0303)	(0.5381)	(0.0840)	(0.0000)	

of other variants is comparatively low – under 15 %. The C variant was common in the Zaanen breed, whereas F alleles have been found only in Italian Teramina, Girgentana and Sarda breeds. Similarly, the D, F, G and E alleles were not found in the Spanish and French breeds (Yahyaoui et al. 2001; Jann et al. 2004).

The AB genotype of Kapa-casein gene locus was the most prevalent in all tested Lithuanian goat breeds. The CC genotype was not found in Czech White and German Czech White breeds. In total, 84 % of goats had the AB genotype and only 5 % had the CC genotype (Table 2).

Beta-lactoglobulin C and T alleles were identified in West European milky goat breeds. The C was obseved to be common among Spanish breeds such as Malagvena (0.75), Pajoja (0.73) and Canaria breed (1.00). In the French Zaanen goat breed this allele was found at a 73 % frequency, in Hungarian milky – 88 % (Veress et al. 2004). In Lithuanian goat breeds the mean C allele frequency was 0.78. Lithuanian Native goats had the highest frequency (0.91). In the Lithuanian Zaanen goat breed, the C allele was at the same frequency as in French goat breeds (Table 1). 65 % of the investigated goats had Beta-lactoglobulin CC genotype, 27 % CT genotype and only 8 % had the TT genotype (Table 2).

In the Native Lithuanian breed, AlfaS1-casein BB genotype was found at a high frequency, while EE genotype at a low frequency (Table 2). 30 % of the Polish white goat breed had the BB genotype; the Italian goat breed had this genotype in 25 - 38 % (Feligini et al. 2002). No BE and EE genotypes were found in the Italian Montefalkone breed (Belivacqua et al. 1999).

Caseins are coded by four related genes that form a cluster: AlfaS1-casein, AlfaS2-casein, Beta-casein and Kapa-casein, found in the 6th goat chromosome. Allele combinations in the casein locus (haplotypes) are closely related and inherited as one genetic unit. Therefore, the casein locus genotype is extremely important in successful selection. In total eight different haplotypes were found in goats bred in Lithuania. The haplotypes B/A/AB were the most common among the Lithuanian goat population. B/B/C and E/A/C haplotypes were found solely in two breeds – Lithuanian local and Zaanen. The haplotype E/B/C was unique, as it was found only in one breed – Zaanen (Table 3).

All Lithuanian goat breeds had a similar level of genetic diversity and comparable number of different alleles found in the milk protein locus. No unique alleles found in one breed can be used as breed genetic markers to serve as marker for goat milk and products of this breed were found. The evaluation of milk protein allele diversity and distribution proved genetic balance in all Lithuanian goat breeds, while observing a lesser heterozygosity than expected by the Hardy-Weinberg equilibrium law. The mean heterozygosity found in the goat group was 0.325 (Table 4). According to literature data, the Kapa-casein locus was found in genetic balance in German (Caroli et al. 2001), Italian and Spanish goat breeds, except Alpine, Frontalakska and Sarda breeds, where a deficiency of heterozygote genotypes was observed (Feligini et al. 2005). In our investigated goat breeds, Lithuanian Native, Zaanen breeds had the Kapa-casein locus in the genetic balance, whereas the Czech White goat breed contained a significant deviation of the Kapa-casein gene locus from the Hardy-Weinberg law as a result of heterozygosity deficiency (Table 5).

The goat breeds can be characterized with a relatively reduced level of artificial selection, in comparison with cattle and sheep breeds. This conditions a high level of genetic variability in goat casein genes. Some breeds or populations might possess unique alleles or allele combinations that no other breeds possess, which might be useful

as the source of genetic diversity for commercial domestic breeds. This emphasises the importance of preservation of a wide genetic diversity in these populations and the need of genetic sources preservation programmes.

High genetic variability, indicated by casein locus, and relation to milk features provide the opportunity to derive goat breeds that produce milk suitable for different manufacture technologies and special consumers needs (Rando et al. 2000).

The application of genetic markers in the selection of agricultural animals opens the possibility to asses animals and fully use the residing beneficial agricultural features. Genetic markers can be used for identifying both one gene or gene group influencing a trait or trait group. One more advantage of the usage of genetic markers in the selection is the fact that this method of assessment is reliable and economical; it allows identifying genes that control selection and technological value of the animal in young age, and to estimate genetic variability in agricultural animals and opportunity to avoid undesirable features. The usage of genetic markers in selection might accelerate the selection process, improve the quality of agricultural production, reduce the production prime cost and make the production more competitive in markets.

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Plasticity of a dune plant *Alyssum gmelinii* in response to sand burial in natural conditions

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Abstract

Sand burial is one of the environmental factors affecting plant distribution on coastal dunes. The aim of the present study was to investigate putative morphological and physiological adaptations to sand burial in *Alyssum gmelinii* plants growing in natural conditions on coastal dunes. Morphological characteristics, photochemistry of photosynthesis and mycorrhizal symbiosis of plants growing on both grey dunes and white dunes with or without recent impact of burial by sand were analyzed. Burial conditions strongly affected the morphology of *A. gmelinii* plants. Plants on fixed dunes with no burial had a shrub-like appearance with lignified main stems. After burial by sand, new branches formed from vegetative buds located at nodes of a burried shoot and intensively elongated above sand surface. At the beginning of the next vegetation season new branches were formed from buds just above the soil surface. In relatively stable sand-level conditions burried stems formed well-rooted ramet-like underground structures. Burial did not affect intensity of mycorrhizal colonization in roots of *A. gmelinii*. No significant differences in mycorrhizal colonization parameters in plants between white and gray dunes were found except for a different trend of intensity of mycorrhizal symbiosis in July and September. Leaf chlorophyll content, potential quantum efficiency of photosystem II and apparent electron transfer rate of photosystem II was not affected by dune type and burial. Non-photochemical quenching was significantly affected by plant location (white or grey dunes) as well as by sand burial of plants growing on white dunes. It is concluded that *A. gmelinii* plants exhibit both morphological (induced clonality) and biochemical adaptations (induced thermal energy dissipation) to maintain high performance after sand burial.

Key words: adaptation, adventitious rooting, *Alyssum gmelinii*, mycorrhiza, photosynthesis, plasticity, sand burial, soil.

Introduction

Sand burial is one of the environmental factors suggested to affect plant distribution on coastal foredunes (white dunes; Maun 1994; Dech, Maun 2005). Apart from the direct effect of sand burial on seed germination and seedling establishment leading to changes in reproduction success (Maun, Lapierre 1986), burial by sand of whole plants or their parts affects physiology and, as a consequence, morphology of burried plants. Burial-induced

response in natural conditions of plants native to foredunes more likely has an adaptive significance. Consequently, in conditions of regular sand accretion plants have developed several physiological and morphological features allowing to withstand burial events.

A level of tolerance to sand burial varies significantly among foredune plants (Kent et al. 2001). Species well adapted to conditions of shifting sand exhibit growth activation in conditions of sand burial (Lee, Ignaciuk 1985; Cheplik, Demetri 1999). As a certain level of sand accretion is necessary for optimal growth of these species (Maun 1998), they can be designated as an "obligate-buried species". For these plants, it is necessary to experience regular episodes of sand burial in order to maintain a high level of fitness or vigour (Eldred, Maun 1982).

Ability to maintain high intensity of photosynthesis during sand burial as well as a capacity to restore photosynthetic activity after the burial event are suggested to represent physiological adaptations to shifting sand conditions (Kent et al. 2005). The exact mechanism of photosynthetic maintenance in buried conditions is not known. Low light intensity reaching buried leaves as well as low rate of oxygen diffusion might be the main problems. For obligate-burried species activation of photosynthesis in unburied parts of partially buried plants can be proposed. One of the indications of this process could be an increase of leaf chlorophyll content (Zhang 1996). Few studies so far have investigated the effect of sand burial on photochemistry of photosynthesis in natural conditions (Perumal, Maun 2006).

Mycorrhizal symbiosis is thought to play a certain role in nutrition of coastal dune plants (Rozema et al. 1985). In particular, mycorrhizal structures in buried roots enhance mineral uptake leading to increase in leaf area and biomass (Perumal, Maun 1999). Due to the established differences in the level of mycorrhizal colonization between different plant species, the benefits from the symbiosis could be also variable. Yet, enhanced mycorrhizal symbiosis is among factors of adaptive character facilitating the growth of sand-buried foredune plants (Maun 1998).

Plants on coastal sand dunes are characterized by a high level of morphological and biochemical plasticity manifested as environmental heterogeneity-induced adaptations (Ievinsh 2006). New model species representing different adaptation strategies to conditions of coastal environment should be established. The aim of the present work was to study putative morphological and physiological adaptations to sand burial in *Alyssum gmelinii* plants growing in natural conditions on coastal dunes. Both plants from foredunes (white dunes) and semi-stabile dunes (grey dunes) were analyzed.

Materials and methods

Study species

Alyssum gmelinii is a perennial coastal plant growing on both foredunes (white dunes) and semi-fixed grey dunes. Particular plants on white dunes are burried by sand early in the spring before the start of the vegetation season due to dune micro-topography dependent wind-driven local sand accretion. As *A. gmelinii* plants flower relatively early in the vegetation season, buried plants have no opportunity to flower in the same vegetation season.

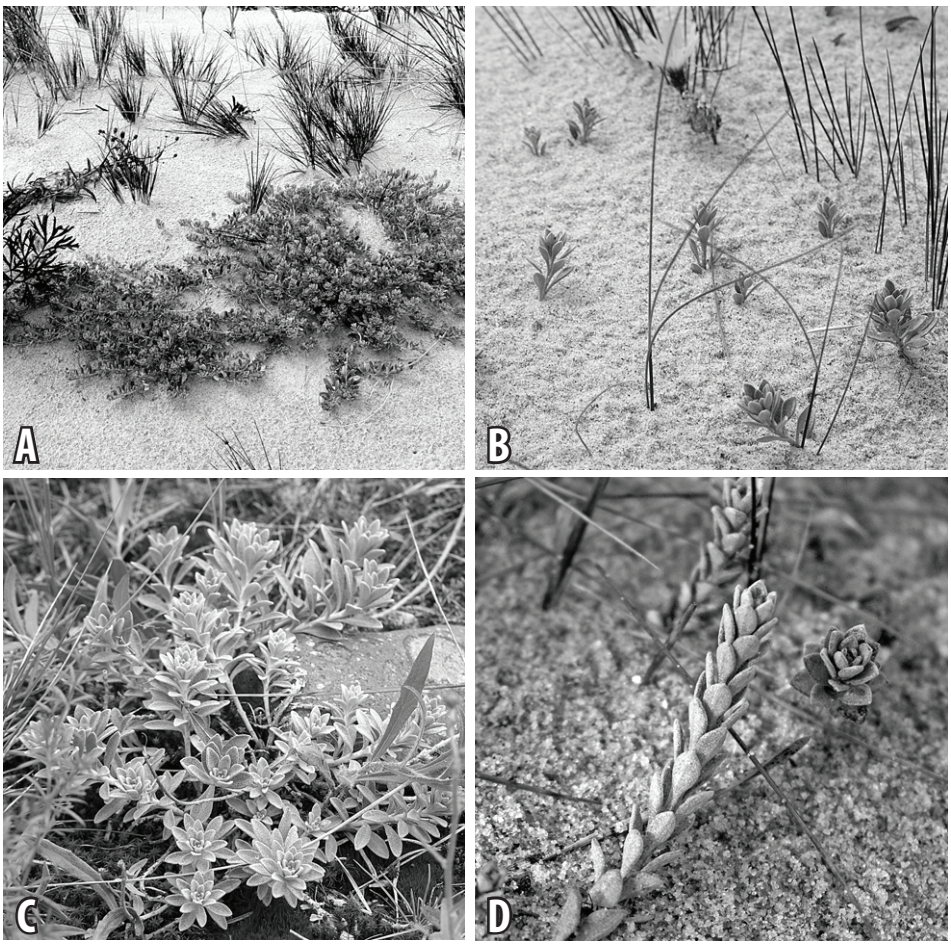

Fig. 1. Typical representatives of *Alyssum gmelinii* plants used in the study. A, unburied plants on white dunes; B, recently buried plants on white dunes; C, unburied plants on grey dunes; D, recently buried "autumn type" plants on white dunes.

Study area and sampling

Plant growing in different habitats at the same site were selected for the study. The site was located near Užava, NW Latvia (57°14' N; 21°25' E). Open sand dunes (foredunes, yellow dunes) were located 50 m from a coast line. The vegetation were periodically affected by wind-driven sand deposition leading to burial of *A. gmelinii* plants. Two types of plants were designated – unburied and recently buried based on observations of plant habit. Typical representative individuals of foredune plants used in the present study are shown in Fig. 1A, B. Fixed dunes (grey dunes) were located 100 m landward. The habitat was protected from wind-driven sand deposition by a group of pine trees (*Pinus sylvestris* L.). A typical representative from grey dunes is shown in Fig. 1C.

Analyses were performed once a month during the vegetation season from May until September. During each month five plants were sampled for leaf chlorophyll content

and chlorophyll *a* fluorescence analysis, and three plants were sampled for analysis of mycorrhizal symbiosis. For chlorophyll and chlorophyll *a* fluorescence analysis, three branches with leaves were picked from every plant. Branches were placed in sealed plastic bags and kept in darkness. Several plants from both habitats with putative different sand-burial history were excavated completely for analysis of morphology.

Measurement of chlorophyll content and chlorophyll a *fluorescence*

Leaf chlorophyll content was measured by a SPAD-502 chlorophyll meter (Konica-Minolta, Osaka, Japan). For chlorophyll measurement, 10 leaves from a branch were measured. For each leaf, five successive readings were made across the leaf surface. The mean of the analysis was calculated using the internal function of the chlorophyll meter.

Chlorophyll *a* fluorescence measurements were performed within 4 h after sampling. For the analysis, 10 leaves were randomly sampled from each branch.

Chlorophyll *a* fluorescence was measured by a pulse amplitude modulated portable fluorometer (PAM 2100, Walz, Germany) and leaf clip holder (2030-B, Walz, Germany) with integrated micro quantum-temperature sensor. A laptop computer equipped with an appropriate software (DA-2000, Walz, Germany) was used to drive the measurements. Leaves were dark adapted for 30 min. The minimal fluorescence level (F_0) was measured by low modulated light and the maximal fluorescence level (F_M) was determined by a saturating pulse on dark-adapted leaves. The ratio F_V/F_M was calculated, where F_V is the difference between the maximum fluorescence and the minimum fluorescence level F_0. The steady-state fluorescence (F_S) was recorded after 6 min light adaptation and then the maximal fluorescence level in the light-adapted state (using a saturating pulse, F'_M) and the minimal fluorescence level (using far-red light, F'_0) was measured.

Maximum apparent electron transport rate (ETR) through photosystem II (PSII) was calculated on the basis of measured overall photochemical quantum yield ($\Delta F / F'_M$; where $\Delta F = F'_M - F_S$) and of photosynthetically active radiation (PAR) according to the equation: $ETR = \Delta F / F'_M \times PAR \times 0.5 \times 0.84$; assuming that transport of one electron requires absorption of two quanta (factor 0.5) and that 84 % of the incident quanta are absorbed by the leaf (factor 0.84).

Chlorophyll *a* fluorescence parameter F_V/F_M measured after a dark adaptation period reflects the potential quantum yield of PSII and thus is indicative of photoinhibition (Maxvell, Johnson 2000). The fluorescence induction curve with quenching analysis at 10 ms p^{-1} was recorded using a built-in standard procedure of DA-2000. Non-photochemical quenching (NPQ) was calculated according to the equation: $NPQ = (F_M - F'_M) / F'_M$ The NPQ emphasizes that part of non-photochemical quenching reflecting heat dissipation of excitation energy in the antenna system.

Root sampling and analysis of mycorrhizal colonization

For analysis of mycorrhizal symbiosis, three root samples were taken from a rhizosphere of a selected plant. Samples were placed in sealed plastic bags, transported to the laboratory and kept at 4 °C until fixation not longer than two months.

Mycorrhizal colonization in root samples was analyzed as described previously (Druva-Lūsīte et al. 2008). Mycorrhizal colonization (abundance of hyphae, vesicles and arbuscules) was measured and both intensity of mycorrhizal colonization in the root system (M%) and frequency of mycorrhiza in the root system (F%) were calculated.

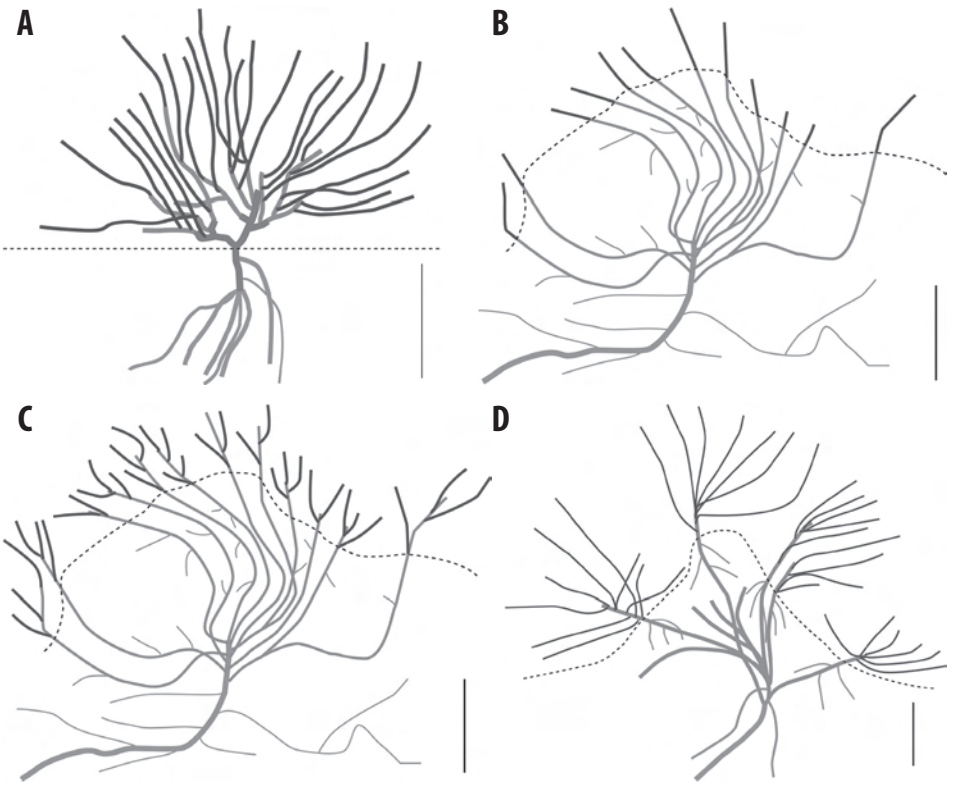

Fig. 2. Morphology of *Alyssum gmelinii* plants as affected by sand burial. A, unburied plants on grey dunes; B, recently buried plants on white dunes; C, plants buried in the past on white dunes. Dotted line indicates level of soil. Bar indicates 50 mm.

Measurement of soil parameters and mineral nutrients

Soil sampling and analysis was performed as described previously (Druva-Lūsīte et al. 2008). Briefly, soil samples were taken from the root zone near *A. gmelinii* plants (0 to 10 cm depth). For each sample five to eight subsamples were collected and throughly mixed to form one sample. Soil pH was measured in 1:2.5 soil to 1M KCl solutions. Soil electrical conductivity was determined in a 1:5 soil to deionized water volume ratio with a conductometer. Soil samples were air-dried and sieved and extracted with 1M HCl. The levels of Ca, Mg, Fe, Cu, Zn, and Mn were measured by an atomic absorption spectrophotometer with an acetylene-air flame. The amount of N, P, Mo and B were assayed by colorimetry, and concentration of S by turbidimetry. K and Na was measured by a flame photometer with an air-propane/butane flame. Chloride was determined by $AgNO_3$ titration.

Results

Burial conditions strongly affected the morphology of *A. gmelinii* plants. Sequential changes of plant habit after the burial event are shown in Fig. 2. Plants on fixed dunes with

no burial in the life history had a shrub-like appearance with lignified main stems (Fig. 2A). Leafy branches were relatively long, with loosely deposited leaves. After burial by sand, new branches were formed from vegetative buds located at nodes of a buried shoot and exhibited intensive elongation above the sand surface (Fig. 2B). At the beginning of the next vegetation season new short branches were formed from buds just above the soil surface (Fig. 2C). Without further burial events in relatively stable sand-level conditions buried stems formed well-rooted ramet-like underground structures with relative independence from the mother plant (Fig. 2D). Burial stimulated abundant adventitious root formation on newly developed underground stems of *A. gmelinii* plants growing on semi-mobile white dunes (Fig. 3).

"Autumn type" plants of *A. gmelinii* appeared only in September, possibly as a result of long-term burial conditions. Morphologically, the "autumn type" plants were characterized by more pubescent leaves that were attached closer to the stem. Consequently, less photosynthetic leaf surface was exposed.

Putative burial events did not affect intensity of mycorrhizal colonization in roots of *A. gmelinii* (data not shown). Also, there were no significant differences in mycorrhizal colonization parameters in plants between white and grey dunes (Fig. 4) except for different trends of intensity of mycorrhizal symbiosis in July (higher in white dunes) and September (lower in white dunes; Fig. 4B). However, a characteristic increase of frequency of mycorrhizal symbiosis was evident during the vegetation season (Fig. 4A).

Leaf chlorophyll content did not exhibit significant changes during a vegetation season (Fig. 5). Also, significant differences in leaf chlorophyll content between white dune / grey dune and buried / unburied white dune plants were not found.

Potential quantum efficiency of PSII (F_V/F_M) in leaves of *A. gmelinii* was not affected by location of plants in white vs. grey dunes nor by burial events (Fig. 6A). However, in May and June a relatively low F_V/F_M indicated the possibility of photoinhibition of photosynthesis. Similarly, there were no differences in relative electron transport rate in PSII throughout a vegetation season (Fig. 6B). In contrast, non-photochemical quenching (NPQ) was significantly affected by plant location (white or grey dunes) as well as by sand

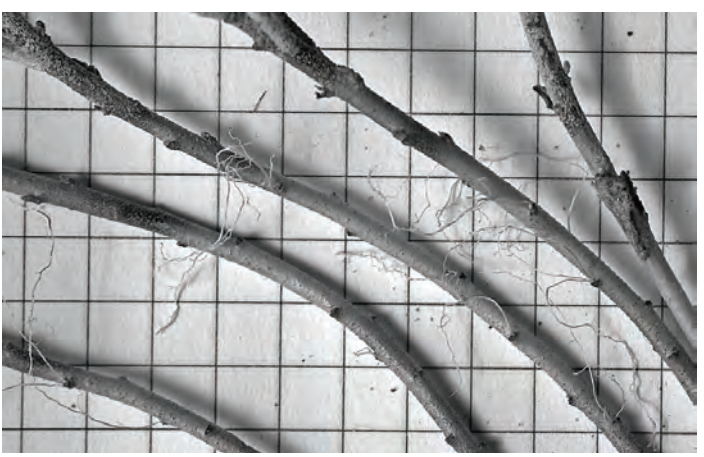

Fig. 3. Formation of adventitious roots on burried stems of recently buried *Alyssum gmelinii* plants on white dunes. A square indicates 5 mm.

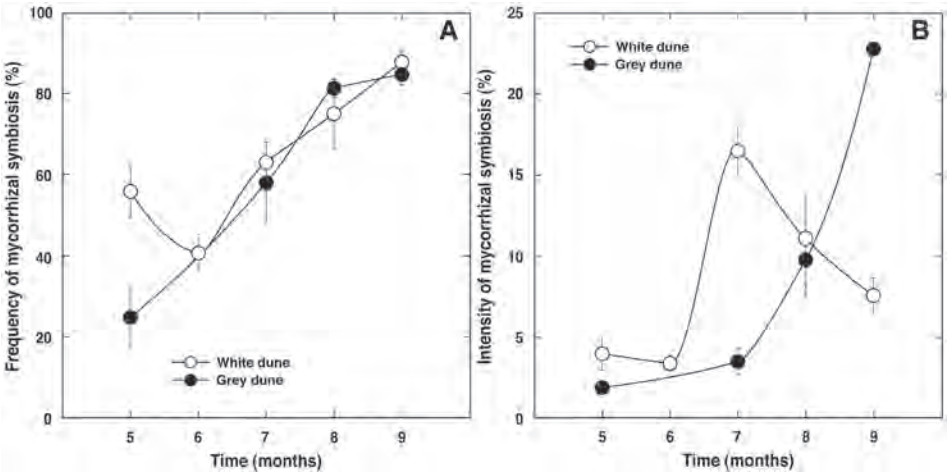

Fig. 4. Seasonal changes of frequency (A) and intensity (B) of mycorrhizal colonization in roots of *Alyssum gmelinii* in different dune types. Data are means ±SE from three independent measurements for each dune type at every time point.

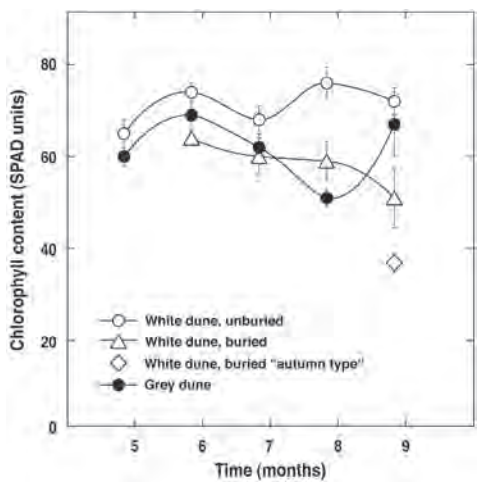

Fig. 5. Seasonal changes of leaf chlorophyll content of *Alyssum gmelinii* in different dune types and sand burial conditions. Data are means ±SE from 20 independent individual leaf measurements for each dune type or sand burial condition at every time point.

burial of plants growing on white dunes (Fig. 7). Grey dune plants had a higher level of NPQ within a season in comparison to white dune plants. For white dune plants, sand burial resulted in increased NPQ in the first part of the vegetation season (June - July). The "autumn type" plants of *A. gmelinii* had no differences in photyochemistry of PSII except higher NPQ (Fig. 7).

Soil macronutrient and micronutrient content was analyzed near *A. gmelinii* plants growing on both white and grey dunes to search for any putative differences in nutrient

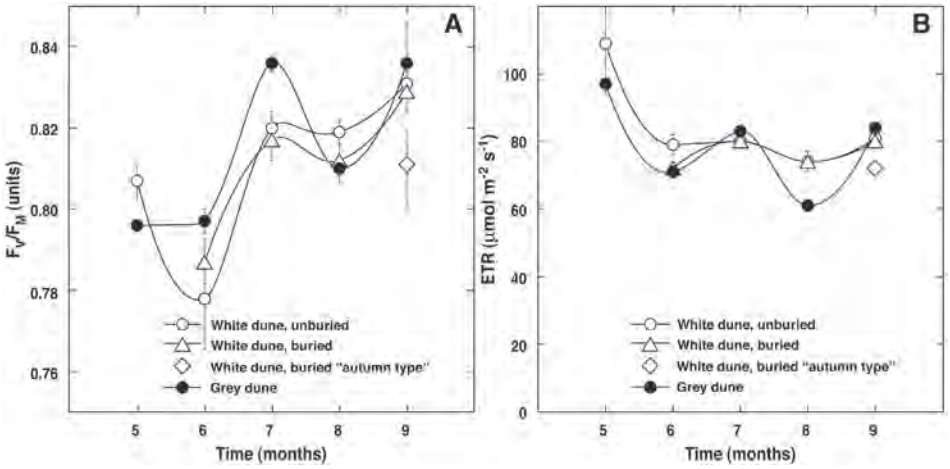

Fig. 6. Seasonal changes of maximum efficiency of PSII (F_V/F_M; A) and relative electron transport rate in PSII (ETR; B) in leaves of *Alyssum gmelinii* in different dune types and sand burial conditions. Data are means ±SE from 10 independent measurements for each dune type or sand burial condition at every time point.

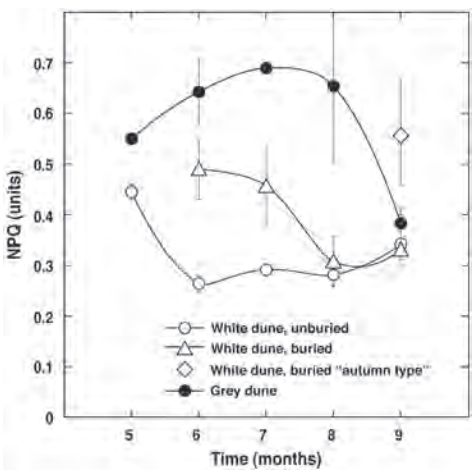

Fig. 7. Seasonal changes of non-photochemical quenching (NPQ) in leaves of *Alyssum gmelinii* in different dune types and sand burial conditions. Data are means ±SE from 10 independent measurements for each dune type or sand burial condition at every time point.

availability. Both sites were characterized by extremely low N and K concentrations. Most of the nutrients showed similar concentrations between the two sites for most of the nutrients. As an exception, grey dune soil was characterized by significantly higher concentration of K and Zn, lower Ca as well as by more acidic pH (Table 1). Given the relatively low soil electrical conductivity as well as low overall Na, Cl, B and S concentrations at both sites, no direct effect of sea water was evident.

Table 1. General soil properties and concentrations of nutrients and Na and Cl (mg L^{-1}) at two sites (white dunes and grey dunes) with *Alyssum gmelinii*. Data are means from five measurements throughout the season ± SE. *, significant differences between the sites ($P < 0.01$)

Parameter or nutrient	White dunes	Grey dunes
pH*	8.6 ± 0.1	7.9 ± 0.1
Electrical conductivity (dS m^{-1})	0.24 ± 0.05	0.26 ± 0.03
N	15.8 ± 2.3	17.7 ± 2.8
P	234 ± 58	206 ±20
K*	16 ± 2	27 ± 5
Ca*	17363 ± 2194	11267 ± 2547
Mg	1097 ± 135	817 ± 134
S	11 ± 1	10 ± 1
Fe	296 ± 27	250 ± 20
Mn	44± 3	40 ± 1
Zn	4.4 ± 0.4	5.3 ± 0.2
Cu	0.500 ± 0.005	0.400 ± 0.130
Mo	0.030 ± 0.006	0.020 ± 0.006
B	0.1	0.1
Na	23 ± 3	18 ± 2
Cl	6 ± 1	9 ± 3

Discussion

Alyssum gmelinii is an interesting model species in sand burial studies as it occurs both on semi-open mobile white dunes with a relatively high frequency of sand burial as well as on fixed grey dunes. Consequently, *A. gmelinii* plants should possess adaptations for sand burial conditions.

Improved growth caharcters (vigour) of individual plants after sand burial is a characteristic feature of foredune perennials well-adapted to frequent sand accretion conditions (Maun 1998). Reemergence from the burial deposits represents a phase of mobilization of stored resources for growth in order to reach the surface and to establish a photosynthetic leaf area. After that, increased photosynthetic capacity would be advantageous to quickly replenish energetic and structural resources necessary for further growth and development. *A. gmeilinii* plants showed characteristics typical for species well adapted to frequent burial events, e.g. elongation of burried stems, abundant formation of adventitious roots on buried stems as well as high intensity of photosystem II photochemistry.

"Obligate-buried" species from foredunes usually have low vigour when growing in conditions without sand burial (Eldred, Maun 1982). Suprisingly, *A. gmelinii* plants also grew well in conditions of grey dunes with no burial events. These plants showed the same maximal potential photochemical efficiency of photosynthesis (F_V/F_M) as plants on white dunes (Fig. 6A) indicating that no environmental changes-related photoinhibition of photosynthesis occurred in either site. However, a certain degree of possible photoinhibition was indicative for all plants earlier in the season (May - June).

No single mechanism of growth stimulation by sand burial has been established so far. More likely, burial itself acts as a signal for a buried plant to use stored resources for growth stimulation. In particular, both oxygen defficiency and absence of light should be considered as signals. When the surface has been reached by elongated plant parts, a positive light signal eventually leads to cessation of increased elongation with subsequent leaf formation and growth.

Morphological mechanisms for adaptation to sand burial are relatively completely described. Formation of adventitious buds and activation of dormant meristems on roots and rhizomes is a prerequisite for any form of growth stimulation in buried conditions when already buried plant parts are concerned. Formation of adventitious roots under the control of decreased internal concentration of oxygen and increased ethylene level (Visser et al. 1996) was noted also in the present study (Fig. 3). New roots are usually produced on higher parts of the buried stem providing increased access to oxygen, water and nutrients (Perumal et al. 2006).

In temperate regions, more than 95 % of sand movement on sea coasts occurs during the fall and winter months (Davidson-Arnott, Law 1990). Consequently, sand deposition in habitats with *A. gmelinii* results in burial of overwintering vegetative buds in a dormant state not on actively growing plants. Due to morphological changes after sand burial, leading to formation of relatively independent daughter plants, *A. gmelinii* represent facultatively clonal plants (clonality induced only after sand burial). On grey dunes, *A. gmelinii* grow as a shrub with lignified stems. A number of coastal plants exhibit clonal growth only after burial by sand. Formation of both adventitious roots and buds on submerged rhizomes of *Honckenya peploides* is a prerequisite for ubiquitious clonal growth during the following vegetation season (Gagne, Houle 2002).

However, biochemical mechanisms leading to adaptation to sand burial have not been extensively studied. An ability to recover high photosynthetic activity after emergence from burial is considered to be an important adaptation for survival of plants on mobile dunes (Kent et al. 2005). Maintainence of a high level of photochemical efficiency of PSII in leaves of *A. gmelinii* plants regrown after burial suggests that plants are effectively protected from adverse environmental effects on photosynthesis. Non-photochemical quenching (NPQ) was significantly increased in buried plants on white dunes in comparison to non-buried plants, indirectly suggesting that these plants exhibit better protection against formation of rective oxygen species. However, grey dune plants exhibited even higher levels of non-photochemical quenching. The latter could be an indication of a certain protection mechanism of photosynthesis in stable substrate level conditions.

Sand burial induced increased non-photochemical quenching of PSII fluorescence (Fig. 7), indicating enhanced loss of absorbed light energy. However, PSII yield parameters did not change accordingly. Consequently, *A. gmelinii* plants after sand burial episode could be better protected against oxygen reactivation by excess absorbed energy by means of thermal energy dissipation through conversion of violoxanthin to antheraxanthin and zeaxanthin (Melis 1999). A similar phenomenon might exist in *A. gmelinii* plants growing on grey dunes. This protection mechanism can explain, at least to a certain extent, high general vigour of burried *A. gmelinii* plants, as there is no need to invest in antioxidative protection. As no photoinhibition of photosynthesis was evident later in the season (indicated by high F_V/F_M), non-buried white dune individuals of *A. gmelinii* might allocate resources towards protection against excessive light-dependent reactive oxygen species.

Increase of mycorrhizal colonization after sand burial is among the factors suggested to lead to enhanced plant vigour (Maun 1998). *Alyssum montanum* has been reported to be a nonmycorrhizal species with no mycorrhizal structures found in roots (Pawlowska et al. 1996). Other species of the genus also have been reported nonmycorrhizal, e.g. *Alyssum szovitsianum* was determined as obligate nonmycorrhizal therophyte on Mediterannean coastal dunes (Cakan, Karatas 2006). In our study, mycorrhizal symbiosis of *A. gmelinii* plants seemed to be not affected by sand burial. However, the results do not support the absence of an adaptive role of mycorrhizal symbiosis for plants growing in sand dunes. As it was argued that the major contribution of mycorrhizal symbiosis after sand burial is related to better exploitation of resources, even a moderate constitutive level of mycorrhizal symbiosis might stabilize general performance of *A. gmelinii* plants, especially after sand burial. Similarly, a coastal marsh plant, *Glaux maritima*, exhibit changes in mycorrhizal symbiosis due to fluctuation in soil salinity indicating an adaptive role of the symbiosis for halophytic species (Druva-Lūsite et al. 2008).

Soil characteristics between white dune and grey dune sites did not differ significantly except higher K and Zn, lower Ca as well as more acidic pH in grey dune soil. It appears that these differences can not count for the observed differences in morphology and photochemistry of photosynthesis of *A. gmelinii* plants, as the levels of N and K were apparently limiting at both sites.

In conclusion, *A. gmelinii* plants show both morphological (induced clonality) and biochemical plasticity (induced thermal energy dissipation) to maintain high performance after sand burial allowing re-establishment of individuals on white coastal dunes.

Acknowledgements

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more than two authors per publication, name only a first author followed by 'et al.'. When a particular author or a group of authors has several publications for the given year, give letters 'a', 'b', 'c' etc. after the year of publication. Separate individual references by a semicolon. As an example, (Collins 1999) or (Collins, Chapper 1999) or (Collins et al. 1999) or (Collins 1999a; Collins 1999b) or (Collins 1999a; Chapper 1999b). It is possible to use also the following format 'It is important to note, that Collins (1999) already described...' As an exception, the reference to Internet may be given without including it in the list of references. Unpublished results should be cited by the name of the author only in the text, e.a., (Collins, unpublished data) or (Chapper, personal communication).

Units of measure. Temperature is expressed in degrees Celsius (°C), time in seconds (s), minutes (min), hours (h), days etc. It is appropriate to use litre (L) as a special designation for 1 dm^3. Consequently, mililitre (mL) and microlitre (μL) also can be used. In all other cases SI units must be used as much as possible. Use negative exponents to indicate units in the denominator, e.g. kg m^{-2}; m m^{-2} s^{-1}. Write out numerals one through nine, except when used with units of measure. Write out all numbers or fractions that begin a sentence, or rephrase the sentence to avoid beginning with a numeral. Use the preposition 'to' between numerals (avoid using a dash): e.g. '8 to 12 h'.

Nomenclature. In the abstract, at first mention in the text, and in *Materials and methods*, include complete biological names for all experimental organisms. Following first mentions, generic names should be abbreviated to the initial, except when confusion could arise by reference to genera with the same initial. Exceptions may be accepted in purely taxonomic papers.

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Acknowledgements. First, provide any details on financial support received. Second, acknowledge any person you need to thank for essential help during experiments or writing your work.

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Monographs

Fahn A. 1979. *Secretory Tissues in Plants.* Academic Press, London. 250 p.

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