*Gametophyte morphology of four subspecies of Asplenium trichomanes L.**

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Resumen:

HERRERO, A., PRADA, C., PANGUA, E., ESCUDERO, A., RUBIO, A. & PAJARON, S. 1993. Morfología del gametófito de cuatro subespecies de Asplenium trichomanes L. *Bot. Complutensis* 18: 67-77

Mediante el cultivo de esporas de *Asplenium trichomanes* L. (subsp. *trichomanes*, subsp. *quadrivalens* D.E. Meyer, subsp. *pachyrachis* (Christ) Lovis & Reichst. y subsp. *inexpectans* Lovis) se ha estudiado el desarrollo de los gametófitos y sus características morfológicas. Excepto en la subsp. *inexpectans* y en dos de las cuatro muestras estudiadas de la subsp. *pachyrachis*, se forman pelos pluricelulares marginales en una proporción variable de protalos según el taxon. Se analiza la longitud, número de pelos por protalo y densidad de los mismos y se discute su valor taxonómico, así como el de otros caracteres morfológicos como el margen y la escotadura.

Abstract:

HERRERO, A., PRADA, C., PANGUA, E., ESCUDERO, A., RUBIO, A. & PAJARON, S. 1993. Gametophyte morphology of four subspecies of Asplenium trichomanes L. *Bot. Complutensis* 18: 67-77

By culturing spores of Asplenium trichomanes L. (subsp. trichomanes, subsp. quadrivalens D.E. Meyer, subsp. pachyrachis (Christ) Lovis & Reichst. and subsp. inexpectans Lovis), we have studied the development of the gamethophytes and their morphological features. Multicellular marginal hairs were produced in different numbers, depending on the taxon, except for subsp. inexpectans and two samples of subsp. pachyrachis. Length, density and number of hairs per gametophyte were analyzed and their taxonomic value is discussed as well as other morphological features such as margin and apical notch.

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INTRODUCTION

Study of fern gametophytes has become essential to complete morphological, ecological and reproductive knowledge and has contributed to a better understanding of taxonomic relationship (STOKEY, 1951; NAYAR & KAUR, 1969 and 1971; ATKINSON, 1973; WINDHAM & HAUFLER, 1986). In spite of the presence of numerous papers on fern gametophytes, the number of species whose gametophytes are know in detail is still small (TAYLOR & MICKEL, 1974). The prothallial development of some Asiatic and European *Aspleniaceae* was studied by MOMOSE (1959, 1960a, 1960b, 1961a and 1961b), NAYAR & al. (1968), HUREL-PY (1950), ARMENISE (1959) and HENRIET (1970).

Among the naturally occurring *Asplenium* species in Spain, we chose *Asplenium trichomanes* L. because of the diversification of this taxon of which four subspecies are recognized: subsp. *trichomanes* and subsp. *inexpectans* Lovis, both diploid, and subsp. *quadrivalens* D.E. Meyer and subsp. *pachyrachis* (Christ) Lovis & Reichst., both tetraploid. The morphological characteristics and the habitat preferences of the sporophytes are well known (LOVIS, 1964; JERMY & PAGE, 1980; LOVIS & REICHSTEIN, 1985; PANGUA & al., 1989); gametophytes, however, have been studied only in part, especially a few details of their morphology (NAYAR & al., 1968; NAYAR & KAUR, 1971). In a previous study (ROA & al., 1987) we examined gametophytes of the two tetraploid subspecies in order to determine if they provide distinctive morphological features; the results pointed to some differences that stimulates us to continue and to complete the study of gametophytes of this group, including the diploid subspecies.

There has been described (RASBACH & al., 1990) a fifth, tetraploid, subspecies, subsp. *coriaceifolium* from Mallorca. This taxon has not been included in our study.

MATERIAL AND METHODS

Spores for gametophyte cultures were obtained from the following plants:

A. trichomanes subsp. *trichomanes*

Spain: Madrid, La Pedriza, sustrato silíceo, 22-IV-1991, *Pangua & Prada* CE181.

Spain: Madrid, La Pedriza, sustrato silíceo, 22-IV-1991, *Pangua & Prada* CE183.

A. trichomanes subsp. *inexpectans*

Austria: Therman-Alpen, bei Gutenstein an der langen Brücke, limestone rocks, 13-VI-1983, leg. *Melzer* s.n., det. *H. Rasbach*. Fronds pressed 15-I-1991.

Switzerland: Tüfels-Cheller, bei Wettingen, Baden, *J. Schneller* 1536. Fronds pressed 16-V-1991.

A. trichomanes subsp. *quadrivalens*

Spain: Orense, entre Córgomo y San Vicente, pizarras, 24-IX-1990, *Prada & al.* CE121.

Spain: Orense, Barco de Valdeorras, Coedo, pizarras, 31-III-1991, *Prada* CE157.

A. trichomanes subsp. *pachyrachis*

Spain: Cuenca, Ciudad Encantada, calizas, 29-IV-1991, *Prada* CE193.

Switzerland: St. Wolgang, Balsthat, Hinterflue, 530 m.s.m., *J. Schneller* 1535. Fronds pressed 16-V-1991.

Spain: Cuenca, Priego, "El Martinete", cerca del río Guadiela, calizas, exposición N., 900 m.s.m., 24-III-1991, *Escudero* CE160.

Spain: Valencia, Benifaró de Valldigna, camino del repetidor, 6-IV-1991, *Prada & al.* CE131.

Spores from each sample were sown in plastic boxes (4,5 x 4,5 x 2,5 cm.) on mineral agar (DYER, 1979) for study of the early stages of development; they were also grown on soil autoclaved twice at 120ºC during 30 minutes in the same kind of boxes, in order to obtain mature prothalli and sporophytes. Four replications of each sample were made. Cultures were maintained under constant environmental conditions in a growth chamber at 23ºC and continuous illumination with white fluorescent tubes. Cultures on soil were watered once a week.

During the first 45 days after sowing, cultures on agar were checked weekly and samples were studied under light microscope; subsequently, about 50 gametophytes growing on soil were removed from each sample every two weeks. All observations on morphology and development were carried out on gametophytes from these laboratory cultures.

We have calculated the number of hairs per gametophyte, the length of hairs and their density (number of hairs/perimeter) by taking ten measurements of each parameter randomly. The perimeter of the prothalli was measured with an image-analysing computer. To test the equality of means both among the individuals and among the taxa, two univariate statistical procedures were used in this study. Analysis of variance (ANOVA) was used when the assumptions of normality, homogeneity of variances and

random sampling were net (Cochran's C test $P<0,05$). When the assumptions of homogeneity of variances was violated, a Kruskal-Wallis test was performed.

RESULTS

All samples started germinating between 5 and 20 days after sowing; subsp. *quadrivalens* and subsp. *pachyrachis* showed a faster development than did subsp. *inexpectans* and subsp. *trichomanes*.

The spore germination pattern follows the *Vittaria* type, and prothallial development seems to follow the *Adiantum* type (NAYAR & KAUR, 1971). Spores of all samples produce a short germ filament of 1 to 3 cells (Fig. 1 a-c) except for subsp. *pachyrachis* which produces 2 to 6 cells (Fig. 1 e). The filaments bear 1 or 2 rhizoids on the basal cell. Development of a bidimensional stage occurs in all samples 8-10 days after spore germination (Fig. 1 d-f), and the apical notch becomes evident three weeks after germination (Fig. 1 g). Mature prothalli are cordate, broader than long as usual in the family (Fig. 1 h-l); in subsp. *inexpectans*, the apical notch is broader than in the other subspecies (Fig. 1 h, 2 a).

The protalli of subsp. *trichomanes*, subsp. *quadrivalens* and subsp. *pachyrachis* (except for the samples CE131 and CE160) bear multicellular marginal hairs with (3)4-5(6) green cells (Fig. 2); the terminal cell is grandular, slightly swollen at the apex and contains a brown secretion. The hairs are straight or curved. The proportion of hairy gametophytes is shown in figure 3. Hairs appear 30-40 days after germination in all hair-bearing samples when the prothalli have become distinctly cordate. The data of parameters studied are shown in table 1.

The number of hairs per gametophyte is variable among the taxa; in 50 days old gametophytes, when sex organs are well developed, subsp. *quadrivalens* has a mean of 8 hairs, whereas subsp. *trichomanes* and *pachyrachis* have only 2 or 3 hairs (Fig. 4 a). In older gametophytes (90 days old), when fertilization has begun to take place in the cultures, the mean number of hairs increases considerably in subsp. *quadrivalens*, but in subsp. *trichomanes* and *pachyrachis* the mean does not change (Fig. 4 b).

In the same way, the density of the hairs varies among the taxa; there are significant differences in density in the 50 and 90 days old stages (Table 1) for all taxa.

The length of the hairs also varies with the taxon; in 50 days old gametophytes the hair length differs significantly among taxa; samples of subsp. *trichomanes* have shorter hairs (about 90 μm), subsp. *quadrivalens* has hairs of about 110 μm, and subsp. *pachyrachis* shows the longest

hairs, about 130 µm (Fig. 4 c). In 90 days old gametophytes hairs are longer and still show significant differences among the taxa (Fig. 4 d), but 15 days later, the hairs of subsp. *pachyrachis* have grown, exceeding slightly the mean length of the hairs of subsp. *quadrivalens*, whereas in subsp. *trichomanes* the mean does not change (Fig. 4 e).

One month after germination all samples produced archegonia, but the diploid subspecies had a lower percentage of prothalli bearing archegonia than the tetraploid ones. Antheridia appeared about two weeks later in all cases.

DISCUSSION

The morphological features of the gametophytes are similar in all four taxa, but subsp. *inexpectans* has a notably broad apical notch and the marginal cells frequently protrude, giving the prothalli a characteristic appearance; the edge is always naked.

NAYAR & al. (1968) found gametophytes of *A. trichomanes* to be naked, but according to our observations in three of the subspecies the prothalli bear hairs in different proportion. In subsp. *quadrivalens* hairy gametophytes are produced at a high rate so that practically all plants of the cultures bear hairs. In this case the higher density of hairs imparts a typical appearance to the prothalli as compared with the other taxa.

An unexpected problem was posed by subsp. *trichomanes* and subsp. *pachyrachis* samples CE160 and CE131; in these cases, both hairy and naked gametophytes were produced. We did not find any reference to this in the consulted literature. Studies on gametophyte morphology are not usually done over a sufficiently long period of time and with enough gametophytic individuals to establish the characteristic variations in each species. In studies of hairy gametophytes, attention is focused mainly on the type of hair, its position and the time of its development, but there are no indications of naked and hairy gametophytes growing together, both produced by spores from the same sporophytic individual. PRAY (1968) mentions morphological variation in a gametophytic population arisen from a single sporophyte of *Pellaea andromedaefolia* (Kaulf.) Fée var. *pubescens* D.C. Eaton; the variation consisted in some differences in the shape of the gametophytes.

We have observed that size, sex, and age of prothalli are not related to the presence of hairs, so that at present we do not have any explanation for this striking feature.

The highest incidence of hairy gametophytes for subsp. *trichomanes* occurs in 60 days old prothalli, later decreasing gradually, being about

10% of the cultures in 135 days old individuals; the behaviour coincidence of both samples studied is remarkable. We have observed in these old prothalli that the basal cell degenerates and is easily ruptured (Fig. 2 e, f). This might explain why the number of hairy gametophytes decreases. Diploid *Phyllitis scolopendrium* from Europe has been reported to have unicellular hairs on the gametophytes, whereas American tetraploids have 1, 2, and 3-celled hairs (ATKINSON & STOKEY, 1964). In our study, the number of cells does not differ significantly both in diploid and tetraploid taxa but the length of the hairs is much less in the diploid.

With regard to subsp. *pachyrachis*, we must emphasize the different behaviour found in the studied samples. As noticed above, two of them bear hairs on some of their gametophytes, the other two being naked. LOVIS & REICHSTEIN (1985) indicated the existence of plants "close but distinct from true *A. trichomanes* subsp. *pachyrachis*"; PANGUA & al. (1989) also mentioned variability within this taxon, as was illustrated in their fig. 1 G, H. Fronds of typical subsp. *pachyrachis* have pinnae that are serratolobate, tend to be symmetrical, 2-4 times longer than wide with biauriculate base; there are plants in which the main morphological characters are the same as in the typical forms, but the pinnae are less than twice as long as wide and they are not always biauriculate. In the four samples of subsp. *pachyrachis* studied, two had typical morphology; gametophytes produced by these plants were always naked. The other two sporophytes, whose pinnae were shorter, produced gametophytic cultures of mixed hairy and naked prothalli. These differences in gametophyte morphology agree with the idea that morphologically atypical plants may represent a different taxon.

The origin of tetraploids in *A. trichomanes* is not well known at present. Hybridization between cytotypes is common; reticulate evolution and possible polytopic origin of the different lineages make it difficult to ascertain relationships in the complex. Electrophoretic analysis of isozymes in gametophytic and sporophytic populations may help in resolving these problems. Our next investigations will be focussed on this topic.

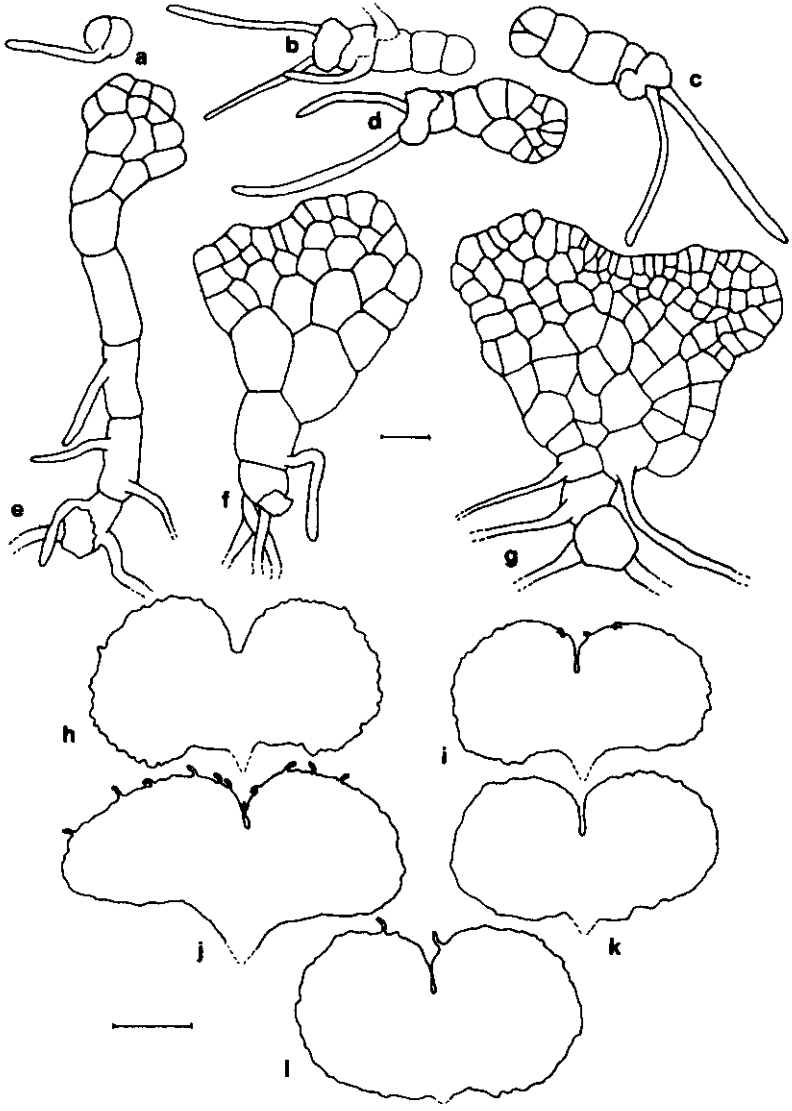

Fig. 1: a-g, Early stages of prothallial development: a, first rhizoid; b, germ filament; c, beginning of laminar stage; e-f, development of meristematic cells; g, beginning of apical notch; h-l, silhouette of mature gametophytes: h, subsp. *inexpectans*; i, subsp. *trichomanes*; j, subsp. *quadrivalens*; k-l, subsp. *pachyrachis*. Scale bars: a-g, 55 µm; h-l, 1mm.

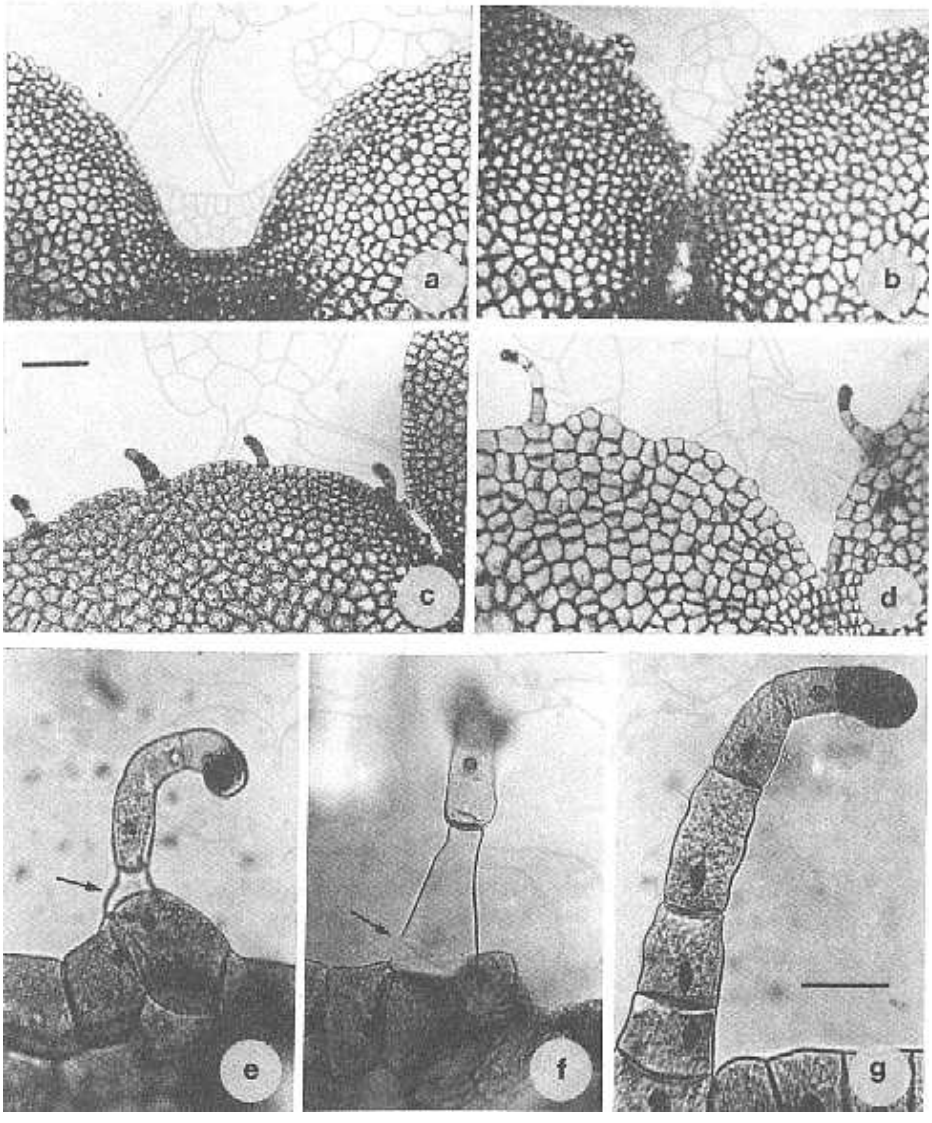

Fig. 2: a-d, Morphological features of margin and apical notch: a, subsp. *inexpectans*; b, subsp. *trichomanes*; c, subsp. *quadrivalens*; d, subsp. *pachyrachis*. e-g, detail of hairs: e-f, subsp. *trichomanes* showing a detaching hair; g, subsp. *pachyrachis*. Scale bars: a-d, 60 µm; e-g, 35 µm. Arrows indicate the point of breaking of hairs.

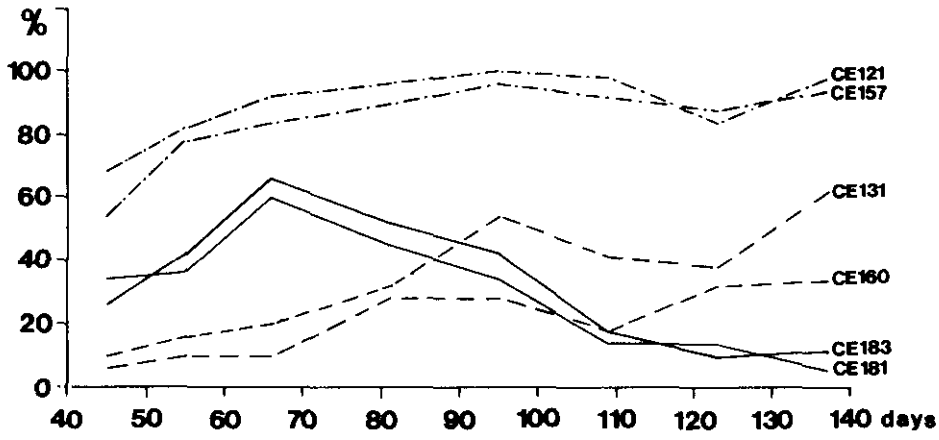

Fig. 3: Percentage of hairy prothalli: subsp. *quadrivalens* (CE121, CE157); subsp. *pachyrachis* (CE131, CE160); subsp. *trichomanes* (CE181, CE183).

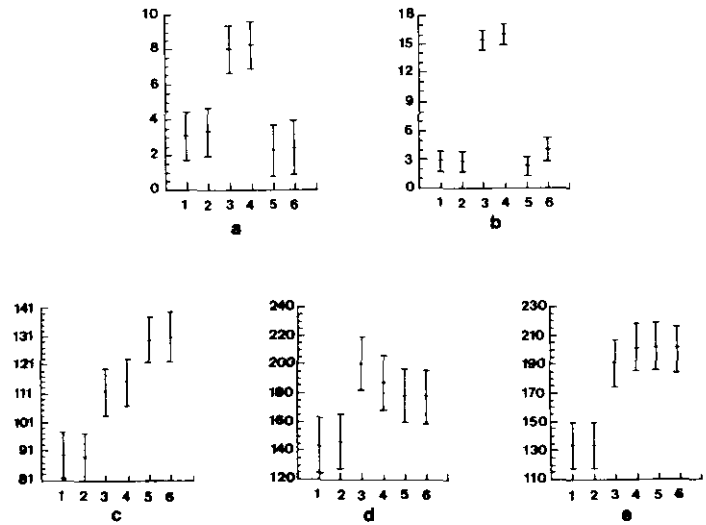

Fig. 4: Mean confidence intervals, LSD (95%) of gametophyte characters. a, number of hairs per prothallus (50 days old); b, number of hairs (90 days old); c, hair length (50 days old); d, hair length (90 days old); e, hair lenth (105 days old). 1, population CE181; 2, CE183; both of subsp. *trichomanes*; 3, CE121; 4, CE157; both of subsp. *quadrivalens*; 5, CE160 and 6, CE131; both subsp. *pachyrachis*. Length of hairs in µm.

Table 1: Means (X) and standard deviations (SD) of gametophyte characters scored for the studied populations. Tests of significance refer to ANOVA except those marked with KW, which indicate results of KW test (*** indicates p<0.001).

Sample size: ten individuals in each population

		trichomanes		*quadrivalens*		*pachyrachis*		
		CE181	**CE183**	**CE121**	**CE157**	**CE160**	**CE131**	
Number of hairs	$\overline{X}$	3,12	3,37	8	8,25	2,28	2,42	***
(50 days old)	SD	1,24	1,40	3,02	2,31	1,11	1,61	
Number of hairs	$\overline{X}$	2,80	2,80	15,40	16	2,40	4,10	***
(90 days old)	SD	1,22	0,76	2,67	2,26	1,34	1,19	
Density	$\overline{X}$	0,33	0,35	0,72	0,89	0,20	0,18	KW***
(50 days old)	SD	0,12	0,12	0,17	0,30	0,09	0,09	
Density	$\overline{X}$	0,17	0,19	0,80	0,89	0,18	0,23	KW***
(90 days old)	SD	0,05	0,06	0,24	0,16	0,13	0,06	
Hair length (µm)	$\overline{X}$	90,10	89,40	112,10	115,40	131,22	130,20	***
(50 days old)	SD	9,07	12,48	11,22	11,91	16,99	14,64	
Hair length (µm)	$\overline{X}$	144	146,90	201,30	187,80	178,40	177,70	***
(90 days old)	SD	22,80	29,10	34,03	24,53	34,81	33,24	
Hair length (µm)	$\overline{X}$	133,90	133,90	190,90	201,80	201,80	199,70	***
(105 days old)	SD	23,47	15,79	27,30	25,35	28,68	30,70	

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