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Rare and endangered terrestrial gastropods of Lower Silesia (SW. Poland) – current status and perspectives

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Abstract

The terrestrial malacofauna of Poland includes 177 species, 18 of which are introduced. The distribution borders of quite many of them cross Poland, and the highest concentration of such borders (41 species) is found in Lower Silesia (Silesian Lowland plus Sudetes). The area is under heavy human impact, hence there is a need to protect, and particularly to study the land mollusc species that are locally or globally important from a conservation viewpoint: *Discus perspectivus* (Megerle von Mühlfeld, 1818), *Eucobresia diaphana* (Draparnaud, 1805), *E. nivalis* (Dumont et Mortillet, 1852), *Semilimax kotulai* (Westerlund, 1883), *S. semilimax* (Férussac, 1802), *Aegopinella epipedostoma* (Fagot, 1879), *Tandonia rustica* (Millet, 1843), *Lehmannia macroflagellata* Grossu et Lupu, 1962, *Bielzia coerulans* (M. Bielz, 1851), *Deroceras praecox* Wiktor, 1966, *Cochlodina costata* (C. Pfeiffer, 1828), *Charpentieria ornata* (Rossmässler, 1836), *Macrogastra badia* (C. Pfeiffer, 1828), *Clausilia parvula* (Férussac, 1807), *Balea perversa* (Linnaeus, 1758), *Helicodonta obvoluta* (O.F. Müller, 1774), *Helicigona lapicida* (Linnaeus, 1758) and *Causa holosericum* (Studer, 1820). For each of these species we present current data on local and European distribution, habitat preferences, conservation status and the state of knowledge of life histories. We also delineate the directions of future studies and necessary conservation actions.

Key words: conservation, Lower Silesia, Poland, terrestrial malacofauna.

Introduction

In recent years the decline in the number and abundance of populations of many European gastropod species has become especially dramatic, even in spite of launching seemingly sensible and well-planned protection programmes in many countries. One of the reasons for this, apart from continuing human interference, is that in many cases threat factors have not been identified. The protection measures taken seem to be targeted at individual species rather than at malacocoenoses of which they form a part, which is due to the still very scanty knowledge of gastropod communities. In this context, it has become crucial to: (1) study all the various aspects of life histories of endangered species in order to identify critical stages in their life cycles, and (2) identify the respective malacocoenoses to protect whole gastropod communities and to use them as indicators of potentially adequate conditions when re-introduction of a species becomes necessary.

Below we discuss the status, perspectives and knowledge of thr ecology and life history of 18 rare and/or endangered species (see also Tables 1 and 2) of Lower Silesia, a region

which due to its specific character favours their survival. Although our paper is entirely based on literature information, many of the publications we cite (Maltz 1999; 2003a; 2003b; 2003c; Cameron, Pokryszko 2004; Pokryszko, Cameron 2005; Pokryszko et al. 2005; Cameron, Pokryszko 2006; Maltz 2006; Maltz, Pokryszko 2006) contain the results of our recent studies regarding the composition of malacocoenoses of which the endangered species form a part, or various aspects of life histories of such species.

Species accounts

Discus perspectivus (Megerle von Mühlfeld, 1818; Fig. 1A, 2) is a Carpathian-Dinaric-East Alpine species with a disjunct distribution. In Poland, apart from the eastern part of the Carpathians and their foothills, it is found in a few isolated localities in the Sudetes. It is a forest-dweller closely associated with rotting timber (Kuźnik-Kowalska 2005). Apart from habitat destruction (clear-felling), the main threat is intensive forest management (removal of dead timber). Its life cycle has been studied both in the field and in the laboratory, which has made it possible to identify the main threat factor (Kuźnik-Kowalska 2005). One of the Lower Silesian localities, the Muszkowicki Las Bukowy Nature Reserve, supports the highest population densities (Kuźnik-Kowalska 2005), and the composition of the associated malacocoenosis there has been thoroughly studied (Wiktor 1972; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data).

Eucobresia diaphana (Draparnaud, 1805; Fig. 2) is an Alpine-Central European montane species, in Poland practically limited to Lower Silesia (Umiński 1983; Wiktor 2004). It prefers humid places, with mainly herbaceous vegetation close to streams. Reasons for it being endangered are unclear, but its populations seem to have declined in recent years. Its life history has been studied in the field only, therefore only population dynamics are known (Umiński 1975, 1983). The composition of its associated malacocoenosis has been studied in five Lower Silesian sites (Pokryszko 1984; Maltz 1999; Pokryszko et al. 2004; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data); four of its localities in Lower Silesia are nature reserves in which it does not seem to be endangered.

Eucobresia nivalis (Dumont et Mortillet, 1852; Fig. 2) is an Alpine-Carpathian species of disjunct and incompletely studied distribution; in Poland it is found in the Carpathians and a part of the Sudetes. Although in the Alps it reaches up to 3,100 m a.s.l., in the Polish part of its range it is usually found below the timberline where it prefers humid places in forests and remains in leaf litter or under stones. In Lower Silesia it seems to be rare; reasons for the decline of some of its populations are not clear. Because its life history has been studied in the field only, only population dynamics are known (Umiński 1979, 1983); the composition of the accompanying malacocoenosis has been studied in one site in Lower Silesia (Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data). It is not protected in any nature reserve in that area.

Semilax kotulai (Westerlund, 1883; Fig. 2) is an Alpine-Carpathian species of disjunct distribution. In Poland, apart from Lower Silesia, it is found in the Carpathians with their foothills, in the Cracow-Częstochowa Upland and the Świętokrzyskie Mts. A mostly montane forest-dweller, it prefers shaded, humid and rather cool places. It is a rare species whose populations seem to be declining for no apparent reason. Its life history has been studied in the field only, thus only the population dynamics are known (Umiński 1975; 1983). It is protected in one nature reserve in Lower Silesia.

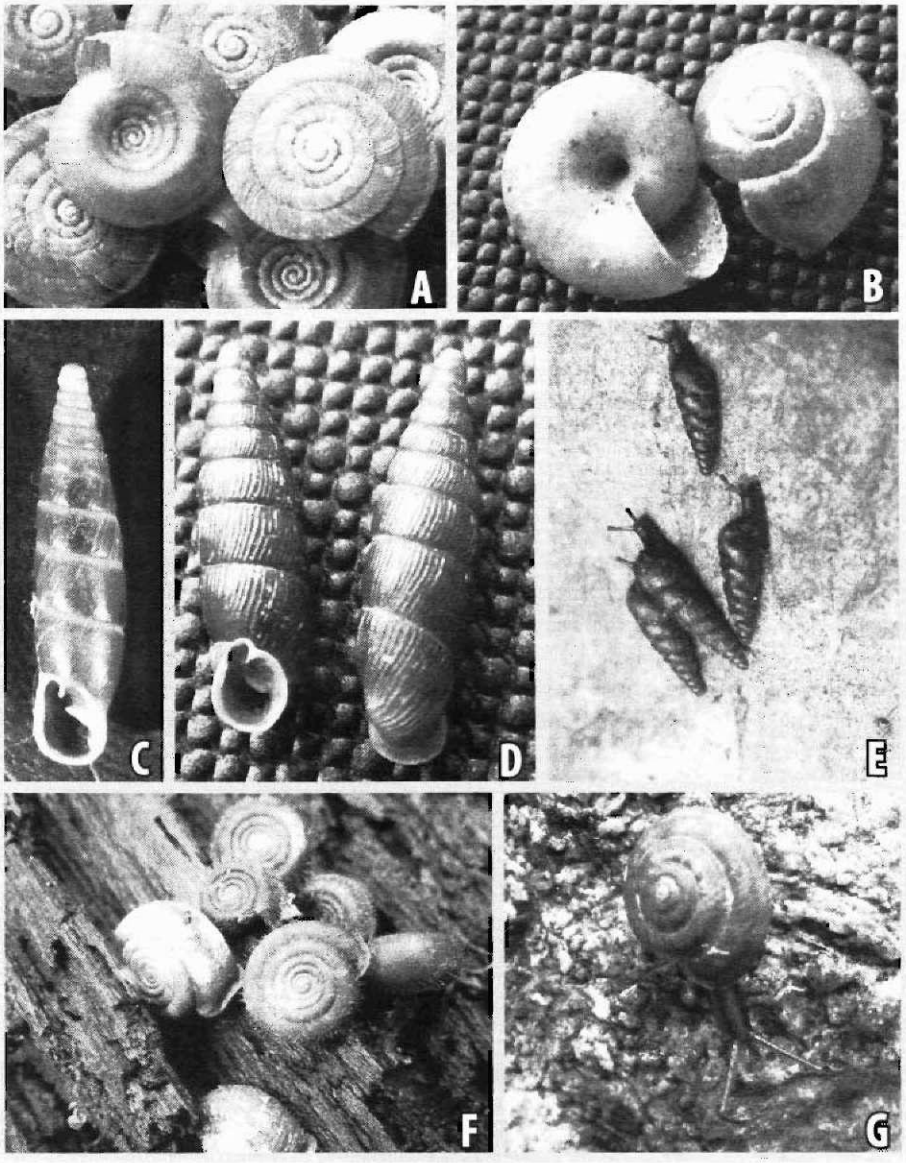

Fig. 1. Some rare and endangered snail species from Lower Silesia. A, *Discus perspectivus*; B, *Aegopinella epipedostoma*; C, *Cochlodina costata*; D, *Macrogastra badia*; E, *Balea perversa*; F, *Helicodonta obvoluta*; G, *Helicigona lapicida* (photo: C – R.A.D. Cameron, all other photos – T.K. Maltz).

Semilimax semilimax (Férussac, 1802; Fig. 2) is a montane Central-European or Alpine-Central European species. In Poland there is an isolated fragment of its range in Lower Silesia and the Carpathians with their foothills. Much like the preceding species, it is mainly a forest-dweller of shaded, humid microhabitats. Like the other three above-mentioned vitrinids, it seems to have increasingly fewer populations, and its local abundance is also

decreasing. The threat factors are not clear. Population dynamics are the only known aspect of its life history (Umiński 1983). The composition of its accompanying malacocoenosis has been studied in five sites in Lower Silesia (Pokryszko 1984; Maltz 1999; Pokryszko et al. 2004; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data); four Lower Silesian localities of the species are nature reserves.

Aegopinella epipedostoma (Fagot, 1879; Fig. 1B, 2) is a montane species of rather little known distribution range. It is found in the Pyrenees and adjacent parts of France, in Germany and Slovenia, and in Poland it occurs in the Carpathians excepting the Tatra and Sudetes. It lives in leaf litter and among rock debris of humid and mesic forests. The small and few isolated populations in Lower Silesia are threatened by random events. Its life cycle is partly known (Kuźnik-Kowalska 2006) and is being currently studied in the field and in the laboratory. The composition of its accompanying fauna has been studied in two of its Lower Silesian sites, both of which are nature reserves; in one it reaches high densities (Pokryszko et al. 2004; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data).

Tandonia rustica (Millet, 1843; Fig. 2) is a South and Central European species, in Poland it is regarded as a thermophile relict of the Atlantic Period, found only in the foothills of the Western Sudetes and inhabiting forested screes and sometimes ruins. Its few and scattered populations are threatened by random events and habitat destruction. Knowledge of its life cycle is very fragmentary (Wiktor 1989). The composition of its associated malacocoenosis has been studied in three sites (Pokryszko 1984; Maltz 1999; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data), two of which are nature reserves where the slug is fairly abundant.

Lehmannia macroflagellata (Grossu et Lupu, 1962; Fig. 2), probably a Carpathian species of imprecisely known range, is a montane arboreal snail, during the vegetation season staying mostly on beech and sycamore trunks. In Poland it is known from higher mountains, in Silesia the Karkonosze and the Śnieżnik Kłodzki Mt. Massif. It feeds on lichens and algae from tree bark (Wiktor 1989). Nothing is known of its life cycle. It is threatened by habitat destruction; some of the populations, being small and scattered may suffer as a result of random events. It is protected in one nature reserve and one national park.

Bielzia coerulans (M. Bielz, 1851; Fig. 2) is a Carpathian, montane species. In Poland, apart from the Carpathians, it is found in the Śnieżnik Kłodzki Massif. A forest-dweller staying mostly on the forest floor, it is sometimes found above the timberline. In Silesia it may be threatened by random events; its populations in two national parks in the east of Poland (Tatra NP and Babia Góra NP) do not seem to be endangered. Its only Silesian population appears to have a very low density; its only locality is a nature reserve. Its life cycle has been studied, though incompletely (Smoleńska 1936; Wiktor 1989).

Deroceras praecox (Wiktor, 1966; Fig. 2) is one of the very few basically Sudetic species, eastwards reaching the Babia Góra Massif. Outisde Poland it has been recorded from the Czech Republic and Slovakia. An inhabitant of deciduous forests and their edges in lower montane and submontane zone, it prefers humid places near streams or springs. Some, but not all, aspects of its life history and genetics have been very thoroughly studied (Reise 1995, 2001). The composition of its associated malacocoenosis has been studied in two sites in Silesia (Wiktor 1972; Maltz 1999; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data). Although rather rare, it does not seem to be threatened in Lower Silesia where it is protected in three nature reserves; in one of them it occurs in very high numbers.

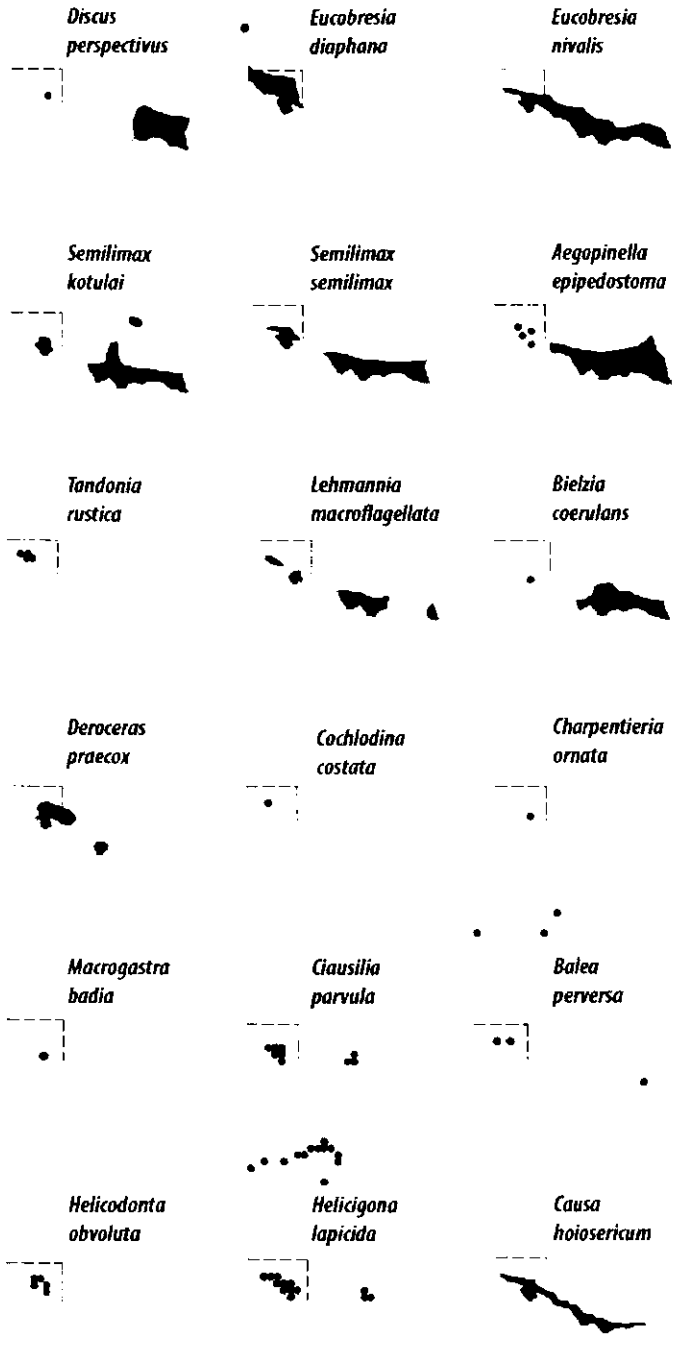

Fig. 2. Distribution of rare and endangered gastropod species in Poland and Lower Silesia.

Table 1. Red-booked, red-listed and protected species in Lower Silesia. Species discussed in this paper indicated in bold. Threat categories (Wiktor, Riedel 2002; Głowaciński 2004) EX – extinct; CR – critically endangered; EN – endangered; VU – vulnerable; NT – near threat; DD – data deficient.

Species	Red book	Red list	Legal status
Pyramidula rupestris		NT	
Vertigo angustior	EN	EN	×
Orcula doliolum		**VU**	
Vallonia enniensis		NT	
Vallonia declivis		**DD**	
Chondrula tridens		NT	
Discus perspectivus		**VU**	
Semilimax kotulai		**NT**	
Semilimax semilimax		**NT**	
Eucobresia diaphana		NT	
Eucobresia nivalis		**NT**	
Aegopinella epipedostoma		NT	
Nesovitrea petronella		NT	
Oxychilus glaber		NT	
Daudebardia brevipes		**VU**	
Tandonia rustica		NT	
Limax bielzi		**DD**	
Lehmannia macroflagellata		NT	
Bielzia coerulans		**NT**	
Deroceras praecox		**NT**	
Cecilioides acicula		**DD**	
Cochlodina costata	EX	**CR**	×
Charpentieria ornata	CR	**CR**	×
Macrogastra badia	CR	CR	×
Macrogastra tumida		NT	
Clausilia parvula		NT	
Clausilia cruciata		**NT**	
Balea perversa	CR	CR	×
Perforatella umbrosa		**NT**	
Trichia tubomirskii		NT	
Trichia unidentata		NT	
Helicodonta obvoluta	CR	CR	×
Helicigona lapicida		NT	×
Causa holosericum		**NT**	

Cochlodina costata (C. Pfeiffer, 1828; Fig. 1C, 2) is an East Alpine species, in Poland reported from several localities in the Sudetes and the Ślęża Massif. After repeated unsuccessful attempts at re-finding the localities, it was pronounced extinct, but was recently re-found on Mt. Miłek near Jelenia Góra (Pokryszko et al. 2004) where it now seems to have

its only population in Poland. It is a forest-dweller, in Poland threatened by random events. Nothing is known of its life cycle. The composition of the associated malacocoenosis has been studied (Pokryszko et al. 2004); its only site is a nature reserve.

Charpentieria ornata (Rossmässler, 1836; Fig. 2) is a South- and East-Alpine species which in Poland has only a small group of closely situated localities in Żelazno near Kłodzko. In these localities, which are its northernmost, it is a species of intermediate stages of forest succession, inhabiting abandoned limestone quarries where it lives on or at the base of half-shaded rock faces. It is threatened by habitat desctruction, random events and ecological succession, and requires active protection (Głowaciński 2004); in spite of this none of its sites is a nature reserve. In at least two sites it is abundant (Maltz 2006; Maltz, Pokryszko 2006). The composition of its associated malacocoenosis has been studied in all its localities; its life cycle is currently under study both in the field and in the laboratory (Maltz 2006; Maltz, Pokryszko 2006), and the obtained information has made it possible to identify the main threat factor – ecological succession.

Macrogastra badia (C. Pfeiffer, 1828; Fig. 1D, 2) is an East-Alpine species with its main range in S. Bavaria and Austria and with very few isolated sites in Poland, in the Orlickie, Bystrzyckie and Stołowe Mts; only one of them has been recently confirmed – Zieleniec (2007: Wiktor, Maltz, Pokryszko unpublished data). It is a forest-dweller of deciduous and deciduous-coniferous stands. Although in the recently confirmed locality it is abundant, the species is threatened by random events and habitat destruction; none of its sites is in a protected area. Its life cycle is being currently studied in the laboratory.

Clausilia parvula (Férussac, 1807; Fig. 2) is a Central European species; in Poland found only in the Sudetes and the Cracow-Częstochowa Upland. It is a forest-dwelling petrophile and calciphile, living mostly in leaf-litter and among rock rubble, sometimes climbing rocks; in non-limestone areas it is associated with old castle ruins (Maltz 1999). It has always been rare and, apart from habitat destruction, it is difficult to envisage any definite threat factors. Its life cycle is being currently studied in the laboratory. The composition of its associated malacocoenosis has been studied in four sites (Maltz 1999; Pokryszko et al. 2004; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data); all of them are nature reserves.

Balea perversa (Linnaeus, 1758; Fig. 1F, 2) is a West-European species of a rather large but much disjunct distribution range. In Poland it has a few scattered sites mainly in Lower Silesia and the Carpathian foothills. Regarded as a thermophile relict from the Atlantic Period, in our climatic zone it inhabits mainly castle ruins. The main threat factor is cleaning and renovation of old castles. Some aspects of its life history have been published (Baur 1990; Baur, Baur 1992), others are being currently studied. None of its localities is a nature reserve.

Helicodonta obvoluta (O. F. Müller, 1774; Fig. 1F, 2) is a Central-European species of disjunct distribution. In Poland, on its northern distribution border, it has several isolated localities in Lower Silesia, many of which support very small populations; only in two localities does it reach a high population density (Maltz 1999; 2003a). An inhabitant of natural forests, in our climatic zone it is dependent on large fragments of dead deciduous logs for egg-laying and hibernation. It is threatened by forest management. Its life history has been studied in detail (Maltz 2003b, c), making it possible to identify the main threat factor. The composition of its malacocoenoses has been studied in two sites with the largest populations (Wiktor 1972; Maltz 1999; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data). The two largest populations are protected in nature reserves.

Table 2. Knowledge of life histories of red-listed, red-booked and protected species of Lower Silesia. Species discussed in this paper indicated in bold.

Species	Knowledge of life cycle	Source
Pyramidula rupestris	no data	
Vertigo angustior	population dynamics	Cameron 2003
Orcula doliolum	no data	
Vallonia enniensis	no data	
Vallonia declivis	no data	
Chondrula tridens	no data	
Discus perspectivus	complete	Kuźnik-Kowalska 2005
Semilimax kotulai	population dynamics	Urniński 1970, 1975, 1983
Semilimax semilimax	population dynamics	
Eucobresia diaphana	population dynamics	
Eucobresia nivalis	population dynamics	
Aegopinella epipedostoma	population dynamics; other aspects currently studied	Kuźnik-Kowalska 2006 and unpublished
Nesovitrea petronella	no data	
Oxychilus glaber	no data	
Daudebardia brevipes	no data	
Tandonia rustica	fragmentary, based on field observations	Wiktor 1989
Limax bielzi	no data	
Lehmannia macroflagellata	no data	
Bielzia coerulans	nearly complete	Smoleńska 1936; Wiktor 1989
Deroceras praecox	nearly complete	Wiktor 1989, Reise 1995; 2001
Cochlodina costata	no data	
Charpentieria ornata	currently studied	Maltz 2006; Maltz, Pokryszko 2006
Macrogastra badia	currently studied	Maltz unpublished
Macrogastra tumida	no data	
Clausilia parvula	currently studied	Maltz unpublished
Clausilia cruciata	no data	
Balea perversa	fragmentary, curently studied	Baur 1990; Baur, Baur 1992
Perforatella umbrosa	no data	
Trichia lubomirskii	no data	
Trichia unidentata	no data	
Helicodonta obvoluta	complete	Maltz 2003a; 2003b; 2003c
Helicigona lapicida	currently studied	Maltz unpublished
Causa holosericum	no data	

Helicigona lapicida (Linnaeus, 1758; Fig. 1G, 2) is a West- and Central-European species, with its eastern distribution border in Poland. Extinct in some parts of Poland, it still has scattered sites in the Cracow-Częstochowa Upland, Western Pomerania and the Sudetes. A forest-dweller (in the north of the country) and petrophile (in the south), it lives under

bark of deciduous tree trunks or on rock faces and in rock crevices. Its life history is being currently studied. It is mainly threatened by climatic changes (aridisation). The composition of the accompanying malacofauna has been studied in five Lower Silesian sites (Pokryszko 1984; Maltz 1999; Pokryszko et al. 2004; Pokryszko, Cameron 2005; Maltz 2006; Maltz, Pokryszko 2006; Pokryszko, Cameron unpublished data). It is protected in four nature reserves.

Causa holosericum (Studer, 1820; Fig. 2) is an Alpine species, with its northern distribution border in Poland where it inhabits the southernmost fringes and is rather rare. It is a montane snail, a forest-dweller preferring rocky substratum and living on the forest floor. Many of its populations seem to have a very low density. Threats, apart from habitat destruction, have not been identified. Nothing is known of the life history of the species. The composition of its malacocoenosis has been studied in three sites (Maltz 1999; Pokryszko, Cameron 2005; Pokryszko, Cameron unpublished data); it is protected in two nature reserves and in one of them is rather abundant.

Discussion

The terrestrial malacofauna of Poland includes 177 species (Riedel 1988; Wiktor 2004 and subsequent corrections: Horsák, Hájek 2005; Juřičková et al. 2005; Reise et al. 2005). The reason for its relative poverty is the Pleistocene glaciation, which, at its maximum, covered virtually the whole country. As a result, the fauna is composed mainly of widely distributed species, many of which have their distribution borders within Poland. Like in the remaining parts of Central Europe, numerous species are in various degrees endangered. The Red List of Endangered Animals in Poland (Wiktor, Riedel 2002) includes 75 species of snails and slugs, and according to a different list (Pawłowska, Pokryszko 1998), the number is even higher (88); thus the proportion of endangered species includes 42 - 50 % of the fauna. Published and unpublished life history and/or associated malacocoenoses data exist for 20 (ca. 23 %) of them, and in most cases are incomplete. The Polish Red Data Book of Animals (Głowaciński 2004) lists 20 species, all of which are also red-listed. The list of legally protected terrestrial molluscs in Poland [Rozporządzenie Ministra Środowiska 2004 (Decree of the Minister of Environment 2004)] includes 28 species, plus one with a legally restricted collecting period; paradoxically, two of them are neither red-booked nor red-listed, while seven are only red-listed. Of these 29 species, life history data exist only for nine, and reasonably complete life history data for two.

Several regions in Poland, such as the calcareous parts of the Carpathians and the Sudetes, and the primeval Białowieża Forest, have preserved especially rich terrestrial malacocoenoses (e.g. Cameron, Pokryszko 2004; Pokryszko, Cameron 2005; Cameron, Pokryszko 2006) with many endangered species. One of these areas is Lower Silesia in the south-west. In a broad sense it includes the Polish part of the Sudetes and the adjacent lowlands. Its malacological uniqueness has two reasons. One is its biogeographical position, as altogether 41 species have their distribution borders there (mostly eastern, north-eastern, northern and, for some Carpathian species, north-western (for distribution data see Riedel 1988; Wiktor 2004). The other is its partly mountainous character, combined with the presence of limestone. This has favoured preservation of pockets of rich natural forests (e.g. Wiktor 1959; 1964; 1972; Pokryszko 1984; Maltz 1999), and in places, castle ruins that now provide refuges for the malacofauna (Juřičková, Kucera 2005).

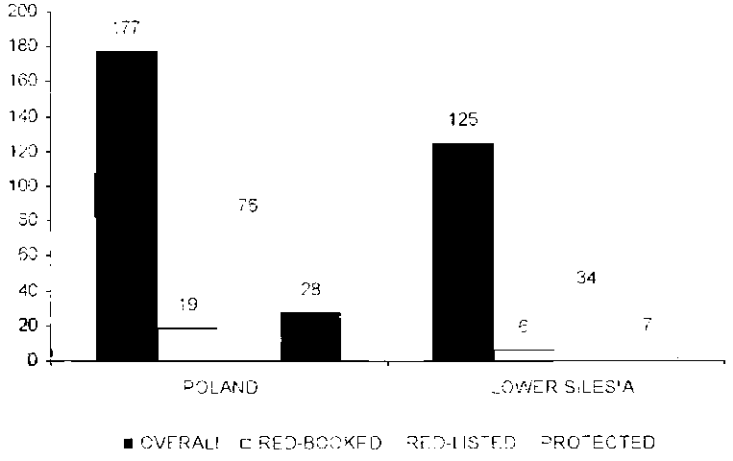

Fig. 3. Graph showing the total number of terrestrial gastropods, number of red-listed, red-booked and protected gastropod species in Poland and Lower Silesia.

Based on the literature, museum collections and unpublished data, in his 1988 catalogue Riedel listed 125 terrestrial gastropod species from Lower Silesia in a broad sense: lowland Lower Silesia plus the Sudetes. This list included more than 70 % of the entire Polish terrestrial malacofauna. However, subsequent verification of distribution data and/or identification revealed that not all of them were actually present in the discussed area. *Columella columella* (Martens, 1830) and *Vertigo arctica* (Wallenberg, 1858), earlier recorded from the Karkonosze Mts. (Wiktor, Wiktor 1968), actually occur only in the Tatra Mts (Pokryszko 1990). The record of *Cernuella neglecta* (Draparnaud, 1805) (Wiktor 1964) was most probably a case of misidentification (Juřičková et al. 2005), and the record of *Trichia plebeia* (Draparnaud, 1805) (Wiktor 1964) pertained to an extreme form of variation of *T. hispida* (Linnaeus, 1758) (Proćków 1997). Thirty four Lower Silesian species are red-listed (Wiktor, Riedel 2002); some are red-booked and/or legally protected (Głowaciński 2004; Rozporządzenie Ministra Środowiska 2004) (Fig. 2, Table 1). Among these, 16 that are rare and/or endangered, are found in various other localities in Poland, for 18 species Lower Silesia is important from a conservation viewpoint: six (*Eucobresia diaphana, Tandonia rustica, Cochlodina costata, Charpentieria ornata, Macrogastra badia, Helicodonta obvoluta*) have their only localities in Poland in that area, another ten (*Discus perspectivus, Eucobresia nivalis, Semilimax kotulai, S. semilimax, Aegopinella epipedostoma, Lehmannia macroflagellata, Bielzia coerulans, Deroceras praecox, Clausilia parvula, Causa holosericum*) have isolated localities there and otherwise are found in SE. Poland, and the localities of two (*Balea perversa* and *Helicigona lapicida*) outside Lower Silesia are very few and scattered.

Of the 18 above species, life histories are completely known for two, for another 12 the knowledge varies from nearly complete (two species) to very fragmentary and/or they are being studied (five species). The threat factors have been identified for 12 species, partly or wholly, and one has been reported as not being endangered in Lower Silesia. Three of the 12 cases (*Discus perspectivus, Charpentieria ornata, Helicodonta obvoluta*) provide good examples of the importance of life history studies for identifying the most

important threat factors and/or critical stages in the life cycle. The main threat to *Discus perspectivus* and *Helicodonta obvoluta* is removal of dead timber; the critical stages in the life cycle are egg-laying, incubation, and hibernation, all requiring the presence of rotting deciduous wood (Maltz 2003a, b; Kuźnik-Kowalska 2005). In the Silesian part of its range, *Charpentieria ornata* is a species of habitats of intermediate succession stages and thus is threatened by forest succession, shown in a recent detailed study on its ecology (Maltz 2006). The composition of the accompanying malacofauna in at least some Lower Silesian sites has been systematically studied for 13 species; only data from random observations exist for the remaining five. The malacocoenoses of which these species form a part are very rich, with species richness ranging from 35 to 45 (Maltz 2003a; Pokryszko, Cameron 2005; Pokryszko et al. 2005; Maltz 2006), thus being comparable to the richest malacocoenoses of primeval forests (Cameron, Pokryszko 2004; Pokryszko, Cameron 2005; Cameron, Pokryszko 2006). Indeed, 14 of the 18 conservationally important species are strict forest-dwellers with distribution limited to the remaining patches of natural forests. Such patches can be indetified based on the species richness of their malacocoenoses. Wherever possible, these patches should be excluded from forest management. Fourteen species are protected in one to four nature reserves (mainly floristic), and the localities of four occur outside protected areas. Fifteen species have one to four healthy populations in Lower Silesia, although one species (*Charpentieria ornata*) is critically endangered. The only Silesian population of *Bielzia coerulans* shows a very low density, and there are no recent estimates for the remaining two species. The following species should be regarded as conservation-priority species in Lower Silesia: *Discus perspectivus*, *Cochlodina costata*, *Charpentieria ornata*, *Macrogastra badia*, *Balea perversa* and *Helicodonta obvoluta*. Two of them (*Discus perspectivus*, *Helicodonta obvoluta*) have been studied with respect to their life cycles, three (*Charpentieria ornata*, *Balea perversa* and *Macrogastra badia*) are being studied, and nothing is known about *Cochlodina costata*. Future research should focus on the ecology and life cycles of the remaining four species, and legislation should aim at improving the conservation status of *Charpentieria ornata*, *Macrogastra badia* and *Balea perversa*. Active protection measures should include preventing forest succession in the localities of *Charpentieria ornata*.

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Reti un apdraudēti zemes gliemeži Lejassilēzijā (dienvidrietumu Polijā) – pašreizējais stāvoklis un perspektīvas

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Kopsavilkums

Polijas zemesgliemežu faunā ir 177 sugas, no kurām 18 ir introducētas. Daudzu sugu izplatības robežas šķērso Polijas teriotiju, lielākā šādu robežu koncentrācija (41 sugai) ir atrodama Lejassilezijā (Silēzijas zemiene un Sudeti). Šis apgabals ir ievērojami pārveidots saimnieciskās darbības rezultātā, tāpēc ir nepieciešams aizsargāt, bet, pirmām kārtām, izpētīt tās molusku sugas, kuras ir lokāli vai globāli svarīgas no saglabāšanas viedokļa: *Discus perspectivus* (Megerle von Mühlfeld, 1818), *Eucobresia diaphana* (Draparnaud, 1805), *E. nivalis* (Dumont et Mortillet, 1852), *Semilimax kotulai* (Westerlund, 1883), *S. semilimax* (Férussac, 1802), *Aegopinella epipedostoma* (Fagot, 1879), *Tandonia rustica* (Millet, 1843), *Lehmannia macroflagellata* Grossu et Lupu, 1962, *Bielzia coerulans* (M. Bielz, 1851), *Deroceras praecox* Wiktor, 1966, *Cochlodina costata* (C. Pfeiffer, 1828), *Charpentieria ornata* (Rossmässler, 1836), *Macrogastra badia* (C. Pfeiffer, 1828), *Clausilia parvula* (Férussac, 1807), *Balea perversa* (Linnaeus, 1758), *Helicodonta obvoluta* (O. F. Müller, 1774), *Helicigona lapicida* (Linnaeus, 1758) un *Causa holosericum* (Studer, 1820). Katrai uzskaitītajai sugai doti pašreizējie dati par vietējo un tālāko izplatību, apdzīvotajiem biotopiem un zināšanu stāvokli par dzīves ciklu. Iezīmēti arī tālāko pētījumu virzieni un darbības, kas nepieciešamas saglabāšanai.

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Investigation of ion selectivity in membranes of muscle cells

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Abstract

The sequences of conductance ratios (G_X/G_K) and permeability ratios (P_X/P_K) for monovalent cations (X = Rb^+, Cs^+, Na^+, Li^+, NH_4^+) were studied in frog skeletal muscle before and after addition of the channel-forming antibiotic gramicidin A. The experiments were carried out under current clamp conditions using a double sucrose gap technique. For inwardly rectifying potassium channels the selectivity measured by membrane conductance ratios before gramicidin treatment was $K^+ > Rb^+ > Cs^+ > NH_4^+ > Na^+ > Li^+$. In gramicidin channels of the same muscle fibre after addition of 5×10^{-7} M or 1×10^{-6} M gramicidin both the permeability and the conductance ratios had the sequence $NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$.

Key words: frog muscle fibre, gramicidin channel, inward rectifier potassium channel, ion conductance, ion selectivity, permeability.

Introduction

The main function of skeletal muscle, contractility, depends on specific membrane ion channel proteins, among other proteins. The two key properties of ion channels are selective ion conduction and gating. Selective conductance reflects a channels ability to select one ionic species among those present in the cellular environment and to catalyze its rapid flow through the pore. The characteristics of selectivity for a monovalent cation in an ion channel can be represented by sequences of conductance ratios (G_X/G_K) and permeability ratios (P_X/P_K), where X = Rb^+, Cs^+, Na^+, Li^+, NH_4^+. Permeability (P) is expressed as the amount of ions transported through the channel in a time unit. Ion conductance (G) is reversely proportional to the membrane resistance (R).

In lipid layers the antibiotic gramicidin A forms selective ionic channels with well-characterized molecular structure (Woolley et al. 1997; Chadwick et al. 2000). Since the gramicidin channel has a number of properties in common with ionic channels of muscle, nerve, and synapse, its study provides useful information about the fundamentals of ion permeation through biological membranes. While selectivity sequences for alkali cations of gramicidin channels in lipid bilayers are well known (Haydon, Hladky 1972; Myers, Haydon 1972; Anderson 1983), corresponding data for biological membranes are scarce.

The aim of our experiments was to measure selectivity for cations in two different kinds of ion channels in the muscle fibre membrane under the same experimental conditions: a natural potassium channel of inward rectification (IRK) and an induced gramicidin channel, a widely used model for cation channels.

Materials and methods

The experiments were performed on single phasic fibres from *iliofibularis* and *semitendinosus* muscles of the frog *Rana esculenta*. After preparation the fibres were incubated in isotonic potassium sulphate solution containing (in mM) 160 K^+, 8 Ca^{2+}, 88 SO_4^{2-}, 2 Tris-malate, pH 7.2. This solution contained only K^+ to carry a substantial current through the membrane, and after 30 min of equilibration the K^+ concentrations inside and outside the cell were nearly identical. In test solutions, K^+ was successively replaced by equimolar amounts of Rb^+, Cs^+, NH_4^+, Na^+ or Li^+. A gramicidin-containing solution was prepared from K_2SO_4 solution by addition of gramicidin A (Serva, Heidelberg; 70 - 85 % gramicidin A) to a final concentration of 5×10^{-7} M and 1×10^{-5} M gramicidin and 0.1 % (v/v) ethanol.

Conductance measurements were performed by means of a double sucrose gap technique described in detail previously (Isenberg, Küchler 1970; Caffier et al. 1980). Hyperpolarizing square wave pulses (0.02 - 0.06 mA) of 300 ms duration were applied to the membrane and the corresponding voltage responses (V_0) recorded (see Fig. 1A, regular vertical lines, hyperpolarization downward). The steady state cord conductance G was obtained as $1/R_m$ where R_m is the membrane resistance (kΩ cm^2) of 1 cm^2 of the outer surface of the muscle fibre membrane, calculated by

$$R_m = V_0 \times S / I \times f^2. \quad (1)$$

V_0 is the voltage measured, I – amplitude of the current pulse, S – the membrane area under investigation, f the short circuit factor given by the relation V_i/V_0 where V_i is the potential change recorded simultaneously with an intracellular microelectrode. To calculate the membrane surface area, S, both the width of the test compartment and the diameter of the preparation were measured under a microscope.

Besides V_0, the changes of membrane resting potential E were continuously measured. In isotonic K_2SO_4 solution E remained at a near-zero resting level within the intervals between the test pulses (Fig. 1A, horizontal straight line). In sulphate solutions containing other cations the resting potential changed to a new value (ΔE). The permeability ratios P_X/P_K were determined from ΔE by the Goldman-Hodgkin-Katz (GHK) equation. The conductance ratio was expressed as G_X/G_K. P_X and G_X are permeability and conductance measured at the same time under asymmetric conditions (K^+ inside, X outside the fibre, where X corresponds to Li^+, Na^+, Rb^+, Cs^+ or NH_4^+). P_K and G_K are permeability and conductance values of potassium.

Results

At the beginning of the experiment the selectivity sequences for the potassium channel of inward rectification were tested. The resting potential in experimental solution (160 mM K^+) was found to be 0.1 ± 0.3 mV and $[K^+]_{in}$ – 159.3 mM (Leech, Stanfield 1981). By substituting cation X for K^+ the resting potential in our experiments reached a new more

Table 1. Conductance ratios (G_X/G_K) and permeability ratios (P_X/P_K) for monovalent cations in potassium and gramicidin channels of muscle fibre membrane

Before gramicidin treatment (potassium channels)

Fibre	K^+	Rb^+	Cs^+	NH_4^+	Na^+	Li^+
No.	G_X/G_K	G_X/G_K	G_X/G_K	G_X/G_K	G_X/G_K	G_X/G_K
1	1				0.27	0.23
2	1	0.21	0.16	0.17	0.15	0.15
3	1	0.21	0.15	0.26	0.14	0.13
4	1	0.47	0.41	0.34	0.30	0.28
5	1	0.48	0.41	0.29	0.21	0.18
6	1	0.28	0.26	0.27	0.21	0.18
Mean	1	0.33	0.28	0.27	0.21	0.19

After gramicidin treatment (gramicidin channels)

Fibre	NH_4^+		Cs^+		Rb^+		K^+		Na^+		Li^+	
No.	G_X/G_K	P_X/P_K	G_X/G_K	P_X/P_K	G_X/G_K	P_X/P_K	G_X/G_K	P_X/P_K	G_X/G_K	P_X/P_K	G_X/G_K	P_X/P_K
3	1.10	1.82	1.15	1.35	1.05	1.19	1	1	0.62	0.40	0.28	0.16
4	1.48	1.79	1.19	1.25	1.10	1.13	1	1	0.54	0.33	0.23	0.17
5							1	1			0.22	0.19
6	1.16	1.43	1.05	1.15	1.03	1.09	1	1	0.69	0.53	0.38	0.22
7	1.23		1.08	1.74	1.07	1.36	1	1	0.72	0.18	0.54	0.04
8	1.03	1.90	1.09	1.41	1.14	1.21	1	1	0.37	0.40	0.24	0.18
Mean	1.20	1.74	1.10	1.38	1.08	1.20	1	1	0.59	0.37	0.32	0.16

hyperpolarized level (ΔE). The hyperpolarization tended to increase in the sequence $Rb^+ < Cs^+ < NH_4^+ < Na^+ < Li^+$ (Fig. 1C). Permeability ratios (relative to K^+) defined by the expression for bi-ionic potentials using the GHK equation

$$E = (R \times T / z \times F) \ln (P_X / P_K) \quad \text{(Myers, Hayden 1972)}, \quad (2)$$

gave a sequence corresponding to $K^+ > Rb^+ > Cs^+ > NH_4^+ > Na^+ > Li^+$. The same sequence was obtained by membrane conductance ratios G_X/G_K, measured in the same experiment before gramicidin treatment (see Table 1). This sequence is commonly referred to as the Eisenman sequence IV (Eisenman, Horn 1983).

The application of gramicidin (1×10^{-6} M) into K_2SO_4 solution resulted in an increase of conductance due to the formation of new gramicidin channels in the membrane. The steady state gramicidin-induced conductance constituted about 80 % of the entire conductance G. Since the gramicidin-induced conductance of the muscle cell membrane was irreversible (i.e. after removal of gramicidin from the solution G remained unchanged or decreased only slightly; Shvinka et al. 1979), G, ΔE and V_0 measured after gramicidin treatment were characteristics of the gramicidin channel. Under these experimental conditions the replacement of external K^+ by equimolar Rb^+, Cs^+ or NH_4^+ caused a deviation of E toward depolarization whereas Na^+ and Li^+ shifted E in the hyperpolarizing direction (Fig. 1A, B). Using Equ. (2) the following sequence of permeability ratios P_X/P_K for the gramicidin channel was obtained (see also Table 1): NH_4^+ (1.74) > Cs^+ (1.38) > Rb^+

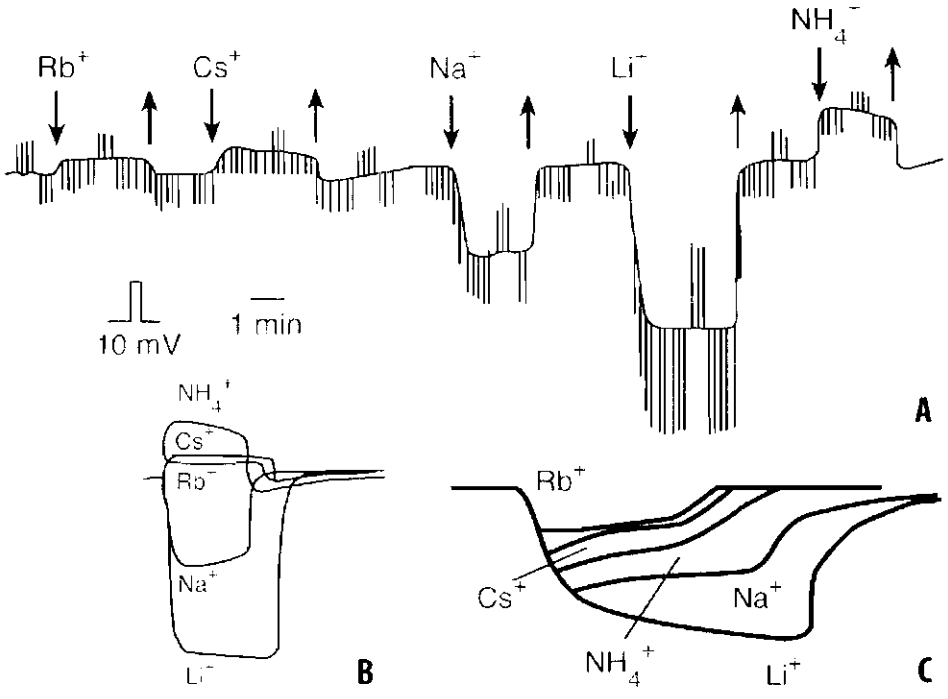

Fig. 1. Effect of monovalent cations on membrane voltage recorded during periodic application of constant current pulses. Measurements with double sucrose gap technique. A typical experiment made on one muscle fibre is shown. A, fibre in isotonic sulfate solution after application of 10^{-5} M gramicidin. Periodic vertical lines are voltage responses to pulses applied once every 10 s at alternate polarities (depolarization upward, hyperpolarization downward). Pulse duration 300 ms, intensity 0.03 μA. Horizontal line, membrane potential (E) in isotonic K_2SO_4 solution. Arrows indicate change from isotonic K_2SO_4 to solutions with different cations and vice versa. B, superimposed changes of resting potential (ΔE) shown in Fig. 1A. C, superimposed ΔE measured before gramicidin treatment on the same fibre.

(1.20) > K^+ (1) > Na^+ (0.37) > Li^+ (0.16). This type of selectivity corresponds to sequence I given by Eisenman and Horn (1983). The same selectivity sequence was obtained from the membrane conductance ratios: NH_4^+ (1.20) > Cs^+ (1.10) > Rb^+ (1.08) > K^+ (1) > Na^+ (0.59) > Li^+ (0.32). It should be mentioned, however, that the numerical values of conductance ratios were not equal to the permeability ratios (see also Table 1).

Discussion

In our experimental conditions (membrane potential near null) hyperpolarizing pulses initiate K^+ outfluxes through the potassium channels of inward rectification (IRK). Rectifier is a term that comes from electronics, referring to devices that conduct electrons only in one direction. In biology, rectification in IRK channels is important because it is used to control the cell resting membrane voltage (Doupnik et al. 1995; Nishida, Mac Kinnon 2002). At voltages favoring the outward flow of K^+ ions the pore becomes blocked

by intracellular Mg^{2+} and polyamines (Aidley, Stanfield 1996). The measurements at the beginning of our experiments prior to gramicidin treatment estimate the activity of this type of K^+ channel. The second measurement on the same muscle fibre after gramicidin treatment demonstrates the selectivity of the gramicidin channels.

The selectivity sequences of gramicidin channels in our experiments are close to those observed for lipid bilayer membranes (Myers, Haydon 1972). The relation of conductance (G) to permeability (P) ratios in the gramicidin channel has been widely discussed (Myers, Haydon 1972; Anderson 1983). In a limiting low ion concentration (10 mM) the ratio of conductance is equal to the permeability ratio when they are measured at the same voltage. Our results are obtained at higher ion concentrations (160 mM) and under asymmetric conditions (i.e. K^+ inside and ion X outside the cell). In this situation the conductance ratio can be computed from the GHK equation by

$$G_X / G_K = \frac{\Delta E + V_0}{V_0 P_K} \left(\frac{P_K e^{(\Delta E + V0)F/RT} - P_X}{e^{(\Delta E + V0)F/RT} - 1} \right), \qquad (3)$$

where ΔE is the polarization which occurs immediately on replacement of the external K^+ by another cation, and V_0 is the voltage recorded at the same time in response to periodic current pulses. After insertion of the experimental values both parts of Equ. (3) became identical. Thus, the inequality of P_X/P_K and G_X/G_K presented in the Table 1 may result from asymmetric conditions of conductance ratio measurements.

Taken together, our results demonstrate the similarity between selectivity sequences of gramicidin channels in muscle cell membrane and in lipid bilayers. In contrast, there is markable difference in the cation transport of natural IRK channels and gramicidin channels induced in the same membrane.

Our study using muscle cell membrane as a model of animal cell cholesterol-containing membranes should aid in elucidating the complex relationship between the structure, biophysics and physiology of ion channels.

Acknowledgements

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Jonu selektivitātes pētījumi muskuļšķiedru membrānās

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Kopsavilkums

Pētīta monovalento katjonu selektivitāte vardes skeleta muskuļu izolētās šķiedrās pirms un pēc kanālveidojošās antibiotikas gramicidīna A pielietošanas. Selektivitāte raksturota gan kā jonu vadītspējas attiecība G_X/G_K, gan arī kā jonu caurlaidības attiecība P_X/P_K ($X = Rb^+, Cs^+, Na^+, Li^+, NH_4^+$). Eksperimenti veikti strāvas fiksācijas režīma, izmantojot divkāršu saharozes tiltiņa izolāciju. Eksperimenta sākumā, pirms gramicidīna iedarbības, selektivitātes rinda $K^+ > Rb^+ > Cs^+ > NH_4^+ > Na^+ > Li^+$ raksturoja anomālās iztaisnošanas K^+ kanālu (IRK). Pēc 5×10^{-7} M vai 1×10^{-6} M gramicidīna pielietošanas taja pat muskuļšķiedrā, katjonu caurlaidības un vadītspējas selektivitāte veidoja rindu $NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, kas raksturīga gramicidīna kanālam māksīgajos lipīdu dubultslāņos.

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Somatic embryogenesis and plant regeneration in immature zygotic embryos of *Brassica napus*

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Abstract

The effect of zygotic embryo age and pH of media on somatic embryogenesis induction of spring rapeseed was investigated. Immature zygotic embryos cultured on a growth regulator-free Murashige & Skoog medium produced primary somatic embryos without an intervening callus phase. Secondary somatic embryo induction was also possible on hormone-free medium. Similar to direct embryogenesis, the efficiency of secondary embryogenesis depended on pH of media. Higher pH (5.0) stimulated the proliferation of primary somatic embryos 25 to 26 days after pollination of zygotic embryos, while a lower pH (3.5) significantly increased the number of responding primary somatic embryos developed 20 to 21 days after pollination of zygotic embryos. Our study showed that primary somatic embryogenesis proceeded at a lower rate than secondary, but the highest number of somatic embryos per responding explant was obtained in primary somatic embryogenesis. Upon transfer to B5 medium, cotyledonary embryos developed into plantlets at a frequency of 7.3 - 30 %.

Key words: age of zygotic embryos, *Brassica napus*, pH, somatic embryogenesis.

Introduction

Oilseed rapes (*Brassica napus*, *B. campestris* and *B. juncea*) are now the third most important source of edible vegetable oil in the world (Kott 1998). Over the last decade, researchers have made great efforts into developing biotechnological methods to facilitate rapeseed breeding. All biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation to improve traits of important crops strongly depend on an efficient recovery of plants through *in vitro* systems. Proliferative embryogenic cultures provide suitable and convenient target tissues for genetic transformation, although initiation and maintenance of these cultures are time-consuming and labor-intensive. Induced somatic embryos develop directly on the explant in a few weeks, and then can be targeted for genetic transformation. Direct somatic embryogenesis from the immature zygotic embryos has been reported: *B. napus* (Koh, Loh 2000), *Arabidopsis thaliana* (Luo, Koop 1997), *Pisum sativum* (Tetu et al. 1990), *Solanum tuberosum* (Pretova, Dedicova 1992) and *Arachis hypogea* (Reddy, Reddy 1993).

Secondary somatic embryogenesis is a phenomenon whereby new somatic embryos are initiated from somatic embryos (Vasic et al. 2001). It has, compared to primary somatic

embryogenesis, advantages such as a high multiplication rate, independence of an explant source and repeatability. Furthermore, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis (Raemakers et al. 1995). Secondary somatic embryogenesis has potential application for both plant breeding practice and research.

In the present study, the influence of zygotic embryo age and pH of media on induction of primary and secondary somatic embryogenesis from immature zygotic embryos of spring rapeseed was investigated.

Materials and methods

Plant material

To study the induction of somatic embryogenesis, the double haploid lines NL-302-01, NL-302-02, NL-302-25 were used. Doubled haploids were generated using the Guelph doubled haploid production method (Fletcher et al. 1998). Donor plants were grown in a growing chamber under controlled conditions: temperature 22 ± 2 °C, light intensity 5000 lx, photoperiod – 16/8 h (day/night).

Isolation of explants and induction of somatic embryogenesis

For the study of somatic embryogenesis, immature zygotic embryos were used. The age of immature zygotic embryos was counted as days after pollination (DAP) – from 14 to 29 days. Explants were sterilized in 70 % ethanol for 2 min, then three times rinsed with sterile distilled water. The embryos under aseptic conditions were transferred to 90 mm Petri dishes containing 25 ml basic Murashige and Skoog (1962) culture medium, supplemented with 2 % sucrose and 8 g l^{-1} Difco-Bacto agar. For each treatment 30 embryos were cultured (10 embryos per Petri plate) and each treatment was done in triplicate. The MS basal medium was adjusted to pH 3.5; 4.0 and 5.0 prior to the addition of agar and autoclaving. Explants were grown under controlled conditions: light intensity 5000 lx, photoperiod 16 h, temperature 25 ± 2 °C. After three days of cultivation, immature zygotic embryos were transferred on the same fresh media and then cultivated for 28 days under the same conditions. The percentage of formed primary somatic embryos and the number of somatic embryos per explant were estimated.

Part of the primary somatic embryos at the cotyledonary stage were carefully separated and transferred to a fresh culture media of the same composition and then cultivated for 28 days under the same conditions. After 28 cultivation days, the percentage of formed secondary somatic embryos and their number per primary embryo were estimated.

Plantlet regeneration

For the regeneration of embryos, a modified B5 (Fletcher et al. 1998) culture medium supplemented with 0.1 mg l^{-1} gibberellic acid (GA_3), 30 g l^{-1} sucrose and 8 g l^{-1} Difco-Bacto agar was used. The percentage of plants regenerated from primary and secondary embryos in B5 medium was estimated.

For statistical analysis, the computer programme STAT 1.55 from "SELEKCIJA" (Tarakanovas 1999) and ANOVA for EXEL, vers. 2.1 were used. Mean values and SE's were calculated based on the number of independent replications

Results

Primary somatic embryogenesis

Immature zygotic embryos cultured on medium with various pH formed primary somatic embryos (SE) without an intervening callus phase. Variable embryogenic responses were expressed by all of the three genotypes tested on the different induction media. Zygotic embryos at the age of 14 to 15 DAP cultivated on pH 3.5 medium did not show any response (Fig. 1A). Higher pH (4.0) improved frequency of embryogenesis of NL-302-01 and NL-302-25 lines (Fig. 1B); however, increasing pH to 5.0 raised the embryogenic potential only in the NL-302-01 line (Fig. 1C). Zygotic embryos at the age of 20 to 21 DAP formed primary somatic embryos on all of the tested media. Explants of NL-302-25 and NL-302-01 lines formed somatic embryos at the highest rate on pH 5.0 medium, whereas pH 4.0 promoted somatic embryogenesis of the NL-302-02 line. Zygotic embryos isolated 25 to 26 days after pollination underwent somatic embryogenesis in all of the media tested, except explants of NL-302-02 line cultivated on pH 3.5 medium. Zygotic embryos of NL-302-02 and NL-302-25 showed the best response on pH 5.0 medium, while a lower pH (4.0) was more suitable for NL-302-01. Immature 28 to 29 DAP zygotic embryos had the highest embryogenic potential on pH 5.0 medium (NL-302-25 and NL-302-02 lines) and pH 4.0 medium (NL-302-01 line).

The number of primary SE produced per responding explant was significantly affected by zygotic embryo age and pH of the medium. Generally, zygotic embryos isolated 20 to 21 days after pollination showed the highest number of primary SE per explant (Fig. 2A-C). Lines NL-302-25 and NL-302-02 formed the highest number of primary SE per responding explant in pH 4.0 media, respectively 18.2 and 7.8. The highest number of primary SE per explant of the NL-302-01 genotype was obtained from zygotic embryos cultivated on pH 5.0 media.

Most primary somatic embryos were formed on the hypocotyls, while some somatic embryos were formed on the cotyledon. Most primary SE had at two cotyledons, but some were multiple with asymmetric cotyledons and had no apical apex. Part of the primary SE after four weeks were transferred to B5 regeneration medium supplemented with 0.1 mg l^{-1} GA_3, and another part were used to induce secondary somatic embryogenesis.

Secondary somatic embryogenesis

Secondary somatic embryos were visible from the root pole of the primary SE within 7 days of culture. The tissue at the root pole of the primary SE proliferated into a small mass of tissue from which several secondary SE emerged. The processes continued in a recurrent manner and by the end of 28 days of culture secondary SE at different developmental stages were observed.

Similar to direct embryogenesis the efficiency of secondary embryogenesis depended on the primary explant age and pH of medium used for inducing the primary embryos. Generally primary SE developed from 14 to 15 DAP zygotic embryos had a very low embryogenic response with some exceptions. Primary SE of NL-302-01 showed the best induced secondary SE on pH 4.0 medium, while secondary SE formation in this line was strongly reduced by pH 5.0 medium and completely inhibited by pH 3.5 medium.

Primary somatic embryos developed from immature zygotic embryos at the age of 20 to 21 DAP exhibited the greatest frequency of secondary embryo formation at pH

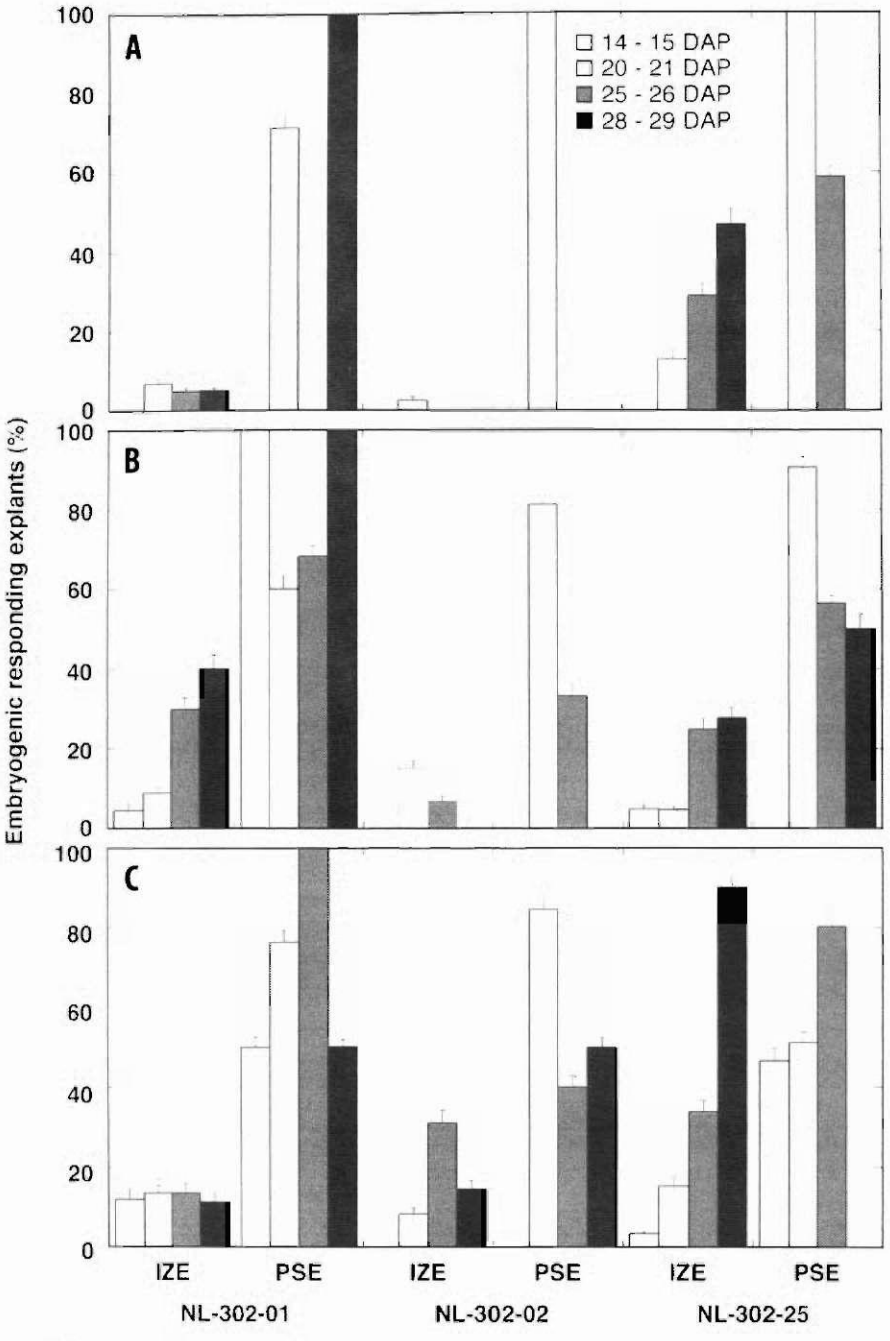

Fig. 1. Effect pH of medium and the age of embryos (days after pollination; DAP) on somatic embryogenesis from immature zygotic embryos (IZE) and primary somatic embryos (PSE) of tested rapeseed lines. A, pH 3.5; B, pH 4.0; C, pH 5.0. White columns – 14 to 15 DAP; light grey columns – 20 to 21 DAP; dark grey columns – 25 to 26 DAP; black columns – 28 to 29 DAP.

3.5 medium (Fig. 1A). In contrast, the higher pH 5.0 of medium promoted secondary embryogenesis of primary SE developed on 25 to 26 DAP zygotic embryos (Fig. 1C).

Cultivating primary SE from 28 to 29 DAP age zygotic embryos, the highest frequency of secondary embryogenesis was observed for the NL-302-01 line in pH 3.5 and 4.0 media, and an increased medium pH up to 5.0 significantly decreased the embryogenetic potential of this line . The efficiency of secondary embryogenesis depended on pH of medium used for the induction of primary embryos, and on the genotype. The highest average number (7.9) of secondary somatic embryos per explant was obtained in pH 4.0 medium from the primary SE of NL-302-01 line, formed from 28 to 29 DAP zygotic embryos.

Plantlet regeneration

Globular somatic embryos developed into cotyledonary embryos after an additional four weeks of culture. Most somatic embryos possessed two cotyledons, some had three or more cotyledons, and a few somatic embryos were fused. Upon transfer to B5 medium, cotyledonary embryos developed into morphologically normal plantlets at a frequency from 57.7 % to 87.3 % (Fig. 3). Experiments did not show significant differences between the capability of primary and secondary somatic embryos to regenerate morphologically normal plants. Somatic embryos that did not develop into plantlets became abnormally enlarged, and frequently formed calluses on their surfaces prior to eventual disorganization. After 28 days, regenerated plantlets bearing three to four leaves were transferred to small cups containing soil, kept for 10 days under a glass cover and finally transferred to a greenhouse.

Discussion

Somatic embryos have shown to be excellent source for secondary embryos. This is associated with loss of integrated group control of cell organization in the somatic embryos. Some cells break away from group control and initiate new somatic embryos. In many species immature zygotic embryos possess and mature zygotic embryos lack the ability to express somatic embryogenesis (Raemakers et al. 1995). In rapeseed it has been reported that immature zygotic embryos had significantly greater embryogenic potential than mature embryos (Koh, Loh 2000; Burbulis, Kupriene 2005). Immature zygotic embryos of various species comprise mitotically arrested pre-embryogenic determined cells, therefore growth regulators in culture medium are required for induction of somatic embryogenesis. It has been reported that exogenous cytokinin is necessary for somatic embryogenesis in immature zygotic embryo cultures of various species, including *Ginkgo biloba* (Laurain et al. 1996) and *Rosa hybrida* (Kim et al. 2003). However, the BAP and other cytokinins have been reported to suppress secondary embryogenesis or cause partial or complete inhibition of embryo development in cell suspension and tissue cultures (Luo, Koop 1997). Other species have been found to require exogenous auxin for embryo induction, while prolonged exposure to auxin in some species inhibits continued development of globular embryos (Merkle et al. 1995). In some species somatic embryos are formed directly from specific explants cultured on medium without growth regulators (Koh, Loh 2000; Zegzouti et al. 2001; Burbulis, Kupriene 2005).

It has been reported that pH of medium or an application of electric field can affect initiation of somatic embryogenesis (Smith, Krikorian, 1990). Exogenous growth regulators

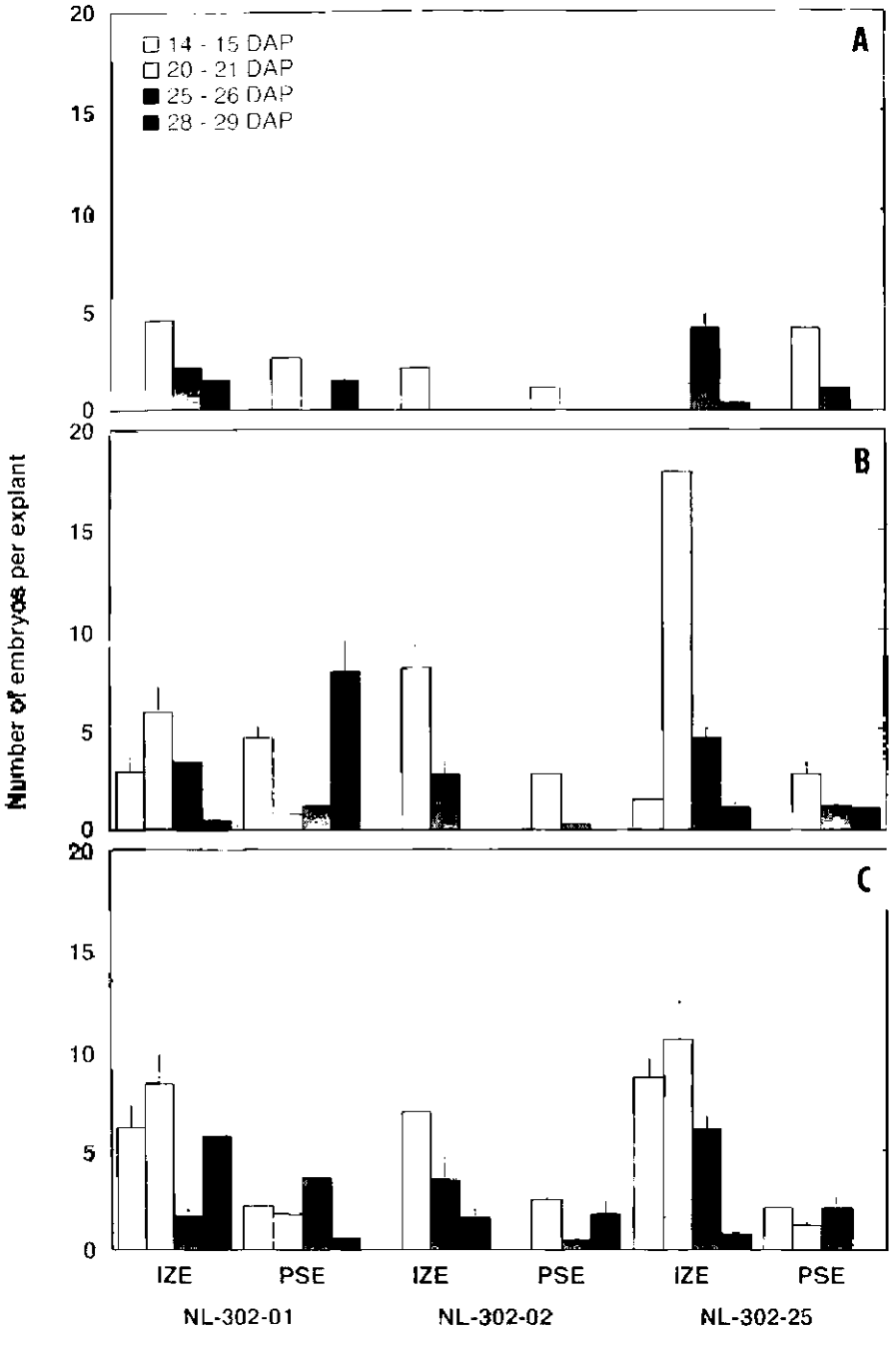

Fig. 2. Effect pH of medium and the age of embryos (days after pollination; DAP) on number somatic embryos per responding immature zygotic embryo (IZE) and primary somatic embryo (PSE) of tested rapeseed lines. A, pH 3.5; B, pH 4.0; C, pH 5.0. White columns – 14 to 15 DAP; light grey columns – 20 to 21 DAP; dark grey columns – 25 to 26 DAP; black columns – 28 to 29 DAP

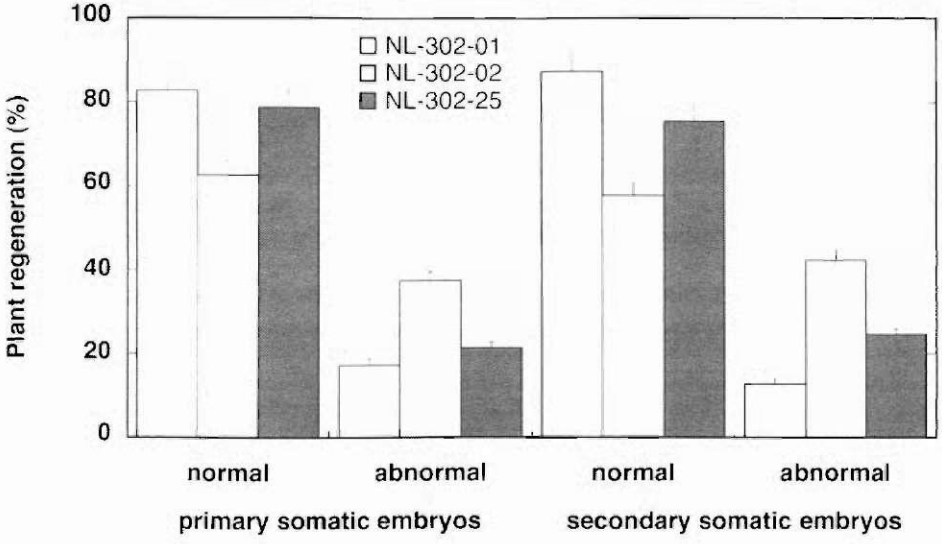

Fig. 3. Plant regeneration from primary and secondary somatic embryos.

probably modify the cell polarity by interfering with the pH gradient or electrical fields around the cells (Dodeman et al. 1997). In soybean it was reported that auxin concentration and pH value influenced somatic embryo production, with optimal levels of 10 mg l^{-1} napthaleneacetic acid and pH 7.0 (Bonacin et al. 2000). However, Hofmann et al. (2004) did not find any significant differences in soybean somatic embryogenesis between 5.7 and 7.0 pH levels.

In the present study somatic embryogenesis of the tested rapeseed lines was induced directly from immature zygotic embryos on hormone-free media. Thus, we suggest that pH of medium can reverse the arrest of pre-embryogenic cells, resulting in somatic embryogenesis. Continued secondary embryogenesis was also possible on hormone-free medium, thereby excluding the complication of exogenous plant grow regulators.

The production of embryos is determined by the number of responding explants and the number of embryos produced per responding explant. It has been documented that in many species the production of embryos in primary embryogenesis is lower than in secondary embryogenesis (Raemakers et al. 1995; Zegzouti et al. 2001). In our study primary somatic embryogenesis proceed at a lower rate than secondary, but a higher amount of somatic embryos per explant was obtained during the primary somatic embryogenesis (Fig. 1, 2).

Detachment of primary SE from the original explant might have stimulated the proliferation of small quantities of the embryogenic tissue attached to its root pole to give a mass of embryogenic tissue or a proembryogenic mass, which ultimately gave rise to further embryos in a cyclic manner. The intensity of secondary embryo formation depended on medium pH used for culturing the explants. Medium pH 5.0 stimulated the proliferation of primary SE from 25 to 26 DAP zygotic embryos, while a lower pH (3.5) significantly increased the number of responding primary SE developed from 20 to 21 DAP zygotic embryos.

In conclusion, a reliable method to induce primary and secondary somatic embryogenesis from new genotypes of rapeseed was established. This system may be highly useful for developing effective transformation systems to improve important economics traits such as oil and protein content and disease resistance.

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Somatiskā embrioģenēze un augu reģenerācija no nenobriedušiem zigotiskajiem embrijiem *Brassica napus* augiem

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Kopsavilkums

Pētīta zigotisko embriju vecuma un vides pH ietekme uz somatiskās embrioģenēzes indukciju vasaras rapsim. Nenobrieduši zigotiskie embriji, kultivēti Murašiges un Skūga vidē bez augšanas regulatoru pievienošanas, veidoja primāros somatiskos embrijus bez kallusa fāzes. Bezhormonu vidē bija iespējama arī sekundāro somatisko embriju indukcija. Līdzīgi tiešajai embrioģenēzei, arī sekundārā embrioģenēze bija atkarīga no vides pH. Augstāks pH (5,0) stimulēja primāro somatisko embriju proliferāciju uz zigotiskajiem embrijiem 25 līdz 26 dienas pec apputeksnēšanas, bet zemāks pH (3,5) ievērojami paaugstināja primāro somatisko embriju veidošanos no zigotiskajiem embrijiem 20 līdz 21 dienu pēc zigotisko embriju apputeksnēšanas. Mūsu pētījumi parādīja, ka primārā somatiskā embrioģenēze notiek ar zemāku atrumu, nekā sekundārā, bet lielāku skaitu somatisko embriju uz reaģētspējīgo eksplantu skaitu iegūst pirmās somatiskās embrioģenēzes procesā. Pēc pārvietošanas uz B5 vidi 7,3 līdz 30 % dīgļlapu embriju attīstījās par mikroaugiem.

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First report on recording of the invasive species *Trachemys scripta elegans*, a potential competitor of *Emys orbicularis* in Latvia

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Abstract

An exotic turtle invasive species *Trachemys scripta elegans* Seidel 2002 was imported to Latvia with commercial purposes for sale in pet-shops. Now illegal introduction has resulted in invasion of the species into the nature of Latvia. In the study, the author checked reports from Latvian local inhabitants about their observation of *Trahemys scripta elegans* in Latvia. In five cases single animals were observed. In one case, for the first time in Latvia a group of six adult *Trahemys scripta elegans* individuals was observed in Sigulda area, village Nitaure. The problems of import of the invasive species *Trahemys scripta elegans* to Latvia and the subsequent illegal introduction of species in nature are examined and possible measures of preventing invasion of *Trahemys scripta elegans* are offered in Latvia.

Key words: *Emydidae*, invasive species, Latvia, *Trachemys scripta elegans*.

Introduction

The natural Latvian fauna includes only one species of turtles: European pond turtle *Emys orbicularis* L. (Silins, Lamsters 1934). Despite some observations of pond turtles in nature during last few years (Pupins, Pupina 1996; Meeske et al 2006) nothing is still known about any stable population of *Emys orbicularis* L. in Latvia (Pupins, Pupina 2005). At the same time, the exotic *Trachemys scripta elegans* Seidel 2002 fresh-water turtle species was massively and uncontrollably imported to Latvia (Pupins, unpublished data), as well as to many other European countries with commercial purposes for sale in pet-shops (Obst 1983; Cadi et al. 2004; Pendlebury 2006). Synonyms: *Chrysemys scripta elegans* Boulenger 1889, *Emys elegans* Wied 1839, *Emys holbrooki* Gray 1844, *Emys sanguinolenta* Gray 1855, *Pseudemys scripta elegans* Stebbins 1985, *Trachemys lineata* Gray 1873 (Obst 1983; Pendlebury 2006).

The natural area of distribution of *Trachemys scripta elegans* is the Mississippi Valley area (USA; Pendlebury 2006). *Trachemys scripta elegans* occupies different types of waters: ponds, rivers, lakes with developed vegetation, warmed up by the sun. The species is omnivorous, feeds on aquatic and terrestrial plants, insects, mollusks, amphibious, fish, crustaceans, and worms. It grows up to 15 - 35 cm long (Obst 1983). It reproduces laying from three to 11 eggs (Pendlebury 2006). Sexual maturity is reached in the 3rd to 4th year (Obst 1983; Pupins, unpublished data).

In XX - XI centuries *Trachemys scripta elegans* was actively grown on farms and massively imported to other countries due to its biological features, attractive appearance and behavior and cultural-historical traditions (raising turtles at home, terrarium keeping, use of turtles in food; Pendlebury 2006) in Europe. According to statistics, 448 000 juvenile *Trachemys scripta elegans* were imported to Poland during 1994 - 1997 (Najbar, Bartlomiej 2001).

For some reason, there occurred an illegal and inadvertent introduction of the species into nature of the importing countries. Where climatic conditions were suitable for the species, it has successfully adapted into nature. According to the data of the Invasive Species Specialist Group which collects and studies the information about invasive species, *Trachemys scripta elegans* is now found in 21 countries of the world: Australia, Bahamas, Bermuda, Brazil, Costa Rica, France, Guam, Italy, Japan, Korea, Republic of Micronesia, Federated States of (FSM), New Zealand, Northern Mariana Islands, Poland, Spain, Taiwan, Thailand, United Kingdom (UK), United States of America (USA), Viet Nam (Vietnam), Virgin Islands, British (Pendlebury 2006), Bangladesh, and India. There is information on the stable existence of *Trachemys scripta elegans* groups and other exotic turtles species in Germany (Schneeweis 2002; Drews 2005) and in cultural habitats of Russia (Pupins, unpublished data).

This wide spread of the species and its influence on local habitats has led to its inclusion in the list of the hundred most invasive biological species of the world (Lowe et al. 2000). In Europe *Trachemys scripta elegans* is a competitor of *Emys orbicularis* in basking places (Cadi, Joly 2003). Spread of invasion of *Trachemys scripta elegans* in Latvia, besides the influence of this species on the habitat, can result in a direct competition of the species with the extremely rare Latvian species of turtles *Emys orbicularis* (Pupins 2005; Pupins, Pupina 2005). This raises a need for research on the occurence of *Trachemys scripta elegans* in Latvia.

Methods

Research on the distribution of turtles was carried out throughout the whole territory of Latvia. Other specific habitats were surveyed additionally, after receiving reports from inhabitants about their observations of *Trachemys scripta elegans* in Latvia.

The search for *Trachemys scripta elegans* was carried out in Latvia together with the study of the distribution and ecology of turtles in Latvia, where the target species of research was European pond turtle *Emys orbicularis* (Pupins 2005).

The basic method of primary research of the distribution of *Trachemys scripta elegans* in Latvia was questioning of local people about observation of turtles in nature. Particular attention was paid to population groups such as: (i) people professionally connected with nature conservation: environmental, forest guards, protected territory workers; (ii) people who regularly use natural resources for their material needs or in their work: amateur hunters and fishermen, forest workers, land owners; (iii) people taking interest in published information concerning nature: the magazine and newspaper articles about nature, radio, television, Internet; (iv) people in contact with nature: tourists, schoolchildren, zoo visitors, etc. The initial question to participants of the survey regarding the observation of turtles in Latvia was asked in the following ways.

(i) Oral form (direct interview of teachers, students, zoo visitors etc) since 1982. The

number of oral questioned people was 30796 in total: 54 in 1982; 52 in 1983; 36 in 1984; 48 in 1985; 123 in 1986; 220 in 1987; 2236 in 1988; 1583 in 1989; 1568 in 1990; 2641 in 1991; 1562 in 1992; 2240 in 1993; 2221 in 1994; 2363 in 1995; 2344 in 1996; 1167 in 1997; 1200 in 1998; 1285 in 1999; 1264 in 2000; 658 in 2001; 356 in 2002; 368 in 2003; 1356 in 2004; 1287 in 2005; 2564 in 2006.

(ii) In written form by direct questions to readers of publications on turtles in Latvia in different local media, radio, TV since 1995.

(iii) Beginning with the year 2005, survey was also conducted by a questionnaire specially designed for the research (Lidaka et al. 2005). The given questionnaire was both preliminary and an independent method of research. It included the following: a question regarding the observation of turtles in Latvia, pictures of *Trahemys scripta elegans*, and recommendations on observation of turtles. The questionnaire included a form for filling in an observation of turtles in Latvia. The form contained questions of geographical-ecological and ethological nature. The survey participants were paid in advance for filling in the survey, which made the work with the population more effective (Lidaka et al. 2005). The number of questioned people in the survey carried out through the newspapers, booklets, TV and radio can not be exactly calculated. After receiving the participant's positive answer, an obligatory telephone or personal interview with the survey participant was organized.

After receiving an affirmative answer to the initial question concerning the observation of turtle in Latvia, the survey participant was offered to choose the animal he saw among the colored pictures of turtles *Emys orbicularis*, *Trahemys scripta elegans* and *Agrionemys horsfieldi* (Lidaka et al. 2005). Also additional questions were given regarding the observation. All the positive answers about the observation of any turtle species were checked.

Depending on the year of the observation and time of receiving the data, an initial examination of the water body and habitat was carried out (Inger 1994; McDiarmid 1994) as well as the examination of the nearest water body if the turtle was seen on the land.

Fig. 1. Observations of *Trahemys scripta elegans* in Latvia in 2006.

Results

We received and checked six reports from Latvian inhabitants about their observation of *Trahemys scripta elegans* in Latvia. Messages were received from the following areas: Daugavpils: (i) N 55°52', E 26°30'; (ii) N 55°52', E 26°35'; (iii) N 55°49', E 26°29'; Sigulda: (iv) N 57°06', E 25°17'; Valmiera: (v) N 57°32', E 25°25', (vi) N 57°31', E 25°24' (Fig. 1). The greatest number of reports (n = 3) was received from the Daugavpils area. In Valmiera District, within an interval of four months, the same animal was repeatedly reported.

In five cases single animals were observed. In one case, in Sigulda area, village Nitaure, a group of adult *Trahemys scripta elegans* individuals was observed for the first time in Latvia (Table 1).

In Sigulda area (Nitaure village), six adult large individuals of *Trahemys scripta elegans* were observed. Five individuals from the given group were captured by local inhabitants. Three of them were examined by the author. All were adult large females (Table 2). The carapaxes of all surveyed turtles were symmetric, without any traces of a scoliosis or other anomalies of development, often found in *Trahemys scripta elegans* grown in terrariums by beginners (Pupins, unpublished data). All turtle carapaxes had numerous fine scratches.

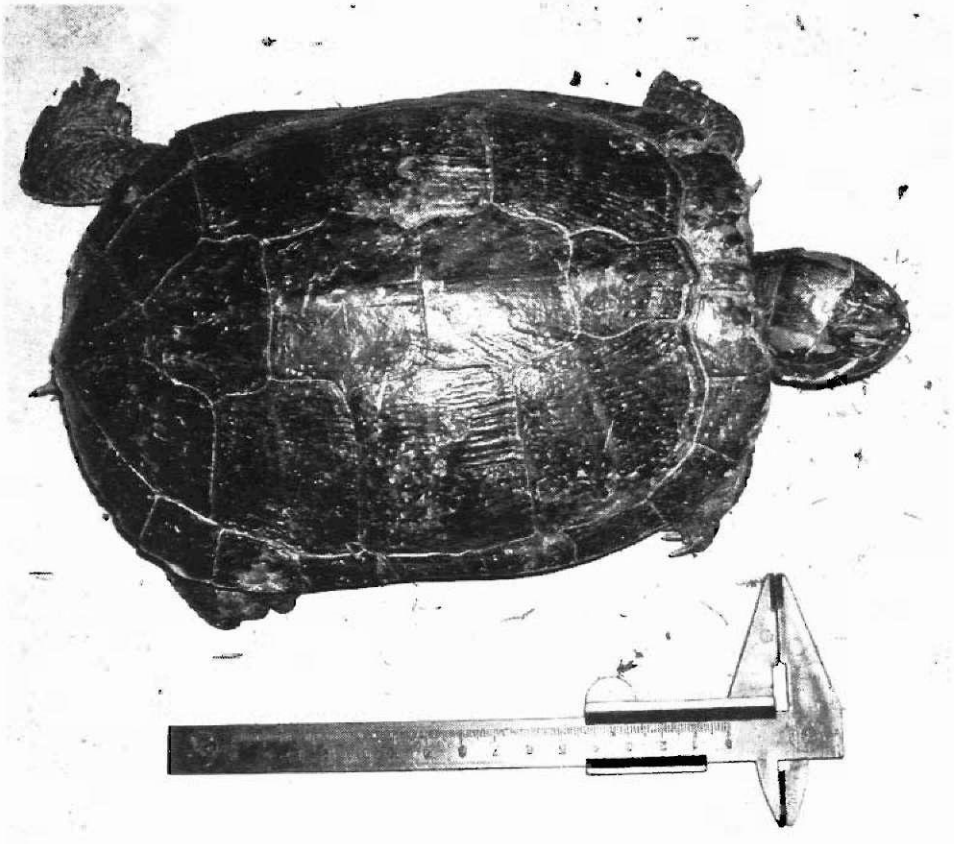

Fig. 2. Female *Trahemys scripta elegans*, captured in Sigulda area, Nitaure village, 2006.

There was a growing wound on the head of one female, presumably from traumatic influence of anthropogenous character (a scar in the form of a direct line; Fig. 2).

Two waterbodies with *Trahemys scripta elegans* records were reported: districts of Daugavpils and Sigulda (village Nitaure).

Waterbody 1. Region: Daugavpils. One adult *Trahemys scripta elegans* was observed in former fish-breeding ponds in the area of Daugavpils (local name of the area "Elektroinstruments", N 55°52', E 26°35'). The habitat represents a system of channels and ponds of artificial origin that were used previously for fish cultivation. The system includes dams and sluices. The depth is up to 3 - 4 m. The eutrophic level is high. The shore zone is warmed and is densely covered with reed and other near-water plants. There are also open shore sites. The shore is bordered by sandy hills. About 20 % of the shore is covered by coniferous and mixed wood. There is plenty of fish, many water birds. There was a report of observation of an adult *Emys orbicularis* individual in 1985 in same location (Pupins, unpublished data). There are abandoned buildings, operating industrial premises and gravel roads at the distance of 20 to 100 m from the shore. Roads also are along shore of the waterbody. The area is actively used by local residents for recreational purposes, walking with dogs and fishing. Due to the length of the shore line, there are sites inaccessible by people (Fig. 3).

Waterbody 2. Region: Sigulda (Nitaure village). A group of six adult *Trahemys scripta elegans* was observed in a pond with local names "Dzirnavinu dikis", "Dzirnavinas" N 57°06', E 25°17'). The habitat is an old pond earlier used for cultivation of carp. The poorly flowing pond formed possibly as a result of blocking a small forest river. The ground is clay. Water depth is up to 3 m. The shore is shaded and eutrophic level is high. The water is densely covered with water plants and the shore with mixed wood and bushes. There are fallen trees in the water. There is an open place on the southern shore used by *Trahemys scripta elegans* for sun basking. There is some household debris on the shore, as the waterbody is used by inhabitants for fishing. There is an operating country farm at the distance of 100 m. There is a gravel road along the shore of the waterbody (Fig. 4).

Table 1. Observations of *Trachemys scripta elegans* in Latvia

District	Year of observation	Reports	Animals observed
Daugavpils	2006	3	3
Sigulda (Nitaure)	2006	1	6
Valmiera	2006	2	2
Total		6	11

Table 2. The sizes of adult *Trachemys scripta elegans* captured in Latvia (Sigulda area, Nitaure village, 2006)

The individual	Sex	Carpax length (mm)	Carpax with (mm)
1	Female	214	144
2	Female	217	164
3	Female	212	174

Fig. 3. Part of a pond system where *Trahemys scripta elegans* was observed in Daugavpils city, Elektroinstruments, 2006.

The above observations confirm records of the invasive species of *Trahemys scripta elegans* in Latvia since 2006. The species is present as individuals and as a group of adult animals.

Discussion

Reliability of records of species by inhabitants and completeness of the received data

Three animals caught in Sigulda (Nitaure village)were examined by the author. Certainly, the received data is incomplete because only those animals about which the inhabitants informed were considered. It is possible to assume the existence of single individuals and groups of *Trahemys scripta elegans* in Latvia that have not been observed by inhabitants or about whom the author had not been informed. Some sellers of pet-shops claimed that during 1990 - 2000 several thousands of juvenile *Trahemys scripta elegans* were imported to Latvia, for sale in pet-shops (Pupins, unpublished data).

The reasons of import of Trahemys scripta elegans *to Latvia and the subsequent illegal introduction of the species in the nature*

Trahemys scripta elegans is not used in food or for other economic purposes in Latvia. This species is imported to Latvia for keeping as pets. Usually they are attractive juvenile animals with 3 - 5 cm carapace length, green carapax and bright red spots on the head. In

Fig. 4. Waterbody where the first group (six individuals) of adult *Trahemys scripta elegans* was found in Latvia, in Sigulda district, Nitaure village, 2006.

some cases, there are also *Trahemys scripta elegans* with a length of carapace 10 - 15 cm on sale, which were given by previous owners for subsequent resale (Pupins, unpublished data). In spite of the fact that education of owners is low and during the first or second year after purchase about 50 % of animals perish, some *Trahemys scripta elegans* grow up to 10 - 15cm (Pupins, unpublished data). The larger individuals have problems of maintenance: need for a greater aquarium volume, better filtration, aggression of adult turtles, need for more forage, greater expenses for electricity for heating (Obst 1983), reduction of animal attractiveness, also comprehension by the owner that this animal will live with him/her for many years. Therefore, many Europeans give their *Trahemys scripta elegans* to zoos, also in Latvia (Riga Zoo, Latgale Zoo), but some turtle owners prefer to release the animal in nature. The author has reports on two cases of successful runaway of *Trahemys scripta elegans* into nature, when owners were walking with them outside (Pupins, unpublished data).

Ability of Trahemys scripta elegans *to adapt to the environment in Latvia*

Obvious limiting factors for the species are low mid year and seasonal temperatures in Latvia. A group of more than 30 *Trahemys scripta elegans* with a length of carapax more than 10 cm has for several years been raised by the author in a pool in the natural climate of Latvia from April until October. Animals that are well adapted for the Latvian climate are active, feed, grow and do attempts to copulate (Pupins, unpublished data). Similar

adaptation of *Trahemys scripta elegans* to the German climate has led to the species being the most often met exotic species in nature (Drews 2005). The ability of *Trahemys scripta elegans* to winter under the ice in waterbodies in Latvia seems obvious as well. However, the author lacks data on wintering *Trahemys scripta elegans* in Latvia, in March - April 1995 - 1999 he repeatedly observed a group of 8 - 12 adult *Trahemys scripta elegans* individuals with a length of carapace not less than 20 cm that were wintering in unwarmed ponds of the Moscow Zoo, Russia, Moscow, N 55°44', E 37°39' (Pupins, unpublished data). *Trahemys scripta elegans* winters successfully in Germany (Schneeweis 2002).

Hatching times are weather dependent: temperatures between 22 to 30 °C for 55 - 80 days are preferred (Pendlebury 2006). Successful reproduction of *Trahemys scripta elegans* is recorded in southern France (Cadi et al. 2004). The average sum of temperature in Latvia is obviously insufficient for incubation of *Trahemys scripta elegans* eggs naturally, as well as for regular successful incubation of egg laying of *Emys orbicularis* in Latvia (Pupins 2005). In this regard there are only single cases of observation of juvenile *Emys orbicularis* in Latvia (Kocane 1999; Pupins, unpublished data). At the same time, regarding potential invasion of *Trahemys scripta elegans* in Latvia, it is necessary to consider the warm autumn-winter periods in Latvia, the general tendency of change of mid-annual temperatures in Europe, and also the long life of *Trahemys scripta elegans*.

Possible measures of preventive maintenance of invasion

Possible measures of preventive maintenance of invasion of *Trahemys scripta elegans* in Latvia are:

(i) education to Latvian inhabitants about the danger of release of *Trahemys scripta elegans* into nature;

(ii) informing Latvian inhabitants about *Trahemys scripta elegans* biology;

(iii) creation of a *Trahemys scripta elegans* shelter in Latvia for receiving turtles from inhabitants;

(iv) further research on *Trahemys scripta elegans* distribution and ecology in Latvia;

(v) removal of *Trahemys scripta elegans* in places of their observation;

(vi) restriction of import of *Trahemys scripta elegans* to Latvia.

Partially the given measures are now implemented by the Rigas Zoo and Latgale Zoo. In 2007 in Latgale Zoo housed 67 adult *Trahemys scripta elegans* received from local inhabitants (Pupins, unpublished data).

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Invazīvās sugas *Trachemys scripta elegans*, potenciālā *Emys orbicularis* konkurenta, pirmais atradums Latvijā

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Kopsavilkums

Eksotisko invazīvo sugu *Trachemys scripta elegans* Seidel 2002 ieved Latvijā komercāliem mērķiem pārdošanai zooveikalos. Notiek nesankcionēta sugas introdukcija un invāzija Latvijas dabā. Pētījuma rezultātā iegūti un pārbaudīti seši ziņojumi no Latvijas iedzīvotajiem par *Trachemys scripta elegans* novērojumiem Latvijā. Piecos gadījumos novēroti atsevišķi īpatņi, bet vienā gadījumā, pirmo reizi Latvijā, novērota *Trachemys scripta elegans* sešu pieaugušu īpatņu grupa Siguldas apkārtnē Nītaures ciema tuvumā. Rakstā analizētas invazīvās sugas *Trachemys scripta elegans* importa Latvijā un sekojošās nesankcionētās sugas introdukcijas dabā problēmas, piedāvāti iespējamie pasākumi, kuri varētu novērst *Trachemys scripta elegans* invāziju Latvijā.

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A new *Bombina bombina* L. population "Demene" in Latvia, Daugavpils District

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Abstract

Bombina bombina L. is a rare and protected Latvian and European animal. Before 2006, three Latvian *Bombina bombina* populations were known: the "Ilgas" and "Ainavas" populations in the Daugavpils District and the "Bauska" population in the Bauska District. On 21 June, 2006 we found a new *Bombina bombina* population in the vicinities of Demene in the Daugavpils District in the southeast part of Latvia. Eight localities were found with ~70 vocalizing males in total. This population "Demene" in 2006 is the largest population of *Bombina bombina* on the northern border of the distribution range of a species. Thus, the given largest Latvian population of *Bombina bombina* plays very important role in conservation of *Bombina bombina* in the Latvian and the European fauna.

Key words: *Bombina bombina*, new population, Latvia.

Introduction

Bombina bombina L. is a rare and protected Latvian animal. *Bombina bombina* is an amphibian species with the small number of individuals in Latvia. Before 2004 only two stable Latvian populations were known: the "Bauska" population in the Bauska District and the population "Ilgas" from the Daugavpils District (Pupina, Pupins 2005a).

The "Ilgas" population is known since the 1970s (G. Kasparsons, personal communication). This population is stable, regularly observed (Pupins, Skute 1992; Barsevskis 2002; Pupina, Pupins 2005b), but there is a decline in their number and in borrowed habitats due to natural overgrowing and degradations of waterbodies, and due to the predatory introduced Far East invasive fish species *Perccottus glenii* Dyb. (Pupina, Pupins 2005b). The "Atlas of Amphibians and Reptiles in Europe" also gives these two *Bombina bombina* sites in Latvia (Gasc et al. 1997). In 2004 a new site of *Bombina bombina* in Latvia was recorded in the Daugavpils District, Kalkunes pagasts, Ainavas, where one vocalizing male was heard (Pupina, Pupins 2005a).

Thus, before 2006 three Latvian *Bombina bombina* L populations from the southern part of Latvia were known, the "Ilgas" and the "Ainavas" populations in the Daugavpils District and the "Bauska" population in the Bauska District. Due to the rarity and small population size of the given species in Latvia, research of its distribution and search of new populations is important.

Materials and methods

Research on *Bombina bombina* distribution was carried out by the authors over the whole territory of Latvia. More carefully were surveyed the southeast and southern parts of Latvia, due to close distance to already known Latvian *Bombina bombina* populations. The search for new *Bombina bombina* populations in Latvia was carried out in two steps.

First, questioning of inhabitants. In 2002 - 2006 questioning of Latvian inhabitants regarding observation of *Bombina bombina* in nature was carried out. Various methods were used: (i) oral interview of local inhabitants, hunters, people interested in nature, visitors of Zoos, schoolchildren, biology teachers, etc.; (ii) written questioning of inhabitants via publication in newspapers and Internet; (iii) questioning of inhabitants during radio and video broadcasts. The characteristic *Bombina bombina* song was also broadcast.

Second, field studies. After receiving a report on *Bombina bombina* the following search methods were used: (i) audible recognition and count of vocalizing males (Zimmerman 1994), also playback of provoking sound imitation of vocalizing action of *Bombina bombina* males; (ii) visual search by surveying the shore zone of waterbodies, also using binoculars; (iii) catching tadpoles with a scoop-net (Shaffer et al. 1994), and checking them for *Bombina bombina*. Audible and visual checking of potentially possible sites of *Bombina bombina* was carried out repeatedly, at different times of the day, with suitable weather conditions. All reports of *Bombina bombina* were followed by interview of reporters concerning the waterbody, length of time of hearing *Bombina bombina* etc.

Results

In our survey a few thousand Latvian inhabitants were directly and indirectly interrogated: zoo visitors (n = 5682), students, participants of seminars (n = 864). Number contacted by questioning carried out through the newspapers, radio and TV can not be calculated exactly, twelve reports were received from inhabitants of *Bombina bombina* in Latvia.

Fig. 1. Known populations of *Bombina bombina* in Latvia in 2006. (○) "Bauska" and "Ilgas" known before 2004. (●) "Ainavas" found in 2004. (▲) "Demene" found in 2006.

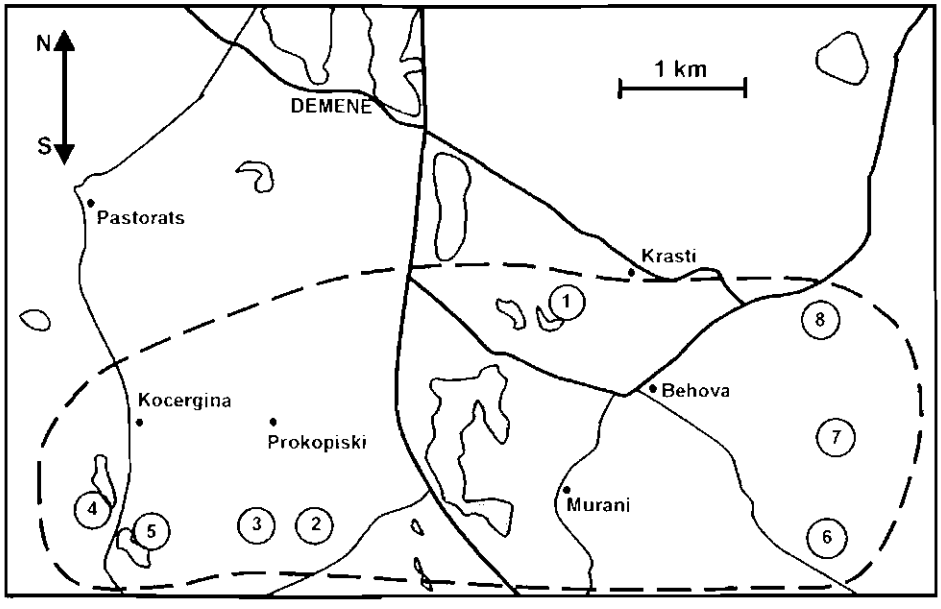

Fig. 2. Map of locations of the "Demene" *Bombina bombina* population in 2006. (---) supposed border of the population.

The reports had different reliability and were checked by the authors. In most cases, the inhabitants were mistaken, considering other species of amphibians and even insects for *Bombina bombina*.

One report of *Bombina bombina* was confirmed. On 21 June, 2006 a *Bombina bombina* population was found in the vicinity of village Demene, Daugavpils area, southeast part of Latvia (Fig. 1). The distance to the "Ilgas" population is 14 km along a straight line through the large lake Ricu (along the coast of lake – 15.31 km), distance to the population "Ainavas" is 16 km, and to the "Bauska" population 165 km. There are rivers, lakes, roads, woods, and settlements between populations.

We observed eight locations of the "Demene" *Bombina bombina* population during 2006: (1) Bebru gravis, (2) Izcirtuma pelke, (3) Mezu dikis, (4) Lauru dikis, (5) Gravu dikis, (6) Cizovkas dikis, (7) Orhideju purvs, (8) Tumsais karjers (Fig. 2). A characteristic *Bombina bombina* habitat at the location "Bebru gravis" is shown in Fig. 3.

We recorded ~70 vocalizing males in the eight locations of the population during June - August 2006 (Table 1). In most cases, the number of males was counted using the audible method, precisely to one individual, but also visually. An exception was made in estimation of male number in the location "Lauru dikis", consisting of two large ponds, and "Mezu dikis". Due to many simultaneously vocalizing males, their number was approximate (±2 animals).

Local residents living in a house 30 m from the location "Mezu dikis" reported that the sounds made by *Bombina bombina* have been heard for more than 30 years. Thus, this Latvian population of *Bombina bombina* has survived not less than 30 years.

The whole "Demene" population is located on unprotected territory, but establishment of a protected area is in process.

Table 1. Found locations and size of the *Bombina bombina* population "Demene" in 2006

Location Nr.	Name of location	Coordinates	Height above sea level (m)	Number of vocalizing males
1	Bebru gravis	N 55°42'70" E 26°34'22"	149	16
2	Izcirtuma pelke	N 55°42'18" E 26°31'51"	141	1
3	Mezu dikis	N 55°42'03" E 26°31'24"	134	~15
4	Lauru dikis	N 55°41'89" E 26°30'47"	132	~25 (~15+~10)
5	Gravu dikis	N 55°41'92" E 26°30'47"	132	5
6	Cizovkas dikis	N 55°41'69" E 26°35'85"	162	3
7	Orhideju purvs	N 55°41'85" E 26°36'54"	169	4
8	Tumsais karjers	N 55°42'46" E 26°36'11"	152	1
Total				~70

Fig. 3. One of characteristic habitats "Bebru gravis" of the *Bombina bombina* population "Demene".

Discussion

Number of vocalizing males in known populations of *Bombina bombina* in Latvia in 2006 were: in the population "Ilgas" - 9, in the population "Bauska" - 7, there are no males in 2006 in the population "Ainavas" (the only male was observed in 2004). Thus, the population of *Bombina bombina* in "Demene" with ~70 vocalizing males in 2006 is the biggest population of *Bombina bombina* on the northern border of the distribution range of a species in

Latvia. Thus, this population probably plays an important role in conservation of *Bombina bombina* in Latvia and Europe.

The distance from known locations of the *Bombina bombina* "Demene" population to populations "Ilgas" and "Ainavas" is about 15 - 16 km. There are urbanized habitats that hinder migrations of animals between these populations: roads, settlements. However, due to the hilly and cut up relief of this part of Latvia, there are plenty of thicksets, lowlands, suitable waterbodies, drainage channels, ponds and tempory pools. These habitats could be used by *Bombina bombina* in migration or territories for forming a metapopulation. Thus, contacts between the Daugavpils area *Bombina bombina* populations are likely possible or might have been possible in the past. Search for intermediate locations of Bombina bombina and genetic research is needed. The same concerns also the possible contacts to Lithuanian and Belarus populations. Also, further research of the given population is necessary to determine its sizes, new locations, habitat conditions, population genotype, possible contacts to other populations, population dynamics etc.

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Jauna *Bombina bombina* populācija "Demene" Daugavpils rajonā, Latvijā

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Kopsavilkums

Sarkanvēdera ugunskrupis *Bombina bombina* L. ir rets un aizsargājams dzīvnieks Latvijā un Eiropā. Līdz 2006. gadam bija zinamas trīs šīs sugas populācijas, divas no kurām – „Ilgas" un „Ainavas" atrodas Daugavpils rajonā, bet populācija „Bauska" – Bauskas rajonā. Jaunu *Bombina bombina* populāciju mēs atradām Demenes apkārtnē, Daugavpils rajonā, Latvijas dienvidaustrumu daļā. Konstatētas astoņas lokalizācijas ar pavisam aptuveni 70 vokalizējošiem tēviņiem. Jaunatrastā „Demene" *Bombina bombina* populācija, 2006.gadā bija vislielākā areāla ziemeļu robežās Latvijas teritorijā. Tādēļ šai populācijai ir butiska nozīme sugas saglabāšanā Latvijā un Eiropā.

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Distribution and habitats of the Sand Lizard (*Lacerta agilis*) in Latvia

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Abstract

The aim of the paper was to summarize the data on distribution and habitats of *Lacerta agilis* in Latvia. Data on distribution of *Lacerta agilis* was collected by the author and other observers in 1990 - 2006. Records were made more frequently in the Coastal Lowland, in stretches of valleys of large rivers, and in South-Eastern Latvia. Habitats were described in the field on circular plots with a radius of 1.5 m for herbs, 5 m for shrubs, and 10 m for trees using a modified Braun-Blanquet method; a total of 32 plots were established. Principal Component Analysis was used to detect natural groups of habitats. Vegetation composition in all the plots was similar, in about 75 % dominated by grasses, and in 25 % by *Calluna vulgaris*. Detailed vegetation description is given.

Key words: distribution, habitat, *Lacerta agilis*, Latvia, Principal Component Analysis, vegetation cover.

Introduction

The Sand Lizard, *Lacerta agilis*, is a medium-sized lizard with a wide distribution range, from the Pyrenean Mountains, Southern England and Southern Scandinavia to Central Asia and Mongolia (Arnold, Ovenden 2002). About ten subspecies of *Lacerta agilis* are recognized (Kalyabina-Hauf et al. 2001). Populations are large in southern and eastern parts of the range (Jablokov 1976), but north-west European populations, belonging to subspecies *L. a. agilis*, and northern populations of *L. a. chersonensis* are generally rare and considered as declining (Edgar, Bird 2006). The population living in the territory of Latvia presumably belongs to the latter subspecies found in Eastern Europe and Western Russia (Jablokov 1976; Kalyabina-Hauf et al. 2001), although study is insufficient. The species is included in the Annex IV (strictly protected species of community interest) of Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora.

Lacerta agilis is considered to be a rare species in Latvia (Andrušaitis 2003), and is included in the strictly protected species list by the Cabinet of Ministers of Latvia regulation Nr. 396/14.11.2001. In spite of this status, the information on the distribution and habitats of *Lacerta agilis* in Latvia is scanty. Arguably the best general description of the species ecology was published more than 70 years ago (Siliņš, Lamsters 1934). All of the published information is either a popular species account (Siliņš, Lamsters 1934; Sloka

1961; Lipsbergs et al. 1990; Andrušaitis 2003), based mostly on other literature sources, annotated checklists of fauna of some protected areas (Barševskis et al. 2002; Čeirāns 2002, 2003), or wider studies on reptile ecology with minor emphasis on *Lacerta agilis* (Čeirāns 2004; 2006). A species distribution map in Latvia has never been published.

The aim of the present paper was to summarize data on the distribution of *Lacerta agilis* in Latvia, and to describe and classify the species habitats, which is necessary for effective habitat management and conservation measures.

Materials and methods

Data on the distribution of *Lacerta agilis* were collected mostly by the author, but was supplemented by communications from other observers, mainly biologists and naturalists. Communications were verified by verbal species descriptions from the correspondents. Data before 1990 are not taken into account as earlier literature lacked exact site locations, and oral communications on the time-span before 1990 were few and probably do not reflect real situation any more. The species distribution map was prepared using the Latvian co-ordinate system (LKS-92) 5 × 5 km grid.

Habitat data were collected in *Lacerta agilis* locations in different regions of Latvia in 2001 - 2005. Only vegetation was described, in circular plots with a centre in the point where the specimen was first spotted. A radius of 1.5 m was used for moss layer and herbs, 5.0 m for shrubs, and 10.0 m for trees. A modified Braun-Blanquet method was used. Vegetation cover was estimated visually, and described separately for five different height classes. A total of 43 taxa and ecological groups were represented. The latter were selected arbitrarily, on the basis of literature (Pētersone, Birkmane 1980; Fitter et al. 1984; Fitter et al. 1996) and author's personal experience. Ecological groups were larger groups of plant species, not necessary taxonomically closely related, with similar habitus and ecological (such as soil and light) demands. Easily identifiable and frequent taxa (tree, undershrub, and some herb species) were treated at a species level. To reduce possible estimation error, the coverage was coded as whole number from 1 to 5 (Table 1). Number of plots in each site was limited to maximum three randomly selected plots from a single location of *Lacerta agilis* to reduce the effect of a few well-studied sites on a total microhabitat data pool. A total of 32 plots were described. Most of them were located in central (Riga district, 12 plots) and southeastern (Daugavpils district, seven plots) Latvia, although plots were made also from other regions (western, north-central). Cluster analysis did not reveal any regional clusters. The length of the first gradient (3.1) in Detrended Component Analysis (DCA) indicated linear response of the vegetation data (Van den Brink et al. 2003). Therefore, Principal Component Analysis (PCA) with inter-sample distances, with no centering or standardization, was selected to detect natural groups of the plots. All statistical analyses were conducted on CANOCO 4.5 for Windows.

Results and discussion

The Sand Lizard *Lacerta agilis* was recorded in 65 squares, or ~2.5 % of the total in Latvia (Fig. 1). Records were more common in three areas: (a) the Coastal Lowland, especially west and south-east from the Gulf of Riga; (b) parts of stretches of valleys of large rivers, particularly the Gauja and Daugava, with neighboring dry pine forest areas; (c) south-

Table 1. Vegetation characteristics in *Lacerta agilis* habitats in Latvia. Unimportant variables (small coverage on few plots) omitted. ([a]) coded as follows (except for mosses): 0 - absent; 1 - scanty (cover 1 - 5 %); 2 - rare (6 - 14 %), 3 - medium (15 - 33 %), 4 - common (34 - 67 %), 5 - abundant (>67 %); the moss layer: 0 - not developed (coverage <10 %), 1 - poor (10 - 32 %), 2 - medium (33 - 67 %), 3 - well developed (>67 %). ([b]) small, tufted grasses on infertile soils with all leaves thread-like (*Koeleria glauca, Nardus stricta, Festuca ovina* agg.). ([c]) medium-sized loosely tufted or tufted grasses on xero-mesic soils with all or some leaves thread-like (*Deschampsia flexuosa, Festuca rubra*). ([d]) mesic grasses with flat leaves (*Festuca pratensis, Poa pratensis, P. trivialis, Dactylis glomerata, Bromus arvensis* etc). ([e]) medium-size herbs with simple narrow to elliptical leaves from *Asteraceae* (*Taraxacum* spp., *Crepis* spp., *Senecio* spp., *Centaurea* spp., *Hieracium* spp. etc), *Campanulaceae* (*Jasione montana*), and *Dipsacaceae* (*Knautia arvensis*) families. ([f]) tall or climbing herbs from the *Fabaceae* family (*Viccia* spp., *Lathyrus* spp., *Astragalus* spp., *Melilotus* spp.). ([g]) small plants on bare places with succulent-like, fleshy leaves (*Sedum acre, Honckenya peploides*)

Variable	'Heath' habitats (n = 8)			'Grassy' habitats (n = 23)		
	% of plots present	Coded[a] average of coverage ± SD (maximum value)	Weighted average of coverage %	% of plots present	Coded[a] average of coverage ± SD (maximum value)	Weighted average of coverage %
Vegetation height layer						
Shrubs & trees (> 1.0 m)	75	1.9 ± 1.4 (3)	14	57	1.3 ±1.6 (5)	12
Tall herbs (> 0.50 m)	25	0.4 ± 0.7 (2)	2	61	1.1 ± 1.2 (4)	7
Medium tall herbs (0.15 - 0.50 m)	100	3.3 ± 1.6 (5)	41	91	2.9 ± 1.3 (5)	30
Low-grown herbs (< 0.15 m)	75	2.3 ± 1.8 (5)	25	43	1.3 ± 1.5 (4)	12
Moss layer	100	1.8 ± 0.7 (3)	44	43	0.7 ± 0.1 (3)	20
Wooded vegetation						
Pinus sylvestris	63	1.4 ± 1.3 (3)	9	57	0.9 ± 1.0 (3)	5
Deciduous trees	37	0.9 ± 1.2 (3)	6	57	1.1 ± 1.4 (5)	9
Grasses						
Xeric grasses[b]	50	1.3 ± 1.6 (4)	11	13	0.2 ± 0.6 (2)	1
Meso-xeric grasses[c]	37	0.8 ± 1.2 (3)	5	22	0.6 ± 1.1 (3)	4
Mesic grasses[d]	13	0.4 ± 1.1 (3)	3	52	1.3 ± 1.4 (4)	10
Calamagrostis spp.	0	0	0	52	1.2 ± 1.3 (4)	9
Undershrubs						
Calluna vulgaris	100	3.1 ± 1.2 (5)	34	13	0.4 ± 1.2 (4)	4
Vaccinium vitis-idaea	37	1.0 ± 1.6 (4)	10	4	0.2 ± 0.8 (4)	2
Other herbs						
Narrow-leaved herbs[e]	0	0	0	43	0.7 ± 1.1 (3)	4
Fabaceae (tall)[f]	0	0	0	30	0.3 ± 0.5 (1)	1
Artemisia spp.	13	0.1 ± 0.4 (1)	1	57	0.8 ± 0.8 (3)	3
Onagraceae	0	0	0	22	0.3 ± 0.7 (3)	2
Galium spp.	0	0	0	30	0.5 ± 0.9 (3)	3
Small 'succulents'[g]	0	0	0	26	0.4 ± 0.6 (2)	1
Moss layer						
Lichens on ground	67	2.4 ± 1.8 (5)	25	4	0.1 ± 0.4 (2)	1

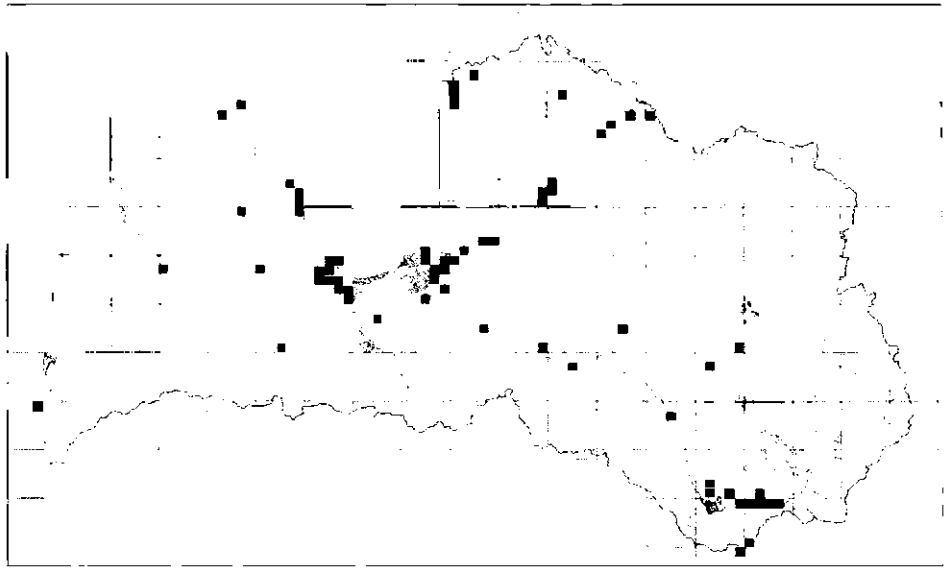

Fig. 1. Distribution of *Lacerta agilis* in Latvia in 1990 - 2006; black are 5 × 5 km squares in LKS-92 co-ordinate system with one or more *Lacerta agilis* records.

eastern part of Latvia. The above distribution can partly be explained by better investigation of some of these areas, particularly the vicinity of Riga.

An uneven distribution pattern, however, is confirmed by a vast field survey conducted in 1999 - 2003 (Čeirāns 2006). *Lacerta agilis* is probably naturally absent in uplands of northern and eastern Latvia due to unfavorable climatic conditions. All the known records are below 100 - 125 m a.s.l., excepting in south-eastern Latvia, where the species is found at elevations about 160 m a.s.l. The latter region also has the most suitable climate for *Lacerta agilis* in Latvia due to particularly high summer temperatures (Čeirāns 2006).

Lacerta agilis was not recorded in south-central Latvia (Zemgale Lowland), an area of intensive agriculture on rich soils that in the past supported mainly broad-leaved forests. Both habitats are not characteristic for *Lacerta agilis* in Latvia. However, the species is recorded in the neighboring Lithuanian part of the Zemgale Lowland (Trakimas 2005), and records in south-central Latvia are still possible.

In Central Sweden, at about the same latitudes as Latvia, species records are confined to open pine (*Pinus sylvestris*) forest areas on sandy glacio-fluvial sediments (Berglind 2005). The present data indicate similar habitat preference in Latvia, as: (a) the species was recorded mainly in an area of various plain habitats on sandy soils (see a map of Latvian landscape types published by Kavacs 1998); (b) disturbed and altered dry pine forests are also the most important among *Lacerta agilis* habitats in Latvia (Čeirāns 2004, 2006), and in neighboring countries – Lithuania (Gruodis 1987) and Belarus (Pikulik et al 1988).

The expected presence of *Lacerta agilis* populations in Latvia is expected to be 10 - 15 % of the 5 × 5 km squares (Čeirāns 2006). Hence, the present distribution of the species is not sufficiently documented, and many new records, especially in lowland areas of western Latvia characterized by dry pine forests on predominantly sandy soils, are expected.

A classification of *Lacerta agilis* sites based on vegetation characteristics was proble-

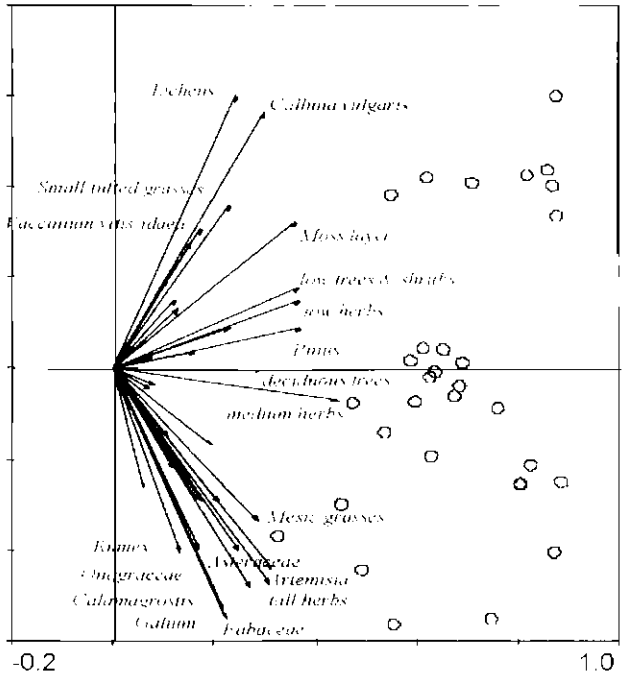

Fig. 2. Principal Component Analysis (PCA) ordination of vegetation plots in *Lacerta agilis* sites; for vegetation explanations see Table 1.

matic, because plots differed in vegetation cover rather than in taxonomic composition. The PCA first two axes explained 58 % of variation. The first axis (eigenvalue 0.48) could be interpreted as a vegetation cover gradient, and the second (eigenvalue 0.10) – as a vegetation composition gradient. All plots fell into two groups (Fig. 2), and only in respect to the second reflected a composition gradient. Vegetation in both groups was rather similar (Table 1), and both could be separated by relative importance of heath (*Calluna vulgaris*) and grass vegetation. In about 75 % of plots vegetation was dominated by sparse swords of grasses (*Calamagrostis*, *Poa*, *Festuca* etc.), with presence of other herbaceous vegetation and some low shrubs. Plots were located on different soils, some even on peat, where *Lacerta agilis* penetrated edges of drained bogs from neighboring dry habitats. In about 25 % of plots vegetation was more closed, dominated by heath (*Calluna vulgaris*) and small, tufted grasses typical for relatively xeric sites (*Festuca ovina* agg., *Koeleria glauca*, *Nardus stricta*) on sand. Trees were sparse, low, and dominated by *Pinus sylvestris*, *Betula* spp. in both cases. *Lacerta agilis* habitats with heath are typical for Northern Europe, indicating a specific, dry environment with mosaic vegetation pattern (Dent, Spellerberg 1987; Berglind 2005). However, sites, dominated by grasses, may be more important (Stumpel 1988), confirmed also by the present study.

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Sila ķirzakas (*Lacerta agilis*) izplatība un dzīvesvietas Latvijā

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Kopsavilkums

Dotā pētījuma mērķis bija apkopot informāciju par sila ķirzakas (*Lacerta agilis*) izplatību un biotopiem Latvijā. Ziņas par sugas izplatību ievāca raksta autors no 1990. līdz 2005. gadam, izmantoti arī ticami citu novērotāju ziņojumi par sugas izplatību dotajā laika periodā. Suga biežāk konstatēta Piejūras zemienē, atsevišķās lielo upju ieleju daļās, kā arī Latvijas dienvidaustrumu daļā. Biotopi (veģetācija) aprakstīti apaļveida parauglaukumos, izmantojot modificētu Brauna-Blanķē metodi. Parauglaukumu skaits – 32, to rādiuss 1,5 m lakstaugu stāvam, 5 m – krūmu un 10 m – koku stāvam. Lai identificētu parauglaukumu grupas biotopu klasifikācijai, izmantota galveno komponenšu analīze. Veģetācija parauglaukumos bija samērā līdzīga, ap 75 % parauglaukumu dominēja graudzāles, 25 % – virši (*Calluna vulgaris*). Rakstā sniegts arī detalizēts veģetācijas apraksts.

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Zooplankton indicators of trophy in Latvian lakes

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Abstract

Studies on the relationship between zooplankton taxonomic composition and environment characteristics are an important issue in the evaluation of the ecological quality of lakes. Data were collected in 1998 - 2004 in 113 lakes of different types. Zooplankton samples were taken simultaneously with the hydrochemical and phytoplankton samples, and field measurement of physical parameters. Redundancy Analysis was used to ordinate zooplankton abundance data against environmental variables, and General Linear Models and XY(Z) plots were used to test and visualize these relationships. A number of taxa increased in abundance with eutrophication, while results on using of zooplankton organisms as low trophy indicators were poor. However, an indirect adverse effect of increased nutrient concentrations through algal blooms was suggested.

Key words: high-trophy indicators, lake trophy, Latvia, low-trophy indicators, Redundancy Analysis, zooplankton.

Introduction

Zooplankton is an important component of lake ecosystems, the composition and abundance of which is related to many factors, such as water hydrochemistry, season, lake morphology, presence of macrophytes, predators etc., and, particularly, to the productivity of the lake – i.e. lake trophic condition (Wetzel 2001). The latter makes it very suitable and popular for monitoring programs devoted to record changes in the lake's ecological quality. Three groups of zooplankton organisms are usually used: rotifers, cladoceran crustaceans, and copepod crustaceans.

Various chemical and biological data on the ecological quality of the lakes have been collected in the period 1998 - 2004 by the Latvian Environment, Geology and Meteorology Agency during several state governed projects. These data included also zooplankton, covered a wide variety of lakes, but also had some shortages. Analyzes were made by different specialists with various levels of knowledge and experience, with taxa identified to various taxonomical levels (genus, species, subspecies, forms), and some possible identification errors. Another problem was the presence of taxonomically uncertain forms. Thus, *Daphnia* and bosminid cladocerans are taxonomically very complicated groups with many morphs, races with intermediate forms, and introgressive processes (Nilssen, Larsson 1980; Leider 1986; Taylor at al. 1996; Taylor et al. 2002). There are differences in identification even among different scientist groups - e.g. between the West European and Russian 'schools'. A third problem is finding the optimal taxonomical level for data used

for interpretation, since different closely related forms may have the same response to lake trophy, and their presence in one or an other lake may be due to other, not trophic factors, or simply by chance (Wetzel 2001).

Taking into account the above, the present study was devoted to identify easily-recognizable, more or less common taxa, not necessarily at the species level, with marked response to the lake trophy. Since zooplankton may respond not only to trophy, but also to many other parameters, including lake morphology or physical-chemistry, the latter were also taken into account.

Materials and methods

Sampling design

Sampling was carried out during summer stratification period from 2nd decade of July to the 3rd decade of August. Lakes covered all types available in Latvia, and were located in the whole territory of Latvia. Usually only one sample per lake was taken. In some cases, a lake was sampled several times in different years, or in different parts if they were well-separated by shore-line configuration and banks. A total of 113 lakes were surveyed, 144 samples processed. Zooplankton, phytoplankton and water chemistry samples were taken simultaneously, from the deepest part of a the lake, and from the epilimnion 0.5 m horizon, in mid-day.

Environmental variables

Relatively few variables with the potentially highest response of the zooplankton community were selected for the analysis. Lake morphology variables included area and average depth, and physical-chemistry variables were a temperature, pH, conductivity, and colour. The total phosphorus concentration (mg l^{-1}) was chosen as the most important productivity-limiting (trophy) factor in inland waters (Wetzel 2001). The total phytoplankton biomass (mg l^{-1}) and the percentage of cyanophyta were both also selected as trophy-related factors, having significant impact on zooplankton communities as food items (Wetzel 2001). These variables were used as predictors in zooplankton taxa ordination, and in subsequent taxa – variable relationship analysis.

Data on area and average depth of the lake were acquired from the on-line data base

Table 1. Environmental characteristics of surveyed sites (n = 144)

Variable	Range	Average ± SD	Median
Area (ha)	0.5 - 5765	366 ± 936	49.9
Average depth (m)	0.3 - 16.5	4.7 ± 3.5	3.5
Temperature (°C)	14.6 - 27.9	21.9 ± 2.9	22.2
pH	4.1 - 9.4	7.7 ± 1.2	8.03
Conductivity (μS cm^{-1})	9 - 793	232.5 ± 140.4	244
Colour (mg Pt^{6} l^{-1})	5 - 610	88.2 ± 108.4	50
P total (mg l^{-1})	0.003 - 0.180	0.034 ± 0.030	0.022
Phytoplankton biomass (mg l^{-1})	0.010 - 122.14	4.1 ± 13.2	0.762
Proportion of *Cyanophyta* (%)	0 - 99.8	29.81 ± 31.55	17.6

(http://www.ezeri.lv). Most of the physical-chemistry parameters – temperature, pH (both + 0.1), conductivity (μS cm^{-1}) were measured on site with a multi-parameter field meter (WTW, Germany). Total phosphorus (mg l^{-1}) and water colour (mg Pt° l^{-1}) analysis was carried out with standardized methods in a laboratory accredited by the Latvian National Accreditation Bureau. Water samples for chemistry and phytoplankton were taken with a bathometer. Phytoplankton samples were preserved with the addition of the Lugol solution, then concentrated and cells were counted in a counting chamber with inverse light microscopy; biomass was calculated from the cell biovolume (Keskitalo, Salonen 1994). Statistical summary of the environmental variables is given in Table 1.

Zooplankton

Sampling was made by filtering of 100 l of water through a plankton net with mesh size of 65 μm. Samples were preserved by addition of 2 - 4 % solution of formaldehyde. During

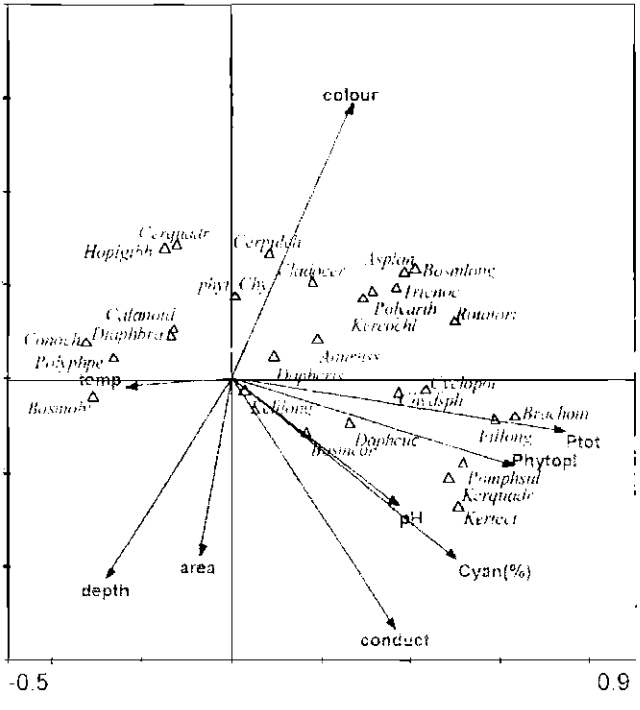

Fig. 1. RDA ordination plot for zooplankton taxa and environmental variables. Taxa in RDA plot indicated with triangles instead of arrows. Anurfiss – *Anuraeopsis fissa*, Asplan – *Asplanchna*, Bosmcor – 'humped' *Eubosmina*, Bosmlong – *Bosmina longirostris*, Bosmobt – non-humped *Eubosmina*, Brachoin – *Brachionus*, Calanoid – calanoid copepods, Cerpulch – *Ceriodaphia pulchella*, Cerquadr – *Ceriodaphnia* cf. *quadrangula*, Chysph – *Chydorus sphaericus*, Cladocer – cladocerans, Conoch – *Conochilus*, Cyclopoi – cyclopoid copepods, Dapheris – *Daphnia* cf. *cristata*, Daphcuc – *Daphnia* cf. *cucullata*, Diaphbra – *Diaphanosoma brucyurum*, Fillong – *Filinia longiseta*, Hoplgibb – *Holopedium gibberum*, Kellong – *Kellicottia longispina*, Kercochl – *Keratella cochlearis* typical forms, Kertect – *Keratella cochlearis* cf. *tecta*, Kerquadr – *Keratella quadrata*, phyt_Chy – phytophilous *Chydoriidae*, Polyarthr – *Polyarthra*, Polyphpe – *Polyphemus pediculus*, Pomphsul – *Pompholyx sulcata*, Rotatori – rotifers, Trichoc – *Trichocerca*.

the counting process, they were sub-sampled and counted twice, and the average was used for calculating the organism density expressed as thousands of per m^3.

Forms similar to *Keratella cochlearis* all were pooled into two groups – (a) typical (including *K. irregularis*, *K. c. hispida*) with a rear spine, and (b) cf. *tecta* (including *K. serrulata*) without a rear spare. *Ceriodaphnia* were treated as: *pulchella* – with a clump of hairs proximally from a row of small teeth on postabdomen, and cf. *quadrangula* – taxa without this feature. All bosminid species and forms were pooled into three groups: (a) *Bosmina longirostris* (subgenus *Bosmina*); (b) non-humped forms of the genus or subgenus *Eubosmina* (*B. obtusirostris*, *B. longispina*, *B. crassicornis*, *B. coregoni* sensu stricto etc.); (c) humped forms of *Eubosmina* (*B. gibbera*, *B. thersites*, *B. lilljeborgii*). *Daphnia* were treated as: cf. *cristata* with 1 + 1 hairs on branches of the second antennae, and cf. *cucullata* – with 2 + 1 hairs on the second antennae.

Other rotifer and cladoceran taxa were analyzed at species or genus level, rare taxa present in few samples were not treated separately. Phytophilous cladocerans of the *Chydoriidae* family (*Acroperus*, *Alona*, *Camptocercus*, *Graptoleberis* etc.) were not rare, but their composition even at the genus level strongly varied from sample to sample, therefore they all were pooled together. Calanoid and cyclopoid copepods were recognized at suborder level. Total densities of all rotifers and all cladocerans (including rare, not separately treated taxa) were also used.

Statistics

Detrended Correspondence Analysis (DCA) was performed to test linear versus unimodal response of the data. The gradient length of the first axis was 2.4, suggesting a linear response. Therefore, Redundancy Analysis (RDA) was chosen as the method for ordination of absolute abundance data against environmental variables (Van den Brink et al 2003). RDA was performed using inter-species correlations, dividing by the standard deviation, and log transformation of the data. The Monte Carlo permutation test was performed under 999 permutations. General Linear Models (GLM) and XY(Z) plots were used to test and visualize taxa relationships with selected environmental variables. All statistics were performed on CANOCO for Windows 4.5.

Results

Eigenvalues of the 1st and 2nd RDA axis were 0.156 and 0.027 respectively, and the cumulative percentage variation of first two axes was 73 %, sum of all canonical eigenvalues 0.252; and consequently, percentage of explained variation was relatively low – 25 %. The Monte Carlo test in all cases was < 0.05. Conditional effect of environmental variables in all cases was significant at $p < 0.05$. The first axis in the ordination can be explained mostly by a trophy gradient, the second by physical-chemistry and lake morphology (Fig. 1). Among environmental variables, the two closest to trophy axis were total phosphorus and phytoplankton biomass, to physical-chemistry axis – colour and conductivity. Area and average depth well characterized lake morphology. These variables were used as pairs of predictors in GLM analysis to verify the response of the taxa to trophy, physical-chemistry, and lake morphology (Table 2). XY(Z) plots with symbol coding were created to visualize all the data for finding threshold values not revealed by GLM.

A group of taxa at the eutrophic end of the ordination had a positive correlation with

Table 2. Statistically significant [(*) $p < 0.05$; (**) $p < 0.01$; (***), $p < 0.001$] regression coefficients (T) for GLM for taxon and environmental variable relationships

Taxon	Trophy		Physical-chemistry		Morphology	
	Ptot	Phytoplankton	Colour	Conductivity	Area	Depth
Anuraeopsis fissa	ns	ns	2.43*	-0.96*	ns	ns
Asplanchna	4.13***	5.75***	2.63*	0.21*	ns	ns
Brachionus	-1.74***	13.13***	ns	ns	ns	ns
Conochilus	ns	ns	ns	ns	ns	ns
Filinia longiseta	1.39***	4.51***	ns	ns	ns	ns
Kellicottia longispina	ns	ns	ns	ns	ns	ns
Keratella cochlearis typical forms	1.64**	2.07**	4.40***	1.62***	ns	ns
Keratella cochlearis cf. *tecta*	2.21**	1.53**	ns	ns	5.10***	-1.92***
Keratella quadrata	3.15**	-0.35**	0.72*	2.67*	ns	ns
Polyarthra	4.32***	-0.90***	2.58*	2.08*	ns	ns
Pompholyx sulcata	4.99***	3.71***	0.95***	4.08***	ns	ns
Trichocerca	ns	ns	ns	ns	ns	ns
Bosmina longirostris	2.97*	-0.97*	3.29**	0.33**	ns	ns
non-humped *Eubosmina*	ns	ns	ns	ns	ns	ns
'humped' *Eubosmina*	3.51**	-1.11**	ns	ns	ns	ns
Chydorus sphaericus	ns	ns	ns	ns	ns	ns
Phytophilous *Chydoridae*	ns	ns	ns	ns	ns	ns
Ceriodaphnia pulchella	ns	ns	ns	ns	-1.22*	-2.16*
Ceriodaphnia cf. *quadrangula*	ns	ns	1.51***	-3.59***	ns	ns
Daphnia cf. *cristata*	3.06**	-1.69**	ns	ns	ns	ns
Daphnia cf. *cucullata*	ns	ns	0.98***	4.09***	ns	ns
Diaphanosoma brachyurum	ns	ns	ns	ns	ns	ns
Holopedium gibberum	ns	ns	4.68***	-2.88***	ns	ns
Polyphemus pediculus	ns	ns	ns	ns	ns	ns
rotifers	6.48***	8.04***	2.55***	3.56***	ns	ns
cladocerans	2.48*	-0.83*	ns	ns	-1.54*	-1.80*
calanoid copepods	ns	ns	ns	ns	ns	ns
cyclopoid copepods	7.11***	-0.45***	3.96***	3.67***	ns	ns

the trophy variables. Some of them had better positive correlation with total phosphorus (*Keratella cochearis* typical forms, *Keratella cochlearis* cf. *tecta*, *Keratella quadrata*, *Polyarthra*, *Bosmina longirostris*, cyclopoid copepods), some with phytoplankton biomass (*Brachionus*, *Filinia longiseta*), and others good with both (*Asplanchna*, *Pompholyx sulcata*, a total of rotifers). Some of the taxa located more in the middle position in ordination (humped *Eubosmina*, *Daphnia* cf. *cristata*, cladocerans in total), also had positive correlations with total phosphorus, but were probably strongly hindered by high algae biomass (Table 2). XY(Z) plots did not reveal any threshold values below which the eutrophic taxa would

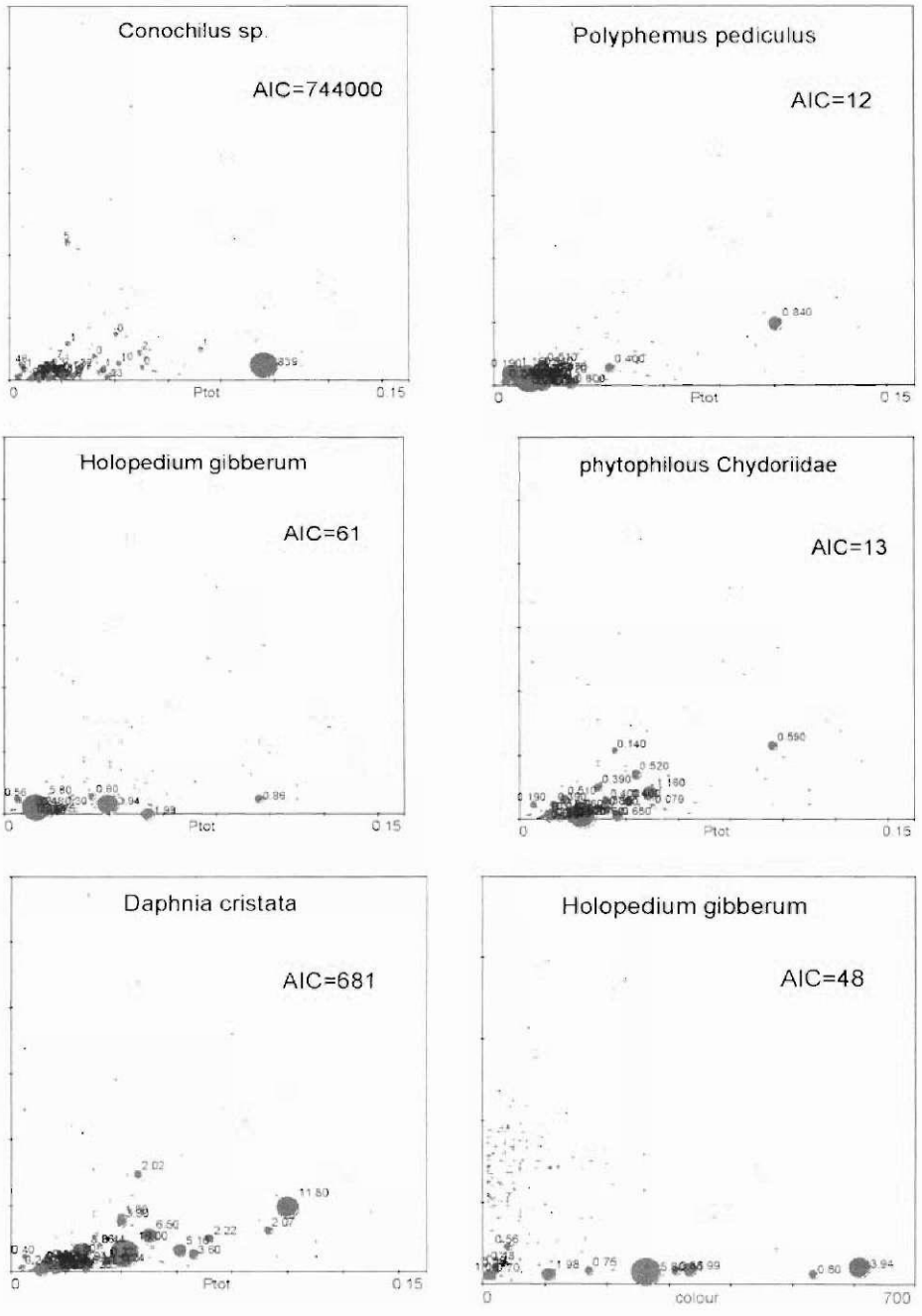

Fig. 2. XY(Z) plots for taxa densities (Z) with significant environment – determined thresholds; abbreviations on axes: Phytopl – phytoplankton, Ptot – total phosphorus concentrations (mg l^{-1}), conduct – conductivity (μS cm^{-1}), colour – colour (mg Pt° l^{-1}); AIC – Akaike information criterion; size of the dot corresponds to the density of taxa in sample, crosses indicate samples where the taxon was absent.

be absent, and all of them were present in small numbers at least in some samples with low P total concentrations (< 0.025 mg l^{-1}) and algae biomass (< 2 mg l^{-1}). Species at the oligotrophic end of the gradient did not show significant negative correlation with the trophy variables. However, among them several taxa (*Conochilus*, *Holopedium gibberum*, *Polyphemus pediculus*), as well as some others of 'indifferent' (phytophilous *Chydoriidae*, *Daphnia* cf. *cristata*) taxa had marked threshold values for phytoplankton biomass, above which they were absent (Fig. 2).

Several taxa were more abundant in a lake type in respect to their physical-chemistry. Colour and conductivity had opposite vectors in ordination, therefore a positive correlation with one and negative with the other should be interpreted with caution. One taxa (*Holopedium gibberum*) had a marked threshold for conductivity, it was found only in soft-water lakes (< 100 µS cm^{-1}), and XY(Z) plots revealed that its 'preference' for brown and soft water lakes (e.g. acid bog lakes) indicated by GLM was coincidental.

Relationships with lake morphology were least significant. Although some taxa (*Ceriodaphnia pulchella*, a total of cladocerans) were somewhere more abundant in smaller lakes, only one (*Keratella cochlearis* cf. *tecta*) had a significant preference for lakes with larger size and smaller average depth.

Discussion

A number of taxa had increased in abundance with eutrophication. Rotifers in general are better indicators than crustaceans, as they are less affected by the summer algae blooms, which in the present study were associated to mass development of blue-green algae. Some taxa (*Brachionus*, *Filinia longiseta*) probably even have an advantage under such conditions. Presumably, they are less or not susceptible to toxic influence of blue-green algae and consume mostly bacteria (Kirk, Gilbert 1992; Agasild, Noges 2005). Presence in the group of high-trophy indicators of taxa such as *Keratella cochlearis* cf. *tecta*, *Keratella quadrata*, *Brachionus*, *Filinia longiseta*, *Pompholyx sulcata*, and total rotifer abundance, was expected from studies in other countries (Hakkari 1972; Maemets 1983; Karabin 1983, 1985; Bērziņš, Pejler 1989). The presence of some other taxa is not so unequivocal. The typical form of *Keratella cochlearis* is usually not regarded as a high-trophy indicator, and various *Polyarthra* species are indicated as preferring different trophy levels (Hakkari 1972; Maemets 1983; Karabin 1985; Bērziņš, Pejler 1989). In the present study, they usually formed the bulk of the rotifer individuals in the samples. The density increased with increasing productivity of the ecosystem. However, they did not dominate in samples from highly eutrophic lakes with clear water colour. One species of *Asplanchna* (i.e. *A. herricki*) is indicated as more characteristic for low trophy conditions (Hakkari 1972; Maemets 1983; Bērziņš, Pejler 1989). However, it was rare in the present study. *Asplanchna* are raptors, and their densities should be higher in more productive ecosystems. Notable was that all of these 'high-trophy' indicator taxa can be present at small numbers in low-trophy conditions as well.

In crustaceans, it is likely that even high trophy indicators are adversely affected by the summer phytoplankton blooms. Cyclopoid copepods were more abundant in brown-water lakes with low transparency and lower algae biomass. Among cladocerans, the relationship with phytoplankton biomass was negative even in some taxa more characteristic of higher trophy conditions – *Bosmina longirostris* and 'humped' *Eubosmina*.

There are probably no good low trophy indicators among the zooplankton taxa – no taxa located in the low trophy area of the ordination had significant negative correlations with trophy variables. High levels of total phosphorus lacked direct adverse impact on any taxa (Table 2; with the exception of probably an occasional correlation with *Brachionus*). However, there was an indirect effect through the algal blooms. Thus, rotifer *Conochilus*, cladocerans *Polyphemus pediculus*, *Holopedium gibberum* were absent above threshold values of 5 - 6 (former two, except the one case for *Conochilus*) to 2 (the latter) mg of algae per liter. Phytophilous cladocerans disappeared also at summer phytoplankton biomass above 6 mg l^{-1}, which was probably associated with a decline of macrophyte vegetation due to the shift from clear-water to turbid state of lake (Wetzel 2001). Large algae-feeding cladocerans of the *Daphnia* genus can be susceptible to the domination of toxic or filamentous blue-green algae (Ghadouani et al 1998; Kurmayer 2001). The present survey indicates possible differences in the susceptibility among taxa, as *Daphnia* cf. *cristata* was absent at algae biomass above 8 mg l^{-1}, but *Daphnia* cf. *cucullata* had relatively high densities even at about 30 mg l^{-1}. In Nordic countries, *Daphnia cristata* is regarded as an oligotrophy indicator versus eutrophy indicator *Daphnia cucullata* (Bērziņš, Bertilsson 1989; Lyche 1990). However, in the present study such a pattern was not observed for samples with an algal biomass below 8 mg l^{-1}.

The remaining surveyed taxa, although some of them occasionally are indicated as low (*Kellicottia longispina*) or high trophy (*Anuraeopsis fissa*, *Chydorus sphaericus*) indicators, should be regarded as 'indifferent' taxa with a wide spectrum of ecological tolerance, sometimes rare or absent at extreme ends of the trophy gradient.

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Zooplankons kā trofiskā stāvokļa indikators Latvijas ezeros

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Kopsavilkums

Pētījumi, kuros zooplanktona taksonomiskais sastāvs tiek saistīts ar vides parametriem, ir būtiski, novērtējot ezeru ekoloģisko kvalitāti. Dati ievākti no 1998. līdz 2004. gadam 113 dažāda tipa ezeros. Zooplanktona paraugi ņemti vienlaicīgi ar ūdens ķīmijas un fitoplanktona paraugiem, paraugu ņemšanas vietā veikti ūdens fizikāli-ķīmisko parametru mērījumi. Zooplanktona taksonu izvietošanai (ordinācijai) attiecībā pret vides rādītājiem izmantota dublēšanas analīze, bet sakarību pārbaudē un vizualizēšanā lietota vispārīgā lineārā analīze un XY(Z) diagrammas. Vairāku taksonu blīvums pieauga ar ezera eitrofikācijas rādītāju pieaugumu, savukārt, saistība ar zemas trofijas rādītājiem nebija pārliecinoša. Domājams, ka barības vielu blīvuma pieaugumam ezera ir netiešs negatīvs efekts, izraisot aļģu masveida ziedēšanas parādību.

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Estimated population dynamics of the Corncrake *Crex crex* in Latvia and Europe in the 20th century by ringing data analysis

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Abstract

We collected data on 2916 Corncrakes ringed in 17 Bird Ringing Schemes in Europe 1908 - 1995 and analyzed the data (n = 643) of former Czechoslovakia, Croatia, Estonia, Hungary, Latvia, Lithuania, Norway, Poland and Switzerland to obtain quantitative estimate (TRIM index) of the Corncrake population decline in Europe during the last century. Our analyses is based on the assumption that Corncrakes were captured randomly: bird ringers used every opportunity to ring any bird, encountered, but no specific search for Corncrakes were made. We also analysed the ringing data collected in Latvia in detail to suggest on the behaviour of the ringers in order to test our assumption. We also analysed data on six passerine farmland bird species in Latvia to evaluate if bird ringers were actively ringing farmland birds later in our period of data analyses, when Corncrake ringing has declined. During the period of 1925 - 1995 there were 215 Corncrakes ringed in Latvia: 190 pulli and 25 adults. A decline of ringed pulli was observed in raw data as well as data adjusted to total number of birds ringed in Latvia in the respective year ($r_s = -0.46$; $p < 0.001$) and data adjusted to active bird ringers in Latvia in the respective year ($r_s = -0.43$; $p < 0.001$). A similar decline of ringed Corncrakes (n = 101) is observed also in data of the former Czechoslovakian ringing scheme in Prague. Index values of combined data for all ringed Corncrakes in Europe show significant decrease during the study period ($r^2 = 0.26$; $p < 0.00001$). If we remove Latvia from data pool, since it contributes roughly one third of the data (n = 215) and therefore drives the index, the observed pattern remains unchanged ($r^2 = 0.11$; $p < 0.01$). Correlation between index of ringed Corncrakes in Europe except Latvia (former Czechoslovakia, Croatia, Estonia, Hungary, Lithuania, Norway, Poland and Switzerland) and number of ringed Corncrakes in Latvia is statistically significant ($r^2 = 0.12$; $p < 0.01$). Similarly to Corncrake, the number of ringed Skylarks ($r_s = -0.30$; $p < 0.001$) and Whinchats ($r_s = -0.23$ $p < 0.05$) have also decreased significantly in Latvia during the period of 1925 - 1995, while number of ringed Meadow Pipits ($r_s = 0.57$), Common Whitethroats ($r_s = 0.68$) and Linnets ($r_s = 0.49$) increased significantly ($p < 0.001$). The only analysed species showing no significant trend was Yellow Wagtail. Qualitatively there is no doubt that ringing data verify the decline of the Corncrake population both in Latvia and in Europe. The average index of ringed Corncrakes of all European countries shows a decrease of about 5.5 times comparing 1925 - 1930 versus 1981 - 1990. Approximation by calculating decrease of available habitat shows a smaller decrease in Latvia by 1.5 - 3 times, but recent population density data for these calculations have been used, which might have decreased as well.

Key words: Corncrake, *Crex crex*, population dynamics, ringing data, TRIM index.

Introduction

Corncrake *Crex crex* is included in the IUCN Red List of Threatened Animals (Hilton-Taylor 2000), because intensive agriculture methods have caused declines in numbers in most of its populations, as well as range contractions in Western Europe (Green et al. 1997). Until the 1990s, when regular and organized annual Corncrake monitoring began (e.g. in Latvia: Keišs 2004; 2005), most of the population dynamic data were obtained only by questionnaires (see overview by Green et al. 1997), "bird of the year" actions (e.g. in Sweden: Enemar 1957; 1969) or breeding bird atlas data (e.g. in Latvia 1980 - 1984: Priednieks et al. 1989). Additionally, most of the data before 1990 come from the surveys with inconsistant area of the sample plots and insonsistant methods over the sites and years. Therefore, there is hardly any reliable estimate of the Corncrake population in any country before 1990, except for those countries with small populations (e.g. United Kingdom: Hudson et al. 1990). Without reliable survey and estimate data it is difficult to analyse the decline quantitatively.

Since the 1990s the decline of the Corncrake populations in Europe has reversed to an increase in numbers (Schäffer, Koffijberg 2004), largely due to changes in agriculture in Eastern Europe (e.g. in Latvia: Keišs 2005). To estimate how this increase compares with historical declines, it is important to understand the quantity of the decrease since its beginning in the 19th century. Since there have been no systematic surveys of the Corncrake in Europe until recent times, the aim of the present study was to evaluate the potential use of data on ringed Corncrakes in Europe (in all existing Bird Ringing Schemes) as a quantitative estimate of the population dynamics of this species in Europe.

Materials and methods

This study is based on the assumption that Corncrakes were captured randomly: bird ringers used every opportunity to ring any bird encountered, but no specific search for Corncrakes was made. Thus, the probability of encountering a Corncrake is higher when there are more Corncrakes present. Therefore, when it was known that this assumption was false (e.g. specific studies involving Corncrake ringing were carried out), ringing data from the respective country were excluded from analysis (see further).

Birds ringed in Latvia

We searched the archives of Latvian Bird ringing center since its foundation in 1925 to identify all ringed Corncrakes, and their age and sex if recorded. A database of all ringed Corncrakes in Latvia was created. We analysed numbers of Corncrakes ringed until 1995, when we started to capture Corncrakes systematically (Keišs et. al. 2004).

To evaluate if the behaviour of the bird ringers changed over time and if ringing data corresponds to population dynamics, we chose seven passerine bird species that inhabit similar habitats as Corncrake: Linnet *Acanthis cannabina*, Meadow Pipit *Anthus pratensis*, Skylark *Alauda arvensis*, Whinchat *Saxicola rubetra*, Whitethroat *Sylvia communis*, Yellow Wagtail *Motacilla flava* and Yellowhammer *Emberiza citrinella*. The assumption here is that, if a ringer was in an open landscape ringing these species, he/she might have a chance

to encounter Corncrake (Corncrake pulli to be more precise) and ring it as well. We used published reports on ringed birds by the Latvian ringing center (von Transehe 1939; 1940; Vilks, Pētersons 1943; Spuris 1962; Kazubiernis 1989) to access the numbers of ringed birds of the seven passerine bird species.

Corncrakes ringed in Europe

We collected data on numbers of ringed Corncrakes from published bird ringing reports. Additionally we used unpublished summaries, kindly provided by the Czech Bird Ringing scheme in Prague (J. Cepak, personal communication), by the Lithuanian Bird Ringing Scheme in Kaunas (R. Patapavičius, personal communication) and by the Swiss Bird Ringing scheme in Sempach (E. Wiprächtiger, personal communication). The Finish Bird Ringing Scheme kindly provided a database with ringed Corncrakes (J. Valkama, personal communication). In total we obtained data on ringed Corncrakes of 17 Bird Ringing Schemes in Europe (Table 1).

Data of several countries include birds ringed in special studies conducted on Corncrakes. Data on numbers of Corncrakes ringed in these countries were not used in the further analysis of the population trends. These countries were Finland (J. Valkama, personal communication), Germany (Prünte 1972), Sweden (Alnås 1974), The Netherlands (van den Bergh 1991) and United Kingdom (Mason 1941; 1947; 1950; Fox 1993). In the studies in Finland and The Netherlands special studies were aimed at capturing only adult birds, therefore we assumed that pulli were still ringed as random events and used number of ringed pulli in the analyses. We also excluded Russia from the analyses, since little data were available and those data available were unreliable.

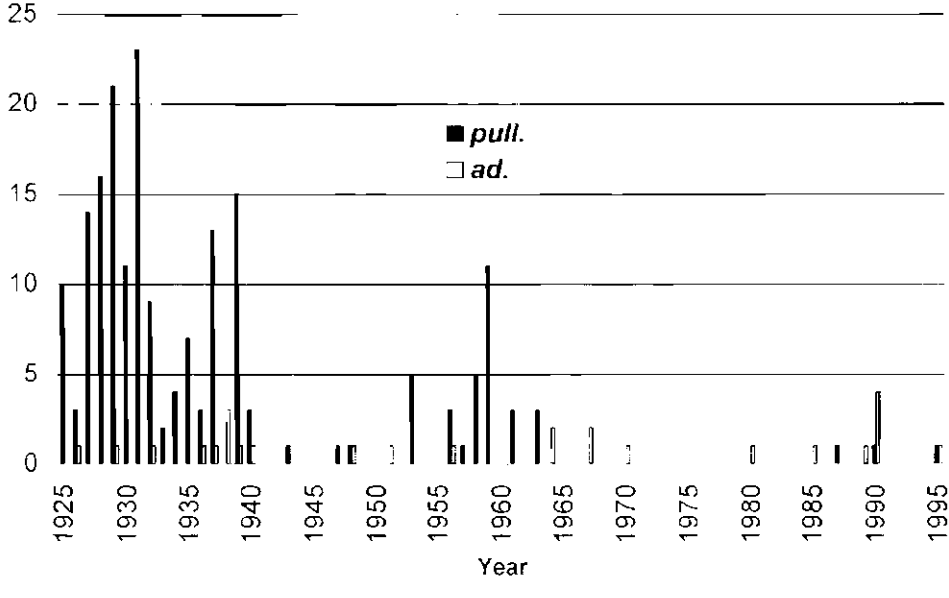

Fig. 1. Number of ringed Corncrakes in Latvia (1925 - 1995; n = 215).

Table 1. Data sources on ringed Corncrakes *Crex crex* in Europe

Ringing Center	Period	Data sources
Czechoslovakia	1934 - 2004	J. Cepak pers. com.; Formánek 1970, 1971/72; Formánek, Škopek 1990, 1991, 1993; Jirsík 1936; Jirsík, Kadlec 1937, 1938; Kadlec 1939, 1940, 1947/48, 1951, 1958, 1959; Kadlec, Bašová 1965; Kadlec, Klůz 1941; Škopek, Formánek 1995
Croatia	1947 - 2000	Čikovič, Kralj 2002; Čiković, Radovič 1999; Igalffy 1950; Kletečki 1988; Kletečki, Sirotič 1976, 1978, 1980, 1982; Kroneisl 1948, 1952; Kroneisl Rucner 1954, 1956, 1957, 1959, 1960; Radovič 1990, 1991; Radovič et al. 1993; Radovič, Sušič 1989; Rucner-Kroneisl 1962; Sirotič, Kletečki 1975, 1977, 1979; Štromar 1963, 1965, 1967, 1968, 1970, 1971, 1972, 1973, 1975, 1977, 1980
Estonia	1922 - 1985; 1987; 1990; 1992; 1994 - 2000	Jõgi 1957; Härms 1939; Kastepõld 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985; Kastepõld et al. 1998a, 1998b; Kastepõld, Kastepõld 1990, 1991, 1992, 1999, 2000, 2001a, 2001b; Kumari, Jõgi 1974
Finland	1928 - 1967 1975 - 1991 1993 - 1997 1999 - 2004	Haapala 2001; Sten 1968, 1969; Nordström 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966; Nordström, Sten 1967; Saurola 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1993, 1994, 1995, 1996, 1997, 1999, 2000; Välikangas, 1931; Välikangas, Huuskonen 1952; Välikangas, Hytönen 1932, 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940; Välikangas, Koskimies 1950; Välikangas, Nordström 1954, 1956, 1957; Välikangas, Siivonen 1942, 1949; Valkama 2002, 2003, 2004
Germany: Helgoland	1957 1962, 1964 1966 - 1998	Bub 1990; Deutsche Ornithologen-Gesellschaft 1992; Foken 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998; Reiser 1971, 1972, 1973, 1974a, 1974b, 1975, 1977; Rogall 1975, 1976, 1977, 1978, 1979, 1980; Rogall et al. 1977, 1978, 1979; Schloss 1968a, 1968b.
Germany: Hiddensee	1975 - 2005	Köppen 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004; Pörner 1979, 1980, 1981, 1982; Siefke 1975, 1976, 1977; Siefke, Pörner 1978.
Germany: Radolfzell	1947 - 1981 1992 - 2002	Fiedler et al. 1999, 2002; Jacoby, Zink 1966; Zink 1959, 1961, 1962, 1964, 1969, 1974, 1975, 1976, 1977, 1978, 1979, 1980a, 1980b, 1981
Hungary	1908 - 1915 1923 - 1932 1945 - 1953 1975 - 1984 1998 - 2002	Halmos et al. 2003; Halmos, Karcza 2004; Harangi 1984; Harangi, Haraszthy 1983; Haraszthy et al. 1980, 1982, 1954; Haraszthy, Schmidt 1977, 1978, 1979; Keve 1954; Pátkai 1955; Schmidt 1975, 1976, 1981; Schenk 1908, 1909, 1910, 1911, 1912, 1913, 1916, 1924, 1926, 1929, 1930, 1934; Simon 2002; Warga 1955

(continued)

Ringing Center	Period	Data sources
Latvia	1924 - 2005	Kazubiernis pers. com.; Kazubiernis 1989; Spuris 1962; von Transehe 1939, 1940; Vilks, Pētersons 1943
Lithuania	1929 - 2004	R. Patapavičius pers. com.
Netherlands	1975 - 1989	Speek 1975, 1976, 1977, 1978, 1979, 1980, 1981, Wassenaar 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989
Norway	1975 - 1982	Holgersen 1975, 1976, 1977, 1978, 1979; Runde 1980, 1981, 1982
Poland	1932 - 1939	Anonymous 1999a, 1999b, 2000, 2001, 2002, 2003, 2004; Buse,
	1945 - 1963	Gromadzki 1966; Czerniakiewicz 1983; Domaniewski 1933, 1934,
	1975 - 1983	1936; Domaniewski, Kreczmer 1937; Gromadska, Kania 1975,
	1998 - 2004	1976; Gromadzka, Kachwicz 1978, 1979, 1980, 1981, 1982; Kania, Surdyk 1977; Rydzewski 1938, 1939, 1949a, 1949b; Szczepski 1951, 1970, 1976; Szczepski, Szczepska 1953, 1956, 1957, 1959, 1963, 1965
Russia	1975 - 1982	Dobrovalskaja et al. 1978; Dobrovalskaja, Litvin 1977; Dobrykina
	1986 - 2004	1979, 1980, 1981, 1982; Gurtovaya 1986; Gurtovaya, Litvin 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996; Vronskii 1975, 1976
Sweden	1917	Österlöf 1973, 1975a, 1975b, 1975c, 1976a, 1976b, 1977a, 1977b,
	1922 - 2004	1978a, 1978b, 1979a, 1979b, 1980a, 1980b, 1981, 1982, 1983, 1984, 1985a, 1985b; Stolt 1989, 1990a, 1990b; Stolt et al. 1996, 1997, 1998, 1991, 1992, 1995; Stolt, Fernholm 1989
Switzerland	1924 - 2005	E. Wiprächtiger pers. com.
United Kingdom	1975 - 1981	Hudson 1977, 1978, 1979, 1980, 1981; Spencer 1975, 1976

Statistical analyses

The raw data from all countries on ringed Corncrakes could not be used to estimate population trends, since numbers of ringers and ringed birds considerably changed over the time period covered. We used the computer program TRIM (*Trends and Indices for Monitoring data*) 3. version (Pannekoek, van Strien 2001) designed by Statistics Netherlands to analyse bird monitoring data (Pannekoek, van Strien 2001; van Strien et al. 2004) and used widely in Europe (Gregory et al. 2005). The TRIM program calculates a yearly index by using a matrix of observations with missing values. The basic model of the program is the following:

$$\ln\mu_{ij} = \alpha_i + \gamma_j,$$

where α_i shows site effect, and γ_j – year effect on the natural logarithm of the expected value of the count μ_{ij} (Pannekoek, van Strien 2001; van Strien et al. 2004).

We treated each ringing scheme as a site with Corncrakes ringed by the scheme as an observed count of the corresponding year. We also used the value adjusted to the number of ringers active in the scheme in the corresponding year or value adjusted to the number of ringed birds by the scheme in the corresponding year, to prepare input values for the TRIM program. Standard statistical procedures (calculation of the Spearman's Rank correlation coefficient and Pearson's product-moment correlation coefficient) were used to analyse data (Zar 1996).

Results

Birds ringed in Latvia

During the period of 1925 - 1995 there were 215 Corncrakes ringed in Latvia: 190 chicks (pulli) and 25 adults (Fig. 1). A decline of ringed pulli were observed in raw data as well as adjusted data: (1) Spearman's rank correlation between year and proportion of ringed Corncrake pulli to total number of birds ringed in Latvia in the respective year ($r = -0.46$; $p < 0.001$; Fig. 2A) and (2) Spearman's rank correlation between year and proportion of

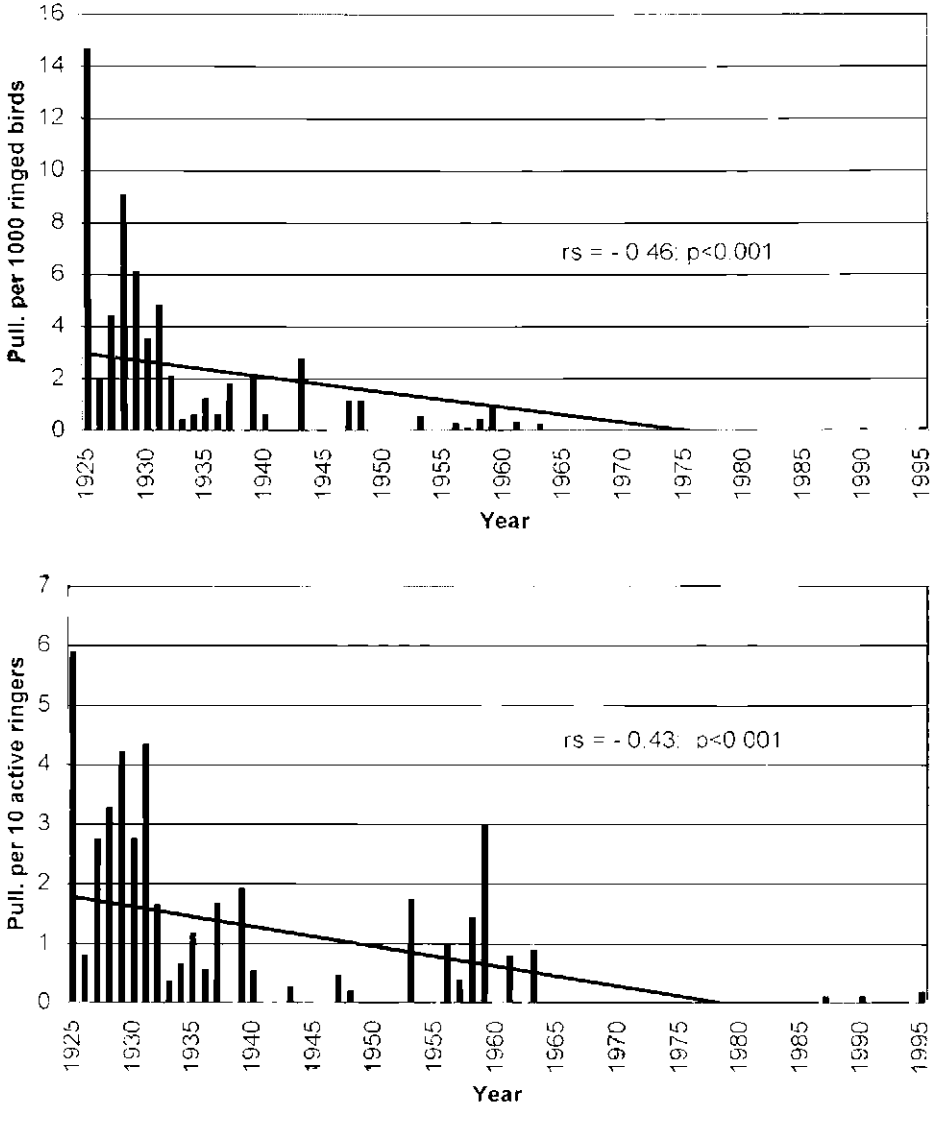

Fig. 2. Decline of ringed Corncrake pulli in Latvia from 1925 to 1995. A, ratio to 1000 birds ringed in Latvia in respective year. B, ratio to 10 bird ringers, active in Latvia in respective year.

Table 2. Collected data of ringed Corncrakes *Crex crex* in Europe 1925 - 1995 (for sources and period covered for each country see Table 1). (*), excluded from analyses; (**), only number of pulli analysed

Country	Pulli	Adults	Age unknown	Total
Croatia	8	12	2	22
Czechoslovakia	0	22	79	101
Estonia	29	2	7	38
Finland**	3	483	8	494
Hungary	4	0	31	35
Germany*	6	217	514	737
Latvia	190	25	0	215
Lithuania	24	8	0	32
Netherlands**	8	0	229	237
Norway	0	16	0	16
Poland	0	0	115	115
Russia*	17	53	1	71
Sweden*	4	245	319	568
Switzerland	23	22	13	58
United Kingdom*	130	47	0	177
Total:	446	1152	1318	2916

ringed Corncrake pulli to the number of active bird ringers in Latvia in the respective year ($r = -0.43$; $p < 0.001$; Fig. 2B).

Similarly to Corncrake, the number of ringed Skylarks and Whinchats also decreased significantly (Spearman Rank correlation coefficient; $p < 0.05$) during the 1925 - 1995 period (Fig. 3); the number of ringed Meadow Pipits, Common Whitethroats and Linnets increased significantly ($p < 0.001$; Fig. 3). The only species, showing no significant trend during the 1925 - 1995 study period was Yellow Wagtail (Fig. 3). The data analysis showed that Yellowhammers were mainly ringed in winter until the 1940s and, since we were interested in the investigation of the breeding population, we did not analyze the trend of ringed birds for this species.

Corncrakes ringed in Europe

We collected data on 2916 Corncrakes ringed in Europe until 1995 (Table 2). Examining ringing data of other Ringing centres in Europe for example, former Czechoslovakia (1934 - 1993), we observed similar decrease in numbers (Fig. 4). Index values of combined data of all ringed Corncrakes ($n = 643$) in former Czechoslovakia, Croatia, Estonia, Hungary, Latvia, Lithuania, Norway, Poland and Switzerland show a significant decrease during the study period (Pearson product-moment correlation; $r^2 = 0.26$; $p < 0.00001$). If we remove Latvia from data pool, since it contributes roughly one third of the data ($n = 215$) and therefore drives the index, the observed pattern remains unchanged (Fig. 5) and a significant decrease is observed (Pearson product-moment correlation; $r^2 = 0.11$; $p < 0.01$). Correlation between index of ringed Corncrakes in Europe, excepting Latvia (former Czechoslovakia, Croatia, Estonia, Hungary, Lithuania, Norway, Poland and Switzerland),

and number of ringed Corncrakes in Latvia, is statistically significant (Pearson product-moment correlation; $r^2 = 0.12$; $p < 0.01$).

Discussion

Criticism of the use of ringing data

Ringing data are the only quantitative data on Corncrake numbers extending back as early as 1908 (Schenk 1908). Because of the advantage of the availability of a long time-series we used the ringing data to access population dynamics. Use of these data, however, has also disadvantages. First, ringing locations are not placed stationary within a country (ringing scheme) as survey sample plots, but might change over time, especially, if our main assumption of ringing as a random event is true. Second, area covered by some ringing schemes have changed in the 20th century due to changes of the political borders of European countries in World War I and World War II (main examples are Germany, Hungary and Poland). Third and most important, changes of the behaviour of ringers over time is impossible to quantify: many of the bird ringers in Latvia in the 1920s and 1930s were students spending their summer in the countryside, where they worked on the farm and might have had opportunity to ring farmland birds. Intensification of agriculture impacted not only bird populations, but also behaviour of people including bird ringers.

We tried to test our assumption of ringing of a Corncrake as a random event, by checking if ringers who ringed Corncrakes also ringed other farmland birds. Ringers who ringed at least one Corncrake ringed 51 % of ringed Meadow Pipits, 40 % of Common Whitethroats, 20 % of Whinchat and 18 % of Linnets. Corncrake pulli were ringed by 57 ringers (identity of ringers of five Corncrake pulli remains unknown). No single ringer has ringed more than 13 Corncrake pulli in a maximum of four years. On average a ringer had ringed Corncrake pulli in 1.3 years or 1.6 occasions. In 68 % of all cases a ringer had ringed only pulli from one clutch. On average one ringer had ringed 3.25 Corncrakes or 2.4 Corncrakes annually. For some ringed birds it was stated on the ringing report that birds were ringed after they have accidentally entered the backyard of the countryside house. The above suggest that most probably Corncrake ringing in Latvia was random – nobody was particularly looking for Corncrake juveniles in order to ring them, confirming our assumption in Latvia.

Table 3. Comparison of farmland bird species trends by the ringing data (1925–1995) in Latvia and expert estimate in Latvia and Europe [+1, small increase (20 - 49 %); –1, small decline (20 - 40 %); –2, large decline (at least 50 %); 0, stable (change < 20 %)]

Species	Ringing in Latvia	1970 - 1990 Heath et al. 2000		1990 - 2000 Burfield, van Bommel 2004	
	1925 - 1995	Latvia	Europe	Latvia	Europe
Alauda arvensis	–	0	–2	0	–1
Anthus pratensis	+	0	0	–1	–1
Motacilla flava	0	0	0	0	–1
Saxicola rubetra	–	0	0	+1	–1
Sylvia communis	+	0	0	+1	+1
Acanthis cannabina	+	0	0	+1	+1

We compared trends of ringed farmland passerine birds in Latvia in 1925 - 1995 (Fig. 3) with expert estimate (Table 3) of the trends for those six species in 1970 - 1990 (Heath et al. 2000) and 1990 - 2000 (Burfield, van Bommel 2004). The only of those six species not showing any significant changes in Latvia both by ringing data and expert opinion is Yellow Wagtail. This might support the use of ringing data in obtaining population trends, since it suggests that the population of Yellow Wagtail has not changed in numbers (expert opinion), which is correctly reflected by ringing data. We assume that ringers do ring Yellow Wagtails (as well as all of the six analysed passerine bird species) randomly, thus no trend in ringing data and no trend in expert opinion might support the assumption that

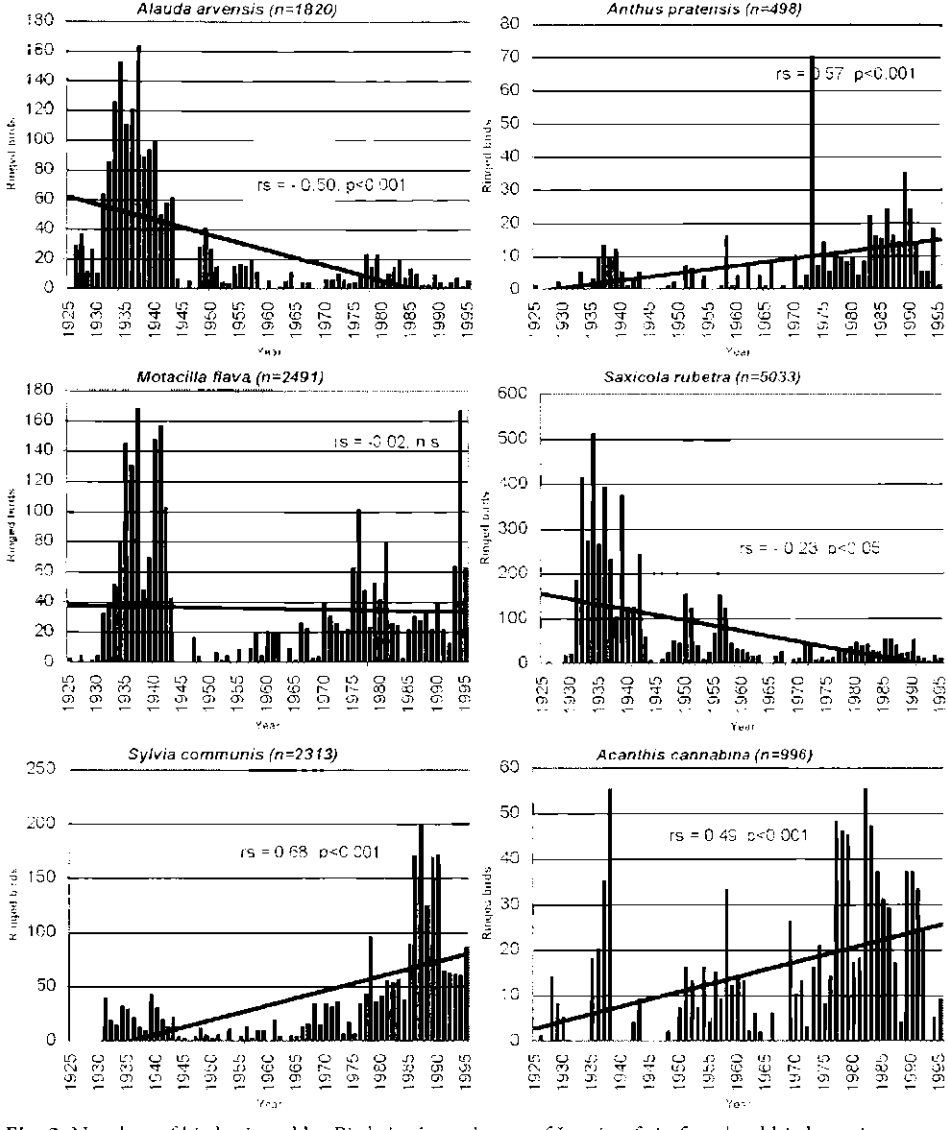

Fig. 3. Number of birds ringed by Bird ringing scheme of Latvia of six farmland bird species.

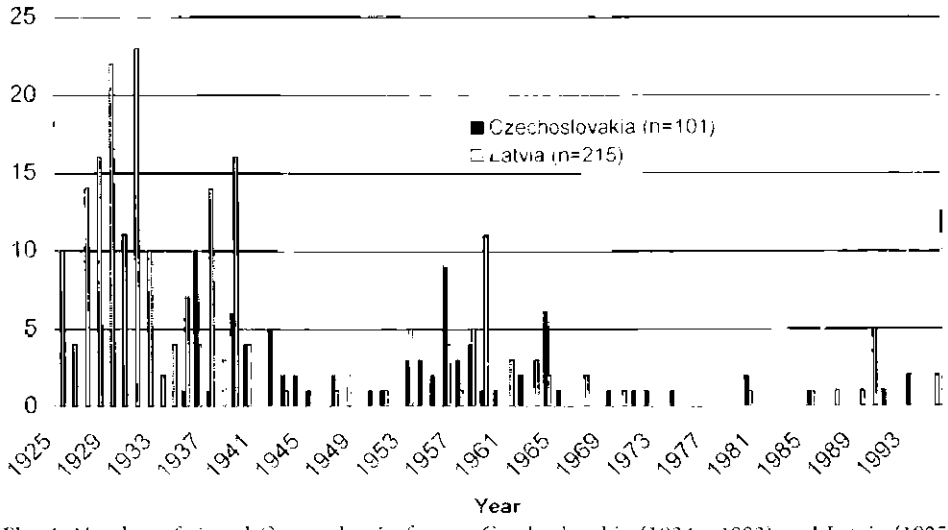

Fig. 4. Number of ringed Corncrakes in former Czechoslovakia (1934 - 1993) and Latvia (1925 - 1995).

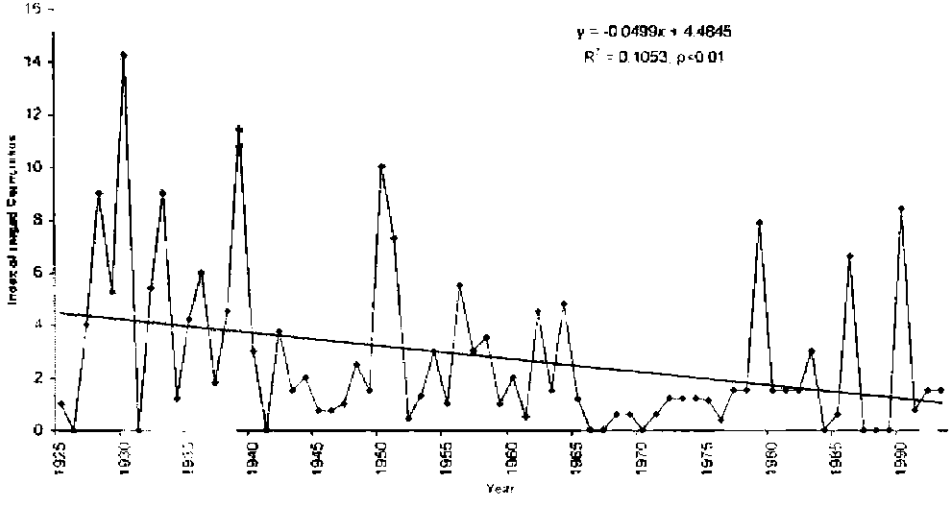

Fig. 5. Index of rimged Corncrakes in Europe (former Czechoslovakia, Croatia, Estonia, Hungary, Lithuania, Norway, Poland and Switzerland) 1925 - 1993.

ringers are still visiting wet meadows and ringing Yellow Wagtails at the same frequency. Therefore the decline in numbers of ringed Corncrakes does not reflect changes in ringer behaviour, but might truly reflect changes in population numbers. Three other species showed the same trend of ringed birds and population trends by the opinion of experts – decreasing for Skylark and increasing for Common Whitethroat and Linnet (Table 3). Meadow Pipit showed increase by the ringing trend, but decrease by expert opinion. In contrast Whinchat decreased by the ringing trend but increased by expert opinion. This might be explained by changes after 1995, which are not reflected in the trend of ringed

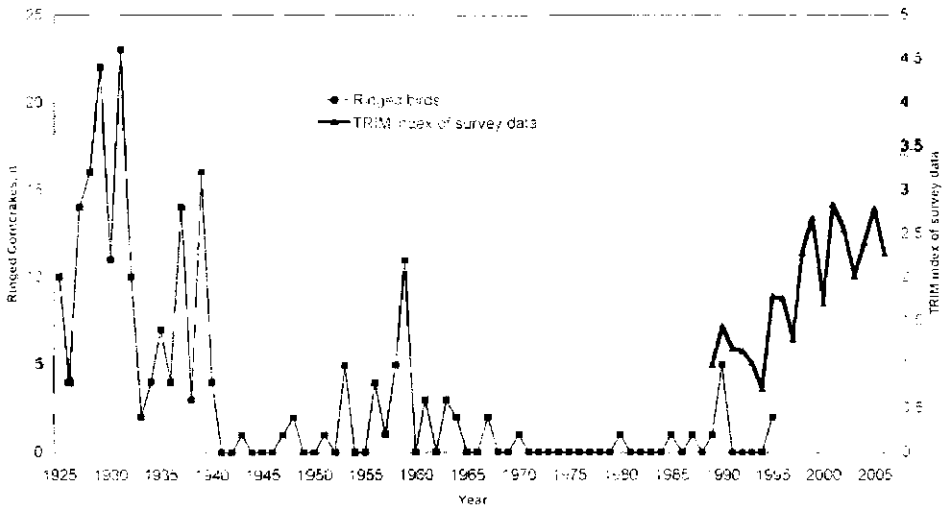

Fig. 6. Corncrakes ringed in Latvia (1925 - 1995) and TRIM index of Corncrake survey data in Latvia (1989 - 2006; Keišs 2006, modified).

birds, since ringing data after 1995 were not analysed. In general it is difficult to prove or disprove the use of ringing data for population trend analyses, since the same factors (intensification of agriculture and abandonment of agricultural lands) influence other species inhabiting similar habitats as Corncrakes and thus it is difficult to distinguish between decrease in population numbers and decrease in ringers activity.

Quantifying the decline of the Corncrake

Qualitatively there is no doubt that ringing data verify that there has been a decline of the Corncrake population both in Latvia and in Europe (Fig. 1, 2, 4, 5) as already reported in the literature (von Transehe 1965; Green et al. 1997). In our data (Fig. 1 - 5) we observed decline of ringing activities of all species during 1941 - 1950, which could be easily explained by World War II. The average index of ringed Corncrakes of all European countries for which data were available (including Latvia) showed a decline by about 5.5 times, if we compare 1925 - 1930 versus 1981 - 1990 (Fig. 6). Thus we can approximate that the population decline might have been the same. The other approximation by calculating decrease of available habitat (Keišs 2005; 2006) showed smaller decrease in Latvia by 1.5 - 3 times, but recent population density data for these calculations have been used, which might have decreased as well (Keišs 2006).

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Griezes *Crex crex* Latvijas un Eiropas populāciju skaita izmaiņu novērtējums XX gadsimtā pēc gredzenošanas datiem

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Kopsavilkums

Līdz pat 1990-iem gadiem griežu populācijas stāvokļa vērtējums lielākajā daļā Eiropas valstu balstījās uz aptuveniem skaitļiem, kas tika ieguti no dažādu anketēšanu, gada putna akciju, atsevišķu novērojumu un ligzdojošo putnu atlantu rezultātiem,bet nevis no griežu uzskaitēm. Šī iemesla dēļ iegūtie dati tikai aptuveni atspoguļoja patieso griezes populācijas stāvokli Eiropā. Mēs apkopojām datus par 2916 apgredzenotām griezēm no 17 Eiropas gredzenošanas centriem no 1908. līdz 1995. gadam un analizējām datus (n = 643) no bijušās Čehoslovākijas, Horvātijas, Igaunijas, Latvijas, Lietuvas, Norvēģijas, Polijas, Šveices un Ungārijas, lai iegūtu kvantitatīvu griezes populācijas samazināšanās novērtējumu (TRIM indeksu) pagājušajā gadsimtā. Šī pētījuma pamatā ir pieņēmums, ka griezes tika gredzenotas nejauši – gredzenotāji izmantoja katru iespēju apgredzenot griezes, kuras tie sastapa, bet īpaša griežu meklēšana, lai tās gredzenotu, nenotika. Mēs analizējām Latvijas gredzenošanas datus sīkāk, lai pārbaudītu savu pieņēmumu par griežu gredzenotāju uzvedību. Papildus mēs analizējām sešu lauku putnu sugu gredzenošanas datus Latvijā, lai novērtētu, vai gredzenotāji gredzenoja lauku putnus vēlāk pētījumu periodā, kad griežu gredzenošana bija apsīkusi. No 1925. līdz 1995. gadam Latvijā apgredzenoja 215 griezes – 190 mazuļus un 25 vecos putnus. Apgredzenoto mazuļu skaita samazināšanos var novērot gan neapstrādātiem datiem, gan kalibrējot tos pret attiecīgajā gadā apgredzenoto putnu kopskaitu ($r_S = -0,46$; $p < 0,001$), gan pret attiecīgajā gadā iesniegto atskaišu skaitu ($r_S = -0,43$; $p < 0,001$). Līdzīgu skaita samazināšanos var novērot arī bijušajā Čehoslovākijā gredzenotajām griezēm (n = 101). Eiropas valstu kopējais indekss pētījumu periodā ir būtiski samazinājies ($r^2 = 0,26$; $p < 0,00001$). Ja no šīs datu kopas atmet Latvijas datus, jo tie sastāda apmēram vienu trešdaļu no visiem datiem un tādēļ ļoti stipri ietekmē indeksu, novērotā tendence saglabājas ($r^2 = 0,11$; $p < 0,01$). Starp Latvijā gredzenoto griežu skaitu un Eiropā (Čehoslovākijā, Horvātijā, Igaunijā, Lietuvā, Norvēģija, Polijā, Šveicē un Ungārija) gredzenoto griežu indeksu ir novērojama statistiski būtiska korelācija ($r^2 = 0,12$; $p < 0,01$). Līdzīgi kā griežu, arī apgredzenoto lauka cīruļu ($r_S = -0,50$; $p < 0,001$) un lukstu čakstīšu ($r_S = -0,23$; $p < 0,05$) skaits Latvija no 1925. līdz 1995. gadam ir būtiski samazinajies, turpretim apgredzenoto pļavu čipstu ($r_S = 0,57$), brūnspārnu ķauķu ($r_S = 0,68$) un kaņepīšu ($r_S = 0,49$) skaits ir būtiski pieaudzis ($p < 0,001$). Vienīgā suga, kurai netika novērota nekāda būtiska tendence, bija dzeltenā cielava. Nav šaubu, ka gredzenošanas dati apstiprina griežu skaita samazināšanos Latvijā un Eiropā. Vidēji Eiropas valstu kopējais griežu indekss ir samazinājies par 5,5 reizēm, ja salīdzina 1925. - 1930. un 1981. - 1990. gadu periodus. Citi aprēķini par griežu skaita samazinašanos Latvijā parāda mazāku skaita samazināšanos - 1,5 - 3 reizes, bet tie ir izdarīti, balstoties uz mūsdienās iegūtiem populācijas blīvuma radītājiem, kuri laika gaitā varētu arī būt samazinājušies.

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Evaluation of past and present sea holly (*Eryngium maritimum*) habitats on Polish coastal dunes

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Abstract

Studies based on morphometric measurements and population assessment of plants on the dunes of the Polish coast of the Baltic Sea carried out since 1997 indicate that sea holly (*Eryngium maritimum*) is now an especially rare species in the area. Sea holly, a perennial plant of the pioneer habitats covering coastal dunes, is under legal protection on the Polish coast, but its habitats are slowly vanishing due to storms and human impact.

Key words: dune habitats, *Eryngium maritimum*, Polish coastal dunes.

Introduction

On many fragments of the Polish coast, dunes are threatened by storm surges; in effect, the dunes are retreating and sometimes are completely destroyed (Łabuz 2005). Another hazardous factor influencing dune habitats is human impact, particularly tourism, coastal protection measures, and forestry (Piotrowska, Stasiak 1984; Piotrowska 1995). All of the natural coastal plant habitats in Poland are threated (Herbich, Warzocha 1999). Abrasion and human impact are completely destroying the foredunes and partly yellow dunes. At present, great numbers of first dune ridges in Poland are covered by pine forest (*Empetro nigri-Pinetum*), affecting the distribution of dune plants. Also sea holly (*Eryngium maritimum*), a perennial plant of pioneer habitats covering coastal dunes, is endangered and protected by Polish law since 1957 (Ćwikliński 1979).

In 1978 an inventory of the whole coast was carried out to assess the distribution of the sea holly. The inventory recorded about 100 specimens of sea holly per one kilometer of coast (Ćwikliński 1979). Later reports indicated the extinction of its habitats due to forestry, the development of tourism and devastation of the coast (Piotrowska, Stasiak 1984; Piotrowska 1995; Piotrowska 1997; Piotrowska 2002). Those results have been confirmed by research on the dynamics and the pace of degradation and retreat of the Polish Baltic coast (Łabuz 2005; http://polishdunes.szc.pl).

The main aim of this work was to survey and describe the condition of Polish coastal dunes and its vegetation with emphasis on sea holly (*Eryngium maritimum*).

Methods

This work was conducted over several years, as it was not possible to check the plant ranges and dune dynamics along the whole coast in one year. Therefore the data on sea holly (*Eryngium maritimum*) distribution are not complete. On the Eastern part of the Polish coast, the investigations were conducted in 2002 and 2004, in the middle part between 2002 and 2006, and on the Western part from 1997 to 2007. The field study included estimation of (i) dune relief changes, (ii) vegetation dynamics, (iii) human or animal influences, (iv) storm surge impact and (v) wind impact.

The relief measurements were taken several times during the year, particularly, but whenever possible, after every major manifestation of the factors impacting the dunes. Relief changes were measured with geodesic devices across the coastal profile in selected sites along the coast, also sometimes in fixed plots (5 × 5 m). Other field studies concentrated on the determination of dune morphodynamics, which provided comprehensive information on relief changes and the condition of the entire environment along the dune coast.

Another important element of this study was defining plant habitats along profiles, including along the coast (especially on the first dune ridge). The dominating plant species for different stages of succession (after Kornaś, 1959; Piotrowska, Gos 1995) such as pioneer habitats (*Elymo-Ammophiletum*), psammophilous sward (*Helichryso-Jasionetum*) and pine forest (*Empetro nigri-Pinetum*) were marked along profiles (see also Fig. 1). Lines were used to mark their presence and ranges. Also, a simplified method of phytosociological plots was used. In the plots the projective cover and plants sociality were determined using a method described by Braun-Blanquet (Pawłowski 1977). Plot size was 5 × 5 m.

The presence of sea holly was marked separately along profiles and in the plots. Also sea holly was searched for on the dunes and marked on topographical maps (scale 1:10 000).

To describe the habitat conditions of sea holly on the examined profiles, the ranges of pioneer grass communities were determined, as well as those of psammophilous sward, mosses cover and pine forests.

The Polish coast is 464 km long. It is divided according to the distance markers applied by the Maritime Office (established for coastal protection). According scale, the border with Russia on the Vistula Spit is the point described as 0 km, and the border with Germany at 428 km. The Hel Peninsula has a separate classification, with 72 km of coast. The scale is applied for the delineation of endangered areas, and in monitoring of coastal phenomena. In this paper, the sites of Sea Holly habitats and drawn profiles were recorded according to the scale used in Poland (see also Fig. 2).

Results and discussion

Habitats of the Polish coastal dunes

The climate of the Polish coast is mild with marine influences. In an Eastern direction, the continental influence increases causing a temperature decrease, and changes in wind and storm surge directions. Very strong northerly as well as westerly winds cause development of the coast and new dunes. In autumn-winter, these winds are responsible for the biggest storm surges with water levels up to 2 m higher than the mean.

Dune coasts constitute about 80 % of the Polish coast. At present, Polish coastal dunes constitute coasts of various types. Dyke foredunes are located on sandspits or the coastal

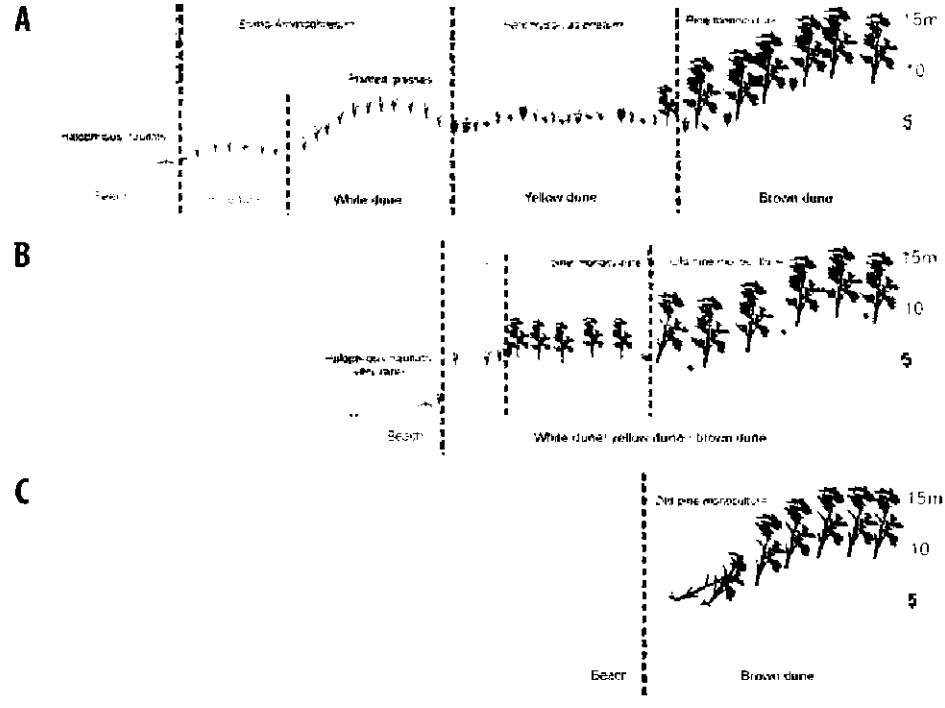
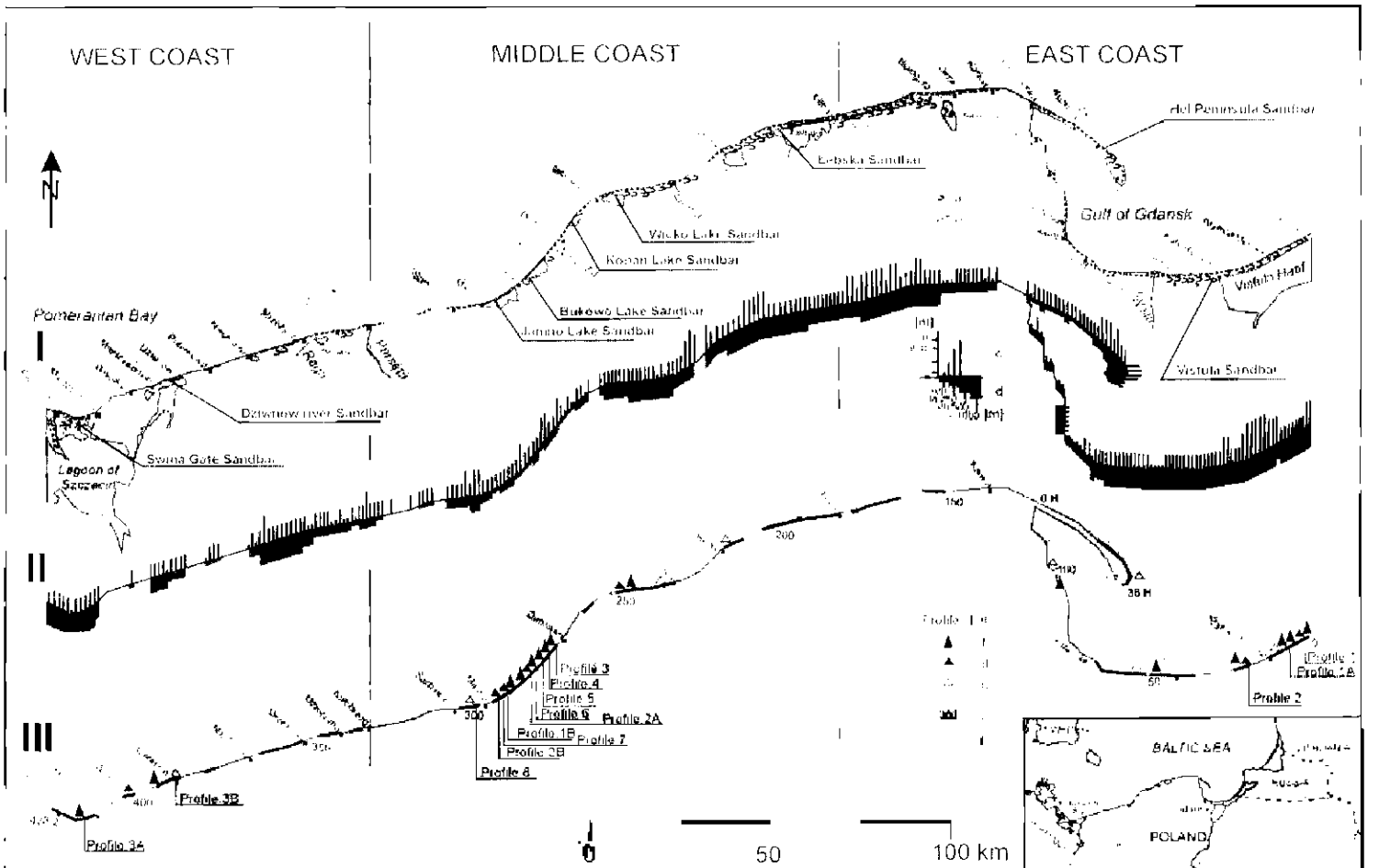


Fig. 1. The history of dune shore management on the Polish coast (simplified after Piotrowska, Gos 1995). A, after 2001 (only sandbar of Świna, Łebsko, Vistula outlet). B, after 1983 (general). C, after 1983 (locally), after 2001 (common).

endings of the river valleys and proglacial valleys. There are also some coastal field dunes, located in coastal areas due to land retreating (Łabuz 2005). The sandspits of the Polish coast and their dunes were formed over the last 5000 years during the relative stabilization of the coast, after the end of the maritime transgression from the Atlantic period (Tomczak 1995).

The sand texture in the foredunes of the Polish coast is between 0.16 and 0.25 mm. It is fine-grained sand, rarely with the addition of medium sand, accumulated during winds blowing at high speed. More than 90 % of the sands consist of light quartz grains (Łabuz 2005). In recent decades, the level of the Baltic Sea has been observed to increase steadily (Zeidler et al. 1995). The fluctuation of the sea level is associated with the oscillation of pressure systems and winds resulting in sea waves.

The amplitude of observed fluctuations in sea level reaches over 3.2 m in Świnoujście, 3.4 m in Kołobrzeg, and 2.6 m in Gdańsk. The greatest number of storm surges is observed in autumn and winter. Most of the storms on the Western coast (with force greater than 6 on the Beaufort scale) are produced by northerly winds (55 %), north-westerly winds (31 %) and north-easterly winds (14 %). The strongest storms, of force 10 to 12 on the Beaufort scale, occur during NE winds. Over the last ten years, strong storms on the Polish coast have been responsible for considerable destruction of coastal dunes and their plant habitats (Łabuz 2005).


WEST COAST
MIDDLE COAST
EAST COAST
N
Pomeranian Bay
Dziwnow river Sandbar
Swina Gate Sandbar
Lagoon of Szczecin
Vicko Lake Sandbar
Kopan Lake Sandbar
Bukowo Lake Sandbar
Lebsko Lake Sandbar
Lebska Sandbar
Hel Peninsula Sandbar
Gulf of Gdansk
Vistula Sandbar
I
II
III
Profile 3
Profile 4
Profile 5
Profile 6
Profile 2A
Profile 1B
Profile 7
Profile 3B
Profile 3A
Profile 2
0
50
100 km
BALTIC SEA
POLAND

The first stage of succession on the upper beach is determined by the halophytic species sea sandwort (*Honckenya peploides*), marram grass (*Ammophila arenaria*) and lyme grass (*Elymus arenarius*), forming a *Elymo-Ammophiletum honckenyetosum* community (Fig. 1; Kornaś 1959; Piotrowska, Celiński 1965). On foredunes (white dunes), psammophilous grasses from *Elymo-Ammophiletum typicum* are the most predominant. Reports indicate that sea holly can be found on foredunes (Piotrowska, Celiński 1965; Ćwikliński 1979; Piotrowska 2002).

Predunes as a poor habitat subject to the activity of strong winds and are affected by autumn and winter storms. Gradually, due to succession, the accumulation sections are invaded by species typical for the next succession stage on the partly fixed dunes characterized by a psammophilous sward from the community *Helichryso-Jasionetum* (Piotrowska, Celiński; Piotrowska, Gos 1995). This is a rich habitat, most frequently covering fixed dunes (yellow dunes) and is a prefered habitat of sea holly (Ćwikliński 1979; Łukasiewicz 1992).

In the area of the coastal towns, yellow dunes are subset to human activity and have been planted since the 19th century for protecting. The last stage of succession on the coastal dunes are the forest communities (*Empetro nigri-Pinetum*): various types of coastal pine forests (Wojterski 1993) in natural conditions covering brown dunes, with a developed podzol soil profile. On the low, marshy backs of the dunes, the forests are characteristic of humid areas with large biomass of plants and broadleaf trees.

The most important cause of degradation of coastal habitats has been planting of dune grasses (*Ammophila arenaria*), willow shrubs (*Salix arenaria*) as fashine fences, and pine trees (*Pinus sylvestris*). Today, most of the pine forests covering coastal dunes have been planted (Piotrowska, Stasiak 1984; Wojterski 1993). Moreover, dune communities are vanishing due to the development of tourist infrastructure.

Coastal towns occupy more and more land for construction purposes. On the Western coast, tourist centers have coalesced in a 30-km long town (from the 361st to the 291st km of the coast, according to the Polish scale). Buildings are not only at the back of the coastal dunes but also more and more on the ridge of the foredune or the beach (Łabuz 2003b).

Recent habitats of sea holly of the Polish coastal dunes

In the past, sea holly was present on the whole dune coast area of the present Polish coast. It was often observed on white dunes (Ćwikliński 1979; Wojterski 1993) and protected only in the Woliński National Park on the West coast (established in 1960) and in the Słowiński National Park on the East coast (established in 1966). Sea holly is a symbol of the Polish dune coast. It is present in the community of pioneer grasses *Elymo-Ammophiletum* and psammophilous sward *Helichryso-Jasionetum*. In the sward community on yellow dunes, sea holly develops better due to the stable substratum and smaller influence of winds and drifting sand. Its seeds grow slowly and, when blown towards the forest, suffer from mortality due to the difficult habitat conditions (Łukasiewicz 1992).

Fig. 2. The characteristics and locations of dunes of the Polish coast (Łabuz 2005) with sea holly (*Eryngium maritimum*) habitats (2002 - 2006). I, dune localisation (a – land dunes, b – foredunes). II, height of dune coast (c) and the width of coastal dune areas (d). III, localisation of sea holly: e – profile localisation; f – abundant sea holly (over five specimens per 200 m); g – site of sea holly (below five specimens per 200 m); h – individuals; i – coastline scale, every 50 km; j – coast nourishing of white and yellow dune (between 2002 and 2006).

Sea holly has adapted to disadvantageous conditions on coastal dunes by developed underground and hard parts, and waxy above ground parts. The woody root of mature specimens may even be 5-m long (Łukasiewicz 1992). The very appearance of the overground parts in their color, stiff and thorny leaves, flowers, makes the holly different from other plants. This appearance may be the reason why tourists remove the plant, collecting it as a beautiful seaside souvenir (Ćwikliński 1979). Another reason for its scarcity is the drifting of the seeds by winds blowing from the sea towards the forest, where this plant does not grow (Łukasiewicz 1992). Moreover, the exposure of its brittle roots (Łukasiewicz 1992) to wind may result in mechanical damage and in effect, lead to the death of individual specimens. In addition, intensive accumulation of sand may be disadvantageous for development.

Studies carried out in the 1960s and in 1970s indicated the highest densities on the middle and East coast of Poland (Piotrowska, Celiński 1965; Ćwikliński 1979; Piotrowska, Stasiak 1984). Investigations of sea holly populations on the Polish coast by Ćwikliński (1979) showed that this plant was most dense on the Middle coast. The highest density was observed on a 50-km-long section of the coast between Mielno and Ustka, with about 720 specimens per kilometer (Ćwikliński 1979). Another sea holly-rich section of the coast was the Vistula Spit, between Kąty Rybackie and the border with Russia. On the Western coast, the species was most numerous on Dziwnow Spit, on both banks of the Dziwna river outlet to the sea (391 km) and on the Wolin Island near Wisełka (about 404 km).

On the Hel Peninsula, holly was most numerous near the town of Chałupy (7 km Hel). Interestingly, the species was particularly rare on a very long section of the East coast, from Hel to Rowy (also in the Słowiński National Park). For example, only 12 specimens of sea holly were observed along a 40-km section in the Park. Ćwikliński (1979) cites other authors when considered that holly had not been present in the area from 1920 to 1957, probably due to destruction by locals and tourists.

In the 1980s, Piotrowska and Stasiak (1984) observed that the species was endangered due to forestation of the dunes. Numbers decreased also by planting grasses and tree overgrowth. On the Vistula Spit, sea holly was present only in a few areas: Piaski (1 to 2, 8 km), Krynica Morska (12 to 13 km), Przebrno (21 km), Stegna (39 to 40 km), Sobieszewo (53 km). Another recorded area was the Łeba Spit in the Słowiński National Park, with rare habitats in high psammophilous grass communities on white dunes and psammophilous swards on yellow dunes near Łebsko Lake (193 km), Dołgie Lake (207 km) and the town of Rowy (215 and 217 km; Piotrowska et al. 1997).

During studies conducted in 1986 and 1994/1996 on the Middle coast dominated by narrow spits, sea holly was observed in the vicinity of Wicko Lake (262 km), Dąbki town (280 to 281 km), Łazy (287 to 288 km) and in the area of the channel outlet from Jamno lake (292 km; Piotrowska 2002). These area previously supported the large populations of sea holly (Ćwikliński 1979). According to other reports conducted in middle coast cliffs, sea holly was present in the area of Gąski (Piotrowska, Chojnacki 1972).

Studies covering the West coast of Poland in the 1960s (Piotrowska, Celiński 1965) and 1970s (Ćwikliński 1979) show that sea holly was most numerous in the *Elymo-Ammophiletum* community in the area of the Woliński National Park, near Grodno, Wisełka villages (402 to 404 km) and on the Dziwnów Spit near Międzywodzie (393 to 395 km) and Dziwnów (391 to 393 km). In most cases, it occurred on a low foredunes at the bottom of the moraine cliff, or was present on the high dune ridge on the spit.

Ćwikliński (1979) reported that sea holly was endangered due to dune afforestation.

Even then, 41 % of the observed habitats on the dunes were planned to be forested. Another 10 % of habitats were in newly sown forest and were likely to dissappear. To protect them, he proposed the creation of reserves: 5th km of coast on the Vistula Spit (540 specimens), the 244th km on Wicko Lake Sandbar (3800 specimens), the 265th km on Kopań Lake Spit (4200 specimens), the 293rd km on Jamno Lake Sandbar (2222 specimens) and the 391st km on Dziwnów Sandbar (357 specimens).

Present sea holly habitats of the Polish coastal dunes

The proposed reserves have not been established to this day. The only sites of dune vegetation protection are in the Słowiński National Park and some locations in the Woliński National Park. Additionally, a strict bird reserve at the Vistula estuary near Mikoszewo has been created. Some hope for dune vegetation and sea holly protection in particular has been given by the Nature 2000 network. On the Polish coast, the network includes the Vistula Spit, Hel Spit, the Słowiński National Park, and the Woliński National Park, a section of the West coast near Kołobrzeg, and a section of the East coast near Białogóra. Unfortunately, except for the Vistula Spit, sea holly does not occur in the mentioned areas (Fig. 2).

Decade-long studies on the dynamics and the morphodynamics of plant habitats indicate a drastic depletion of white and yellow dunes (Łabuz 2005; see Fig. 1). The white dunes on the Vistula Spit have disappeared due to abrasion; moreover, pine planting is still being used for dune consolidation (Łabuz, unpublished data). The Hel Peninsula is under strong adverse human impact due to protection of the coast and the development of tourist infrastructure. Most of the dunes and beaches on the peninsula, except the tip, have been

Table 1. The present distribution of sea holly (*Eryngium maritimum*) on the Vistula Spit

Kilometre scale of the coast	Location	Habitat description
2 to 3 km	State border	Some individuals on the undisturbed dune ridge and on its south slope
4 km	Piaski	Few individuals at the south slope of the abraded foredune, along pine forest border
9 to 10 km	Piaski, west	Few individuals on the abraded dune top, close to the pine plantation on the south slope; also between low pine trees
11 to 13 km	Close to Krynica Morska town	Many specimens on the top and south slope of the dune. In some places dying within young pine plantation (trees now 1 m high). Only few individuals in pine forest (trees 4 - 5 m high)
22 to 23 km	Przebrno	10 specimens on the first ridge not covered by pine
23.4 to 25 km	Skowronki	8 specimens on the uncovered by pine first ridge. 5 withered between pine trees
46 to 47 km	Vistula outlet	Some behind first dune hidden between willow shrubs. Protected against pine forest by a wider dune belt

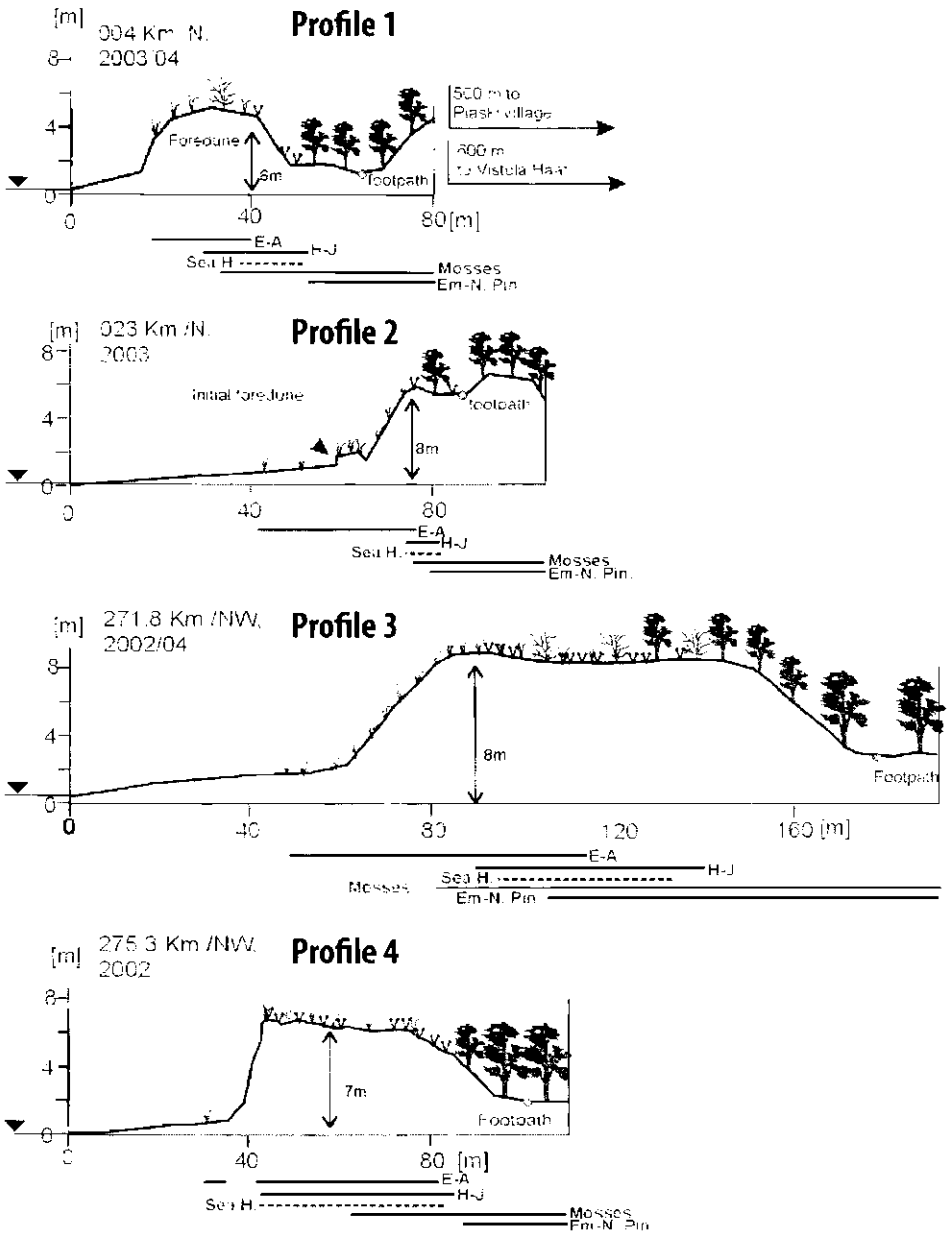


Fig. 3. Sea holly (*Eryngium maritimum*) distribution along coastal dunes in Poland between 2002 and 2006 (see Fig. 2 for location of profiles). Symbols for plant communities: E-A – *Elymo-Ammophiletum* habitats, H-J – *Helichryso-Jasionetum* habitats, Em-N. Pin. – *Empetro-Nigri pinetum*, Sea h. – sea holly habitats. Profile 1, foredune covered by grasses, pine monoculture at the back, sea holly specimens on the dune and on south dune slope, small tourist impact. Profile 2, seasonal low foredune with pine monoculture on the higher, narrow psammophilous habitats. Profile 3, very wide and high foredune with accumulation tendences, wide natural dune habitats with numerous sea holly individuals. Profile 4, wide clipped foredune, wide habitats of grasses and other dune plants, large number of sea holy specimens. *(continued)*

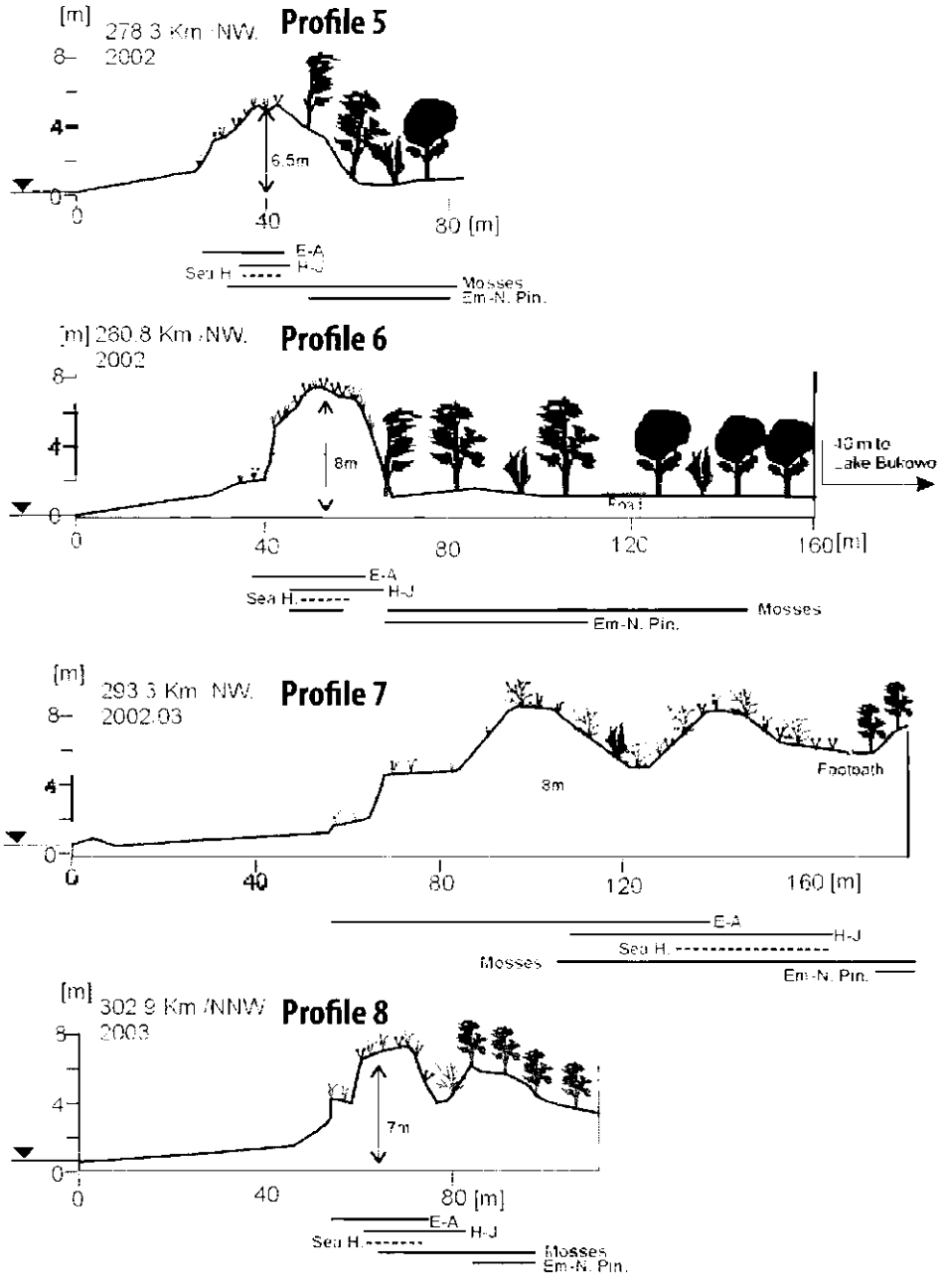


Fig. 3. *(continued)* Profile 5, narrow dune covered by dune grasses, at the back and low area covered by forest, narrow sea holly habitats. Profile 6, very narrow part of sandbar, narrow single dune covered by dune grasses, a few sea holly individuals, dune retreat in the south is burying forest. Profile 7, cliffed first dune with dune grasses, on the second ridge psammophilous greensward, vanishing dune habitats caused by tourist activities, sea holly close to the forest. Profile 8, abraded foredune with narrow psammophilous habitats, pine monoculture at the back, a few individuals of sea holly, also on abraded dune top.

reconstructed artificially. On the section between Hel Peninsula and Łeba Spit, the first dune from the sea is covered with pine forest (Piotrowska 2002).

Foredunes and a belt of dunes covered with psammophilous grasses can only be found in the area of the Słowiński National Park. On the middle coast, where sea holly used to be most numerous, the spit is retreating at a rate of 1 to 2 m a year (Łabuz 2005). In some places, the dune does not exist any more (the Kopań Lake Spit) or has been artificially reconstructed (the Bukowo Lake Spit). Only on the spit of Wicko Lake is the dune vegetation developing, in the area of the military training ground. In Jamno Lake Spit, tourist infrastructure is growing despite the retreat of the coast, resulting in an almost complete loss of non-forested dunes between 1998 to 2002 (Łabuz 2003a).

Spits on the West coast, apart from the Świna Gate Sandbar, tend to retreat at a rate of 2 to 4 m a year (locally even up to 6 m; Łabuz 2005). Between Kołobrzeg and Mrzeżyno, the shore has retreated and the first ridge is covered by pine forest. The foredune is developing only on some short sections of the coast. On the Dziwna River Spit, abrasion tendencies are predominant where dunes with pioneer grass habitats occur only on the Eastern shore of the river's estuary and near Międzywodzie. Sand accumulation tendencies and vegetation succession are found only on the Świna Gate Sandbar (Łabuz 2005; Łabuz, Grunewald 2007).

East coast

Studies carried out in 2003 and 2004 indicate that on the Vistula Spit sea holly was present only on the ridge of the yellow dune (second or third, looking from the beach) not covered by forest (Fig. 2, Fig. 3, profiles 1, 2; Fig. 4, profile 1A). Sometimes, only one specimen was found on a very long section of the coast, sometimes one per 50 m. Its presence was observed only in few sites of the spit in the psammophilous sward community (Table 1).

Another place where sea holly was abundant is a short part of the coast between Rewa Mew Peninsula and the Gdynia cliff (Mechelinki village). Along 2.5 km of the low dune coast (up to 1.5 m high) a large number of sea holly plants were found, mostly on a meadow (up to 20 m wide) between the sea and surrounding swamps. Some of them were present on a narrow beach (20 m wide). This part of the coast is not under significant touristic impact (data from 2007).

Sea holly was not found on the remaining sections of the coast, apart from two specimens on the Hel Peninsula (35th km of Hel Spit classification) and near Reda (Gulf of Gdańsk).

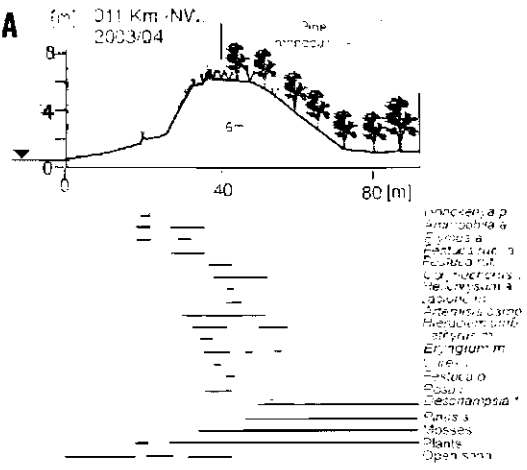
Middle coast

On a section of the coast in Słowiński National Park, sea holly was found only in the western part of the park near the town of Rowy.

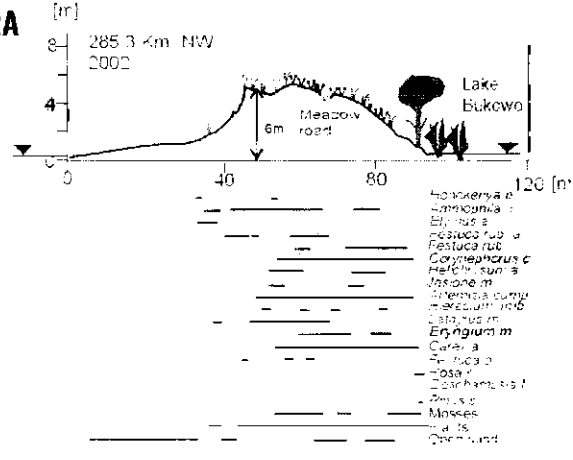
The greatest number of sea holly specimens was found from 2002 to 2006 on the section between Ustka and Mielno. Presently sea holly is very frequent on the coastal dunes of

Fig. 4. Example of localisation of sea holly individuals in respect to distribution of other plants (Braun-Blanquet method used after Pawłowski 1977). Profile 1A – East coast, Vistula Spit. Narrow accumulative dune coast with sea holly individuals decreasing due to pine forest growth. Profile 2A – Middle coast, Lake Bukowo Spit. Very narrow part of sandbar. Lake begins just at the back of the dune. Whole dune covered by dune grasses. Wide sea holly habitats. Profile 3A – West coast, Świna Gate Spit. Wide accumulative dune coast with sea holly individuals increasing in number every year.

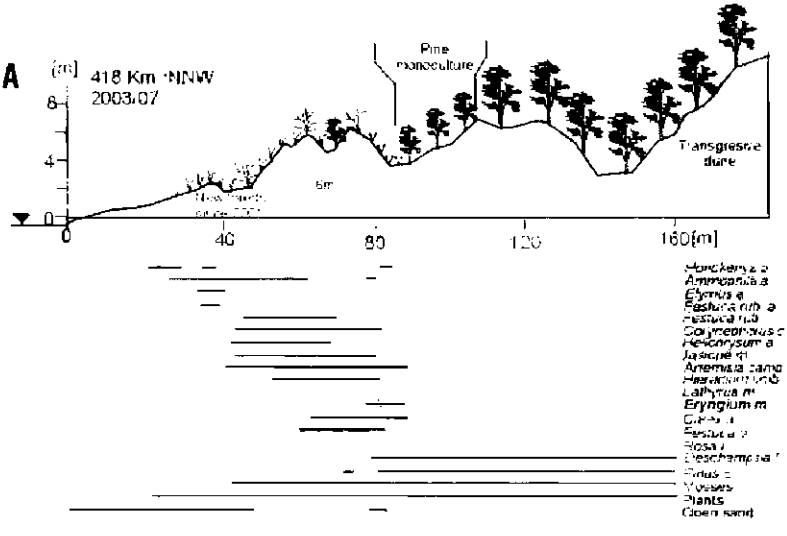
Profile 1A



Profile 2A



Profile 3A



Wicko Lake Spit (military area) and west of Darłówek until Dąbki (military areas). On the latter section, sea holly densely populates the wide coastal and non-forested dune (Fig. 3, profiles 3 and 4). A few dozen sea holly specimens were observed there, on a high non-forested dune by the border of the psammophilous grasses and sward (271 to 275 km). Also, further west on the particularly protected dunes of the narrow spit of Bukowo Lake from Dąbki to Łazy, sea holly was often observed on narrow dune dykes destroyed by the sea (Fig. 3, profiles 5, 6; Fig. 4, profile 2A), where dunes are covered by mostly psammophilous sward. Further, the section between Łazy and Unieście (on the Jamno Lake Spit) used to be under military administration, which limited tourist activities. On this section, sea holly was present in the community of *Helichryso-Jasionetum* on the non-forested dune (Fig. 3, profile 7). Sea holly was present even on the upper beach, where between 1997 and 2001 a new foredune was developing (293 to 294.5 km; Łabuz 2003a). After the storms of 2001 and 2003, the habitats became extinct. Only on the crown of the non-forested dune has sea holly been present until today (about one specimen per 50 m of the shore). Due to the retreat of the spit's shore, the dunes are being stabilized by planting dune grasses and pines, which results in the narrowing of sea holly habitats (Fig. 5, profile 1B). Moreover, the channel connecting Jamno Lake with the sea and the area further west is heavily exposed to tourists in summer months.

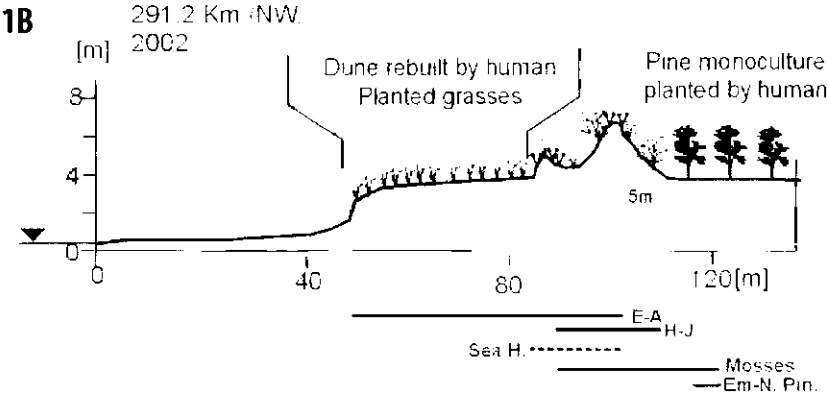
Similarly to many natural sites, the area lacks basic infrastructure, marked paths or boardwalks preventing walking on the dunes. In the area from the channel to the town of Mielno, a few sites of sea holly habitats on the ridge and the southern slope of the non-forested dunes (294.6, 296 and 297 km of the spit) were recorded between 1997 and 2001. This section is also intensely affected by the sea, which is indicated by abrasively cut dunes with steep slopes, devoid of plants. Between 1999 and 2003, all sea holly specimens in this area became extinct due to the retreat of the coast and the destruction of the dunes by tourists. The dune vegetation is aggressively destroyed by beach-goers and storms abrasing the dunes (Łabuz 2003b).

An example of the effects of abrasion on sea holly habitats is the western bank of the channel connecting Jamno Lake with the Baltic Sea. Over the last four years the base and the ridge of the dune have retreated by 10 to 15 meters. Holly habitats on the slopes of the dyke were destroyed and the plant emerged on the low back of the dune in the surroundings of a low and sparse pine forest (profile 2B). Eight young specimens were observed (8th May 2007) close to the channel on an illegal parking place created on the degraded ground indicating the drift of seeds towards the land. Probably during the tourist season, all these specimens will be damaged by cars or trampled by tourists.

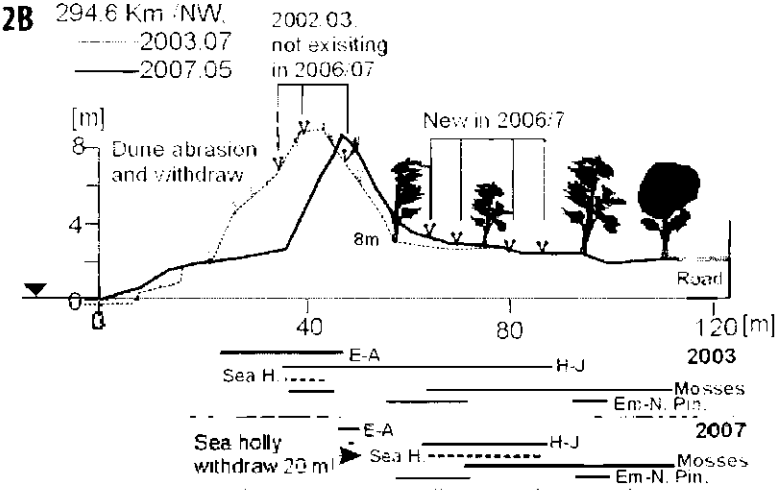
During studies in 2003 west of Mielno (to Sarbinowo), sea holly was observed on the non-forested dunes distant from the seaside resorts; a few specimens on the 303rd km (Fig. 3, profile 8). Further west, beyond Sarbinowo, no sea holly was found.

Fig. 5. Example of the vanishing sea holly specimens due to the main affecting factors. Profile 1B – dune stabilisation by planting. Vanishing sea holly caused by grass and pine plantations. Profile 2B – dune retreat. Between 2003 and 2007 dune moved back about 10 m, sea holly vanished completely in 2004. In 2007 sea holly reappeared on the low lying back side of the dune covered by degraded greensward. Profile 3B – dune improvement. Whole natural part of dune habitats were artificially covered by sand. Symbols of the plant communities: E-A – *Elymo-Ammophiletum* habitats; H-J *Helichryso-Jasionetum* habitats, Em N. Pin. – *Empetro nigri-Pinetum* habitats, Sea h. – sea holly habitats.

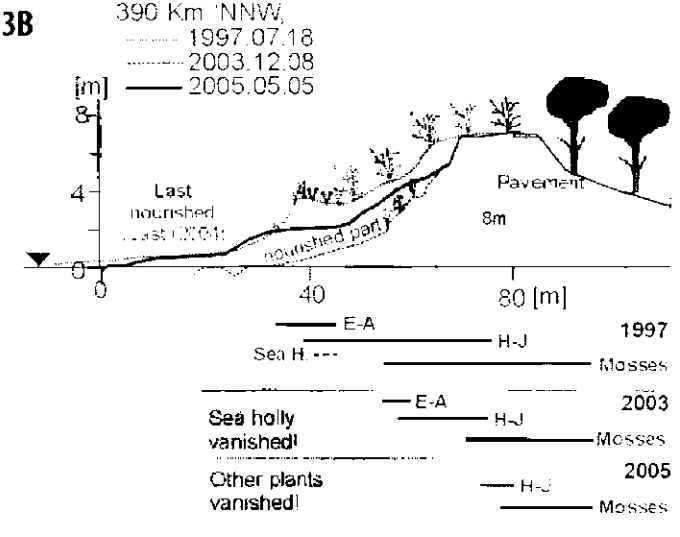
Profile 1B



Profile 2B



Profile 3B



West coast

On the West coast, sea holly specimens are even less frequent. The main habitat previously was on Dziwnów Spit between the towns of Dziwnów and Międzywodzie. Between 1997 and 2002 its presence on the dunes was limited to several sites. It was found in Dziwnów on the 389th to 391st km and in Międzywodzie on the 395th to 396th km. Since 2002 sea holly has not been observed in Dziwnów due to tourist pressure and action taken for protection of the coast from the sea. Strong storm surges from the winters of 2001 and 2003 resulted in the retreat of the dune dyke by 4 to 6 m and the destruction of plant cover and slope of the foredune. With this, whole habitats of natural dune vegetation were utterly destroyed (Fig. 5, profile 3B). The last two specimens of sea holly were found in 2005 on the 391st km, where the coast is growing and the dunes are developing due to accumulation of deposits by the breakwaters of the Dziwna River outlet. Between 1997 and 2004 individual specimens were found in the area of Międzywodzie and Wisełka (at present no data is available).

On the most westerly situated Świna Gate Spit (412 to 428 km of the coast), where plant succession is observed (Łabuz, Grunewald 2007) sea holly was present only on the third dune from the sea, which is non-forested and covered with a species-rich community of psammophilous sward (*Helichryso-Jasionetum*). It was found there between 2002 and 2007 on the 418th km of the coast (Fig. 4, profile 3A); 25 specimens were observed in an area of about 100 m^2. Another 14 specimens grew 100 m to the west. In 2004 willow bushes were cut there to obtain material for the fashine fences, which resulted in a threefold increase in specimens compared with 2005. However, the pine monoculture on the south side is threating its development; five dead specimens were found on the tree line. Flowering specimens grew on the southern slope of the dune ridge. This area has no tourist pressure, the coast is growing and new dunes are developing. It is possible that at this site sea holly will develop without any serious threat.

Conclusions and perspectives

Studies on the distribution of sea holly on the Polish coast indicate that its habitats are vanishing (Ćwikliński 1979; Piotrowska, Stasiak 1984, Piotrowska 2002). The main threats are seaside tourism and coastal protection measures, along with the natural abrasion of the coast, which together destroy coastal dunes and their plant habitats.

In recent years, threats have intensified and the number of habitats has decreased dramatically (Piotrowska 2002). The abrasive character of the Polish coast results in the destruction and retreat of costal dunes, the habitats for sea holly. This is confirmed by studies on the Middle coast (since 2001 to 2004) where numerous specimens of sea holly have disappeared from Kopań Lake Spit and partly from the spits of Vistula, Jamno, Bukowo and Dziwnów. Strong winds from the sea do not facilitate the development of new specimens. Seeds drift to the forests neighboring the non-forested dunes and sea holly grows only in rare cases in very sparse forests (Łukasiewicz 1992). Due to the development of seaside resorts along the coast and infrastructure on the ridges of the dunes, areas with a natural environment are becoming increasingly scarce.

Sea holly is no longer present on the dunes neighboring seaside resorts (Fig. 2). The explotation of dunes by tourists has resulted in the destruction of dune vegetation. Individual specimens observed in 2002 do not exist at present. The plant may be found frequently in coastal areas situated further from the towns and on the dunes next to military areas (Wicko Lake Spit, Dąbki, and, until recently, Jamno Lake Spit). In these

areas, the plant is protected from tourists by a ban on approaching military areas, very often well-fenced and secured by the army. Planting grasses and bushes on the dunes and afforestation contribute to the decrease in biodiversity and the extinction of numerous species, i.e. sea holly. Planting pines quickly eliminates sea holly from its primary habitats. This has been shown by processes observed on the Vistula Spit and Jamno Lake Spit where dead specimens were found in the pine forest, and dying individuals observed in newly planted areas. Interestingly, sea holly is not present on dune sections with predominant rebuilding processes and the development of dunes, despite the fact that these sections are not affected by tourists.

In field studies, the longest sections of coast with sea holly habitat were observed on the Middle coast from Darłówek to Łazy. On the spits of the Jamno and Bukowo lakes sea holly covers dunes quite remote from seaside towns where the coast is virtually free from human activity. Sea holly is observed mainly on gray dunes covered by psammophilous communities with a predominance of *Carex arenaria* (Fig. 4). This is mainly a stable dune substratum where the supply of sand is not large, there are southern slopes or vast and flat dune ridges.

On the Polish coast sea holly is seriously endangered. At present, sea holly is not present in any great numbers in any of the existing Nature 2000 areas, apart from the Vistula Spit. To protect the species a reserve should be established; the most suitable location is the area of Jamno Lake Spit (290th to 294th km) or the section between Darłówek and Dąbki (271st to 275th km) where sea holly is most numerous.

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Kādreizējo un esošo jūrmalas zilpodzes (*Eryngium maritimum*) biotopu izvērtējums Polijas piekrastes kāpās

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Kopsavilkums

Pētījumi par jūrmalas zilpodzes (*Eryngium maritimum*) izplatību Baltijas jūras Polijas piekrastē, kas veikti no 1997. gada, izmantojot morfometriskas analīzes un populāciju apsekošanu, liecina, ka zilpodze ir kļuvusi par īpaši retu sugu. Jūrmalas zilpodze, daudzgadīgs piekrastes kāpu biotopu augs, Polijas teritorijā ir juridiski aizsargāts. Tomēr, zilpodzes augšanai piemēroto biotopu platības lenam samazinās vētru un cilvēka darbības ietekmes rezultātā.

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Interacting influence of cold stratification treatment and osmotic potential on seed germination of *Triglochin maritima* L.

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Abstract

The effect of cold stratification treatment and isoosmotic NaCl and polyethyleneglycol (PEG) concentrations on germination of *Triglochin maritima* seeds was assessed. The highest (400 mM) NaCl concentration caused a significant decrease in germination percentage and no germination was observed at the two highest (64 and 128 mM) PEG concentrations. The large difference in germination among experiment replications suggests that time of collection and length of storage may have influenced germination. Stratification treatment had a significant positive effect on seed germination and there was a significant interaction between PEG, but not NaCl, concentration and duration of cold stratification during germination. Further research is required to determine the importance of the degree of seed ripeness as well as the possible effect of after-ripening or induction of secondary dormancy of undispersed seeds on seed germination.

Key words: cold stratification, dormancy, NaCl, polyethyleneglycol, seed germination, *Triglochin maritima*.

Introduction

Seeds of most of the plants growing in the temperate climate zone possess dormancy mechanisms, the main function of which is to prevent seed germination in an inappropriate season (Vleeshouwers et al. 1995; Baskin, Baskin 1998). Apart from the innate mechanisms, various external factors such as water availability, temperature, light and other determine the ability of seeds to germinate. Endogenous seed dormancy mechanisms interact with external factors throughout the dormancy period (Finch-Savage, Leubner-Metzger 2006).

In regions with cold winter seeds generally germinate in spring, therefore seed dormancy is often broken during a certain period of low temperature, provided that water is available (Baskin, Baskin 1998). Such conditions can be imposed artificially, by storing imbibed seeds at low temperatures, a treatment known as a cold stratification. It has been noted that cold stratification widens the range of temperature at which seeds can germinate, and that the same effect takes place under other environmental factors, including salinity (Baskin, Baskin 1998).

Salinity of the soil solution is an important environmental factor affecting seed

germination of plants growing in the coastal zone. However, comparatively few studies have investigated the interaction of salinity and low temperature treatments. Salinity affects plants in at least two ways: by lowering the osmotic potential in the ambient solution and by ion toxicity (Munns 2002). Low osmotic potential hinders water intake by seeds, thus slowing or altogether inhibiting germination. However, temporary exposure to a substance that lowers osmotic potential to a certain value and subsequent rehydration is known to produce a germination enhancing, or priming, effect (Obroucheva 1999). Treatment with neutral osmotica, such as polyethyleneglycol (PEG) is most often used in promoting germination, however in natural conditions seeds are more likely to be exposed to NaCl and other mineral salts that are toxic at high concentrations. Woodell (1985) classified seed germination that is enhanced after a period of exposure to high salinity as a type three, that is, most salinity-tolerant, seeds. Apart from a priming effect, it has been noted that low salinity levels as such can sometimes enhance seed germination (Baskin, Baskin 1998).

Triglochin maritima L. is a halophytic species distributed throughout the Northern hemisphere and restricted to coastal areas and inland saline habitats (Davy, Bishop 1991). Although certain characteristics of seed germination of this species have been described before (Masuda et al. 1999; Khan, Ungar 2001), there is no information available on aspects of the biology of *T. maritima* in the Baltic region. The habitats where *T. maritima* occurs on the coast of the Baltic sea may differ considerably from habitats elsewhere in respect to salinity levels. In addition, *T. maritima* is not a homogeneous species but a species complex (Davy, Bishop 1991). Therefore, considerable differences may exist between different populations. *Triglochin maritima* is listed in the Red Data Book of Latvian flora and is a protected species, which makes it important to understand aspects of reproduction and ecophysiological demands of this species. Seeds of *T. maritima* are characterized by innate dormancy, which can be broken by cold stratification treatment (Masuda et al. 1999).

The aim of this study was to investigate the combined effects of cold stratification and osmotica, in order to determine whether an interaction takes place between them. The use of two different osmotically active substances – potentially toxic NaCl and neutral PEG – was aimed at discerning the effect of lowering the osmotic potential of the solution and that of ion toxicity.

Materials and methods

Seeds of *Triglochin maritima* were collected in August 2005 and July 2007 near lake Liepaja, Latvia. Seeds were stored dry at 2 - 3 °C before germination experiments were carried out in January 2006 - January 2007 with seeds collected in 2005 and in August 2007 with seeds collected in 2007; germination was pre-assessed shortly after the collection. Seeds were germinated in Petri dishes on top of a double layer of filter paper moistened with distilled water or NaCl or PEG-4000 solution. Concentrations of solutions used were 25, 50, 100, 200 or 400 mM NaCl, and 8, 16, 32, 64 and 128 mM isoosmotic PEG. To test germination recovery after exposure to NaCl solution, at the end of the germination period (14 days) ungerminated seeds were rinsed with distilled water, re-imbibed during 0.5 h and germinated on filter paper moistened with distilled water for another 14 days. Germination was carried out at 20 - 25 °C with a 16 h photoperiod. Germinated seeds were counted three times a week. The effect of cold stratification on seed dormancy was tested

Table 1. Germination (%) of *Triglochin maritima* seeds collected in August 2005 and July 2007 imbibed for either 0.5 or 5 h in distilled water. n.d., not determined

Time of collection	Time of analysis	Imbibition time (h)	
		0.5	5
August 2005	January 2006	49 ± 6	n.d.
August 2005	January 2007	n.d.	76 ± 4
July 2007	August 2007	78 ± 3	81 ± 1

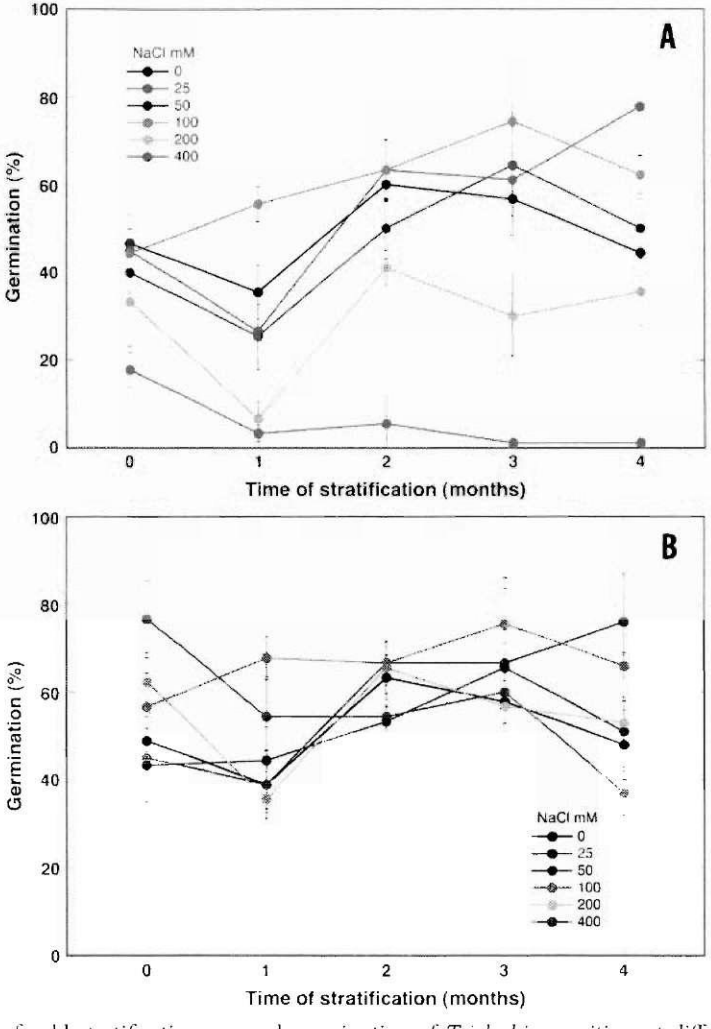

Fig. 1. Effect of cold stratification on seed germination of *Triglochin maritima* at different NaCl concentrations before (A) and after (B) seed rinsing with distilled water. Germination percentage after rinsing is a sum of the number of seeds germinated within 14 days before and within 14 days after the rinsing. Seed imbibition time before germination: 0.5 h. Data are means of three replications (30 seeds per replication) for each NaCl concentration, vertical bars denote standard error.

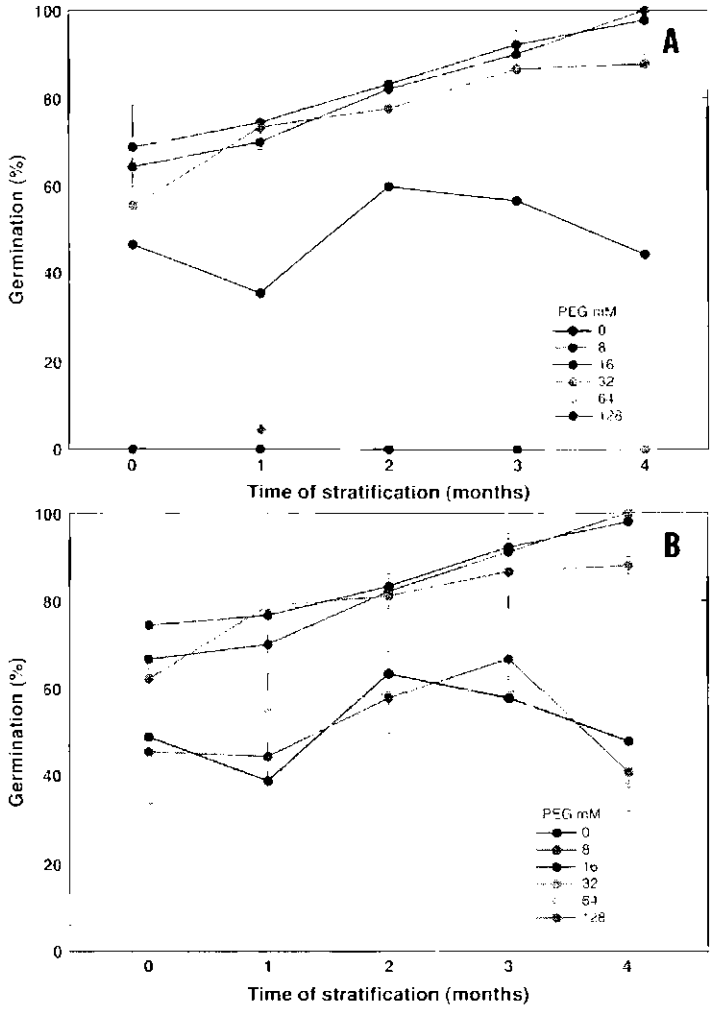

Fig. 2. Effect of cold stratification on seed germination of *Triglochin maritima* at different PEG concentrations before (A) and after (B) seed rinsing with distilled water. Germination percentage after rinsing is a sum of the number of seeds germinated within 14 days before and within 14 days after the rinsing). Seed imbibition time before germination: 0.5 h. Data are means of three replications (30 seeds per replication) for each NaCl concentration, vertical bars denote standard error.

by storing fully imbibed seeds at 2 - 3 °C. Originally seeds were imbibed in distilled water or a respective solution for 0.5 h and stratified for one to four months. This experiment was replicated with seeds colleted in 2005, imbibed for 5 h using both NaCl and PEG in variants without a one and two-month-long stratification treatment, but only NaCl in the variants with stratification treatment, as well as with seeds collected in 2007, imbibed for either 0.5 or 5 h and germinated without a stratification pre-treatment (both NaCl and PEG were used). There were three replications of 30 seeds or four replications of 20 seeds (August 2007). Seeds tested for germination immediately after imbibing were used as a control.

Statistical analysis (ANOVA and multiple range tests using 95 % Scheffe interval) was performed using Statgraphics Plus for Windows 4.1.

Results

The maximum germination percentage observed varied widely among the control variants (Table 1). Nevertheless, the impact of NaCl and PEG was similar in all replications. The number of germinated seeds was significantly reduced when NaCl concentration reached 400 mM; no germination was observed at 64 and 128 mM PEG. There was an interaction between length of cold stratification and PEG concentration ($P < 0.0001$): germination percentage at 8 and 16 mM PEG increased up to 100 ± 0 % and 98 ± 2 %, respectively, after four months of cold stratification (Fig. 2). In contrast, no interaction between cold stratification and NaCl concentration was observed neither in the first, nor in the second replication, although the main effect of cold stratification in the second replication (seeds imbibed for 5 h) was significant ($P < 0.01$; Fig. 1). After re-imbibing seeds in distilled water germination recovered up to the level of the control variant (Fig. 1), except in variants where seeds collected in 2007 were used.

There was a significant difference between variants in which NaCl and PEG were used. In the variants with a higher osmotic potential (25 - 50 mM NaCl and 8 - 16 mM PEG), seed germination was higher if PEG was used as an osmoticum for seeds collected in 2005, but not for seeds collected in 2007, when similar germination percentages were obtained (results not shown). However, in the variants with low osmotic potential (200 - 400 mM NaCl and 64 - 128 mM PEG) germination was always higher if NaCl was used (Fig. 1, 2). It is noteworthy that while the germination percentage was low in the control variant without stratification treatment, a priming effect was observed when seeds germinated at 200 and 400 mM NaCl were re-imbibed in distilled water (Fig. 1).

Discussion

The difference in final percentage of germinated seeds in the two experiments may have been caused by different time period of storage as well as difference in time when seeds were collected. Storage of dry seeds at low temperature cannot guarantee that no changes occur in the physiological state of the seeds (Baskin et al. 2006). Seeds collected earlier or later in the vegetation season may differ in the degree of ripeness. Moreover, environmental conditions faced by the mother plant were shown to influence dormancy and germination patterns of the ripening seeds (Donohue et al. 2007). Seeds of *T. maritima* ripen at different times during vegetation season, mainly in August and September (Davy, Bishop 1991). Factors such as photoperiod and temperature experienced by mother plants during seed ripening, which obviously vary during vegetation season, can influence the degree of dormancy in mature seeds, creating a heterogeneous seed population (Finch-Savage, Leubner-Metzger 2006). In addition, ripe seeds tend to persist in the spikes and can be dispersed as late as in February (Davy, Bishop 1991), thus seeds may undergo after-ripening or enter secondary dormancy before dispersal. It is also possibile that seeds collected in late July were not fully ripe, which may account for the differences in germination responses. On the one hand, unripe seeds may have not entered dormancy period, which would explain the high germination percentage. On the other hand, unripe seeds may

be more vulnerable when subjected to treatment with osmotica. When seeds collected in July 2007 were germinated at 128 mM PEG, a high proportion of seeds were infected by fungi (Nečajeva, unpublished results). Fungal infection can serve as an indicator of seed mortality (Baskin, Baskin 1998), and high mortality suggests that the seeds were strongly affected by a low osmotic potential.

It is important to know at which time the seeds had ripened, in addition to the collection time, in order to determine what kind of processes could have taken place before collection. Testing germination of seeds which ripen at different times during several years may shed light on the impact of environmental factors on resulting seeds dormancy, as well as help to reveal the stage at which innate or secondary dormancy is imposed.

It is supposed that seed coats of *T. maritima* contain germination inhibitors (Davy, Bishop 1991). Therefore, lengthy imbibition could lead to enhanced germination by leaching of such substances. However, the imbibition time appears to be an insignificant factor for *T. maritima* seeds, despite a slightly higher germination percentage observed in variants imbibed for 5 h (Table 1). During stratification treatment, the negative effect of the inhibitors, if there was any, may have been overpowered by the mechanisms promoting germination.

In the present experiments, there was a significant difference between the variants where NaCl or PEG were used as an osmoticum (Fig. 1, 2). It is possible that even at high concentrations of NaCl seeds of *T. maritima* are able to imbibe more solution that at isoosmotic concentrations of PEG, which can explain the higher germination percentage at the lowest osmotic potentials. Possibly, this effect accounts for the significant interaction with stratification treatment observed in variants where PEG was used as an osmoticum. Almansouri et al. (2001) found that PEG solution inhibits germination of durum wheat more strongly than NaCl or mannitol, suggesting that intake of NaCl and mannitol occurs to a certain degree, whereas PEG cannot penetrate seeds which as a result do not imbibe. Apparently, NaCl was not toxic to *T. martima* seeds at concentrations used in the experiments, as complete recovery of germination was observed after rinsing (Fig. 1, 2). The priming effect observed when seeds germinated at 400 mM NaCl were re-imbibed in distilled water further supports that osmotic potential in general and ambient concentration of NaCl in particular is among those environmental factors which interact with internal factors in the process of germination. Duan et al. (2004) reported that the effect of priming was greater in *Chenopodium glaucum* seeds when using NaCl, rather than PEG. Enhanced germination after alleviation of salinity effect can be explained as an adaptation to germinate in the rainy season, in the temperate climate - in spring, when rain and thawing of snow and ice reduce salinity (Baskin, Baskin 1998). Seeds of certain halophytes were shown to be able to intake water at elevated salinity levels owing to specfic adaptations, such as high NaCl content in the testa (Song et al. 2005). However, it is not as yet clear what inner physiological mechanisms take part in promoting germination after alleviation of salinity and whether other environmental factors or physiological state of the seed determine the strength of the priming effect.

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Aukstās stratifikācijas un osmotiskā potenciāla mijietekme uz *Triglochin maritima* L. sēklu dīgšanu

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Kopsavilkums

Pētīja aukstās stratifikācijas un izoosmotisku NaCl un polietilēnglikola (PEG) koncentrāciju ietekmi uz *Triglochin maritima* sēklu dīgšanu. Augstākā (400 mM) NaCl koncentrācija izsauca butisku dīgšanas procenta samazinašanos, un dīgšana nenotika divu augstāko PEG koncentrāciju klātbūtnē (64 un 120 mM). Ievērojamās dīgšanas atšķirības eksperimentālo atkārtojumu starpā liecina, ka sēklu ievākšanas laiks un to uzglabāšanas ilgums iespējami ietekmējuši dīgšanu. Stratifikācijai bija būtiska pozitīvā ietekme uz sēklu dīgšanu un varēja novērot mijiedarbību starp PEG (bet ne NaCl) koncentrāciju un aukstuma stratifikācijas ilgumu dīgšanas laikā. Nepieciešami tālāki pētījumi, lai noteiktu sēklu gatavības pakāpes un iespējamā pēcgatavības vai sekundārā miera perioda indukcijas ietekmi neizbirušām sēklām uz sēklu dīgšanu.

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Is vaccine against HCV possible?

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Abstract

Natural immunity against the hepatitis C virus (HCV) was discovered not long ago. Some infected individuals are able to clear the virus, while other patients need intensive antiviral drug treatment that is expensive and not always effective. For this reason, many efforts are directed towards development of an effective vaccine in order to improve treatment outcome by stimulating humoral and cellular immune responses against HCV proteins or their immunodominant epitopes. Several HCV vaccine candidates have been tested in chimpanzees and promising results have allowed optimism about the development of at least partially effective vaccine against highly heterogeneous HCV pathogen. Hepatitis B virus core particles can be used successfully as a carrier of HCV epitopes. Chimeric HBc/HCV virus-like particles represent an interesting and valuable tool in the development of HCV vaccine.

Key words: hepatitis C virus, immune response, vaccine, virus-like particles.

Introduction

An estimated 3 % of the world's population (more than 170 million people) are infected by the hepatitis C virus (HCV). Most infections have become chronic: a condition that is incurable in many patients, leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC). There were 1339 cases of chronic hepatitis C and 105 cases of acute hepatitis C in Latvia in the period from January 2006 to December 2006 (http://www.sva.lv/epidemiologija/statistika/). The number of chronic HCV patients increased by 30 % compared to year 2005, and by 76 % compared to statistical data acquired during the last five years (http://www.sva.lv/epidemiologija/statistika/). Current medical treatment options are limited. Chronic HCV infection is the most common cause of liver transplantation. Despite the discovery of the virus by molecular biological methods more than 15 years ago, and the sequencing of its entire genome, our knowledge of the virus and the nature of the protective immune responses is limited. Researchers have been hampered by the lack of a robust cell-culture system yielding infectious virus until very recently, and by the absence of a non-primate animal model. The situation clearly emphasizes the need for novel prophylactic/therapeutic approaches that can prevent spread of HCV and provide more efficient antiviral therapy of individuals suffering from chronic hepatitis C.

It is assumed that induction of vigorous, long-lasting, and cross-reactive antiviral antibodies as well as a multispecific cellular immune response that includes both helper and cytotoxic T lymphocytes (CTL) are necessary for an effective HCV vaccine (Rollier et al. 2004; Torresi et al. 2004; Neumann-Haefelin et al. 2005; Bowen, Walker 2005; Rehermann, Nascimbeni 2005). The development of such a vaccine meets, however, with many difficulties. The natural course of HCV infection and mechanisms of HCV interaction with an infected host are very complicated and still poorly understood, and the immunologic correlates associated with disease resolution and protection remains to be precisely defined. In addition, HCV is characterized by high genome sequence variability and a quasispecies distribution in an infected patient. The rare and expensive chimpanzee remains the only available animal model for HCV. Despite all these difficulties and restrictions some progress has been achieved during the last few years and several vaccine candidates are being explored. Among them are a recombinant protein subunit vaccine, a peptide vaccine, a live recombinant vaccine, virus-like particles (VLPs), and a DNA vaccine (Lechner et al. 2002; Inchauspe, Feinstone 2003; Duenas-Carrera 2004; Torresi et al. 2004; Houghton, Abrignani 2005; Encke et al. 2005). All these approaches are promising, although each of them has some shortcomings and limitations, and their applicability in medical practice remains to be determined. Most probably, the efficient HCV vaccine of the future will be based on a combination of two or several immunogens, one of which might be multi-epitope virus-like particles. One of the most promising VLP candidates is a chimeric particle on the basis of hepatitis B virus core antigen (HBcAg). The major intrinsic advantage of recombinant HBc particles is their improved immunogenicity due to formation of a covalent link between B and T helper (Th) epitopes, and the ability of HBcAg to act as both T-cell-dependent and independent antigen. As a result, the HBc particles induce high titers of antibodies and vigorous T-cell proliferative responses (Ulrich et al. 1998; Pumpens, Grens 2001). Perspectives of using HBc particles were demonstrated most recently in a successful clinical phase I trial of a malaria vaccine based on the HBcAg-platform (Oliveira et al. 2005).

Biology of the HCV virus

HCV is a noncytopathic hepatotropic member of the *Flaviviridae*, genus *Hepacivirus*, and is most closely related to the pestiviruses, Bovine viral diarrhea virus and GB virus B. All members of this family are small-sized, enveloped viruses containing a positive-strand RNA genome encoding a viral polyprotein. The viral genome of HCV is ~9.6 kilobase-long containing a single open reading frame (ORF). The ORF is flanked by 5' and 3' untranslated regions (UTRs). The highly conserved 5' UTR is 341-344 nucleotides long. Several stem-loop structures in this region contribute to an internal ribosome-binding site (IRES) that mediates the CAP-independent translation of the viral RNA. The ORF encodes a polyprotein of 3,010 or 3,011 amino acids, which is processed into structural and non-structural proteins (Fig. 1). The structural proteins forming the viral particle include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include the p7 ion channel, the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase (Moradpour et al. 2007; Fig. 1). The HCV life cycle is entirely cytoplasmic. Replication occurs through a minus-strand intermediate in a membrane-

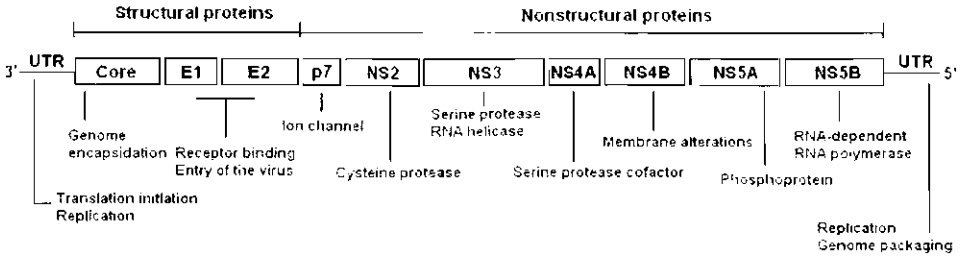


Fig. 1. HCV genes and gene products. The structure of the viral genome with the long open reading frame encoding structural and nonstructural proteins, and 5' and 3' untranslated regions (UTRs).

bounded compartment (Moradpour et al. 2004), yielding double-stranded RNA (dsRNA) intermediates. The replicative intermediates are fully exposed to the cell dsRNA-sensing machinery (Samuel 2001; Yoneyama et al. 2004) and induce strong innate cellular responses following infection. HCV infects only humans and chimpanzees.

HCV isolates can be classified into genotypes and subtypes (Simmonds et al. 2005). There are six major genotypes of HCV worldwide that differ by up to 30 - 35 % in sequence. Patients infected with genotype 1 do not respond as well to interferon-α-based therapy as those infected with genotype 2 or 3. Within an HCV genotype, several subtypes (designated a, b, c and so on) can be defined that differ in their nucleotide sequence by 20 - 25 % (Moradpour et al. 2007). The term quasispecies refers to the genetic heterogeneity of the population of HCV genomes that coexist in an infected individual (Simmonds et al. 2005).

Characterization of HCV infection

The hepatitis C virus causes acute and chronic hepatitis, and hepatocellular carcinoma (Hoofnagle 2002). The liver is its primary target organ, and the hepatocyte is its primary target cell. Acute infection is usually asymptomatic, making early diagnosis difficult. A notable feature of HCV infection is its tendency towards chronicity: ~70 % of acute infections become persistent, and chronic cases are often associated with serious liver disease (Hoofnagle 2002). As a result, HCV infection is a leading killer worldwide and the commonest cause of liver failure. In common with hepatitis B and human immunodeficiency (HIV) viruses, HCV is primarily transmitted parenterally (Alter 1996). Before the development of diagnostic tests, the infection was commonly passed on through blood and related products (Alter 1997), haemodialysis (Alter 1999) and organ transplantation (Alter 2002). Today, HCV primarily affects injecting drug users and their sexual partners (Alter 2002). It is a particular problem in correctional facilities, where 20 - 40 % of inmates are infected, in contrast to ~2 % of the general population (Spaulding 1999). It is opportunistic in HIV-infected individuals, ~25 % of whom are co-infected with HCV (this figure raises to 50 - 90 % among injecting drug users; Sulkowski 2000). Co-infection causes higher HCV titres and a more rapid progression to cirrhosis (Sulkowski 2000).

Immune response to HCV

In common with other persistent viruses, HCV does not kill the cells it infects, but triggers an immune-mediated inflammatory response (hepatitis) that either rapidly clears the infection or slowly destroys the liver, causing the development of HCC. The outcome is largely determined by the efficiency of the antiviral immune response. Host-virus interactions are ideally investigated in cell culture and small-animal models; the former are only now becoming available.

Implication of host determinants

Innate immune response. HCV spreads rapidly in the liver after inoculation (Rehermann, Nascimbeni 2005; Wieland and Chisari 2005), and thus the innate immune response might be expected to influence the outcome of infection. Indeed, prospective genomic analysis of the intrahepatic innate immune response in acutely infected chimpanzees suggests that HCV triggers a strong type-1 interferon (IFN-α/β) response as it spreads (Bigger et al. 2004; Wieland, Chisari 2005), but resists the effector functions of the downstream antiviral target genes that it induces. Importantly, the response is similar in animals that clear the infection and those that become persistently infected (Bigger et al. 2004; Wieland, Chisari 2005), implying that any influence on the outcome is indirect or obscure. Whatever its function, the innate intracellular immune response probably has a role in controlling HCV infection because the virus has developed several strategies to evade it. Several groups have shown an association between certain human leukocyte antigen (HLA) alleles and the outcome of HCV infection (Shoukry et al. 2004). These differences might influence the breadth of the T-cell receptor (TCR) repertoire and the ease with which the virus can escape. Confirmation of this hypothesis would be facilitated by an inbred mouse model of HCV infection.

Adaptive immune response. The clearest determinants of the outcome of HCV infection are the magnitude, diversity and quality of the adaptive immune response. Viral clearance during self-limited infection is characterized by vigorous polyclonal $CD4^+$ and $CD8^+$ T-cell responses that are relatively weak and narrowly focused in chronically infected humans and chimpanzees. Moreover, the onset of viral clearance and liver disease coincide with that of the T-cell response and the entry of virus-specific T cells into the liver; primary failure to induce a T-cell response or functional exhaustion of an initially vigorous response predict viral persistence (Shoukry et al. 2004; Rehermann, Nascimbeni 2005). However, the basis for variable immunological responsiveness to HCV has largely remained elusive. Indeed, it is not known whether the failure to respond vigorously in persistently infected subjects is caused by antigen overload during immunological priming, virus-induced defects in antigen presentation, hyperinduction of regulatory T cells, genetically determined restriction of the virus-specific T-cell repertoire or other causes (Bowen, Walker 2005). Therefore, whereas both primary and secondary immunological hyporesponsiveness to HCV seem to contribute to the establishment and maintenance of persistent infection, the reasons why they occur in selected subjects remain to be determined. Moreover, the virus can persist despite a multispecific $CD4^+$ and $CD8^+$ T-cell response (Shoukry et al. 2004; Rehermann, Nascimbeni 2005) by progressive mutational escape, which confirms the importance of the immune response in viral clearance and disease pathogenesis.

Implication of viral determinants

The six distinct genotypes of HCV show marked differences in geographic distribution, disease progression and response to therapy. However, the complex epidemiological differences in patient groups infected with each genotype make it difficult to ascribe variability in outcome to the virus instead of the host (Feld, Hoofnagle 2005). The mutation rate of HCV is high (10^{-3} per nucleotide per generation), as is its replication rate ($\sim 10^{12}$ virions per day in humans; Neumann et al. 1998). This results in explosive expansion of the virus after inoculation and in the evolution of numerous viral quasispecies in each infected subject, which could influence the magnitude and efficacy of the antiviral immune response. Moreover, the virus produces a constant stream of escape variants that outrun the immune response and can eventually produce mutants with no corresponding receptors in the immunological repertoire (Shoukry et al. 2004; Rehermann, Nascimbeni 2005). The influence of these parameters on the outcome of infection has been studied in a few acutely infected humans and chimpanzees and in many chronically infected individuals. The results show that B- and T-cell escape mutants are selected by the immune response during HCV infection and probably contribute to viral persistence. Regarding the second strategy, mutational inactivation of B- and T-cell epitopes is common in HCV infection (Bowen, Walker 2005). B-cell epitopes are concentrated in the hypervariable region 1 (HVR1) of the E2 protein (Mondelli et al. 2001), probably allowing the virus to persist in the presence of antibody that is neutralizing for its ancestors.

The T-cell epitope mutations span the viral polyprotein (Bowen, Walker 2005), often in residues that bind to major histocompatibility complex (MHC) molecules or are otherwise involved in antigen presentation. Mutations also occur in residues engaged by the TCR, making infected cells invisible to T cells expressing the corresponding TCR (Meyer-Olson et al. 2004). Although mutational escape probably contributes to the persistence of the virus, it is less clear whether it determines the outcome.

Development of vaccine against HCV

A decade ago, an effective vaccination against HCV was considered only a remote possibility. Three factors contributed to this: the high propensity of HCV to promote chronic persistent infections (Alter, Seeff 2000); evidence that convalescent humans and chimpanzees could be reinfected following re-exposure (Lai et al. 1994); and the considerable genetic heterogeneity of this positive-stranded RNA virus (Simmonds 2004). The situation today is more positive for two reasons. First, we now know that spontaneous eradication of the virus occurs in up to 30 % of acute infections (Seeff 2002) and that this viral clearance is associated with specific immune responses to the virus. Recapitulation of such immune responses by appropriate vaccination is therefore a realistic option. Second, clear evidence for at least some natural immunity has emerged recently in both humans (Mehta et al. 2002) and chimpanzees (Bassett et al. 2001; Weiner et al. 2001; Lanford et al. 2004). Chimpanzees are the only animal model available and develop only mild clinical sequelae. Convalescent humans and chimpanzees are protected against re-exposure to the virus in the majority of cases, even against very divergent viral strains. Importantly, protection is usually at the level of prevention of progression to chronic, persistent infection following re-exposure rather than prevention of acute reinfection, but this could translate to effective prophylaxis because, in humans, it is the chronic, persistent nature

of HCV infection that is mainly associated with viral pathogenicity (Alter, Seeff 2000; Seeff 2002). Although some re-exposed individuals develop chronic infection (Farci et al. 1992), most do not (Bassett et al. 2001; Weiner et al. 2001; Mehta et al. 2002; Lanford et al. 2004). This suggests that the generation of at least a partly effective vaccine against HCV is feasible. Indeed, emerging vaccine efficacy data from the chimpanzee challenge model indicate that it is possible to impede the progression to chronic infection in vaccinees. Until very recently (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005), it was not possible to grow HCV efficiently in cell culture, and thus the use of inactivated or live attenuated viral vaccines has not yet been evaluated. Vaccine approaches have therefore included the use of adjuvanted recombinant polypeptide subunits of the virus in attempts to prime viral neutralizing antibodies to the envelope glycoproteins 1 and 2 (gpE1 and gpE2), as well as priming MHC class-II-restricted $CD4^+$ Th and MHC class-I-restricted $CD8^+$ CTL responses to these and other viral proteins. Both types of T cell can secrete antiviral cytokines such as interferon-α (IFN-α), and $CD8^+$ CTLs have the potential to kill infected cells.

Results from the recent studies have shown optimistic observation of successfully vaccination against HCV. These studies involved the use of the recombinant HCV envelope glycoproteins gpE1 and gpE2 as vaccine antigens. Derived from mammalian cells, the two glycoproteins associate together to from a non-disulphide linked gpE1-gpE2 heterodimer that is thought to resemble the pre-virion envelope structure (Ralston et al. 1993). When combined with oil/water-based adjuvants and used to vaccinate naive chimpanzees, this vaccine candidate elicits anti-envelope antibodies as well as Th cell responses to gpE1 and gpE2. Some earlier experiments showed that when vaccinated animals were challenged experimentally with homologous viral inocula, the highest responding animals (in terms of anti-gpE1/gpE2 antibody titres) were completely protected against infection (Choo et al. 1994). Using sensitive reverse transcription polymerase chain reaction (RT-PCR) assays, no viraemia was detected in blood or liver samples at any time after challenge in these seemingly 'sterilized' animals. This apparent sterilizing immunity correlated directly with anti-gpE2 antibody titres that prevent the binding of gpE2 (or the virus itself) to CD81 (Rosa et al. 1996), which has been shown to be an important receptor component for binding of infectious HCV (Pileri et al. 1998; Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005) and for cell entry of lentiviral/HCV pseudoparticles (McKeating et al. 2004). Furthermore, although lower-responding animals became infected, the majority underwent an abortive acute infection that did not result in the persistently infected carrier state (Choo et al. 1994) that in humans can be associated with chronic liver disease (Alter, Seeff 2000; Seeff 2002). Overall, these data showed that the carrier rate in vaccinees was significantly lower than in unimmunized controls (Choo et al. 1994; Houghton, Abrignani 2005). A crucial question that remained for the authors to solve was whether the vaccine derived from strain HCV-1 would protect against heterologous strains of the virus. For that purpose they challenged nine chimpanzee vaccinees with the HCV-H strain that, like the vaccine strain HCV-1, was of the 1a genotype. Although none of the vaccinated animals was protected against acute infection, all but one vaccinee resolved the acute infection and failed to progress to the carrier state (Houghton, Abrignani 2005). By contrast, the majority of control animals became carriers when challenged with HCV-H, indicating that the vaccine significantly reduced chronic, persistent infection (Houghton, Abrignani 2005). These pre-clinical data [and supporting data from other small studies

exploring various gpE1/gpE2 vaccine formulations (Forns et al. 2000; Puig et al. 2004; Rollier et al. 2004)] supported the authors to initiate a clinical prophylactic programme using adjuvanted gpE1/gpE2 that is currently in phase 1 testing.

It is difficult to prime $CD8^+$ CTLs using polypeptide subunit vaccines, although certain adjuvants are capable of eliciting such responses (Polakos et al. 2001; Pearse, Drane 2005). Various forms of plasmid DNA vaccine (Table 1) are also being explored to elicit HCV-specific humoral and cellular immune responses to encoded antigens which, by virtue of being newly synthesized in the cytosol of transfected cells, can be particularly effective at priming $CD8^+$ CTLs. DNA vaccines also include immunostimulatory deoxycytosine-deoxyguanosine (CpG)-containing motifs capable of activating antigen-presenting dendritic cells (Houghton, Abrignani 2005). This would lead to stimulation of innate immune responses (such as the synthesis of type 1 interferons and natural killer (NK) cells) as well as adaptive B- and T-cell responses to vaccine antigens. Various live attenuated

Table 1. Prophylactic HCV vaccine candidates

Vaccine	Potency	Stages
Recombinant gpE1/gpE2 in oil/water adjuvants (Choo et al. 1994; Houghton, Abrignani 2005)	Protects chimpanzees against chronic infection	Phase 1 clinical trials
DNA prime and protein boost (using C, gpE1, gpE2 and NS3) (Rollier et al. 2004)	Protection or amelioration in chimpanzee challenge model	Pre-clinical
Recombinant VLPs containing C, gpE1, gpE2 (Jeong et al. 2004)	Highly immunogenic in mice and baboons	Pre-clinical
Recombinant gpE1 in alum (Leroux-Roels et al. 2004)	Primes humoral and cellular immune responses in humans	Phase 1/2 clinical trials
Modified vaccinia ankara expressing gpE1/gpE2 (Abraham et al. 2004)	Induces Th1 response in HLA A2.1 mice	Pre-clinical
Semliki forest virus expressing NS3 (Brinster et al. 2002)	Induces NS3-specific CTLs in mice	Pre-clinical
DNA encoding gpE1/gpE2 in poly-lactide-co-glycolide particles (O'Hagan et al. 2004)	Substantial increase in anti-gpE1/E2 titre in mice compared with naked DNA	Pre-clinical
Defective ovine atadenovirus expressing NS3 (Wuest et al. 2004)	Strong Th1 cellular response in mice	Pre-clinical
DNA prime and canary pox boost (encoding all HCV genes) (Pancholi et al. 2003)	Broad Th1 cellular immune responses in mice	Pre-clinical
Defective alphaviral particles expressing gpE1/gpE2 and NS genes (Perri et al. 2003)	Mouse studies in progress	Pre-clinical
Recombinant NS3 protein with polyriboinosinic: polyribocytidylic acid emulsified in Montanide ISA 720 (Jin et al. 2007)	Strong and persistent Th1 cellular immune responses in mice	Pre-clinical
Recombinant core protein (aa 1-122) formulated in Montanide ISA 720 with CpG oligodeoxynucleotides (Roohvand et al. 2007)	Strong humoral and cellular immune response in mice	Pre-clinical

or defective viral or bacterial vectors expressing HCV genes (Table 1) are also being investigated because improved vaccine immunogenicity can result from more efficient expression and delivery of HCV antigens. This may include the targeting of antigen-presenting cells in some cases. The use of various prime/boost immunization modes and regimens (Table 1) are also being explored to optimize vaccine immunogenicity and potency (Houghton, Abrignani 2005).

Potential for therapeutic HCV vaccination

The current standard-of-care therapy for chronically infected HCV patients is a combination of pegylated IFN-α and ribavirin, which is costly, lengthy (6 to 12 months), associated with significant side effects and results in sustained viral response in only ~50 % of patients. In patients infected with genotype 1 response rates are even lower (Saadeh, Davis 2004). With an estimated 170 million HCV carriers worldwide, it is clearly important to develop better therapeutic options. With our increasing knowledge of the virus-encoded enzymes and genetic elements vital to the life-cycle of HCV, much attention is now being focused on the development of HCV protease, replicase, helicase, antisense, silencing RNA and other specific inhibitors. However, preliminary data have directly linked responses to IFN-α and ribavirin with pretreatment titres of viral antibodies (Baumert et al. 2000; presumed to be against the envelope glycoproteins), peripheral Th cell responses to the HCV core and other antigens (Cramp et al. 2000), as well as to intrahepatic $CD8^+$ CTL responses to the virus (Nelson et al. 1998). Total pretreatment $CD8^+$ T-cell counts in the liver have also been correlated with sustained responses to standard-of-care therapy (Vrolijk et al. 2003). Therefore, it may be possible to boost such immune responses in patients by appropriate vaccination and thereby improve the response rate to the standard-of-care therapy. Such immunotherapy may also help control the emergence of escape mutants that would be predicted to arise from any future use of HCV protease or replicase inhibitors, for example, given the extreme fluidity and heterogeneity of the HCV genome (Simmonds 2004). Many therapeutic vaccine trials are planned or are already in progress and use diverse delivery methods and formulations (summarized in Table 2) but little information is available about their efficacy at present. What is known, however, is that use of an alum-adjuvanted recombinant gpE1 antigen was able to boost humoral and cellular immune responses to gpE1 in viraemic patients, providing encouragement that vaccination can increase immune responses in pre-existing carriers (Nevens et al. 2003). It remains to be seen whether boosting viral-neutralizing antibody titres or broad $CD4^+$ Th responses or broad $CD8^+$ T-cell responses will have the greatest impact on reducing viral load and in the response to antiviral therapy. But, as may be the case for optimal prophylaxis, boosting all of these immune responses may be ideal for immunotherapy. HCV tries to counter innate immunity by inhibiting the induction of type-1 interferons (Foy et al. 2005; Li et al. 2005) and downregulating NK cell activity (Crotta et al. 2002; Tseng, Klimpel 2002).

Therefore, therapeutic vaccine formulations could benefit by inclusion of molecules capable of triggering innate immune responses. Such molecules include oligonucleotides containing CpG motifs that trigger Toll-like receptor 9 within dendritic cells and that also enhance adaptive immune responses to vaccine antigens (Abel et al. 2005). If successful, vaccination for the treatment of chronic hepatitis C would be one of the first demonstrations of immunotherapeutic intervention in chronic viral infections, although,

Table 2. HCV immunotherapeutic vaccine candidates

Vaccine	Potency	Stage
Alum-adjuvanted E1 glycoprotein (Nevens et al. 2003)	Boosts humoral and cellular immune responses to gpE1 in HCV patients. May ameliorate hepatitis	Phase 1/2 patient trials
Oil/water-adjuvanted gpE1/gpE2 proteins (Choo et al. 1994; Houghton, Abrignani 2005)	Prophylactic efficacy in chimpanzees. Boosts anti-gpE1/gpE2 antibody titres in chronically infected HCV chimpanzees	Phase 1b patient trials
ISCOMATRIX-adjuvanted (Polakos et al. 2001; Pearse et al. 2005) core protein	Primes Th1-type $CD4^+$ and $CD8^+$ CTL responses in macaques and uninfected humans to conserved epitopes within core antigen	Phase 1b patient trials
ISCOMATRIX-adjuvanted (Polakos et al. 2001; Pearse et al. 2005) NS3-NS4-NS5-C polyprotein	Primes broad Th1-type $CD4^+$ and $CD8^+$ CTL responses in chimpanzees which when challenged with heterologous HCV have reduced viraemia and hepatitis relative to control	Pre-clinical
Heat-killed yeast expressing C and NS3 (Franzusoff et al. 2005)	Primes specific $CD4^+$ and $CD8^+$ T cells in mice	Pre-clinical

very recently, such an approach has been used successfully to inhibit the age-related emergence of herpes zoster infections and disease in carriers (Oxman et al. 2005).

Hepatitis B virus core for display of foreign epitopes

Hepatitis B virus (HBV) core protein or antigen (HBcAg) was first reported as a promising VLP carrier in 1986 (Newton 1987) and published in 1987 (Clarke et al. 1987). The HBcAg particles were the beginning of a long list of structurally well-defined icosahedral VLP carriers and to this day remain one of the most flexible and immunologically most powerful epitope carrier candidates. HBcAg consists of 180 or 240 copies of identical polypeptide subunits. The multifunctional character of HBcAg seems to be responsible for the unusual flexibility of the core protein. The HBc polypeptide is able to self-assemble and was therefore selected as a target for protein engineering manipulations.

In many ways HBcAg holds a unique position among other VLP carriers because of its high expression level and efficient particle formation in mostly all known homologous and heterologous expression systems, including bacteria. Over-expressed HBc protein showed correct self-assembly into naturally-shaped particles in the absence of any other viral component. Electron microscopy revealed the ultrastructural identity of the HBc particles derived either from HBV virions and infected hepatocytes, or from *Escherichia coli* (Cohen, Richmond 1982) or yeast (Yamaguchi et al. 1988).

The fine structure of HBc particles (Fig. 2) was revealed by electron cryomicroscopy and image reconstruction (Crowther et al. 1994; Bottcher et al. 1997; Conway et al. 1997). Finally, this three-dimensional structure was confirmed by X-ray crystallography at 3.3-Å

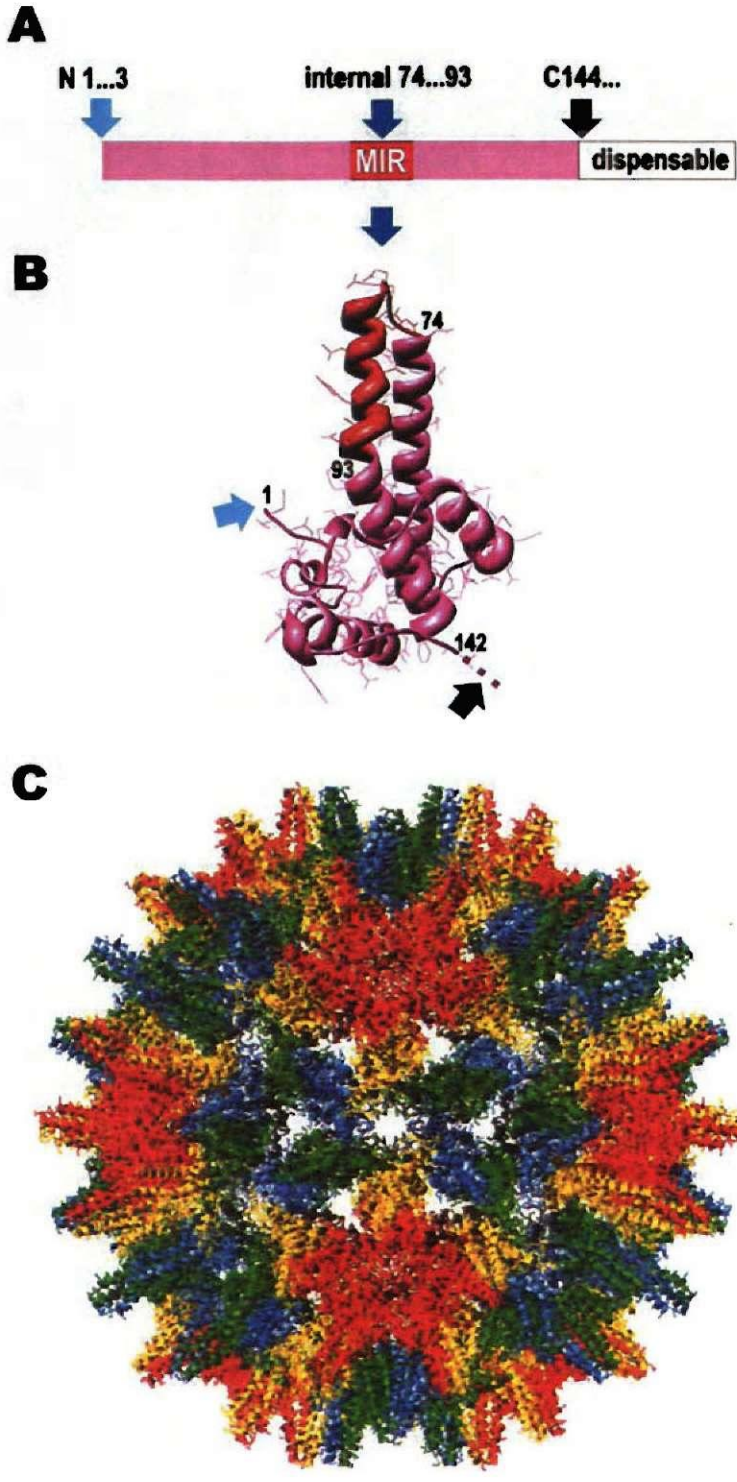
resolution (Wynne et al. 1982). Organization of HBc particles was found largely α-helical (Fig. 2B, C) and quite different from previously known viral capsid proteins with β-sheet jelly-roll packings (Wynne et al. 1982; Crowther et al. 1994). The HBc monomer fold is stabilized by a hydrophobic core that is highly conserved among human viral variants. Association of two amphipathic α-helical hairpins results in the formation of a dimer with a four-helix bundle as the major central feature. The dimers are able to assemble into two types of particles, large and small, that are 34 and 30 nm in diameter and correspond to triangulation number T = 4 and T = 3 packings, containing 240 and 180 HBc molecules, respectively. The major immunodominant region (MIR) with the central positions amino acids (aa) 76-81 is located at the tips of the α-helical hairpins (Fig. 2B) that form spikes on the capsid surface (Wynne et al. 1982).

In addition to MIR, the region aa 127-133 is the next exposed and accessible epitope on the particle surface. This region is located at the end of the C-terminal α-helix and forms small protrusions on the HBcAg surface.

Of special structural value was the clear demonstration of dispensability of the C-terminal protamine-like arginine-rich domain of the HBc protein (aa 150-183) for its self-assembly capabilities in the so-called HBcΔ particles (Borisova et al. 1988; Gallina et al. 1989; Inada et al. 1989). The HBcΔ particles formed by C-terminally truncated polypeptides were almost indistinguishable from the HBc particles formed by full-length HBc polypeptides, as shown by electron cryomicroscopy (Crowther et al. 1994). However, unlike the full-length HBc particles, HBcΔ particles were less stable, failed to encapsidate nucleic acid, and usually accumulated as empty shells (Borisova et al. 1988; Birnbaum, Nassal 1990; Bundule et al. 1990; Hatton et al. 1992; Ulrich et al. 1992; Crowther et al. 1994). The C-terminal limit for self-assembly of HBcΔ particles was mapped experimentally between aa residues 139 and 144 (Inada et al. 1989; Birnbaum, Nassal 1990; Seifer, Standring 1995).

The extremely high immunogenicity of HBcAg particles has been known for a long time. Thus, HBV patients develop a strong and long-lasting humoral anti-HBc response (Hoofnagle et al. 1973). Among the HBV polypeptides, HBc induces the strongest B-cell, T-cell, and CTL response (Chisari, Ferrari 1995). HBcAg is known to function as both a T-cell-dependent and T-cell-independent antigen (Milich, McLachlan 1986). Following immunization, it primes preferentially Th1 cells, does not require an adjuvant (Milich et al. 1997a), and is able to mediate anti-HBs response (Milich et al. 1987). Recently, enhanced immunogenicity of HBcAg was explained by its ability to be presented by B cells as the primary antigen to T cells in mice (Milich et al. 1997b). HBcAg elicits a strong CTL response

Fig. 2. General structural features of the HBc protein as a putative VLP carrier. A linear presentation of the HBc gene with localization of the (i) MIR (major immunodominant region), (ii) self-assembly dispensable protamine-like C-terminal region (aa 145-183), and (iii) preferable sites for the insertion of foreign epitopes (shown by blue arrows) (A). A three-dimensional presentation of the HBc monomer (chain A) derived from the crystal structure (Wynne et al. 1999) with localization of alpha-helices and MIR (colored red), insertion sites for foreign epitopes are marked by the arrows as in the (A). The N- and C-terminal, as well as MIR border amino acid residues of the map are deciphered (B). The T = 4 HBc capsid viewed down an icosahedral threefold axis (C). HBc chains are colored as follows: A - orange red, B - gold, C - green, D - blue. The maps are the generous gift of R. Anthony Crowther. Molecular graphics images were produced using the UCSF Chimera package (Pettersen et al. 2004) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).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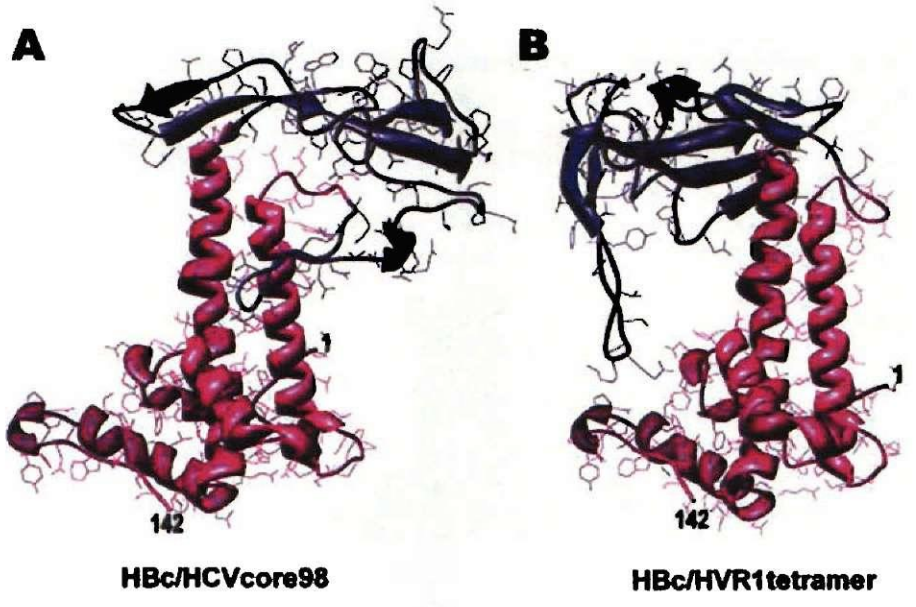

Fig. 3. Prediction of the spatial structure of the chimeric HBc/HCV monomers with insertions at MIR: HBcMIR/HCVcore98 (A) and HBcMIR/HVR1tetramer (B). An X-ray based three-dimensional map of the molecules was calculated on the basis of the HBc carrier X-ray data (T = 4, resolution 3.3 Å; Wynne et al. 1999) by the 3D-JIGSAW program (Contreras-Moreira, Bates 2002) and presented by the Chimera program (see Fig. 2). HCV inserted sequences are colored blue. The N- and C-terminal amino acid residues of the maps are deciphered.

during HBV infection (Mondelli et al. 1982), and this response is maintained for decades following clinical recovery, apparently keeping the virus under control (Rehermann et al. 1996).

Experimental search for appropriate target sites for foreign insertions pointed to the MIR region at the tip of the spike and to the N and C termini of the HBc molecule (Milich et al. 1995; Pumpens et al. 1995; Schodel et al. 1996; Ulrich et al. 1998; Murray, Shiau 1999; Pumpens, Grens 1999; Karpenko et al. 2000; Pumpens, Grens 2001). These findings are in general agreement with the X-ray data because these regions do not participate in the critical intra- and intermolecular interactions (Wynne et al. 1999).

HBc/HCV chimeric proteins as a model for an HCV vaccine

Several experiments on insertion of HCV protein fragments into HBc vectors were already done, but the purpose of these experiments was different. C-terminal insertions of the HCV core protein demonstrated the extraordinary capacity of the HBc particle as a VLP carrier: a 559-aa-long insertion did not prevent self-assembly of chimeras, and even a 741-aa-long insertion allowed production and self-assembly of chimeras to some extent (Yoshikawa et al. 1993). C-terminally added HCV core (Wu et al. 1999) and NS3 (Claeys et al. 1995) sequences were used successfully for detection of specific antibodies in HCV enzyme immunoassay.

Important practical advantage of the HBc model lies in the fact that chimeric HBc-derived particles due to their particulate nature are easy to purify by gel filtration or sucrose gradient centrifugation (Pumpens, Grens 2001). C-terminally truncated variants can be subjected to dissociation with subsequent re-association, in order to remove internal impurities and produce nucleic acid-free preparations. A special purification protocol for preparation of HBc derivatives of vaccine quality was elaborated by addition of a 6 histidine tag to the truncated C-terminus of the HBc protein (Wizemann, von Brunn 1999). On the other hand, the ability of full-length or special chimeric HBc derivatives to controlled encapsidation of nucleic acids may be used for the further development of this carrier for gene therapy experiments (Pumpens, Grens 2001).

One of the crucial questions in construction of chimeric VLPs is whether they will retain the ability of formation of complete, well-structured and stable capsids after insertion of foreign sequences. There are some modeling programs which allow prediction to some extend or at least visualization how chimeras could look like. But nevertheless computer modeling is not able to provide an answer to this important problem, and the only path is to try everything in practice. For the construction of HBc/HCV immunogen we have chosen two HCV determinants: HCV core aa 1-98 and HCV HVR1 region. HCV core 1-98 and HVR1 insert (created of four HVR1 variants; HVR1tetramer) were inserted at MIR as well as fused to C terminus of truncated HBc. Detailed information on description of chimeric HBc/HCV VLP construction, purification and analysis was published by Mihailova et al. 2006a. Chimeric proteins with insertions at MIR (HBcMIR/HCVcore98 and HBcMIR/HVR1tetramer) failed to form VLPs in both cases. Based on the predictions made by the 3D-JIGSAW program (Contreras-Moreira, Bates 2002) for these two chimeric constructs (Fig. 3), foreign inserts made of predominantly β-sheets do not form compact structure on the tip of the spike of the monomer of HBc protein but somewhat flat formation. Considering that basic unit of HBc VLPs is the dimer of HBc polipeptides such flat formation could be the reason for disruption of VLP organization. Indeed, electron microscopy analysis confirmed that HCV core 1-98 and HVR1tetramer inserts at MIR of HBc formed only clumps of uncompleted or disrupted capsids (Fig.4 B,D). Our experiments show that computer modeling can be successfully used for prediction of VLP formation; although these data should be confirmed experimentally. Two other constructs containing inserts at C terminal part of truncated HBc (HBcCterm/HCVcore98 and HBcCterm/HVR1tetramer) formed complete virus-like particles (Fig.4 C, E). Although construct with HCV core insert at C terminus failed to induce strong HCV specific immune response in mice, the other construct bearing HVR1 multi-epitope showed very promising results. In the study we used not a single HCV HVR1 sequence but a combination of four HVR1 variants in order to construct a model of a multi-epitope immunogen capable of inducing antibodies reacting with a large number of HVR1 sequence variants. As a result, the sera of mice immunized with the chimeric HBc VLP bearing such a tetrameric molecule were able to react with 55 % of variants of HVR1 peptides derived from 172 field HCV isolates of different genotypes (Mihailova et al., unpublished data). The same sera were also able to inhibit the binding of HCV-like particles (comprised of E1/E2 proteins, derived using baculovirus expression system in insect cells) of different HCV subtypes (1a and 1b) to Huh7 cells in surrogate "neutralization" assay (Mihailova et al., unpublished data). HCV HVR1 tetramer as a Histidine tagged protein also showed very interesting results. The method describing HVR1tetramer construct, protein purification and immunization of

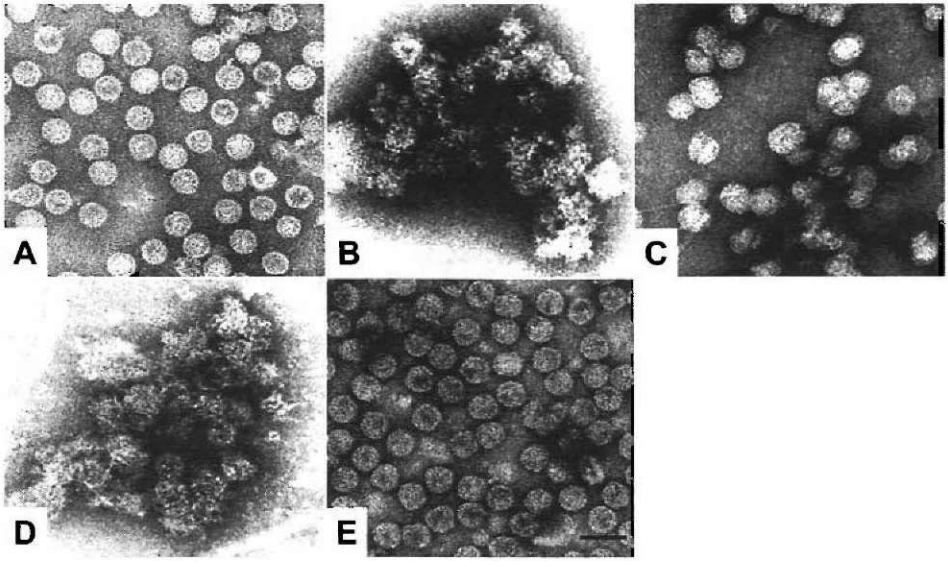

Fig. 4. Comparison of electron microphotographs of purified chimeric HBc/HCV particles with insertions at MIR or C terminus of HBc protein. HBc particles made of original truncated HBc protein (A) and of chimeric HBc/HCV proteins: HBcMIR/HCVcore98 (B), HBcCterm/HCVcore98 (C), HBcMIR/HVR1 tetramer (D), and HBcCterm/HVR1tetramer (E). Bar = 50 nm.

mice is available in a paper published by Mihailova et al. (2006b). The HVR1 tetramer complex was reactive with 75 % of chronically infected patients' sera including sera from patients infected with HCV subtypes 1a, 1d, 2b, 3a and 4. At the same time, the single component of our tetrameric HVR1 construct, the mimotope R9, alone was recognized only by 45 % of the sera and showed much more narrow subtype recognition (Mihailova et al. 2006b). Our finding indicates that it is possible to induce relatively broad and cross-reactive immune response against HCV.

Future directions

In the future, it will be important to use the chimpanzee model to further define correlates of protection, duration of vaccine-mediated protection, the extent of cross-protection against diverse genotypes and mechanisms of chronicity and to determine optimal vaccine formulations for prophylactic and immunotherapeutic efficacy. In addition, human cohorts at high risk of infection need to be identified and characterized for efficacy trials. The huge burden of chronically infected HCV patients facilitates the testing of various immunotherapeutic vaccine formulations that, most probably, will be especially useful when used as adjunct therapy with antiviral drugs, including pegylated IFN-α and ribavirin as well as the new class of HCV drugs currently under development that inhibit viral enzymes and other elements crucial to the viral life-cycle. It will also be important to understand the mechanisms involved in immune dysfunction and evasion during chronic HCV infections so as to facilitate the design of further immunotherapies.

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Vai vakcīna pret HCV ir iespējama?

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Kopsavilkums

Pavisam nesen tika atklāti fakti par iedzimto imunitāti pret hepatīta C vīrusu (HCV). Daži inficēti indivīdi tiek galā ar vīrusu, turpretī citiem pacientiem ir nepieciešama intensīva ārstēšana ar pretvīrusu preparātiem, kas ir dārga un ne vienmēr efektīva. Tādēļ, daudz pūļu velta tādas efektīvas vakcīnas izveidošanai, kas stimulētu humorālu un šūnu imūno atbildi pret HCV proteīniem un to imundominantiem epitopiem un ļautu uzlabot infekcijas iznākumu. Vairāki HCV vakcīnu kandidāti pārbaudīti šimpanzēs, un iegūtie rezultāti ļauj optimistiski skatīties uz vismaz daļēji efektīvas vakcīnas izstrādi pret tādu augsti heterogēnu patogēnu kā HCV. Hepatīta B vīrusa kora daļiņas var veiksmīgi izmantot kā HCV epitopu nesējas. Himerās HBc/HCV vīrusveidīgās daļiņas ir interesants un cerīgs līdzeklis HCV vakcīnu izveidē.

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Nondestructive methods in plant biology: an accurate measurement of chlorophyll content by a chlorophyll meter

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Abstract

Nondestructive methods of plant analysis are becoming increasingly popular in plant biology. Optical measurement of leaf chlorophyll content allows to produce reliable results without destructive sampling. The aim of the present investigations was to validate the use of an absorbance-based chlorophyll meter in different fields of plant biology. A near-linear relationship was found between spectrophotometrically determined total chlorophyll content on fresh mass basis and SPAD values measured by a chlorophyll meter for all of the tested plant species. In experiments with galled plant tissues localized changes in chlorophyll content in the infested leaf indicated a significant effect of the gall-former on photosynthesis *Ulmus laevis*. A contrasting effect of the level of gall infestation on photosynthesis-related characteristics was revealed in studies with *Salix fragilis* and *Tilia platyphyllos*. A pronounced rhythmicity of chlorophyll content in leaves of bean seedlings allowed to characterize an endogenous circadian rhythm in photosynthetic activity. Changes in chlorophyll content in leaves of wheat plants were used as an early indicator to predict the effectivity of application of different foliar fertilizers. The obtained results support the potential usage of nondestructive chlorophyll measurement by chorophyll meter in various branches of plant biology, including ecophysiology, plant physiology, and agricultural sciences, when appropriate accurate calibration of SPAD readings against spectrophotometrically determined total chlorophyll content is made for every particular species.

Key words: chlorophyll, nondestructive methods, plant biology.

Introduction

During the last twenty years, nondestructive methods of plant analysis have become increasingly popular in plant biology. These methods allow to characterize important physiological processes of intact plants without injurous sampling of the plant tissues. Chlorophyll *a* fluorescence has been widely used to assess plant adaptation to an environment as well as to measure the stress level experienced by a plant (Oxborough 2004). Nuclear magnetic resonance micro-imaging allows to map plant tissue metabolites using intact plants (Köckenberger 2001). One of the most widely used group of nondestructive methods in plant sciences is optical measurement of leaf chlorophyll content.

A traditional measurement of chlorophyll amount involves extraction of plant tissues

with a solvent with subsequent spectrophotometric measurement of the absorbance (Lichtenthaler, Wellburn 1983). The major drawbacks of the method are destructive sampling and a time-consuming protocol. Several nondestructive optical methods for chlorophyll measurement have been developed in recent years based on absorbance or reflectance of chlorophyll molecules by leaf tissues at particular wavelengths (Richardson et al. 2002). Absorbance-based chlorophyll measurement has become accepted mostly in small-scale ecophysiological experiments (Neufeld et al. 2006). Commercially available portable chlorophyll absorbance meters measure difference in absorbance at two wavelengths: near 660 nm (absorbed by chlorophyll) and near 940 nm (a reference to adjust for differences in leaf structure). However, reflectance method has been used in more specific fields of ecological studies, e.a. remote sensing (Gitelson, Merzlyak 1997).

The absorbance method is fast, easy to use and produce reliable estimates of relative chlorophyll content. It is of special importance in studies where repetitive chlorophyll measurement of the same plant material over prolonged period of time is necessary or when nondestructive methods are preferred. However in the majority of physiological studies traditional methods of chlorophyll measuremet are still used. This can be related to certain possible problems with the absorbance method e.g. non-linearity of the optically measured chlorophyll amount relative to spectrophotometrical measurements (Richardson et al. 2002; Uddling et al. 2007), side effects produced by environmental conditions (Martínez, Guiamet 2004; Neufeld et al. 2006) or others.

The aim of the present experiments was to elaborate a startegy for calibration of chlorophyll meter measurements by means of chemical chlorophyll analysis. Examples for use of the method in ecophysiology, plant physiology, and agriculture are given.

Materials and methods

Plant material

Gall former effects on leaf chlorophyll content were studied using *Ulmus laevis* Pall. infested with *Colopha compressa* Koch. (Homoptera: Aphididae), *Tilia platyphyllos* L. infested with *Eriophyes tiliae* Pgst. (Acarina: Eriophydae), and *Salix fragilis* L. infested with *Pontania vesicator* Br. (Hymenoptera: Tenthredinidae) growing at the National Botanical Garden of Latvia, Salaspils. Leaves of *Ulmus laevis* were analyzed four times during a growth season. Chlorophyll content in leaves of *Tilia platyphyllos* was estimated twice during the season. Leaves of *Salix fragilis* were analyzed in the second half of the growth season in August.

Daily changes in chlorophyll content were studied in primary leaves of *Phaseolus vulgaris* L.. Plants were sown in individual 250 ml containers with commercial neutralized peat moss and watered with tap water. Plants were kept in laboratory in natural light conditions with average photosynthetic photon flux density of 150 μmol s^{-1} m^{-2}. Simultaneously ten 8-day-old plants were measured. Chlorophyll content was analyzed repetitively every hour within 24 h in both secondary leaves. The experiment was repeated after four days using the same plants.

Effect of foliar fertilizers on leaf chlorophyll content was studied with *Triticum aestivum* L. cv. Jasna plants grown in containers with soil at photosynthetic photon flux density of 150 μmol s^{-1} m^{-2} at the plant level, 16-h-photoperiod. As a substrate, a humus podzolic glay soil was used. Plants were grown in 2-l-containers, with 10 plants per container, four containers per treatment. Plants were sprayed with micronutrient solution

DDMn (Kemira GrowHow) or phosphite-containing foliar macronutrient solution *Phosfik* (Kemira GrowHow) at the stage of fully grown secondary leaves (21 days after sowing). Chlorophyll content was measured repetitively in secondary leaves.

Chlorophyll measurement by a chlorophyll meter

For individual leaves or leaf parts, five to ten successive readings (depending on the area) in SPAD units were taken by a chlorophyll meter SPAD-502 (Konica Minolta, Osaka, Japan) across the whole surface of leaves. The mean of the measurement was calculated using the internal function of the chlrophyll meter.

Spectrophotometric chlorophyll analysis and calibration

Calibration was performed with leaves of an appropriate plant species with different content of chlorophyll. First, chlorophyll amount of the particular leaf was tested by a chlorophyll meter performing ten measurements throughout the leaf. Second, leaves with the known SPAD values were analyzed for total chlorophyll content by means of spectrophotometric analysis.

Ten leaf discs were prepared by a cork borer from a particular leaf. Pigments were

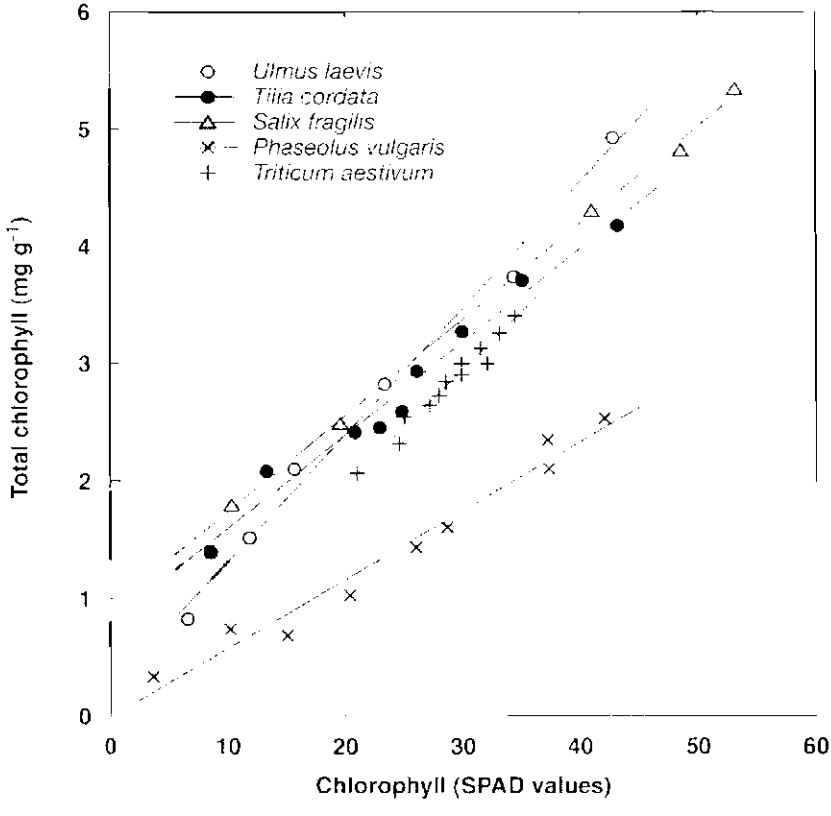

Fig. 1. Calibration of relative chlorophyll units (SPAD values) against spectrophotometrically measured total amount of chlorophyll of the same material. For every data point, SPAD was mesured five to ten times and chlorophyll content was analyzed in triplicate. Mean values are shown.

extracted from a fresh plant material with 96 % ethanol in the presence of $CaCO_3$. Spectrophotometrical readings were made in filtered solutions at 663 and 646 nm (Lichtenthaler, Wellburn, 1983). Data were expressed on a fresh mass basis.

Results

Calibration of SPAD measurements by spectrophometrical chlorophyll analysis

A near-linear relationship was found between spectrophotometrically determined total chlorophyll content on fresh mass basis and SPAD values measured by a chlorophyll meter for all of the tested plant species (Fig. 1). However, for the individual species, characteristic features of the relationship were found. Thus, a characteristic shift of the chlorophyll calibration line off zero was evident for tree leaves, especially, *Tilia platyphyllos* and *Salix fragilis*, indicating that at extremely low leaf chlorophyll levels (below 1.5 mg g^{-1}) the optical chlorophyll measurement method could give uncorrect results. In addition variations in the calibration line slope for different species were evident.

Application of nondestructive chlorophyll measurement in plant ecophysiology

Galls on leaves of *Ulmus laevis* induced by *Colopha compressa* started to develop on May 13. Already two weeks later (May 27) chlorophyll content in the infested leaf below the gall was significantly lower than that above it or in non-infested leaves (Fig. 2). The difference in chlorophyll content due to the gall activity remained significant throughout the vegetation season.

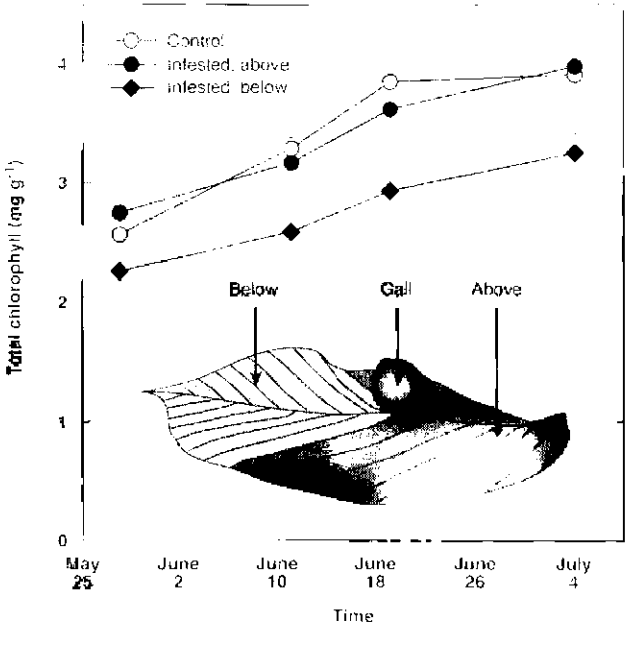

Fig. 2. Time course of chlorophyll content in leaves of *Ulmus laevis* infested with a gall-forming aphid *Colopha compressa*. Five leaves for every data point were measured, with seven SPAD measurements per leaf section. Mean values ±SE are shown.

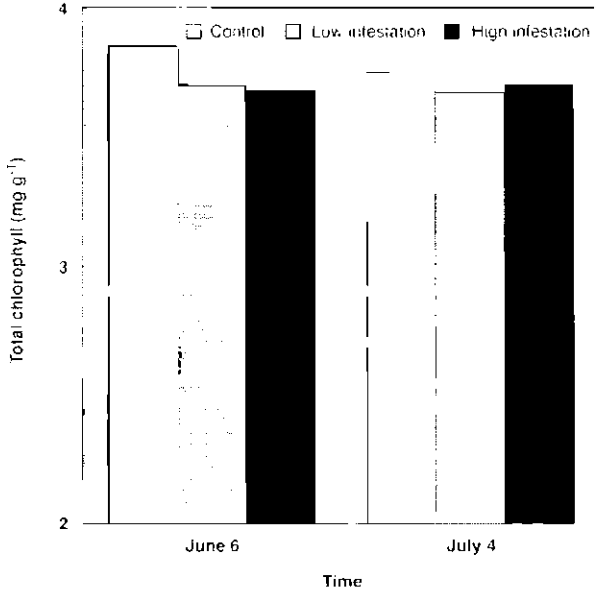

Fig. 3. Chlorophyll content in leaves of *Tilia platyphyllos* with a different level of infestation with a gall-forming mite *Eriophyes tiliae*. Low infestation, 5 to 10 galls per leaf; high infestation, 20 to 30 galls per leaf. Five leaves for every data point were measured, with ten SPAD measurements per leaf. Mean values ±SE are shown.

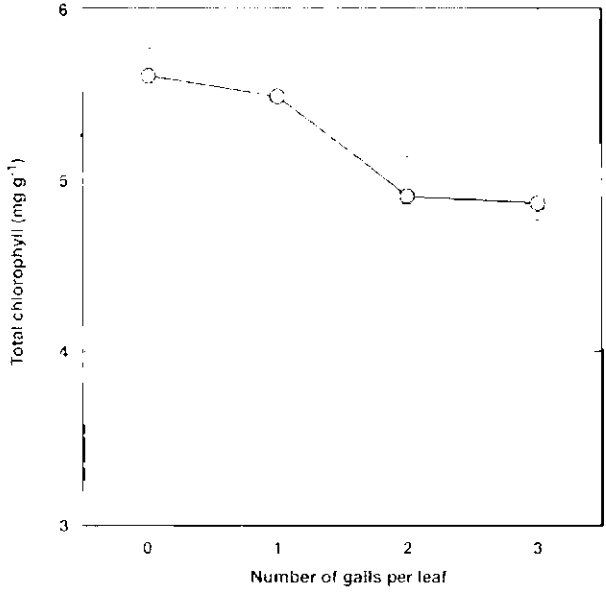

Fig. 4. Effect of number of galls per leaf on chlorophyll content in leaves of *Salix fragilis* infested by gall-wasp *Pontania vesicator*. For every data point, five appropriate leaves were measured, with five SPAD measurements per leaf. Mean values ±SE are shown.

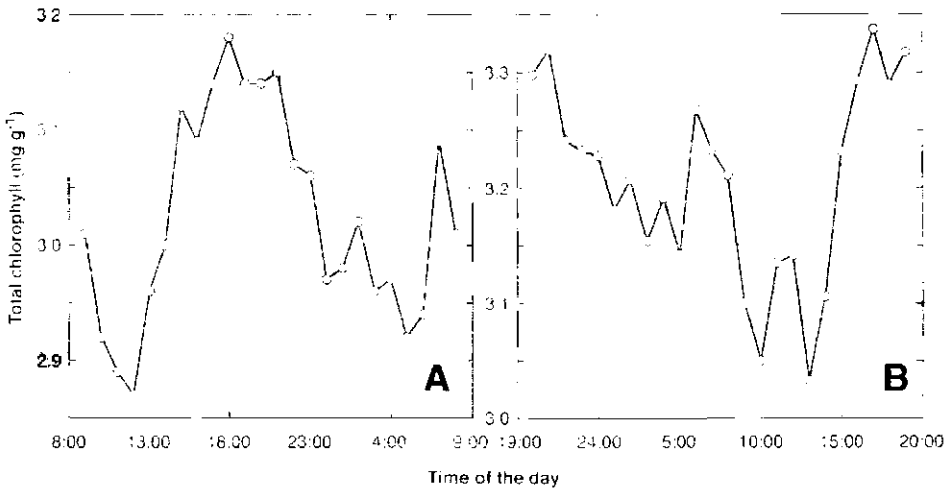

Fig. 5. Daily rhythm of chlorophyll content of *Phaseolus vulgaris* plants. A, 8-day-old plants; B, 12-day-old plants. For every data point, a pair of primary leaves from ten plants was measured, with five SPAD measurements per leaf. Mean values are shown. The gray line corresponds to the mathematically weighed curve (smoothing factor 65 %).

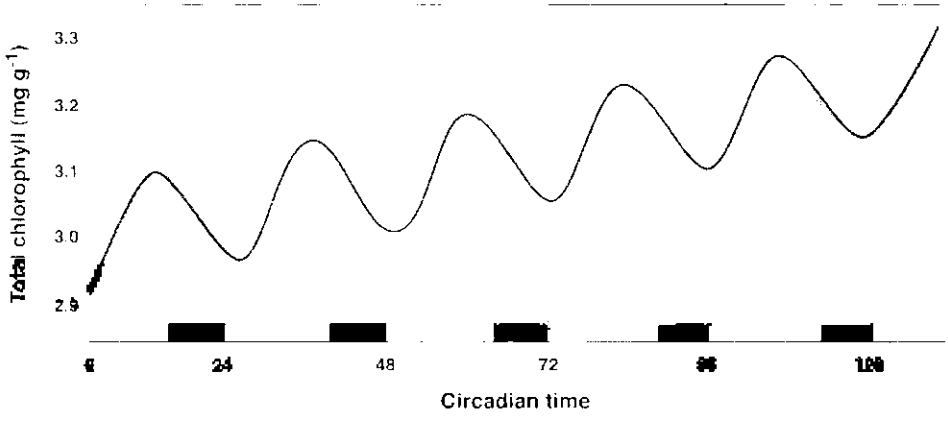

Fig. 6. Interpolated circadian rhythm of chlorophyll content in primary leaves of *Phaseolus vulgaris* plants. Gray line corresponds to the experimental data from Fig. 5. Solid bars at the bottom of the graph indicate the dark period.

In leaves of *Tilia platyphyllos* chlorophyll content was not significantly affected by infestation with a gall-forming mite *Eriophyes tiliae* (Fig. 3). However, there was a tendency to have a statistically significant ($P > 0.05$) lower content of total chlorophyll in the infested leaves early in the season.

Relatively high level of infestation (two to three galls per leaf) with a gall-wasp *Pontania vesicator* resulted in decreased content of chlorophyll in leaves of *Salix fragilis* when measured late in the growth season (Fig. 4).

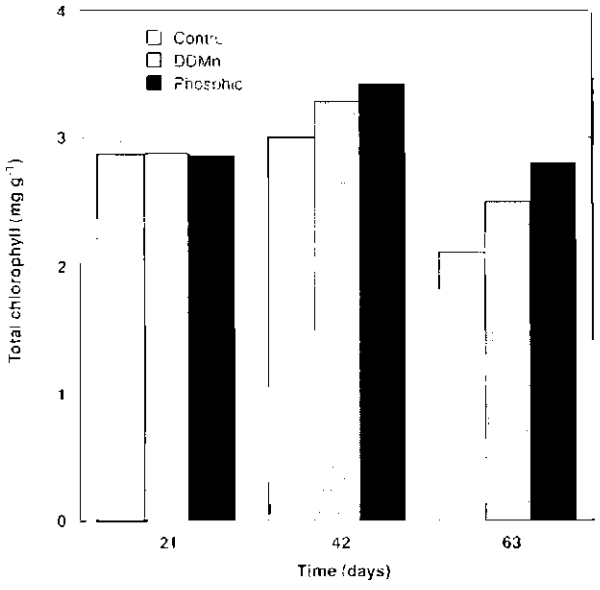

Fig. 7. Effect of foliar fertilizers *DDMn* and *Phosfik* on chlorophyll content in secondary leaves of *Triticum aestivum* plants at different times after sowing. Plants were sprayed with appropriate fertilizer 21 days after sowing. For every data value, five leaves from five plants were measured, with five SPAD measurements per leaf. Mean values ±SE are shown.

Application of nondestrucrive chlorophyll measurement in plant physiology

When a chlorophyll level was measured repetitively every hour within 24 h in primary leaves of 8-day-old bean plants, a pronounced rhythmicity in cholrophyll content was found (Fig. 5A). When the measurement was repeated four days later, a similar rhythm in chlorophyll content was evident, although with a higher absolute level (Fig. 5B). Interpolating both sets of data in time resulted in a pronounced circadian rhythm in chlorophyll content with a daily minimum corresponding to the beginning of a light period and a maximum towards the end (Fig. 6).

Application of nondestrucrive chlorophyll measurement in agricultural sciences

Effect of mineral supply through leaves on changes of chlorophyll content in wheat plants was studied in controlled conditions as an addition to the appropriate field tests. Both micronutrient (*DDMn*) and macronutrient (*Phosfik*) foliar fertilizers applied 21 days after sowing effectively increased total chlorophyll content in the second leaves of both 42- and 63-day-old wheat plants (Fig. 7).

Discussion

Chlorophyll content of leaves is a useful indicator of both potential photosynthetic productivity and general plant vigour (Alonso et al. 2002; Zarco-Tejada et al. 2002). In addition, changes in the amount of chlorophyll may be a part of adaptive responses (Morales et al. 2002). Consequently, the use of non-destructive methods of chlorophyll measurement

provides reliable and effective means of plant analysis in a wide range of biological context. In the present experiments, measurement of leaf chlorophyll content was used for different purposes in the context of various problems of plant biology. First, during studies of gall-forming aphid-host tree relationship localized changes in chlorophyll content in the infested leaf indicated a significant effect of the gall-former on photosynthesis (Fig. 2). Different effect of the level of gall infestation on photosynthesis-related characteristics was revealed in studies with *Salix fragilis* (Fig. 3) and *Tilia platyphyllos* (Fig. 4). Second, a pronounced rhythmicity of chlorophyll content in leaves of bean seedlings (Fig. 5) allowed to characterize an endogenous circadian rhythm in photosynthetic activity (Fig. 6). Third, changes in chlorophyll content in leaves of wheat plants were used as an early indicator to predict the effectivity of application of different foliar fertilizers (Fig. 7).

Changes in leaf chlorophyll content often has been regarded as a relatively late mechanism of photosynthetic adaptation (Anderson et al. 1995). Other mechanisms e.a. regulation of CO_2 supply by stomatal limitation and shifts in photochemistry of photosynthesis are thought to be the primary responses to a changing environment. In the present experiments, change of leaf chlorophyll content was evident in plants in different time scales under the effect of various factors including endogenous rhythm (Fig. 5), mineral nutrition (Fig. 7), and biotic interactions (Fig. 2 and 4). The data collectively indicate that changes in chlorophyll content are a part of an adaptive regulative system of photosynthesis to changes in internal and external environment, presumably acting together with the other means of photosynthesis regulation.

As the relationship between SPAD measurements and extractable chlorophyll concentration had species-specific characteristics, individual calibration for every particular species should be performed. In addition it appears that the SPAD measurements on a plant material with relatively low total chlorophyll content (less than 1.5 mg g^{-1}) should be interpreted with precaution do to a possible nonlinearity and a low accuracy of the measurement, especially for tree leaves. Similar observations have been described earlier for various plant species (Gratani 1992; Monje, Bugbee 1992).

Deviation from linearity in the high and low SPAD range was shown to be caused by a non-uniform distribution of chlorophyll across the leaf surface and multiple scatering, respectively (Uddling et al. 2007). The first can be easily overcome by appropriate multiple SPAD measurements across the leaf and by sampling of an adequate number of leaves. It was shown also that irradiance, leaf water status and time of the day when the measurements were performed may affect a correlation between SPAD readings and extractable leaf chlorophyll content (Martinez, Guiamet 2004). However, it is difficult to generalize the above findings as only two crop plant species (e.g., wheat and maize) were used. In addition a shift by about 2 to 4 SPAD units was usually found caused by the above-mentioned factors, which is within the ordinar biological variability of chlorophyll content within and between individual leaves for most plant species (Ievinsh et al., unpublished data).

Still, the optical method does not allow for a direct comparison of chlorophyll content between different plant species. Most importantly, species-specific leaf traits affecting a correlation between SPAD measurements and extractable chlorophyll content should be taken into account. Leaf surface characteristics (wax, trichomes etc.) as well as presence of microbial agents on leaves and inside them are among important factors in this respect. Thus, age-dependent development of wax layer on leaves of dune xerophyte *Eryngium*

maritimum leads to significant overestimation of chlorophyll content measured as SPAD units (Ievinsh et al., unpublished data).

Our results support the usage of nondestructive chlorophyll measurement by chorophyll meter in various branches of plant biology, including ecophysiology, plant physiology, and agriculture, when appropriate accurate calibration of SPAD readings against spectrophotometrically determined total chlorophyll content is made for every particular species. Together with the other nondestructive methods, e.a. chlorophyll a fluorescence measurements, chlorophyll analysis represents a valuable tool for studies in natural habitats or field experiments allowing continuous measurement of the same plant material. These methods have a special importance in biodiversity studies e.a. ecophysiology of wild plants in native habitats and investigation of genetic resources of agricultural plants.

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Nedestruktīvās metodes augu bioloģijā: precīza hlorofila satura noteikšana ar hlorofilmetru

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Kopsavilkums

Nedestruktīvās augu analīzes metodes augu bioloģijā kļūst arvien populārākas. Lapu hlorofila satura optiskā noteikšana ļauj iegūt ticamus rezultātus bez destruktīvas paraugu ievākšanas. Aprakstīto pētījumu mērķis bija apstiprināt uz absorbcijas pamata izveidota hlorofila mērītāja izmantošanas iespējas dažadās augu bioloģijas nozarēs. Visām analizētajām sugām varēja novērot sakarību starp spektrofotometriski noteiktu kopēja hlorofila daudzumu uz dzīvas masas vienību un ar hlorofila mērītāju noteiktām SPAD mērvienībām, kas bija tuvu lineārai. Pētījumos ar augu pangām lokālas hlorofila izmaiņas infestētajā lapā norādīja uz pangu veidotāja būtisku ietekmi uz saimniekauga *Ulmus laevis* fizioloģiju. Pretrunīga pangu infestācijas pakāpes ietekme uz fotosintēzes īpašībām atklāta pētījumos ar *Salix fragilis* un *Tilia platyphyllos*. Izteikts hlorofila daudzuma ritms pupiņu dīgstu lapās deva iespēju raksturot fotosintēzes aktivitātes endogēno cirkādo ritmu. Hlorofila satura izmaiņas kviešu lapās izmantoja kā agrīnu indikatoru, lai paredzētu dažādu foliāro mēslošanas līdzekļu lietošanas efektivitāti. Iegūtie rezultāti apstiprina iespēju dažādās augu bioloģijas apakšnozarēs (ekofizioloģijā, augu fizioloģijā, lauksaimniecības zinātnēs) izmantot nedestruktīvo hlorofila analīzes metodi, katrai konkrētajai sugai veicot atbilstošu SPAD mērvienību kalibrēšanu attiecībā pret spektrofotometriski noteiktu kopējo hlorofila daudzumu.

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Simultaneous graft union and adventitious root formation during vegetative propagation in elepidote rhododendrons

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Abstract

Light microscopy was used to carry out a detailed study of cutting graft (grafting on unrooted cuttings) in simultaneous graft union and adventitious root formation using elepidote rhododendron cultivars 'Cunningham's White' as rootstock and 'Catawbiense Grandiflorum' as scion. The first visible reaction was the appearance of a necrotic layer in scion, rootstock and the base of rootstock, observed on day 2 after experimental initiation. The next observable reaction was callus formation from cells in the cambial region. The wound vascular cambium was first recognizable on day 21, and the cambium bridge (a continued strand of wound vascular cambium in callus tissue) between rootstock and scion on day 30. Simultaneously, root initial formation from phloem ray was observed on day 30. Root initials differentiated into root primordias and they continued to develop till emergence at the surface of the rootstock base. Cutting graft is a quick propagation technique that reduces substantially time from unrooted cutting graft to field plant. It may be useful as a method of rooting of varieties showing poor rooting potential.

Key words: grafting, rhododendron, rooting, vegetative propagation.

Introduction

Rhododendrons can be propagated both by generative and vegetative methods. Vegetative propagation is an important commercial method of regenerating large quantities of genetically uniform plant material. One method of rhododendron vegetative propagation is grafting on unrooted cuttings (cutting graft), which involves simultaneous graft union and adventitious root formation. Cutting graft is a quick propagation technique, which reduces substantially the time from unrooted cutting graft to field plant (Ackerman et al. 1997).

The development of graft union is a process of forming a functional unit through the interaction of organs, tissues or cells from the same or different plants (Shanfa 2000). Several authors have defined the sequence of structural events during the healing of the grafts both in woody and herbaceous plants. Hartman et al. (2002) provides a review of the sequence of events: the necrotic layer formation, proliferation of new parenchymatous cells from both rootstock and scion that soon intermingle and interlock, filling up the space between the scion and rootstock forming the callus bridge. The wound-repair xylem

and phloem, as well as new cambial cells differentiate from the newly formed callus. New cambium forms a continuous cambial connection between scion and rootstock – cambium bridge. In the last step of the graft establishment process, the newly formed cambial layer in the callus bridge begins typical cambial activity forming new vascular tissue.

In general adventitious roots develop either directly from the stem or indirectly via wound tissue (Grönroos, Arnold 1987). A direct and indirect pattern of root formation may be present in both herbaceous and woody systems (Altamura 1996). Adventitious roots in woody plant stem cuttings usually originate from living parenchyma cells such as phloem, vascular ray and cambium (Hartmann et al. 2002). In most species that are difficult-to-root, and initiation of roots occurs from callus tissue (Hamman 1998).

Simultaneous rooting and grafting has been successfully used in propogation of *Rhododendron* (Eichelser 1967), *Rosa* (Macdonald 1986), *Leucospermium* and *Leucadendron* (Ackerman et al. 1997). However, detailed information about anatomical changes during the simultaneous adventitious root and graft union formation is not available. The objective of the present research was to determine the anatomical changes in elepidote rhododendron cutting grafts during simultaneous graft union and adventitious root formation.

Materials and methods

The investigation was carried out from October 2003 to February 2004 using the rhododendron cultivar 'Cunningham's White' as a rootstock and 'Catawbiense Grandiflorum' as a scion. The plant material was obtained from the Experimental Nursery of Rhododendron Breeding "Babite", University of Latvia. Rootstock and scion materials for grafting were approximately 9 to 12-cm-long current year shoots collected from shrubs of rhododendron shortly before grafting. Scions were side veneer graft to the rootstocks. During the grafting procedure a long shallow cut was made into one side of the rootstock and reverse side of the scion. The scion and rootstock were fitted together and wrapped with woolen yarn. The rooting medium consisting of peat moss and pine needles (1:1) was placed in plastic beds, and the bases of cutting grafts were inserted to a depth of 20 mm. Plastic beds were covered with a polyethylene tent and kept at 23 °C during the day and 20 °C the night, with 16-h photoperiod in the growth chamber.

Two to three graft unions were collected within 58 days after experiment initiation. Cutting-grafts were fixed in solution containing 37 % formaldehyde, glacial acetic acid, 95 % ethanol and distilled water (10:5:50:35, v/v/v/v). Transverse sections were cut by a manual microtome and a razor, or by a rotary microtome (Leica RM2145). The hand sections were stained with Astra Blue - Safranin, rinsed in water, dehydrated in an ethanol-xylol series and permanently embedded in Canada balsam (Braune et al. 1999). At the same time, serial cross sections (25 μm in thickness) were obtained by rotary microtome, dehydrated in an ethanol/tert-butyl alcohol series, embedded in Histowax (Ruzin 1999), stained with Astra Blue - Safranin (Braune et al. 1999) and mounted on glass slides in Canada balsam.

Sections were examined with an Olympus CH30RF200 light microscope and photographed using a Leica DM2000 light microscope equipped with a digital camera Canon Power Shot S40.

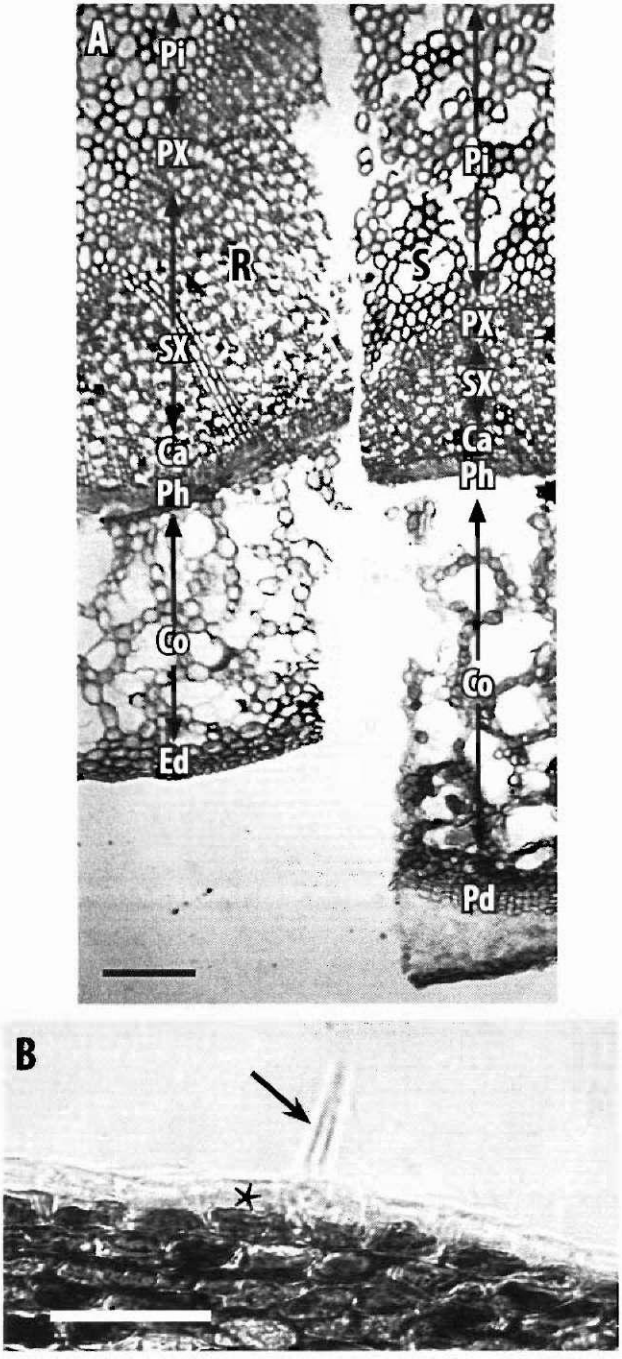


Fig. 1. Transverse sections through a 2-day-old cutting graft. A, anatomy of graft union. Ed, epidermis; Pd, periderm; Co, cortex; Ph, phloem; Ca, cambium; SX, secondary xylem; PX, primary xylem; Pi, pith; R, rootstock; S, scion; B, trichomes (arrows) of scion epidermis (*). Bars 200 μm (A) and 50 μm (B).

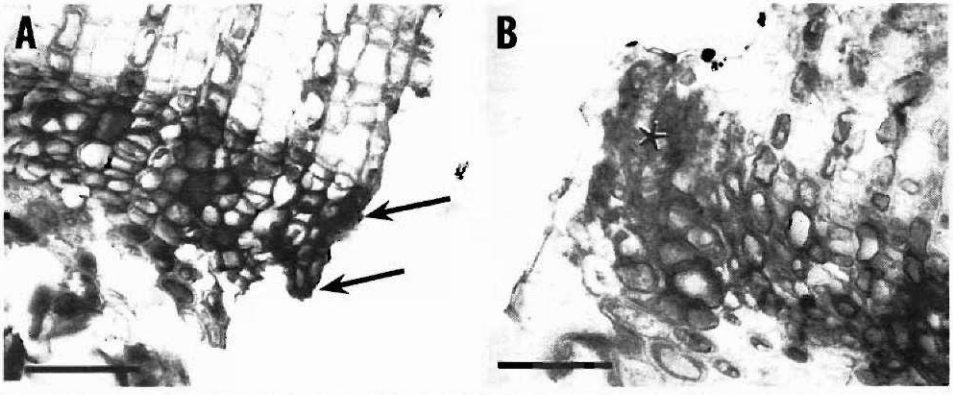


Fig. 2. A, transverse section through a 2-day-old cutting graft shows layer of necrotic tissues (arrows). B, callus formation(*) from the cambial region in a 4-day-old cutting graft. Bars 50 μm.

Results

For successful grafts, it is necessary to match like tissue of stock and scion precisely as it is possible. However, due to small variation in the diameter of the scion and rootstock, improper matching of corresponding tissue often occurs in the graft union. We investigated graft unions with well matched vascular tissues in this study.

The stem anatomy of rootstock and scion were quite similar and typical for woody stem consisting of epidermis, cortex, phloem, cambium, secondary xylem, primary xylem and pith (Fig. 1A). Scion was distinguishable from rootstock only by the presence of trichomes on the epidermis (Fig. 1B). The appearance of a necrotic layer consisting of fragmented and compressed cells was observed on day 2 after experimental initiation in scion, rootstock and the base of rootstock (Fig. 2A).

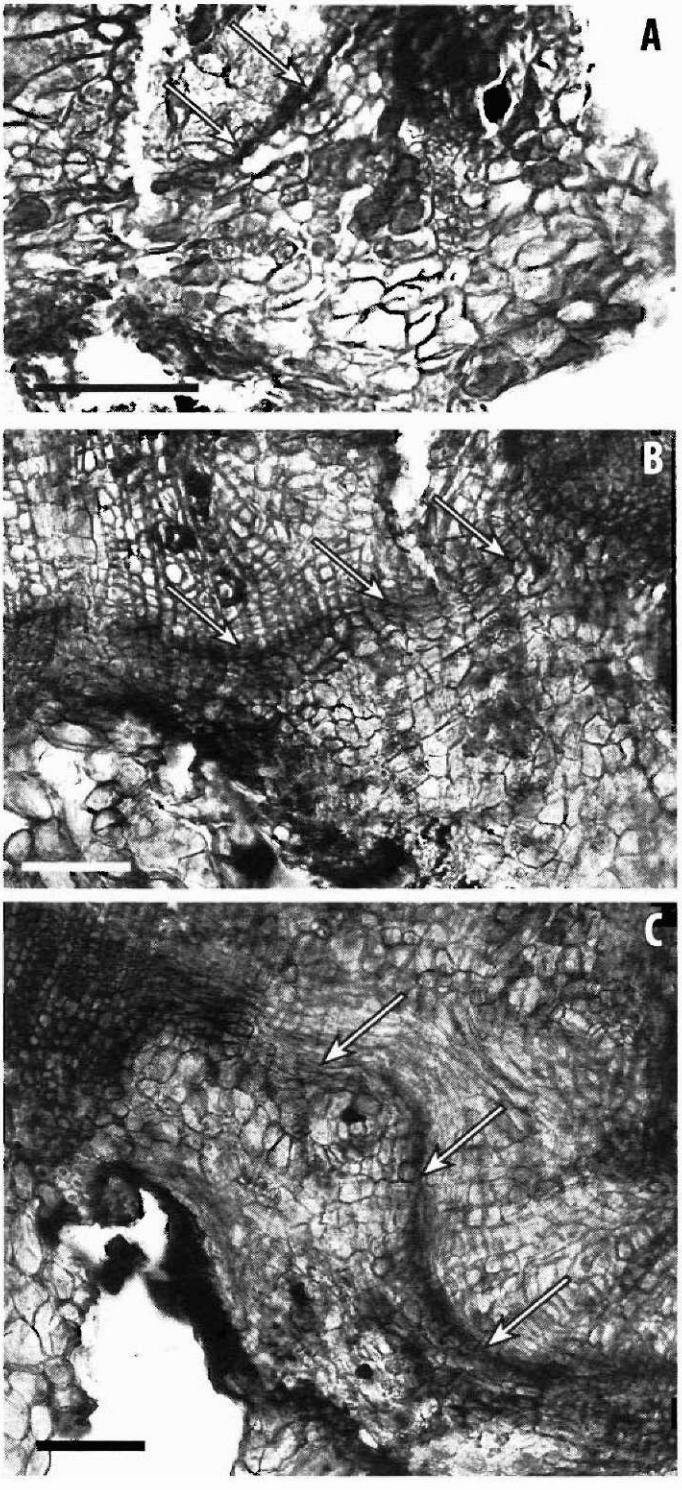
The next observable reaction was callus formation from cells of cambium region (Fig. 2B) on day 4 in graft union and later in the base of rootstock. Callus continued to proliferate and filled the space between rootstock and scion forming a callus bridge. Simultaneously, callus developed at the base of rootstock.

The wound vascular cambium was first recognizable on day 21 (Fig. 3A), and a cambium bridge between rootstock and scion was observed on day 30 (Fig. 3B). Cambium bridge shapes of well-matched graft partners were variable: slightly curved (Fig. 3B) or S-shape (Fig. 3C). Simultaneously, root initial formation from phloem ray was observed on day 30 (Fig. 4A). Subsequently, root initials differentiated into root primordias (Fig. 4B). The root primordia continued to develop until the emergence at the surface of the rootstock base.

Discussion

The graft unit development and adventitious root formation of cutting graft is comparable to graft unit formation and rooting of other woody cuttings. It is known that success

Fig. 3. Different forms of cambium bridge. A, wound vascular cambium (arrows) within callus in a 21-day-old cutting graft; B, a slightly curved cambium bridge (arrows) in a 30-day-old cutting graft; C, S-shape cambium bridge (arrows) in a 36-day-old cutting graft. Bars 100 μm.


A
B
C

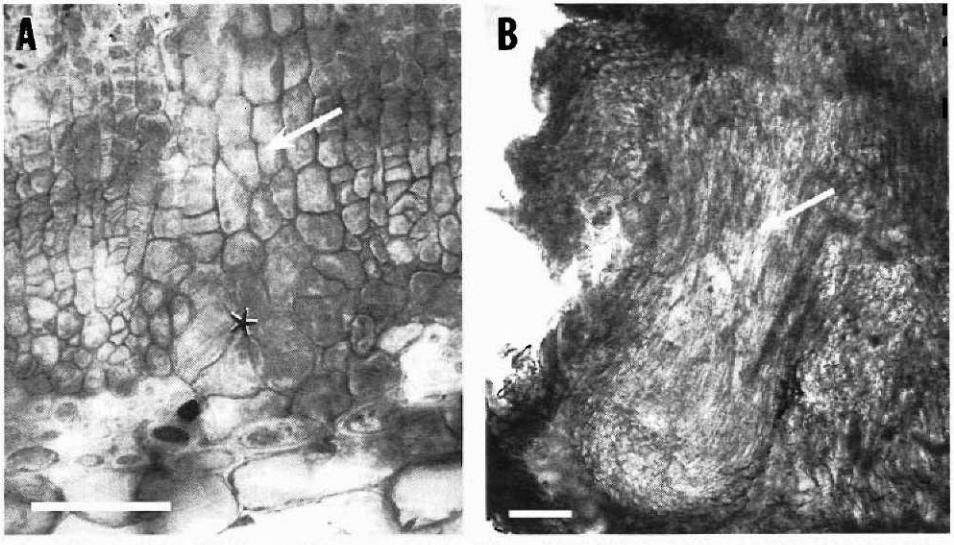


Fig. 4. Adventitious root formation. A, root initial formation (*) from phloem ray cells (arrow) in a 30-day-old cutting graft; B, root primordia (arrow), development in a 44-day-old cutting graft. Bars 50 μm (A) and 100 μm (B).

in grafting of higher plants mainly depends on two essential factors: the physiological compatibility between rootstock and scion, and proper matching of the different tissue of graft partners. (Kollmann et al.1985). At the same time, success in rooting of cuttings depends on two factors: the cutting being in a favourable physiological state at the time of excision and environmental conditions that support the expression of the inherent rooting potential (Cameron et al. 2003).

Grafting includes the formation of a necrotic layer and its subsequent reduction or elimination (Stoddard, McCully 1980). Our results confirm this observation. The formation of callus tissue at the graft interface is the first response to grafting (Pina, Erra 2005). New callus formation is a primary response of wounding that occurs in compatible and incompatible grafts (Moore, Walker 1981) and callus formation is independent of other events in graft development (Pina, Erra 2005). Different views of the origin of callus in various species are present in the literature. Callus can be formed from the cambial region, cortex, pith and xylem ray parenchyma (Hartmann et al. 2002). Our study showed that the cambial region is the main callus producer in cutting grafts of elepidote rhododendrons.

An important step of graft formation is wound vascular cambium formation within callus and the following vascular tissue development. Cambium bridge shapes were variable, depending on rootstock and scion tissue matching (Megre et al. 2004). If the graft partners were well matched, the wound cambium bridge shape can be slightly curved or S-shape. This shape suggests the existence of a high degree of coordinated activity between the cut edges of the pre-existing cambia (Soumelidou et al 1994).

Formation of adventitious roots and wound vascular cambium in the elepidote rhododendron occurred simultaneously. We observed adventitious root formation from phloem ray cells (direct rooting) but callus formation on the base of rootstock and indirect rooting was not seen. Similarly, leaf bud cuttings of *Ficus pumila* form adventitious roots

from phloem ray cells (Davies et al. 1982). However, root primordia can be differentiated from basal callus of some individual mature leaf bud cuttings, and neither these nor the few primordia elongate into well-developed roots (Davies et al. 1982). Direct root formation has been described for *Ficus pumila* (Davies et al. 1982), apple rootstock M26 (Zhou et al. 1992), *Pinus taeda* (Diaz-Sala et al 1996), and *Rhododendron* (Kondratovics, Megre 1999).

The used method is useful for propagating of those woody varieties, which have poor rooting potential, but show histological and physiological compatibility with the rootstock. The studies will be continued to find optimal graft partner combinations.

Acknowledgements

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Mūžzaļo rododendru potējumu saaugšana un adventīvo sakņu veidošanās to veģetatīvās pavairošanas gaitā

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Kopsavilkums

Izmantojot gaismas mikroskopiju, pētīja vienlaicīgu potcelma apsakņošanos un potējuma saaugšanu mūžzaļo rododendru šķirnēm 'Cunningham's White' (neapsakņots potcelms) un 'Catawbiense Grandiflorum' (potzars). Pirmā pamanāmā izmaiņa bija nekrotisku šūnu slāņa izveidošanas potcelma un potzara potejuma griezuma vietā un potcelma pamatnē otrajā dienā pēc eksperimenta sākšanas. Nākamā atbildes reakcija bija kallusa veidošanās kambija tuvumā vaskulārais rētu kambijs pirmoreiz tika novērots 21. dienā, bet kambija tilts (potcelma un potzara veidotā vaskulara retu kambija apvienošanās rezultāts) starp potcelmu un potzaru – 30. dienā. Vienlaicīgi 30. dienā novēroja adventīvo sakņu iniciāļu veidošanos potcelma pamatnē no lūksnes stariem. Turpmākajā gaitā sakņu iniciāļi diferencējas par sakņu aizmetņiem, kuri attīstījas, līdz kļuva redzami uz potcelma pamatnes virsmas. Potēšana uz neapsakņotiem spraudeņiem ir kombinēts veģetatīvās pavairošanas paņēmiens, ko var izmantot, lai ātri iegūtu kokaugu stādāmo materiālu. To var izmantot kā metodi, lai pavairotu tās šķirnes, kurām ir zems apsakņošanās potenciāls, bet kuras labi saaug ar potcelmu.

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Scots pine pointer-years in northwestern Latvia and their relationship with climatic factors

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Abstract

At six sampling sites in northwestern Latvia Scots pines (*Pinus sylvestris* L.) were cored to obtain tree-ring width data and determine site pointer-year values (years with markedly wider or narrower tree-ring width compared to neighboring tree-rings). Correlation analysis was performed between pointer-year values and climatic factors (mean temperatures and precipitation sum) to determine significant relationships. There were only three years (negative 1940 and 1969, positive 1957), when all sampling sites had common pointer-years. The main climatic factors influencing development of pointer-years were February mean temperatures and June precipitation sum. Pointer-year development is mainly determined by local factors.

Key words: dendroclimatology, *Pinus sylvestris*, pointer-years, tree-rings.

Introduction

It is well known that tree-rings contain extensive information about environment changes during the time when the tree has lived (Schweingruber 1996) and one of the features to assess those changes is pointer-years (years with markedly wider or narrower tree-ring width compared to neighboring tree-rings) that indicate regional and local influence of environmental changes (Schweingruber 1990). The suitability of pointer-years to determine extreme growth reactions has been shown already in other studies (Neuwirth et al. 2004; Lebourgeois et al. 2005; Neuwirth et al. 2007).

Scots pine (*Pinus sylvestris* L.) growth depends on growing conditions [pines growing on dunes have narrower tree-rings compared to those growing on firm parental soil (Rigling et al. 2001)], stand structure [density (Tegelmark 1999)] and climatic factors [climatic factors explain up to 50 % of pine tree-ring width variation in Finland (Lindholm et al. 2000)], indicating that it is suitable for pointer-year analysis. As Scots pine is the oldest present-day tree species in Latvia and its area covers the whole Latvia territory (Prieditis 1999) it should be well adapted to local conditions and should show major changes in those conditions.

The aim of this work was to identify pointer-years in tree-ring series of Scots pine at three locations and to determine their relationship with climatic factors. It was hypothesized that there would be common pointer-years between sites.

Materials and methods

Study sites

Sampling sites were located in the northwestern part of Latvia in dry forests. The Kolka 1, Kolka 2, Kolka 3 and Kolka 4 sampling sites were located near the Baltic seashore in Slitere National Park (Fig. 1), the Engure sampling site was located near Engure lake and Spare near Gulbju lake. Sampling site Kolka 1 was located on an inland dune ridge beside a bog, about 6 km from the Baltic Sea; Kolka 2 and Kolka 3 sampling sites about 300 m from the sea on a bluff, some trees partly buried by sand; and the Kolka 4 sampling site on a parallel dune ridges (Brumelis et al. 2005). The Spare sampling site was located on a slope near a lakeshore and the Engure sampling site on low land between Engure Lake and the Riga Gulf.

Climatological data

Climatological data were obtained from the Latvian Environment, Geology and Meteorology Agency for the Kolka, Mersrags and Stende Meteorological Stations. For the Kolka Meteorological Station data were available beginning from 1925 (temperatures) and 1891 (precipitation), for Mersrags from 1896 (temperatures and precipitation), and for Stende from 1924 (temperatures and precipitation). Data from the Kolka Meteorological Station was used for the Kolka 1, Kolka 2, Kolka 3, Kolka 4 sampling sites, Mersrags Meteorological Station data for the Engure sampling site, and Stende Meteorological Station data for the Spare sampling site.

Mean January temperature in northwestern Latvia (Kolka Station) is –2.5 °C and in July 16.7 °C, yearly mean precipitation sum is about 584 mm. At the Spare sampling site

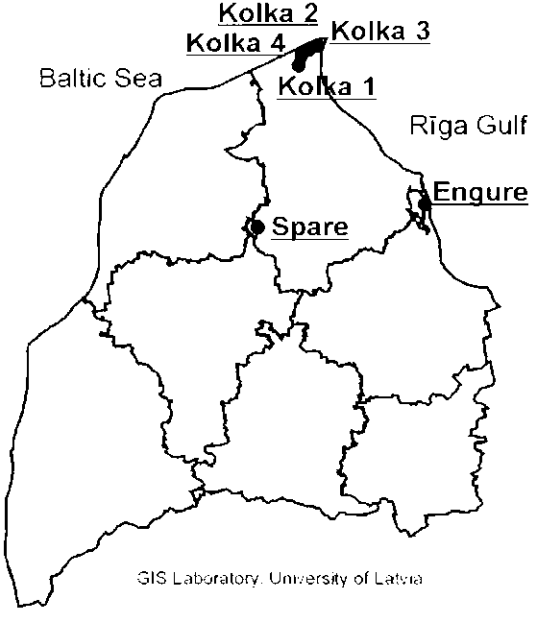

Fig. 1. Location of sampling sites in Latvia.

(Stende Station) winter temperatures are lower (mean January temperature –4.1 °C) and precipitation sum higher (668 mm).

In total 34 climatic factors were used – mean monthly temperatures and precipitation sum (from prior year October till September); autumn (prior year September - November), winter (prior year December - February), spring (March - May), summer (June - August) and growing season (prior year October - September) mean temperatures and precipitation sum.

Sample collection and measurement

Sample collection took place in 2001 (Kolka 1, Kolka 2, Kolka 3, Kolka 4) and 2006 (Engure, Spare). Tree-ring width samples (wood cores) were taken from the oldest trees. One (Kolka 1, Kolka 2, Kolka 3, Kolka 4) or two (Engure, Spare) tree-ring samples per tree were taken with an increment borer at breast height (1.3 m); overall 20 trees at Engure, 20 trees at Spare, 20 trees at Kolka 1, 14 trees at Kolka 2, 29 trees at Kolka 3 and 26 trees at Kolka 4 were sampled. For the tree-ring width measurement a LINTAB "measuring table" connected with TSAP software (Rinn 1996) was used. Cross-dating and quality control of tree-ring measurement were performed using the program COFECHA (Holmes 1983). After tree-ring width measurement and quality control for Engure and Spare sampling sites average tree-ting width was calculated for each tree that had two cores.

Statistical analysis

Analysis was performed for the time period from 1890 till 2006. Pointer-year intensity values were calculated using a modified Skeleton-plot method (Neuwirth et al. 2004). Tree-ring width of each tree was compared to the previous 5 year mean tree-ring width and the difference was expressed as intensity classes (5th intensity class was reached for a difference greater than 80 %; 1st intensity class for a difference less than 20 %). Site pointer-year intensity (I) was calculated using formula:

$$I = \frac{100}{k \times n} \sum_{i=1}^{k} h_i \times i_i ,$$

where k – number of intensity classes; n – total number of trees; h_i – number of trees in intensity class; i_i – value of intensity class.

Correlation analysis was performed using SPSS software (Morgan et al. 2004) between sampling site pointer-year intensity values and climatic factors (mean temperature and precipitation sum) in two ways: (a) all pointer-year intensity values for each sampling site were used; (b) only significant pointer-year intensity values (intensity values greater than 25 % or less than –25 %) were used. Climatic factors were expressed as standard deviation units – from each climatic factor value the mean value for the analyzed time period was subtracted and the result was divided by the standard deviation for that time period.

Results

After quality checking and cross-dating, 10 trees at Kolka 1, 12 trees at Kolka 2, 18 trees each at Engure, Kolka 3, and Spare and 20 trees at the Kolka 4 sampling sites were used for further analysis. In the time period from 1890 till 2006, Kolka 2 site had the largest number of significant pointer-years per 10 years (4.0; Table 1), Kolka 3 site had 3.9 but

Table 1. Number of positive, negative, total and 10-year average significant pointer-years (pointer-year intensity value above 25 % or below -25 %) at sampling-sites

Sampling site	Engure	Kolka 1	Kolka 2	Kolka 3	Kolka 4	Spare
Positive	13	19	22	21	14	19
Negative	18	19	25	25	19	22
Total	31	38	47	46	33	41
Average in 10 years	2.6	3.2	4.0	3.9	2.8	3.5

Table 2. Cases of statistically significant Pearson correlation (at $\alpha = 0.05$) between all pointer-year intensity values, only significant pointer-year intensity values (intensity values greater than 25 % or less than -25 %) at sampling-sites and mean temperatures at corresponding meteorological stations

Sampling site	October	January	February	March	April	September	Winter	Spring	Season
All pointer-years included									
Engure			0.200			0.215	0.211		0.260
Kolka 1			0.362	0.311				0.264	0.261
Kolka 2		0.328	0.447	0.376	0.236		0.379	0.334	0.374
Kolka 3		0.257	0.418	0.289			0.300	0.252	0.298
Kolka 4			0.293						
Spare	0.238		0.321	0.273			0.291	0.221	0.302
Only significant pointer-years included									
Engure		0.476	0.409				0.464		0.470
Kolka 1			0.440	0.430				0.379	
Kolka 2		0.428	0.537	0.435			0.440	0.428	0.451
Kolka 3		0.356	0.505						
Kolka 4									
Spare	0.343		0.484	0.451	0.422		0.462	0.431	0.515

Engure site only 2.6 significant pointer-years. In years 1940 and 1969 trees at all sampling sites had significant negative pointer-years and in 1957 represented a significant positive pointer-year (Fig. 2). As pointer-year values and standard deviation units of climatic factors corresponded to normal distribution, the Pearson correlation coefficient was used.

When all pointer-year intensity values were included in analysis, all of the sampling sites pointer-year values had statistically significant correlation with mean February temperature (Table 2) and all except Kolka 4 with growing season mean temperature. There was no statistically significant correlation between pointer-years and six-month, autumn and summer mean temperatures. Excepting the Spare sampling site, pointer-year values had statistically significant correlation with June precipitation sum (Table 3). There was no statistically significant correlation between pointer-years and nine-month, autumn,

winter, season precipitation sum. Kolka 4 sampling site pointer-year values had statistically significant correlation only with February mean temperature and June precipitation sum.

Using only significant pointer-year intensity values (intensity values greater than 25 % or less than –25 %) in the analysis, excepting the Kolka 4 sampling site, pointer-year values

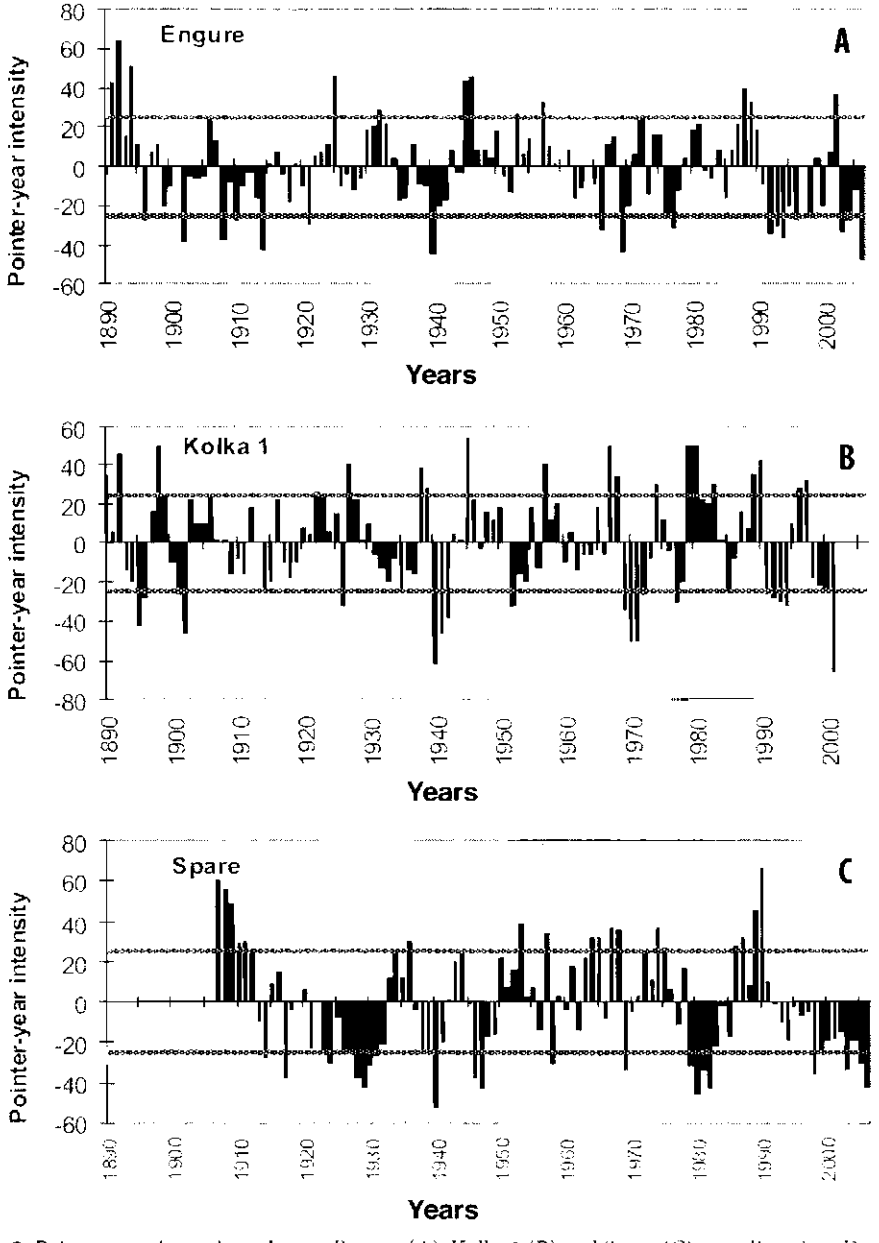

Fig. 2. Pointer-year intensity values at Engure (A), Kolka 1 (B) and Spare (C) sampling sites. Dashed lines show upper (25 %) and lower (–25 %) limits for significant pointer-years.

Table 3. Cases of statistically significant Pearson correlation (at $\alpha = 0.05$) between all pointer-year intensity values, only significant pointer-year intensity values (intensity values greater than 25 % or less than −25 %) at sampling-sites and precipitation sum at corresponding meteorological stations

Sampling site	December	May	June	September	Spring	Summer
All pointer-years included						
Engure			0.195			0.203
Kolka 1			0.189			
Kolka 2		0.211	0.279		0.221	
Kolka 3			0.229			
Kolka 4			0.348			
Spare				0.233		
Only significant pointer-years included						
Engure			0.534			
Kolka 1						
Kolka 2		0.279	0.410		0.320	
Kolka 3			0.423			
Kolka 4	0.345		0.595			
Spare				0.359		

had statistically significant correlation with February mean temperature. Kolka 4 sampling site pointer-year values had no statistically significant correlation with mean temperatures and Kolka 1 with precipitation sum. There was no statistically significant correlation between sampling site pointer-year values and seven-month, autumn mean temperatures, eight months, autumn, winter, season precipitation sum.

Discussion

Although there were only three cases when all sampling sites had common pointer-years, there were common features between sampling sites. One common feature was correlation with February mean temperature, which has been observed in other studies on Scots pine tree-ring chronologies, not only in Latvia (Elferts 2007) but also in Poland (Cedro 2001) and Estonia (Pärn 2003). Abrupt changes in February mean temperatures – decline or increase compared to long-term mean temperatures – causes formation of respectively narrower or wider tree-rings. Significant positive correlation coefficients between most of sampling site pointer-year values with March, winter and spring mean temperatures show that the growth of Scots pine is determined by temperatures during the winter and beginning of spring. One explanation could be that if winter temperatures are higher then the frozen soil layer is shallow and trees can start the growing season earlier.

On the dry soil sites higher June precipitation sum had a positive effect on the Scots pine growth not only in the studied areas but also in Southern Finland (Lindholm et al. 2000) and Poland (Cedro 2001). Higher temperatures in June might lead to increase of evapotranspiration and decline of moisture in soil. Precipitation in other months did not show any common trend between sampling sites, indicating that in this region precipitation has minimal impact on Scots pine growth and that there exists optimal moisture availability

in the soil (Linderholm 2001; Elferts 2007). Lack of a significant correlation between Spare sampling site pointer-year values and June precipitation sum could be explained by location near lake shore, which influences the moisture level in soil.

The low number (three) of cases with common pointer-years at all sampling sites suggests that development of pointer-years was mainly determined by local factors, for example, local climatic conditions, stand factors, or possibly insect outbreaks. Neuwirth et al. (2007) found that also in a much larger scale (Central Europe) common pointer-years occurred in years of extreme climatic conditions. All Kolka sampling sites had eight common pointer-years (five negative, three positive) showing that closer sampling sites had more common pointer-years. The years when all sampling sites showed significant pointer-years were determined by climatic factors. In 1940 at all sampling sites there was a very significant negative pointer-year (very narrow tree-ring), in the same year February the mean temperature at Kolka station was –12.5 °C, which is 9 °C lower than the long-term mean temperature. Also a lower temperature was observed in March and growing season, and also precipitation sum in June was lower. Previously it was shown that these factors are the most important in relation to tree growth. The year 1969 also was characterized by lower February and March temperatures and low precipitation sum in June (at Kolka station only 2 mm). In 1957, when all sampling sites showed a positive pointer-year, winter was very mild, and mean temperatures at Kolka station in December, January and February were above 0 °C, which positively influenced tree growth.

In conclusion, this study showed that pointer-year development mainly is determined by local factors, except years when abrupt changes in climatic conditions are observed. Also February temperature is the main climatic factor associated with Scots pine growth in Latvia.

Acknowledgements

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Parastās priedes zīmīgie gadi Latvijas ziemeļrietumos un to saistība ar klimatiskajiem faktoriem

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Kopsavilkums

Sešos parauglaukumos Latvijas ziemeļrietumos ņēma parastās priedes (*Pinus sylvestris* L.) urbumus, lai iegūtu gadskārtu platumu datus un varētu noteikt zīmīgo gadu vērtības (gads, kurā gadskārta ir izteikti šaurāka vai platāka, salīdzinot ar citām tuvākajam gadskārtām). Starp zīmīgo gadu vērtībām un klimatiskajiem faktoriem (vidējā temperatūra un nokrišņu daudzums) veica korelācijas analīzi, lai noteiktu būtiskās saistības. Konstatēja tikai trīs gadus (negatīvi – 1940. un 1969., pozitīvs – 1969.), kuri visos parauglaukumos bija zīmīgie gadi. Galvenie klimatiskie faktori, kas ietekmē zīmīgo gadu rašanos, ir februara vidējā temperatūr un jūnija nokrišņu daudzums. Zīmīgo gadu rašanās galvenokārt ir saistīta ar lokālajiem faktoriem.

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Influence of chemical preservatives on the quality and composition indices of raw milk samples

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Abstract

The experiment was conducted to assess the feasibility of sodium azide, hydrogen peroxide, bronopol, azidiol, boric acid and potassium sorbate as milk preservatives before estimation of the indicators of the content and quality of raw milk using instrumental methods. The milk samples were collected from fresh bulked milk of the dairy farm in Riga District. Milk samples were stored at 4 °C and 20 °C. Untreated milk samples were considered as control samples and were tested against samples treated with 0.02 % sodium azide, 0.06 % hydrogen peroxide, 0.04 % bronopol, 0.4 % azidiol, 1.0 % boric acid and 0.5 % potassium sorbate. Microbiological and chemical parameters (total bacteria count, fat and protein content, somatic cell count) of raw milk samples were measured in all samples just before preservation and then regularly after specific time interval. The results showed that the most suitable preservatives for storing milk before the estimation of the indicators of the content and quality of raw milk by using instrumental methods are bronopol, sodium azide and azidiol. To ensure stable milk quality starting from the time of sample collection till the analysis, it is advisable to preserve the samples with the above-mentioned compounds and storage at 4 °C not longer than 96 hours.

Key words: chemical preservatives, milk preservation, milk quality, raw milk.

Introduction

The problem of preservation of milk samples is common in many countries because the diagnostic laboratories are generally far away from the dairy farming communities, transport of the samples to the laboratory for diagnosis is inadequate (Dunham, Kroger 1985). These problems are aggravated by the need for facilities to keep the milk cool in order to minimize bacterial proliferation and sample spoilage prior to examination, as they are generally lacking. In such situations, it is necessary that other means of milk sample preservation, for example by use of chemicals, are explored. Recently scientists have used various milk preservatives (hydrogen peroxide, sodium azide, bronopol, potassium dichromate, boric acid, *Milkofix*, azidiol, ortobor acid) to overcome this problem (Ng-Kwai-Hang, Hayes 1982; Hanus et al. 1992a; Hanus et al. 1992b; Heeschen et al. 1994; Saha et al. 2003; FOSS Electric 2005).

Applying instrumental methods in testing raw milk it is allowed to use preservative agents (FOSS Electric 2005). In the literature, it is possible to find various preservatives for each indicator (total bacteria count, fat and protein content, somatic cell count). For the

optimization of instrumental methods and precise estimation of milk content and quality indicators, it is necessary to find a preservative that could be used to estimate all of the indicators, mentioned above from one sample vial.

The aim of the present experiments was to evaluate the efficiency of various chemical preservatives and determine their influence on the quality and composition indices of raw milk samples performed by instrumental methods.

Materials and methods

The experiments were conducted at the testing laboratory of Piensaimnieku Laboratorija Ltd. and at the Institute of Biology, University of Latvia during the spring period 2006.

Whole milk was collected from one farm located in Riga District in Latvia. Milk samples were collected in the morning, 3 to 5 h after milking. Milk was brought to the laboratory on ice and used for experiments within 1 to 2 h of receipt.

Preservation procedures

This study was conducted over a period of three weeks. Each week a five litre cow milk sample was collected from fresh bulked milk of the dairy farm. The collected milk samples after thorough mixing were divided into three equal parts. Of the three parts, one part was kept as untreated milk (fresh) and two portions were treated with 0.02 % sodium azide (NaN_3), 0.06 % hydrogen peroxide (H_2O_2), 0.04 % bronopol ($C_3H_6BrNO_4$), 0.4 % azidiol, 1.0 % boric acid (H_3BO_3) and 0.5 % potassium sorbate ($C_6H_7KO_2$), each week with specific preservative. Plastic containers were used in milk preservation. Each of the three treatments was further divided into two equal portions. One portion from each treatment was stored at 4 °C and the other portion at 20 °C. The untreated milk samples (control samples) were analyzed immediately after delivery to the laboratory and then after 24 h, as the standard LVS 175:1999 requires raw milk without preservation can be used for analysis only within 36 h. Analysis of preservative-treated milk samples was conducted after 24, 48, 72 and 96 h, respectively. All parameters used to monitor the quality of milk were determined in ten replications.

Microbiological analyses

Somatic cell counts were performed using fluorescent opto-electronic method on a Fossomatic™ FC configured as a CombiFoss™ 6000FC together with a MilkoScan™ FT6000 (FOSS Electric A/S, Denmark) according to LVS EN ISO 13366-3:1997 and manufacturer's recommended procedures (FOSS Electric 2005). Total bacteria counts were determined using flow cytometry method on a BactoScan™ FC analyzer (FOSS Electric A/S, Denmark) and manufacturer's recommended procedures (FOSS Electric 2001a; FOSS Electric 2001b).

Chemical analyses

Fat and protein tests were made using infrared spectroscopy on a MilkoScan™ FT6000 configured as a CombiFoss™ 6000FC together with a Fossomatic™ FC (FOSS Electric A/S, Denmark) according to ISO 9622:1999(E) and manufacturer's recommended procedures (FOSS Electric 2005).

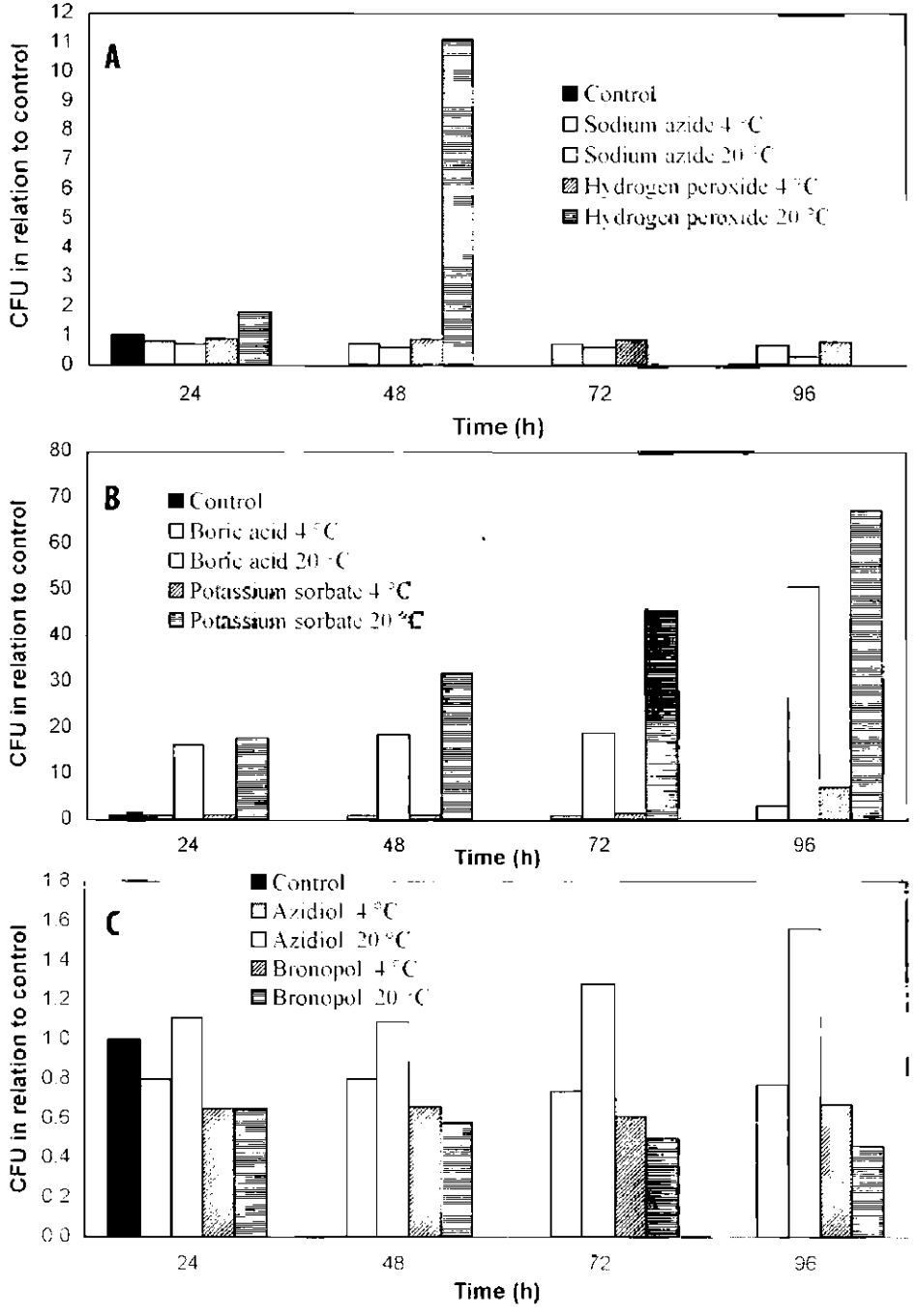

Fig. 1. Total bacteria count (CFU) in the samples of raw milk in relation to chemical preservative treatment estimated using flow cytometry approach. A, 0.02 % NaN_3, 0.06 % H_2O_2. Control value was 397×10^3 CFU ml^{-1}. B, 1.0 % H_3BO_3, 0.5 % $C_6H_7KO_2$. Control value was 250×10^3 CFU ml^{-1}. C, 0.4 % azidiol, 0.04 % $C_4H_4BrNO_2$. Control value was 324×10^3 CFU ml^{-1}. Control samples were analyzed immediately after transport to the laboratory.

Statistical analyses

Data were analyzed using Microsoft Excel and FOSS Electric software – System 4000 version 4.1.8. and FOSS Integrator version 1.3.7. For each count series the average value and standard error (SE) was calculated. The upper and lower 99 % confidence limits (α = 0.01) were estimated.

Results and discussion

Tests with non-preserved milk samples stored at 4 °C for 24 h and 48 h were performed using flow cytometry (FCM). The results indicated differences in ratio of individual bacteria count (IBC) and colony forming units (CFU) depending on the period of sample storage (i.e. 24 h or 48 h). The IBC in the samples after 48-h storage was three to seven times higher, compared to the samples stored for 24 h (Sešķēna, unpublished data). After 24-h storage at 4 °C, the IBC number in the samples increased 1.5 to four times. These results confirm the necessity to develop a preservation approach for infected milk samples with the aim to prolong the quality control period and to provide method precision, because it is impossible to calibrate equipment de novo for every samples series.

The effect of various preservatives was evaluated using criteria of milk content and quality such as the total bacteria count, somatic cell count, and fat and protein content.

The influence of storage temperature on the preservation efficiency was investigated by comparing the quality of raw milk samples stored at refrigeration temperature (4 °C) and ambient temperature (20 °C). The control samples (without addition of preservative), stored at 20 °C for 24 h was fermented and could not be used for analysis. The mean bacteria counts in control samples were higher than 1×10^7 CFU ml^{-1}. For this reason, control samples were tested at time of samples were delivery to the laboratory. Raw milk samples containing preservative were analyzed after 24-, 48-, 72- and 96-h storage. An exception was for samples with H_2O_2 stored at 20 °C, which were tested after 24- and 48-h storage only, because during longer periods total bacteria count exceeded 8×10^6 CFU ml^{-1} and milk was fermented. Thus, a lower stability of hydrogen peroxide at 20 °C is indicated.

The effect of the various preservatives on total bacteria count estimated using flow cytometry in raw milk samples is shown in Fig. 1. Bacteria proliferation during the 96-h period was inhibited in the raw milk samples amended with NaN_3, H_2O_2, $C_3H_6BrNO_4$ and azidiol, when stored at 4 °C. Bacteriostatic effect of H_3BO_3 and $C_6H_7KO_2$ was observed during 72-h storage at 4 °C. In all tested samples, variability of total bacteria count measured by FCM, did not exceeded confidence interval (± 49.02 %, α = 0.01), corresponding to the precision of microbiological methods and not considered as significant.

The antiseptic properties of H_2O_2, H_3BO_3 and $C_6H_7KO_2$ were lost at 20 °C as the total bacteria count was considerably higher already after 24 h storage: 1.8, 16.2 and 17.7 times, correspondingly.

The obtained results indicate a strong dependence of preservative efficiency on the storage conditions, i.e. temperature. It was concluded that the tested preservatives at 4 °C retain their antiseptic properties for a longer period as compared to 20 °C. The most important factors reported to influenc efficiency of preservatives include an initial microbial count in the product, microbial species, temperature and pH of environment (Baltess 1998). In our study, temperature conditions had a the strong effect on preservative

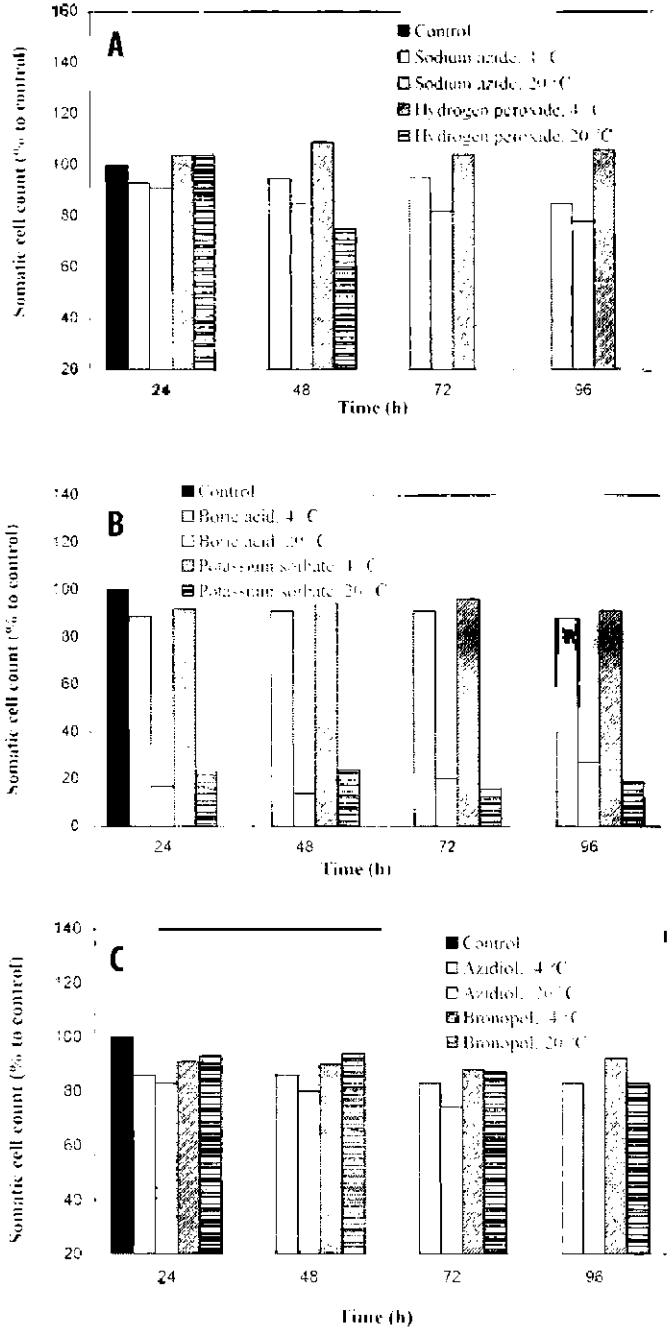

Fig. 2. Somatic cell count in the samples of raw milk in relation to chemical preservative treatment estimated using fluorescent optoelectronic method. A, 0.02 % NaN_3, 0.06 % H_2O_2; B, 1.0 % H_3BO_3, 0.5 % $C_7H_5KO_2$; C, 0.4 % azidiol, 0.04 % $C_4H_6BrNO_2$. Control values were 138×10^3 ml^{-1} (A), 212×10^3 ml^{-1} (B), 159×10^3 ml^{-1} (C). Control samples were analyzed immediately after transport to the laboratory.

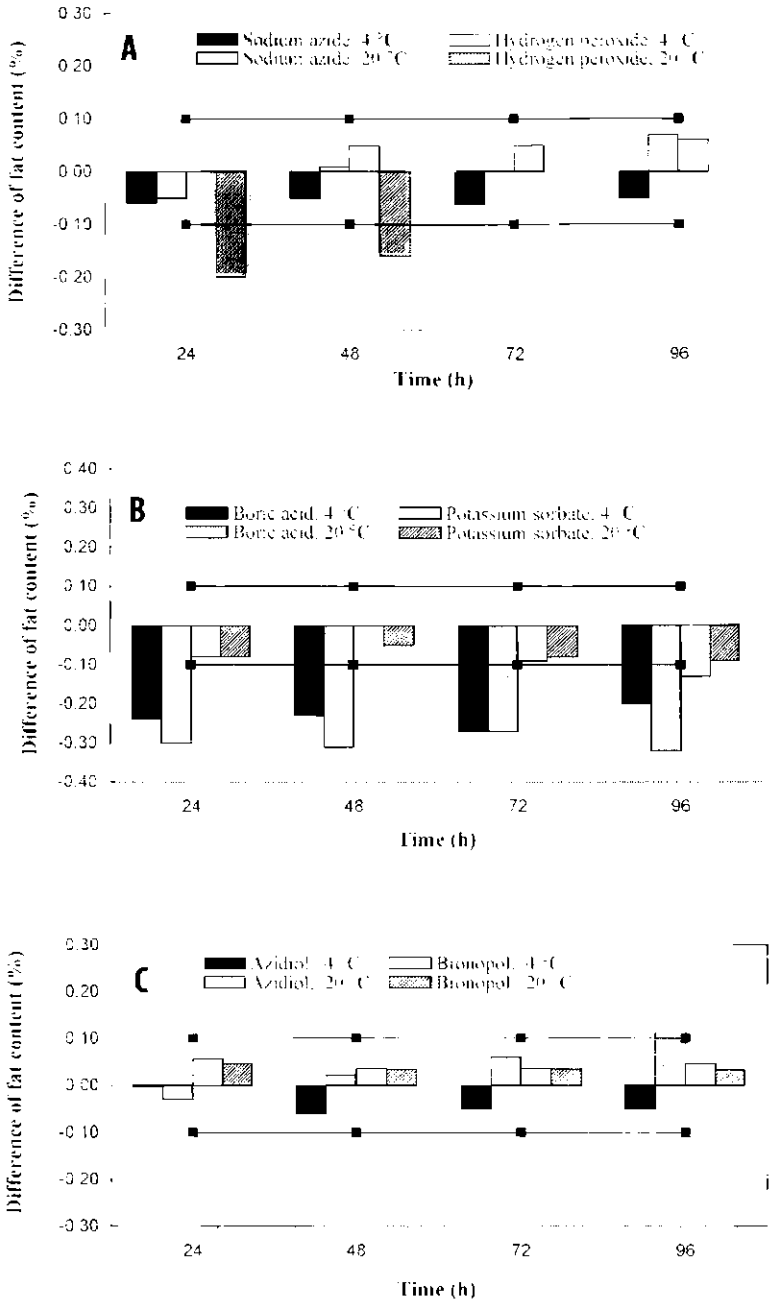

Fig. 3. Fat content in the samples of raw milk in relation to chemical preservative treatment estimated using infrared analysis. A, 0.02 % NaN_3, 0.06 % H_2O_2. B, 1.0 % H_3BO_3, 0.5 % $C_6H_7KO_2$. C, 0.4 % azidiol, 0.04 % $C_3H_4BrNO_2$. Control samples were analyzed immediately after being transported to the laboratory. Control values were 5.84 % (A), 5.14 % (B), 5.78 % (C). Horizontal line represents 99 % confidence interval ($\alpha = 0.01$) of characterized control samples.

efficiency, especially this effect was shown for H_3BO_3, $C_6H_7KO_2$, and H_2O_2.

The influence of NaN_3, H_2O_2, $C_3H_6BrNO_4$, H_3BO_3, $C_6H_7KO_2$ and azidiol on somatic cell count was studied using fluorescent optoelectronic method (Fig. 2). Using these preservatives, the somatic cell count did not change significantly during 96-h storage at 4 °C and did not exceeded confidence interval (± 21.6 %, $\alpha = 0.01$). Our results differ from those obtained by Heeschen et al. (1994), who found that milk preservation with NaN_3 caused significantly lower somatic cell counts. We observed that the somatic cell count in the samples treated with H_3BO_3 and $C_6H_7KO_2$ and stored at 20 °C greatly decreased, i.e. 5.2 and 4.8 times, correspondingly. A decrease of the somatic cell count was detected during the entire period of the experiment. This effect can be explained by decreased cell envelope permeability of somatic cells caused by preservatives at 20 °C, which leads to poor nuclear DNA staining and weak fluorescent optoelectronic detection.

The effect of preservatives on the fat content of raw milk using infrared spectroscopy method is shown in Fig. 3. There were no any considerable changes in fat content in the samples treated with NaN_3, $C_3H_6BrNO_4$, $C_6H_7KO_2$, and azidiol at 4 °C and 20 °C and H_2O_2 at 4 °C during the 72-h period. We observed a significant decrease of fat content after 24 h and 48 h in samples preserved with H_2O_2 and stored at 20 °C, and after 72 h these samples spoiled. The most considerable changes in the fat content were detected in the samples amended with H_3BO_3: fat content decreased up to 0.31 % of the control sample. This effect can be explained by the reaction between H_3BO_3 and CH-groups of lipid molecules, resulted in their altered properties. CH-groups do not absorb light with the wavelength 3.5 μm used for fat content detection by MilkoScan™ FT6000.

The effect of preservatives on the protein content in raw milk using infrared spectroscopy method is shown in Fig. 4. Azidiol, $C_3H_6BrNO_4$, NaN_3 and H_2O_2 did not noticeably affect the milk protein content. The protein content in the samples treated with H_3BO_3, and stored at 4 °C and 20 °C for 96 h, decreased by 0.74 % and 0.87 %, correspondingly. This can be explained by the ability of H_3BO_3 to bind not only with CH-groups of lipid molecules, but also with N-H groups of peptide bounds, thus influencing absorption intensity. H_3BO_3 thus affects the estimation of fat and protein content in raw milk obtained by infrared spectroscopy. The effect of $C_6H_7KO_2$ on protein content measurement was the reverse, i.e. protein content in tested samples stored at 4 °C and 20 °C increased by 0.20 % and 0.39 %, correspondingly. Most probably, the ability of some sites in the potassium sorbate molecule to absorb light at wavelength 6.5 μm interferes with peptide N-H sites, which are known to absorb light at the same wavelength. The literature data indicates light absorption at 6.5 μm also for citric acid [ISO 9622:1999(E)].

The results obtained in this study show that the use of bronopol, sodium azide or azidiol for raw milk preservation could provide stable milk quality and rational use of up-to-date equipment in cases when the samples were immediately refrigerated and stored within the period of 96 h. Similar types of results are obtained by FOSS Electric (2001a; 2005) and Gonzalo et al. (2004) who observed that azidiol and NaN_3 can be used successfully in preserving milk samples for bacteriological analysis on a BactoScan™ FC analyzer, and bronopol is the optimal preservative of milk samples for the CombiFoss™ 6000FC method.

Evaluation of the compounds mentioned above ($C_3H_6BrNO_4$, NaN_3 and azidiol) from the commercial point of view, suggest bronopol as the most appropriate preservative that is commercially available in tablet form. The use of this preservative does not require

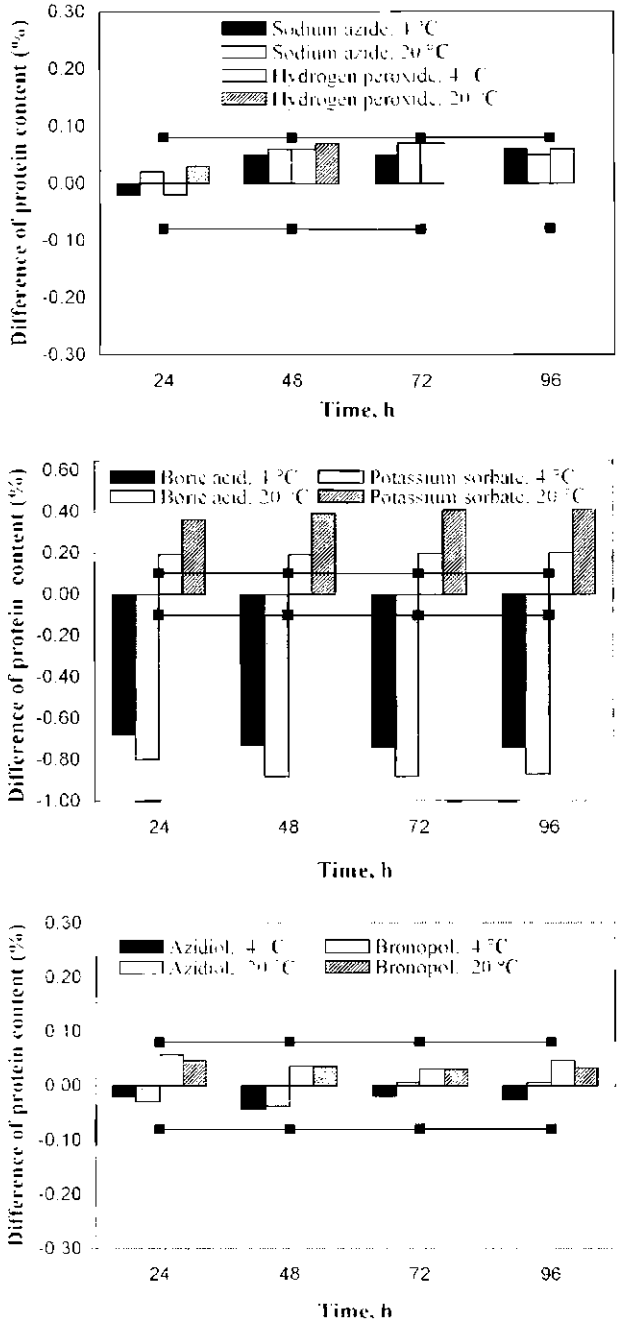

Fig. 4. Protein content in the samples of raw milk in relation to chemical preservative treatment estimated using infrared analysis. A, 0.02 % NaN_3, 0.06 % H_2O_2; B, 1.0 % H_3BO_3, 0.5 % $C_6H_7KO_2$; C, 0.4 % azidiol, 0.04 % $C_4H_6BrNO_2$. Control samples were analyzed immediately after being transported to the laboratory. Control values were 4.21 % (A), 4.86 % (B), 4.34 % (C). Horizontal line represents 99 % confidence interval ($\alpha = 0.01$) of characterized control samples.

additional time for unit-dose packaging. Sodium azide and azidiol are known to be toxic and these compounds do not degrade in the environment, therefore these preservatives are not offered to consumers. These chemicals can be used only for laboratory analyses. However to avoid the potential risk for laboratory personnel and contamination of environment it is necessary to search for new, less harmful agents for raw milk preservation.

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Ķīmisko konservantu ietekme uz koppiena paraugu kvalitātes un sastāva rādītājiem

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Kopsavilkums

Eksperiments veikts, lai noskaidrotu un izvērtētu nātrija azīda, ūdeņraža peroksīda, bronopola, azidiola, borskābes un kālija sorbāta ietekmi uz koppiena paraugu sastāva un kvalitātes rādītājiem, kas noteikti, pielietojot testēšanā instrumentālās metodes. Analīzēm izmantots koppiens, kas iegūts Rīgas rajonā esošā zemnieku saimniecībā. Piena paraugi uzglabāti 4 °C vai 20 °C temperatūrā. Ar 0,02 % nātrija azīdu, 0,06 % ūdeņraža peroksīdu, 0,04 % bronopolu, 0,4 % azidiolu, 1,0 % borskābi un 0,5 % kālija sorbātu konservēti paraugi analizēti paralēli nekonservētiem koppiena paraugiem, kuri izmantoti kontrolei. Kontroles paraugiem mikrobioloģiskie un ķīmiskie rādītāji (baktēriju kopskaits, tauku un olbaltumvielu saturs, somatisko šūnu skaits) noteikti tūlīt pēc koppiena piegādes laboratorijā, bet konservētiem paraugiem – pēc 24, 48, 72 un 96 stundām. Noskaidrots, ka vispiemērotākie konservanti koppiena paraugu kvalitātes un sastāva rādītāju noteikšanai ar instrumentālām metodēm ir bronopols, nātrija azīds un azidiols. Lai nodrošinātu nemainīgu piena kvalitāti no paraugu noņemšanas brīža līdz testēšanai, koppiena paraugus ieteicams konservēt ar šiem savienojumiem un uzglabāt 4 °C temperatūrā ne ilgāk par 96 stundām.

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Isolation, characterization and analysis of *TGFβ* gene expression in adipose-derived precursor cells cultured in different growth media

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Abstract

Human adult (autologous) stem cells which are capable of self-renewal and differentiation into other cell types, can be isolated from various tissue and are a promising source for cell therapy, since there are no ethical and rejection problems as in the case of embryonic stem cells. The human body contains a great amount of adipose tissue which consists also of several mezenchymal stem cell populations. According to the existing data Transforming Growth Factor β (TGFβ) is one of the molecules involved in MSC differentiation. The aim of this work was to study the capablility of human adipose-derived precursor cells to differentiate into other cell types and to study the effect of various cell media on the expression of *TGFβ* in adipoze-derived precursor cells. Here we show that in serum-free medium the morphology of cells differs and the expression level of *TGFβ* is significantly higher than in serum-supplemented media. Our work also shows that cell populations isolated from human adipose tissue can differentiate not only into mezenchymal lineage cells but also into neuronal lineage cells, prooving the plasticity of adipose derived cells. We conclude that cell populations isolated from human adipose tissue contain a number of precursor cells with characteristic features of stem cells – ability to differentiate into more than one cell type. Cell cultivation medium affects population growth and morphology. Cells in serum-containing medium grow faster and develop a monolayer, while cells in serum-free medium grow slower and tend to form spheres. *TGFβ* expression is significantly higher in cells grown in serum-free medium which is more suitable for maintenance of cells than for promoting rapid growth.

Key words: adipose derived precursors, stem cells, transforming growth factor

Introduction

Stem cells can continue cell division and renewal for long periods by stable maintenance of unspecialized status and by differentiaion into specialized cell types (Fuch et al. 2004; Toma et al. 2005). Future stem cell therapy is focused on using the patient's own adult stem cells to regenerate tissue and to treat a variety of disorders. The human body is a good source of adult (or autologous) stem cells, since almost every organ contains smaller or larger populations of immature cells capable of differentiation into some cell types

(Aejaz et al. 2007). Adipose tissue, like bone marrow, is derived from the mezenchyme and contains a supportive stroma that is easily isolated. Considering the above adipose tissue may represent a source of stem cells that could have far-reaching effects in several fields of research and medicine (Zuk et al. 2002). The many investigations on adipose tissue-derived mezenchymal stem cell populations have shown their potential to differentiate into diverse cell types, such as adipogenic, osteogenic, chondrogenic and myogenic lineages. The transforming growth factor beta (TGFβ) signaling pathway induces adipoze tissue cells to differentiate into smooth muscle-like cells (Jeon et al. 2006).

The multifunctional cytokine superfamily of the TGFβ is a large group of structurally related proteins that are major regulators of normal growth and development in multicellular eukaryotic organisms (Piek et al. 1999; Chang et al. 2002). Almost all cells produce TGFβ and its cell surface receptors, and convey their signals to the intracellular mediators, the Smads (Shi, Massague 2003). Several growth factors belonging to the TGFβ superfamily have the potential to regulate cell proliferation, differentiation and migration and their interaction with extracellular matrix molecules (Roberts et al. 1992; Shi, Massague 2003). The TGFβ isoforms 1, 2 and 3 each have been proposed to have specific, non-overlapping roles in development of eykariotic organisms (Dickson et al. 1993; Kaartinen et al. 1995; Proetzel et al. 1995).

TGFβ1 performs many cellular functions, including control of cell growth, cell proliferation, cell differentiation and apoptosis. There is evidence that TGFβ1 is involved in wound healing processes (Assoian et al. 1983). TGFβ1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells, and its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of these immune cells (Letterion, Roberts 1998).

In animal models TGFβ3 regulates molecules involved in cellular adhesion and extracellular matrix (ECM) formation during the process of palate development as well as controls wound healing by regulating the movements of epidermal and dermal cells in injured skin (Taya et al. 1999; Bandyopadhyay et al. 2006).

TGFβ2 is the isoform most strongly linked to heart morphogenesis (Yamagishi et al. 1999), supported by the involvement of TGFβ2 signaling in epithelial-mezenchymal cell transformation during avian and mouse heart development (Mercado-Pimentel, Runyan 2007). It has been reported that TGFβ2 also plays a role in normal hematopoiesis (Majka et al. 2001) and is expressed in multipotent neural progenitor cells (Klassen et al. 2003). Recent work (Im et al. 2003; Wang et al. 2003; Lou et al. 2007) has shown the role of TGFβ2 in neocartilage formation from predifferentiated human adipose-derived stem cells in vivo, as well as in human bone marrow-derived mezenchymal progenitor cells. Therefore, TGFβ2 is one of the key molecules in the cell differentiation, which is an important process in stem cell research field.

Since TGFβ is involved in so many cellular processes, it is important to understand its role and molecular mechanisms within the different cell populations and various growth conditions. In this paper we investigate the effect of different growth conditions on the cell morphology and expression of cellular *TGFβ* within adipose-derived precursor cell (hADPC).

Materials and methods

hADPC isolation and cultivation

Subcutaneous adipose tissue was obtained from elective surgery with patient consent as approved by the Central Ethical committe of Latvia.

Human adipose-derived precursor cells (hADPC) were isolated as previously described (Lee et al. 2004) with minor modification (Hoogdjuin M., personal communication). Briefly, adipose tissue was mechanically disrupted with a scalpel knife and, after two washes with phosphate - buffered saline (PBS), digested with sterile filtered 0.5 mg ml^{-1} collagenase type XI in DMEM/F12 (3:1, v/v) for 30 min at 37 °C with intermittent shaking. Medium with 10 % fetal bovine serum (FBS) and 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin was then added and the floating adipocytes were separated from the stromal - vascular fraction by centrifugation at 500 × g for 5 min. The cell pellet was resuspended in DMEM:F12 medium with serum as described above, and cells were plated in 25-cm^2 tissue culture flasks (T-25; Sarstedt) and grown 2 - 3 weeks in 37 °C, 5 % CO_2 until 80 % confluence. The passage number of human adipose-derived precursor cells used in these experiments was 5 - 7. Different growth medium were used starting with passage number 3. Cells were counted using a hemacytometer.

Media

Serum medium was based on DMEM/F12 (3:1, v/v) supplemented with 1 % antibiotic mix (penicillin/streptamycin, Invitrogen) and fetal bovine serum (10 % and 30 %) alone, or in some samples, with 5 ng ml^{-1} basic fibroblast growth factor (bFGF; R&D Systems). Cells were grown also in serum medium diluted with serum-free medium (described below) supplemented with leukemia inhibitory factor (LIF) as 1:1 and 3:1, respectively.

Serum-free medium consisted of DMEM/F12 (3:1, v/v) 1 % antibiotic mix supplemented with 40 ng ml^{-1} bFGF, 20 ng ml^{-1} epidermal growth factor (EGF; R&D Systems), 2 % B27 (Invitrogen) and 10 ng ml^{-1} LIF (Millipore/Chemicon).

In vitro *differentiation of hADPC*

For induction of neuronal differentiation, 5 × 10^4 cells per well were seeded onto four-well chamberslides (Nunc). Cells were cultured in basal neuronal differentiation medium containing 40 ng ml^{-1} bFGF, 20 ng ml^{-1} EGF and 2 % B27. To induce oligodendritic and astrocyte differentiation, 48 h after initial plating, cell culture medium was replaced with basal medium supplemented with neural differentiation supplement (Human Neural Stem cell Functional Identification Kit, R&D Systems). Cells were fixed for immunohistochemical analysis of nestin expression 48 h after initial plating; immunohistochemical analysis of tubulin βIII, O4 and GFAP expression was performed after seven-day incubation in the neural differentiation medium.

Immunocytochemical analysis

Neural lineage markers were determined using monoclonal antibodies against human neural lineage markers nestin, GFAP, O4, tubulin βIII (all included in Human Neural Stem Cell Functional Identification Kit, R&D Systems) following manufacturer instructions. Cells, when confluence of 70 % was reached, were fixed in 4 % paraformaldehyde in DMEM for 20 min at room temperature. Monoclonal antibodies were applied to specimens and

incubated overnight at 4 °C.

Following incubation with primary antibodies, horse-readish peroxidase-conjugated secondary reagent and diaminobenzidine substrate was applied to all specimens according to manufacturer instructions (DakoCytomation LSAB+ System-HRP). Cells were counterstained with Mayer's hematoxylin (Lilie's modification, DakoCytomation), mounted (DakoCytomation) and examined under microscope (Leica DMI4000 B). Images were made using Image-Pro® Express software.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted by the TRIzol method (Sigma) and the concentrations were obtained using a Nanodrop spectrophotometer. cDNA synthesis was performed as described previously (Lemos et al. 2003). Total RNA was denatured for 5 min at 80 °C and then chilled on ice. The reaction took place at 42 °C for 90 min in a volume of 50 ml. The reaction mixture contained 10 ml of 5 × first-strand buffer (Life Technologies-BRL), 25 pmol of each dNTP (Amersham Pharmacia Biotech Benelux), 0.25 mg of random primers (Promega), 500 U of M-MLV reverse transcriptase (Life Technologies-BRL), 20 U of RNAse inhibitor (Promega), and 0.5 nM of dithiothreitol (Life Technologies-BRL). After the cDNA reaction, the M-MLV reverse transcriptase was inactivated by a 5 min incubation at 95 °C.

Real-time quantitative PCR

Real-time PCR analysis was conducted as previously described (Lemos et al. 2003). The mRNA levels of TGFβ and the housekeeping gene GAPDH were quantified in the hADPC using real-time PCR in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Predeveloped TaqMan assays (Applied Biosystems) were used for *TGFβ* and *GAPDH*. Amounts of primers and probes of the predeveloped kits were added according to manufacturer instructions. PCR conditions were incubation for 2 min at 50 °C, incubation for 10 min at 95 °C, followed by 40 cycles of 15 sec denaturation at 95 °C, 1 min annealing and extension at optimal temperature 60 °C.

Relative quantification was made against the expression levels of *GAPDH* used as a housekeeping gene. Copy numbers were calculated for 20 ng of total RNA for each sample.

Results

hADPC isolation and viability

Isolated cells from subcutaneous adipose tissue were transferred to culture flasks. After three to 10 days, cells with fibroblast morphology appeared. Within a week it was observed that the number of cells rapidly increased in serum containing medium. Isolated adipose-derived precursor cells were cultured in defined FBS-containing medium. The medium was first changed after one week, when together with the medium dead cells were washed away from the cultures. Over the first two weeks most cells had adhered to the tissue flasks and a significant amount of dead cells was no longer noticed. Approximately 35 0000 - 80 0000 viable cells per T-25 flask were observed when 80 % confluence was reached. The estimated cell doubling time was seven to 10 days according to cell counts under the microscope. Cells were grown continuously in culture for six months and passaged more

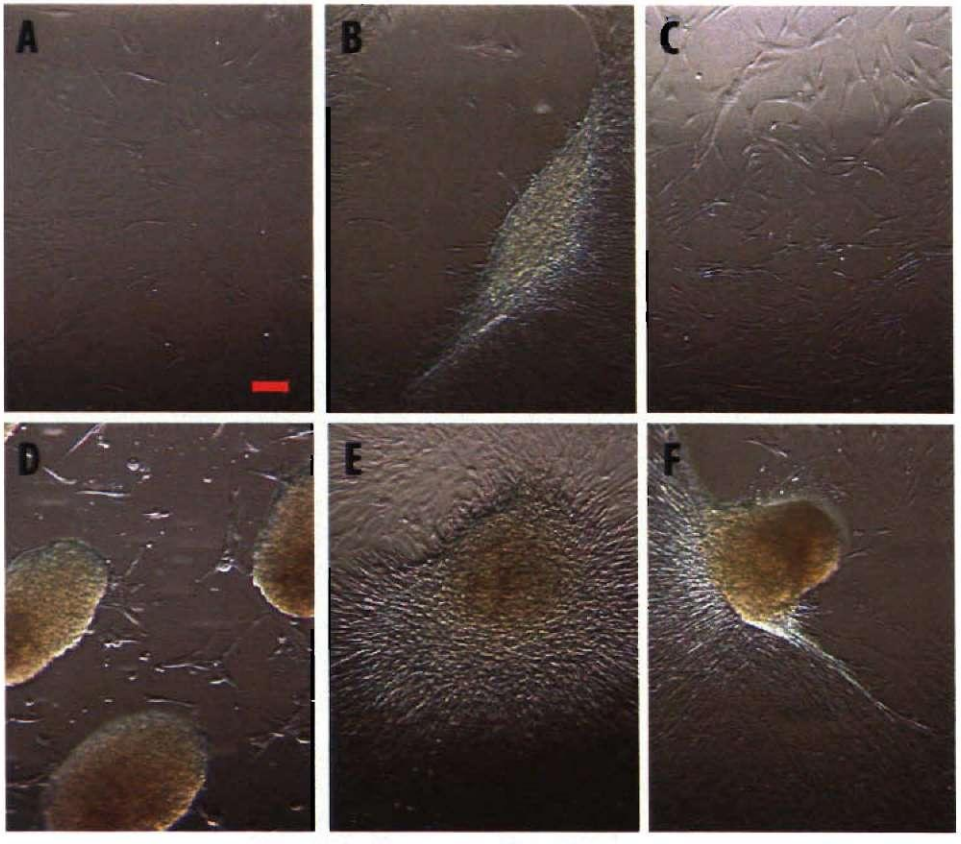

Fig. 1. Morphology of adipose derived precursor cells used for TGFß expression analysis. A, 10 % FBS; B, 30 % FBS; C, 10 % FBS + FGF; D, LIF spheres; E, 10 % FBS + LIF (1:1); F, 10 % FBS + LIF (3:1). The bar represents 100 μM.

than seven times. Comparison of subcutaneous adipose tissue cells to those from heart, spleen, and cultured bone marrow-derived MSCs obtained from Dr. Martin Hoogdjuin (Erasmus Medical Center, Rotterdam, Netherlands) showed similar morphology.

Visual examination of adipose-derived cell cultures revealed significant differences in dependence of medium used for cultivation (Fig. 1). In serum medium with different concentrations of FBS cells had fibroblast-like morphology (Fig. 1 A - C). In serum-free medium supplemented with LIF cells resembled floating spheres (Fig. 1 D), and in serum media diluted with serum-free media supplemented with LIF in different dilutions (1:1 and 3:1, Fig. 1 E - F) – rounded cell bodies and dendritic-like sprouts with some floating spheres, respectively. In some cases cells tended to overgrow themselves forming clumps as in the case of 30 % FBS. Cells forming spheres observed in serum-free medium culture supplemented with LIF grew much slower than cells in serum medium. Nevertheless, when cell spheres from serum-free media supplemented with LIF were seeded back in serum medium (even when diluted with serum-free media supplemented with LIF), they tended to attach to the surface and cells from the spheres grew forming a monolayer (Fig. 1 E - F), suggesting that serum-free media supplemented with LIF could serve as stem

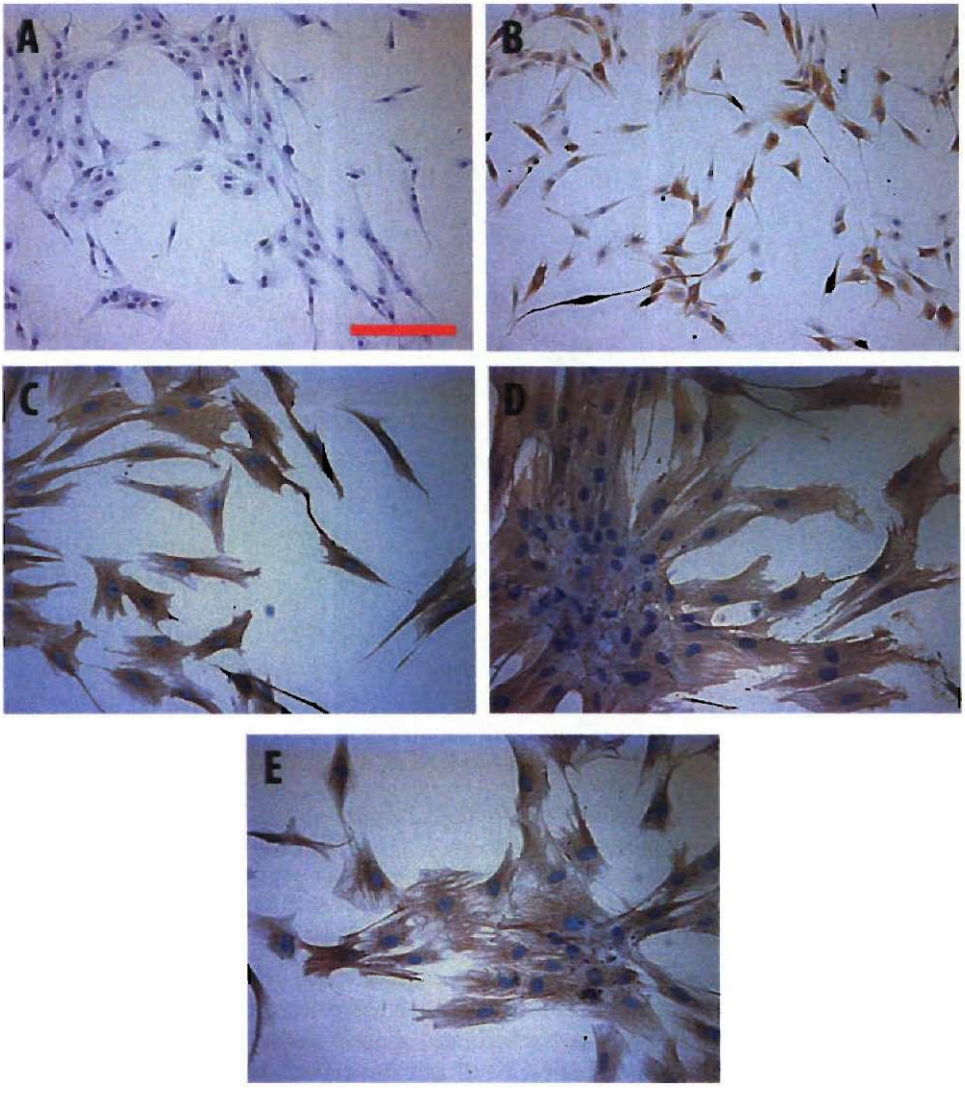

Fig. 2. *In vitro* differentiation of adipose-derived precursor cells. The following markers were used for immunocytochemical staining: control (A), nestin (B), βII tubulin (C), GFAP (D), O4 (E). The bar represents 100 μM.

cell culture-maintenance medium. To investigate whether the isolated cells had stem cell characteristics, their ability to differentitate into mezenchymal and other cell types were examined by *in vitro* diferentiation to neuronal lineage cells. The expression levels of *TGFβ* were examined by real-time PCR analysis.

In vitro *differentiation potential hADPC*

Human adipose-derived precursor cells tested for nestin, a neuronal precursor marker, expressed strongly positive phenotype after 48 h growth (Fig. 2).

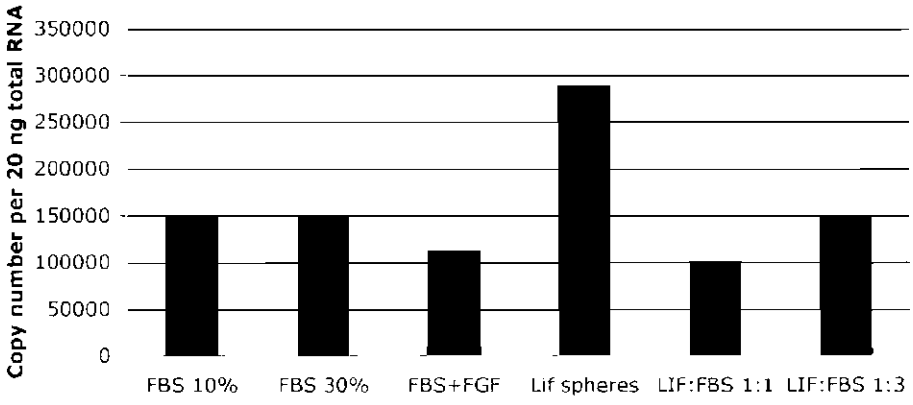

Fig. 3. Expression level of intracellular *TGFβ* in adipose-derived precursor cell line cultivated under different medium.

Nestin staining was observed in approximately 70 % of the cells in an observed area. Following seven-day differentiation into neural differentiation medium, all adipose-derived precursor cell samples tested were positive for neuronal marker tubulin βIII astrocyte marker GFAP and oligodendrocyte marker O4 (Fig. 2). We suspect that under these culture conditions adipose-derived precursor cells probably differentiate to a higher extent towards a neuronal precursor phenotype. Simultaneous expression of nestin, tubulin βIII, GFAP and O4 would suggest an immature neuronal precursor phenotype of these cells.

Real-time quantitative PCR analysis of the expression of TGFβ

Real-time PCR analysis was conducted to determine the level of expression of intracellular *TGFβ*. The expression levels were synchronized to the houskeeping gene *GAPDH* expression level for each sample. Since the cell count was not made before harvesting cells for the RNA isolation, real-time-PCR results were calculated as copy number per 20 ng of total RNA (Fig. 3).

Interestingly, the expression level of the *TGFβ* was highest in serum-free media supplemented with LIF cultivated cells, but did not change in serum medium with different FBS concentrations and in serum medium diluted with serum-free media supplemented with LIF 3:1. In cells cultured in serum-free media supplemented with LIF 1:1, the level of *TGFβ* was significantly lower than in cells cultured in FBS medium containing FGF, suggesting that perhaps FGF could have a negative regulatory effect on the gene expression of *TGFβ*.

Discussion

In this work we showed that some populations of cells isolated from subcutaneous fat can grow *in vitro* for many passages. Under different media conditions they develop specific morphology as in the case of serum and serum-free media. They can not only grow in culture for longer periods but they also can differentiate into cell types other than mezenchymal cells, such as into neuronal cells, which is consistent with the literature (Kokai

et al. 2005; Jeon et al. 2006), indicating that isolated hADPC have features characteristic to stem cells.

In this work we showed that some of the hADPC cultivated in various medium not only changed their phenotypical appearance, but also differed in gene expression level, as in the case of *TGFβ* and other genes (*GAPDH*, *CXCL12* and *CXCR4*; Cakstina et al., unpublished data) in this cell line. Real-time PCR analysis of *TGFβ* also showed that there was no difference between 10 % FBS of 30 % FBS cell cultivation in terms of *TGFβ* (and also *GAPDH*) expression levels. However, *TGFβ* expression in cells cultivated in serum-free medium supplemented with LIF, in which the cells had a tendency to form neurospheres, was significantly higher than in cells grown in serum containing media (Fig. 3). We suggest that FBS may contain some factors that negatively regulate the expression of *TGFβ*, particularly in the case when endogenous FGF is added to the serum medium. There is some evidence in the literature of interaction between *TGFβ* and FGF, suggesting a negative effect of FGF on *TGFβ* expression in quail heart tubulogenesis (Holifield et al. 2004).

Nevertheless, more research is needed in order to characterize isolated cells and to study the role of exogenous TGFβ in hADPC and other putative stem cell lines isolated from different tissue. Therefore, we first needed to examine the level of endogenous TGFβ and the influence of various media on the expression of *TGFβ*. The global aim of the project is to investigate the role and possible use of TGFβ and other factors in controled differentiation towards other important cell types for which the adipoze-derived precursor cells hold a great potential (Roche et al. 2007). Further research is aimed towards characterization of other cell lines isolated from various tissue samples by using not only FACS but also looking at different important signaling pathway and cell cycle regulatory gene expression patterns, such as *TGFβ*.

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Dažādās vidēs kultivētu no taukaudiem iegūto priekšteču šūnu izolēšana, raksturošana un *TGFβ* gēna ekspresijas analīze

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Kopsavilkums

Pieauguša cilvēka, jeb autologās, cilmes šūnas, kas ir spējīgas pašatjaunoties un diferencēties dažādos šūnu tipos, ir perspektīvs izejmateriāls šūnu terapijā, jo tās var izolēt no visdažādākajiem pieauguša cilvēka audiem, kā arī to pētīšana un turpmāka izmantošana terapijā (piem., mazāks atgrūšanas risks) saskaras ar ievērojami mazāk ētikas dabas jautājumiem nekā darbs ar embrionālajām cilmes šūnām. Mūsu ķermenī ir daudz taukaudi, kas satur vairākas mezenhimālo cilmes šūnu populācijas. Pēc literatūras datiem, transformējošais augšanas faktors beta (TGFβ) ir viena no daudzaja molekulām, kas saistīta ar mezenhimālo cilmes šūnu diferenciāciju. Šī darba mērķis ir izpētīt no cilvēka taukaudiem izdalīto mazdiferencēto aizmetņa šūnu spēju diferencēties citos šunu tipos, kā arī pētīt šūnu kultivēšanas vides ietekmi uz šūnu morfoloģijas izmaiņām un *TGFβ* gēna ekspresiju. Darbā iegūtie dati parāda, ka, kultivējot no taukaudiem izdalītās šūnas serumu saturoša un bezseruma vidē, šūnu morfoloģija, kā arī *TGFβ* ekspresijas līmenis ievērojami izmainas. Pētījuma gaitā veikti eksperimenti attaino iegūto šūnu populāciju spēju diferencēties ne tikai mezenhimalās līnijas šūnās, bet arī neironālās līnijas šūnās, kas norāda uz šo populāciju diferenciācijas plastiskumu. Mēs varam secināt, ka no cilvēka taukaudiem izdalīto šūnu populācijas satur noteiktu daudzumu šūnu ar cilmes šūnām raksturīgām īpašībām – spēju diferencēties dažādos šūnu tipos. Šūnu kultivēšanas videi ir liela nozīme populācijas augšana un morfoloģijas izmaiņas – serumu saturošā vidē šūnas aug ātrāk un veido monoslāni, savukārt bez seruma vide šūnām ir tendence veidot sfēras un vairoties daudz lēnāk. *TGFβ* ekspresija ir augstāka tieši šūnās, kas vairojas lenām un ir kultivētas bez seruma vidē.

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