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Original article

Workers of honey bee (*apis mellifera* l.) reared in small-cell combs in apiary conditions show higher activity of the proteolytic system and lower protein concentrations on the cuticle surface than workers reared in standard-cell combs

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Abstract

This study is a continuation of innovative research on the impact of the simultaneous use of standard- and small-cell combs in bee colonies on the characteristics of worker bees and bee colonies. The nests of these colonies had two types of combs: small-cell (approximate cell width/size of 4.90 mm) and standard-cell (approximate cell width/size of 5.50 mm). The aim of the study was to compare the activities of proteases and their inhibitors present on the cuticle of workers reared in small-cell combs (SMC workers) and standard-cell combs (STC workers) in colonies kept simultaneously in standard- and small-cell combs.

The width/size of comb cells in which the workers were reared had a significant effect on protein concentrations and activities of the proteolytic system, i.e. proteases and their inhibitors, on the cuticle surface. Regardless of the age of the workers (1 day, 7 days, 14 days, 21 days, and 28 days), the protein concentrations were statistically significantly higher ($p \leq 0.01$) in the STC than SMC workers. The opposite was found in the case of the activities of proteases and their inhibitors: regardless of the age of the bees, the activities were significantly higher in the SMC workers.

The differences between workers reared in small-cell combs and those reared in standard-cell combs may be responsible for their predispositions to perform different tasks in the colony. In our opinion, workers reared in small-cell combs are more predisposed to work as foragers outside the nest. However, this hypothesis requires confirmation in further research.

Keywords: small-cell combs, *Apis mellifera*, body surface proteins,; proteolytic enzymes, protease inhibitors

Introduction

A very high density of honey bees in the colony nest creates favorable conditions for the growth of pathogens and parasites. To resist their pressure, bees have evolved behavioral defenses at the colony level. Individual apians have biochemical mechanisms for fighting pathogens (Cremer et al. 2007, Evans and Spivak 2010, Strachecka et al. 2018). The first barrier against pathogens trying to penetrate the bee body is a system of active cuticle surface proteins, i.e. the proteolytic system (Strachecka et al. 2018). This system consists of proteases and protease inhibitors constituting the basis of humoral immunity barriers against diseases and pathogens (Strachecka et al. 2018). Its proteolytic components are responsible for the lysis of pathogen proteins and pathogenic factors preventing their entry into the bee organism (Bode et al. 1999, Evans et al. 2006, Strachecka and Demetraki-Paleolog 2011, Migdał et al. 2021, Skowronek et al. 2021). Moreover, proteases are involved in the release of hormones and activation of proenzymes, thereby exerting an impact on body homeostasis (Strachecka and Grzywnowicz 2008, Strachecka et al. 2018). In turn, protease inhibitors defend the organisms of bees against pathogen proteases through blockage or "depletion" of their lytic properties (Leung et al. 2000). They also protect honey bees against redundant activation of phenoloxidase activity mechanisms (Kanost and Clarke 2005, Strachecka and Grzywnowicz 2008). The activity of the proteolytic system in hemolymph may be promoted by biostimulants, e.g. caffein (Strachecka et al. 2014) and curcumin (Strachecka et al. 2015), or may be affected negatively by pesticides present on the bee's body surface and in their hemolymph or organs (Blacquière et al. 2012, Cullen et al. 2019, Paleolog et al. 2020). Such impairment activity is also revealed by acaricides used against *Varroa destructor* as well (Strachecka et al. 2016). The latest research has confirmed that the proteolytic system in bees is influenced by the caste status (Strachecka et al. 2021) and inanimate components of the bee colony nest, i.e. worker combs or, more precisely, the width/size of comb cells (Dziechciarz et al. 2022a, 2023a).

Honey bees build nests where they live for many years. The nest is composed of wax combs, whose cells are used for storage of food and rearing subsequent generations of bees. Although the bee comb is an inanimate element, it is the largest organ of the bee colony superorganism, as suggested by Tautz (2007). Therefore, it can be assumed that, through the size of their cells, combs influence the traits of reared workers and the characteristics of the bee colony as a superorganism. Currently, the use of plastic or wax foundation templates in apiaries forces bees to construct combs with almost the same standardized cell width (McMullan and Brown 2006). In Europe, the standard width/size of the cells in the wax foundation is usually approximately 5.40-5.50 mm (McMullan and Brown 2006, Coffey et al. 2010, Singer et al. 2019). In feral bee colony nests, the cell width of combs built without the wax foundation exhibits substantial variability. As reported by Maggi et al. (2010), feral bee colonies rear worker brood in 4.17-6.75 mm wide cells. Although the standardization of honeycomb cells in Europe was initiated in the second half of the 19th century with the introduction of the wax foundation, the impact of this process on the traits of bees and bee colonies is still poorly known. The first attempts to use a smaller width of comb cells were prompted by research results showing that rearing workers in small-cell combs, in contrast to standard-cell combs, limits the growth of populations of the *V. destructor* mite, i.e. a common bee pest (Message and Goncalves 1995, Piccirillo and De Jong 2003, Taylor et al. 2008, Ellis et al. 2009, Berry et al. 2010, Coffey et al. 2010, Maggi et al. 2010, Seeley and Griffin 2011, Singer et al. 2019). In Europe, combs with a cell size/width of 4.90 mm are usually referred to as small-cell combs (McMullan and Brown 2006, Coffey et al. 2010, Singer et al. 2019).

It has been found to date that keeping colonies in small-cell combs has a considerable effect on the morphological traits of bee workers and the biology of the bee colony (McMullan and Brown 2006, Seeley and Griffin 2011, Dziechciarz et al. 2021). The change in the value of the morphometric parameters of worker bees was found not to be proportional to changes in the width of the comb cells where they were reared. Workers reared in small-cell combs were characterized by a significant increase in the value of the fill factor (thorax width to cell width ratio) (Dziechciarz et al. 2021). Additionally, colonies kept in small-cell combs exhibited greater intensity of hygienic behavior (Olszewski et al. 2014a), and workers reared in small-cell combs had a longer lifespan (Olszewski et al. 2014b).

However, studies conducted to date compare colonies maintained only in small-cell combs with those maintained only in standard-cell combs. Our team has been carrying out innovative research on the impact of variability in the comb cell width on the characteristics of bees and bee colonies. We compare the characteristics of bees and bee colonies kept only in one type of combs (small- or standard-cell combs) with those kept simultaneously in both types of combs, i.e. small-cell and standard-cell combs, in one nest.

To date, we have demonstrated the impact of the size of worker cells on the activity of the proteolytic

system in the hemolymph in apiary (Dziechciarz et al. 2022a) and laboratory (Dziechciarz et al. 2023a) conditions. Regardless of the conditions (apiary/laboratory), the activity of the proteolytic system (proteases and their inhibitors) was higher in workers reared in small-cell combs. Higher protein concentrations were determined in workers reared in standard-cell combs in apiary conditions (Dziechciarz et al. 2022a) and in workers reared in small-cell combs in laboratory conditions (Dziechciarz et al. 2023a). The present study is a continuation of our previous research on the effect of the width/size of comb cells on the proteolytic activities in worker bees.

The aim of the investigations was to determine the impact of the comb cell width on the proteolytic system activities and protein concentrations on the cuticle/body surface of honeybee workers reared in small- and standard-cell combs in apiary conditions.

Materials and Methods

The apiary part of the experiment was carried out in the apiary of the University of Life Sciences in Lublin (Poland) (51.224039 N-22.634649 E). Due to the considerable impact of random environmental factors on the results of the apiary study, the investigations were continued for three consecutive years (2020, 2021, and 2022).

Ethical review and approval were waived for this study, because honey bees (*Apis mellifera*) are not included in the European directive 2010/63/EU on the protection of animals used for scientific purposes.

Acquisition of bees

In each of the consecutive years (2020, 2021, and 2022), five foster colonies with similar strength and structure were used. The nest of each colony consisted of nine worker combs and one drone comb. One of the nine worker combs was usually filled with food (honey and bee breed) and served as a storage comb. The other worker combs were almost entirely occupied by brood. All colonies were fully populated by workers and were headed by naturally mated sister queens of the same age (Dziechciarz et al. 2021, Dziechciarz et al. 2022b). The nest of each foster colony contained two types of worker combs: 5 standard-cell combs (cell width: approx. 5.50 mm) and 4 small-cell combs (cell width: approx. 4.90 mm) (Dziechciarz et al. 2022a). The arrangement of the small- and standard-cell combs in the brood chamber was consistent with that reported previously (Dziechciarz et al. 2022b). The workers in each foster colony were reared in experimental combs, i.e. in one small-cell comb (SMC) and one standard-cell comb (STC) according to the method proposed by Dziechciarz et al. (2022a). After 20 days of oviposition, each experimental comb was placed in a separate mesh frame cage and kept in an incubator until the emergence of workers. Approximately 1500 workers from the pool emerging from each experimental comb in each of the five foster colonies were labeled (POSCA PC-3M marker). Workers reared in the small-cell experimental combs (SMC) were labeled with a different color than those reared in the standard cell experimental combs (STC). The labeled workers were placed in five colonies kept in hives with six combs. The colonies had similar strength and structure; each had a properly ovipositing queen, five combs with different aged brood, and one comb with honey and bee breed. The colonies were headed by naturally mated sister queens of the same age. Workers from different foster colonies were not mixed, and the workers reared in each of the foster colonies were allocated to a separate colony. We used colonies kept on six combs, as it was easier to collect the labeled workers.

Body surface protein collection and analysis

The 1-day-old SMC and STC workers were collected on the labeling day. Next, after 7, 14, 21, and 28 days, the labeled SMC and STC workers were selected randomly from each of the five colonies kept on the six combs and collected for analyses. On each collection day, selected worker was collected using tweezers and locked in a 5 mL plastic sterile tube. Samples were then immediately frozen and stored at -80 °C. In the laboratory, each worker was washed by 2-min vortexing of each sample into a 2-mL plastic tube with 1.5 mL of a 1% Triton X-100 solution (Łoś and Strachecka 2018). The worker's cuticle surface elutions were then frozen and stored at −80 °C until further analysis. Total protein concentrations were assayed with the Lowry et al. (1951) method modified by Schacterle and Pollack (1973). The activities of acidic (pH 2.4), neutral (pH 7.0), and alkaline (pH 11.2) proteases on the cuticle/body surfaces were analyzed using the method proposed by Anson (1938) and modified by Strachecka and Demetraki-Paleolog, (2011). Protease inhibitor activities were determined as in Lee and Lin (1995). The number of samples collected in the consecutive years is shown in Table 1.

Measurements of comb cell size/width

Each small- and standard-cell comb where the workers were reared was photographed in the center of each comb half on one side of the comb. Next, in each half, the widths of 10 adjacent cells in contact with vertical side walls were measured following the procedure used by Dziechciarz et al. (2021). Each year (2020,

Table 1. Number of samples of cuticle surface elutions in each bee age group (1 d, 7 d, 14 d, 21 d, and 28 d) in two worker groups in the three consecutive years. Each sample was taken from one worker.

Age Group	Worker group	Year		
		2020	2021	2022
1 day	SMC	70	50	50
	STC	70	50	50
7 days	SMC	50	50	50
	STC	40	50	50
14 days	SMC	50	50	50
	STC	50	50	50
21 days	SMC	40	50	45
	STC	40	50	45
28 days	SMC	20	15	25
	STC	20	15	25

SMC – workers reared in small-cell combs, STC – workers reared in standard-cell combs.

2021, and 2022), 100 cells (5 combs × 2 measurements of 10 cells per combs) were measured in each type of the comb (small- and standard-cell comb) (Dziechciarz et al. 2022a).

Statistical analysis

The statistical analysis of the results was carried out using Statistica software formulas, version 13.3 (2017) for Windows, StatSoft Inc., Tulsa, OK, USA.

To assess the effect of the year (2020, 2021, and 2022) and age (1 d, 7 d, 14 d, 21 d, and 28 d) in each study year on the protein concentrations and activities of the analyzed types of proteases and their inhibitors separately for the SMC and STC workers, the Kruskal--Wallis test was used, as the data were not normally distributed. The distribution of these data was analyzed using the Shapiro-Wilk test.

The protein concentrations and the activities of each type of protease (acidic, neutral, and alkaline) and their inhibitors in 1-day-old bees were compared between the SMC and STC workers using the pairwise Wilcoxon test (data with non-normal distribution). The distribution of these data was analyzed using the Shapiro-Wilk test. The protein concentrations and the activities of each type of proteases and their inhibitors within the age groups (7 d, 14 d, 21 d, and 28 d) were compared between the SMC and SMC groups with the Mann--Whitney U test (data with non-normal distribution). The distribution of these data was analyzed using the Shapiro-Wilk test.

The effect of the year (2020, 2021, and 2022) on the width of the comb cells in the foster colonies was analyzed separately for the small-cell combs (n=300) and the standard-cell combs (n=300) using the Kruskal--Wallis test (data with non-normal distribution). The distribution of these data was analyzed using the Kolmogorov-Smirnoff test.

In the foster colonies, the width of the cells in the small-cell combs (n=300) and the width of the cells in the standard-cell combs (n=300) was compared collectively for the three years using the Mann-Whitney U test (data with non-normal distribution), as the effect of the year was not significant for either the small- or standard-cell combs (Kruskal-Wallis test). The distribution of these data was analyzed using the Kolmogorov--Smirnoff test.

Results

Comb cell width

The cell width in the small-cell combs and in the standard-cell combs in the foster colonies did not differ statistically significantly between the years (respectively: $H_2 = 0.279$, $p = 0.869$, $n = 300$; $H_2 = 2.359$, $p = 0.302$, $n = 300$; Kruskal-Wallis test).

The width of the small-cells was significantly smaller ($p \leq 0.01$; $n = 300$; Mann-Whitney U test) than that of the standard-cells. The mean cell width was 4.97 mm (SD = 0.042) in the small-cell combs and 5.56 mm (SD = 0.048) in the standard-cell combs.

Protein concentrations and activities of proteases and protease inhibitors

In the SMC workers, the year (2020, 2021, and 2022) had a statistically significant effect on all the biochemical parameters of the cuticle surface (Table 2). In the STC workers, no statistically significant effect of the year was shown only in the case of the activities of acidic proteases and neutral protease inhibitors. In all the study years, the age (1 d, 7 d, 14 d, 21 d, and 28 d) of both the SMC and STC workers had a statistically significant effect on all the analyzed cuticle surface parameters (Table 2).

Table 2. Effect of the year (2020, 2021, and 2022) and age (1 d, 7 d, 14 d, 21 d, and 28 d) on cuticle surface parameters in workers reared in small- and standard-cell combs

Cuticle Surface Elution Parameters	Effect of Year		Effect of Age					
			2020		2021		2022	
	SMC	STC	SMC	STC	SMC	STC	SMC	STC
protein concentrations	H = 0.77	H = 26.81	H = 204.42	H = 193.10	H = 200.89	H= 203.26	H = 189.29	H = 189.05
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.68	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of acidic proteases	H = 17.31	H = 4.75	H = 216.56	H = 206.66	H = 203.17	H= 203.17	H = 189.18	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.00	*p* = 0.09	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of neutral proteases	H = 302.90	H = 323.77	H = 216.55	H = 206.67	H = 203.17	H = 203.16	H = 189.19	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of alkaline proteases	H = 42.54	H = 90.35	H = 216.58	H = 206.65	H = 203.17	H =203.16	H = 189.19	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of acidic protease inhibitors	H = 135.94	H = 130.21	H = 216.48	H = 206.58	H = 202.50	H= 203.17	H = 189.18	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of neutral protease inhibitors	H = 9.01	H = 4.72	H = 216.48	H = 206.58	H = 203.17	H= 203.17	H = 189.18	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.01	*p* = 0.09	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00
activities of alkaline protease inhibitors	H = 301.16	H = 321.90	H = 216.49	H = 206.58	H = 203.17	H= 199.84	H = 189.18	H = 189.18
	df = 2	df = 2	df = 4	df = 4	df = 4	df = 4	df = 4	df = 4
	p = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00	*p* = 0.00

SMC – workers reared in small-cell combs, STC – workers reared in standard-cell combs, H – value of statistics for the Kruska-Wallis test, df – number of degrees of freedom, *p* – probability value. The effect of the year and the effect of the age in the SMC and STC groups are significant at p≤0.01.

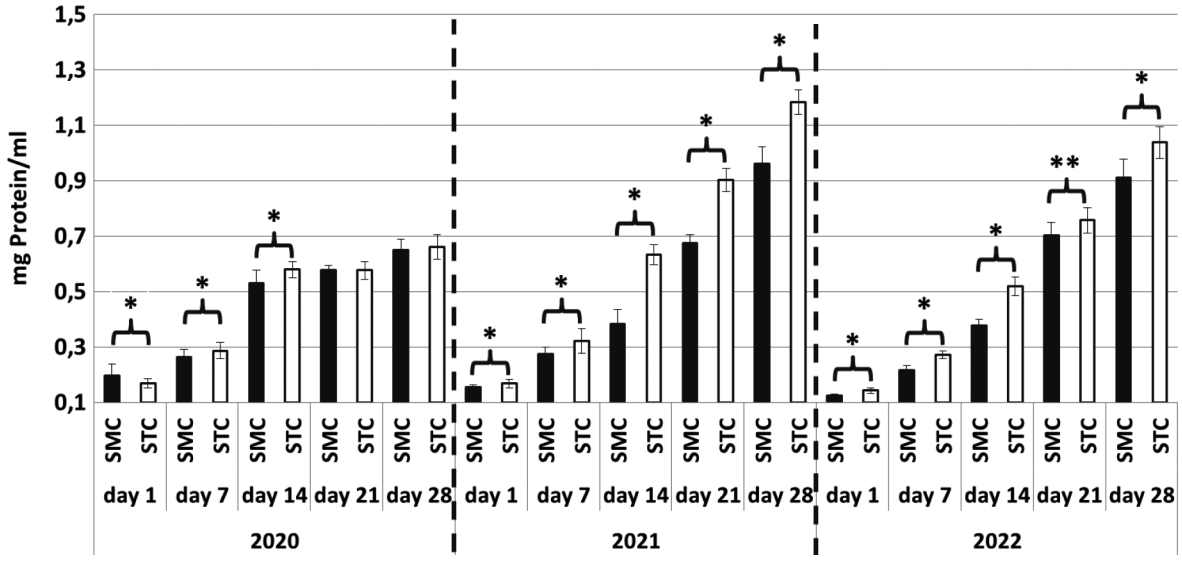

Fig. 1. Protein concentrations on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs; STC – workers reared in standard-cell combs.
* differences between the SMC and STC workers within the age group are significant at p≤0.01, vertical bars indicate standard deviation.

In all the age groups of workers (1 d, 7 d, 14 d, 21 d, and 28 d) examined in 2021 and 2022, the protein concentrations on the cuticle surface were statistically significantly higher (p≤0.01) in the STC workers than in the SMC group (Fig.1, Table 3). Differences were observed in 2020, i.e. the protein concentrations in the one-day-old worker bees were statistically significantly higher (p≤0.01) in the SMC group, and there were no statistically significant differences in this parameter between the STC and SMC workers in the groups of the 21- and 28-day-old bees (Fig. 1, Table 3).

In all the years and worker age groups, the activities of all types of proteases (acidic, neutral, and alkaline) and all types of protease inhibitors (acidic, neutral, and

Table 3. Summary table of cuticle surface protein concentrations and activities of proteases and protease inhibitors in three pH variants in three consecutive years (2020, 2021, and 2022) of workers reared in small- and standard-cell combs.

Year	Day	Group	Protein concentration	Activities of proteases			Activities of protease inhibitors		
				pH 2,4	pH 7,0	pH 11,2	pH 2,4	pH 7,0	pH 11,2
2020	day 1	SMC	0,2±0,04	16,05±0,02	22,9±0,02	35,49±0,02	1,96±0,09	4,88±0,05	1,68±0,08
		STC	0,17±0,02	15,34±0,02	20,38±0,02	34,36±0,02	1,71±0,09	4,66±0,08	1,02±0,12
	day 7	SMC	0,26±0,03	19±0,01	25,86±0,01	39±0,01	2,5±0,04	5,68±0,05	3,49±0,07
		STC	0,29±0,03	17,25±0,11	24,35±0,01	36,39±0,01	2,14±0,05	5,14±0,05	2,15±0,05
	day 14	SMC	0,53±0,05	23,17±0,01	26,68±0,01	39,7±0,01	3,89±0,02	9,79±0,02	4,61±0,02
		STC	0,58±0,03	21,13±0,01	26,67±0,01	37,11±0,01	3,5±0,03	8,08±0,03	3,98±0,03
	day 21	SMC	0,58±0,02	25,17±0,01	28,03±0,01	40,42±0,01	4,68±0,02	10,22±0,02	6,03±0,02
		STC	0,58±0,03	24,17±0,01	27,17±0,01	38,27±0,01	4,11±0,01	9,52±0,01	5,76±0,02
	day 28	SMC	0,65±0,04	26,05±0,5	34,15±0,14	41,38±0,01	6,05±0,04	11,2±0,09	8,53±0,11
		STC	0,66±0,04	26,05±0,5	30,66±0,05	39,19±0,01	5,37±0,03	10,12±0,03	6,05±0,03
2021	day 1	SMC	0,16±0,01	13,33±0,01	25,19±0,01	25,03±0,01	2,47±0,15	4,74±0,14	2,14±0,14
		STC	0,17±0,01	11,38±0,01	24,83±0,01	22,22±0,01	2,16±0,1	4,67±0,1	2,07±0,11
	day 7	SMC	0,28±0,02	17,25±0,01	29,12±0,01	30,1±0,01	2,81±0,07	6,99±0,07	2,61±0,08
		STC	0,32±0,04	15,88±0,01	28,51±0,01	29,94±0,01	2,49±0,05	6,3±0,05	2,26±0,05
	day 14	SMC	0,38±0,05	19,22±0,01	35,82±0,01	37,63±0,01	4,58±0,08	7,28±0,06	4,09±0,05
		STC	0,63±0,04	17,73±0,01	35,52±0,01	37,46±0,01	4,39±0,03	7±0,04	3,55±0,03
	day 21	SMC	0,68±0,01	25,05±0,01	45,92±0,01	46,46±0,01	5,6±0,03	9,57±0,03	6,15±0,03
		STC	0,9±0,01	24,7±0,01	42,19±0,01	41,29±0,01	5,46±0,02	8,48±0,02	5,46±0,02
	day 28	SMC	0,96±0,04	32,14±0,01	52,18±0,01	47,25±0,01	6,22±0,02	11,21±0,02	6,88±0,02
		STC	1,18±0,02	29,5±0,01	48,02±0,01	44,07±0,01	6,23±0,03	9,13±0,02	6,13±0,03
2022	day 1	SMC	0,13±0,01	14,48±0,01	29,78±0,01	34,77±0,01	3,03±0,19	4,1±0,15	5,02±0,18
		STC	0,14±0,01	12,34±0,01	28,63±0,01	31,41±0,01	2,2±0,11	3,37±0,14	5,07±0,14
	day 7	SMC	0,22±0,01	16,33±0,01	29,89±0,01	37,93±0,01	3,9±0,07	5,83±0,12	6,98±0,08
		STC	0,27±0,01	16,11±0,01	29,66±0,01	37,52±0,01	3,79±0,09	4,99±0,09	6,46±0,09
	day 14	SMC	0,38±0,02	18,82±0,01	38,08±0,01	40,43±0,01	5±0,06	9,05±0,06	8,55±0,06
		STC	0,52±0,03	18,53±0,01	36,47±0,01	40,41±0,01	4,31±0,04	8,05±0,04	7,94±0,05
	day 21	SMC	0,7±0,03	22,58±0,01	49,04±0,01	42,55±0,01	6,96±0,04	11,89±0,03	9,01±0,05
		STC	0,69±0,03	22,1±0,01	48,03±0,01	42,18±0,04	6,56±0,04	11,57±0,04	8,93±0,04
	day 28	SMC	0,91±0,04	29,44±0,01	54,17±0,01	43,87±0,01	7,22±0,03	15,79±0,02	9,45±0,02
		STC	1,04±0,03	27,87±0,01	54,16±0,01	43,43±0,01	7,03±0,02	14,01±0,02	9,24±0,02

Values represent mean±standard deviation, SMC – workers reared in small-cell combs, STC – workers reared in standard-cell combs.

alkaline) on the cuticle surface were statistically significantly higher (usually at $p \leq 0.01$) in the SMC than STC workers (Figs. 2-7, Table 3). An exception was found in the group of the 1-day-old workers in 2022, where the activities of alkaline protease inhibitors did not differ statistically significantly between the SMC and STC bees (Fig. 7, Table 3).

Discussion

Our previous studies on the activity of the proteolytic system in the hemolymph of worker bees assessed in both apiary and laboratory experiments showed a significant effect of the size of comb cells on protein concentrations and activities of proteases and their inhibitors (Dziechciarz et al. 2022a, 2023a). On the first day of life, contrasting trends were observed in the protein concentrations on the cuticle surface and in the hemolymph. Higher values of this parameter in the hemolymph were determined in SMC workers in both apiary (Dziechciarz et al. 2022a) and laboratory conditions (Dziechciarz et al. 2023a). In 2021 and 2022, the protein concentrations on the cuticle surface were higher in the STC workers (Fig.1, Table 3). These dif-

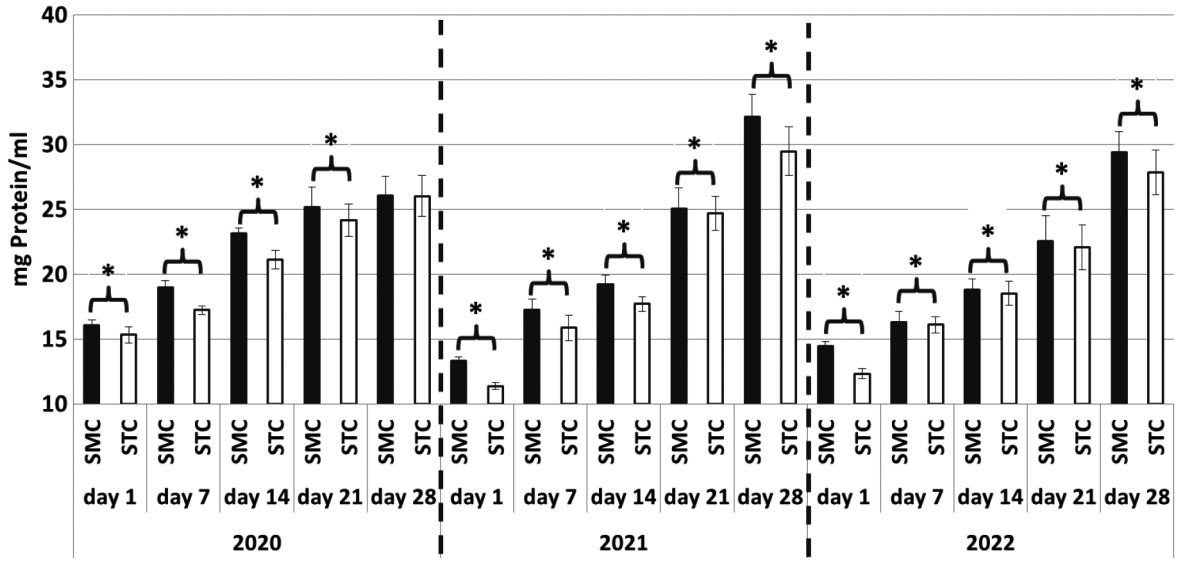

Fig. 2. Acidic protease activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs; STC – workers reared in standard-cell combs.
* differences between the SMC and STC workers within the age group are significant at p≤0.05, ** the differences between the SMC and STC workers within the age group are significant at p≤0.01, vertical bars indicate standard deviation.

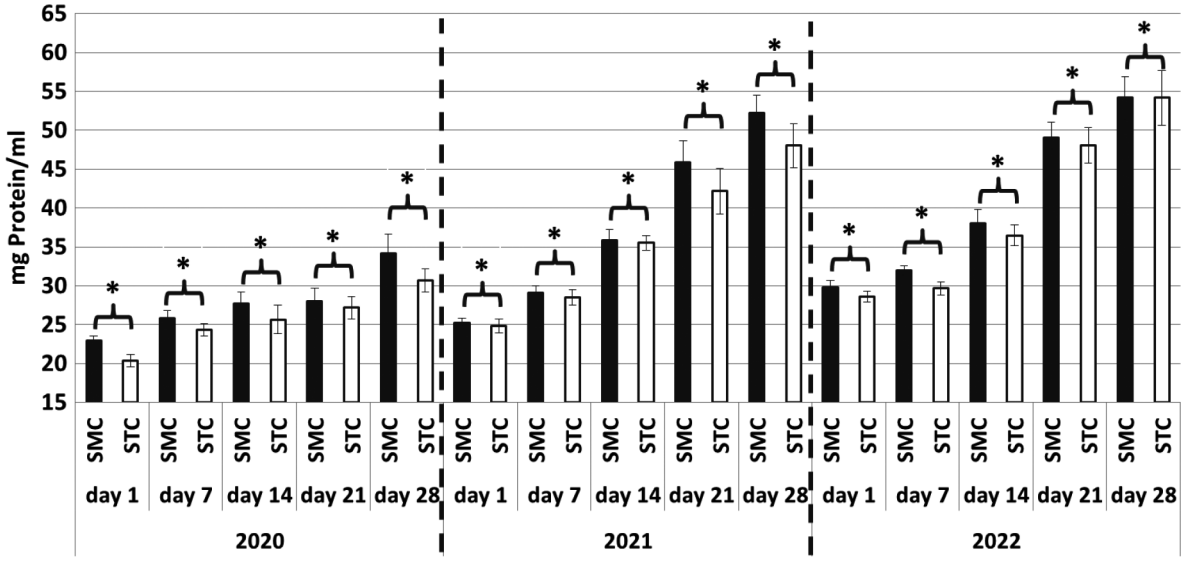

Fig. 3. Neutral protease activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs, STC – workers reared in standard-cell combs.
* differences between the SMC and STC workers within the age group are significant at p≤0.05, ** differences between the SMC and STC workers within the age group are significant at p≤0.01, vertical bars indicate standard deviation.

ferences may indicate different immune mechanisms operating on the body surface than in the hemolymph. In the groups of the older workers (7 d, 14 d, 21 d, and 28 d), the results were consistent only in the apiary conditions, as the protein concentrations in the hemolymph (Dziechciarz et al. 2022a) and on the cuticle surface were higher in the STC than SMC workers (Fig.1, Table 3). The opposite was found in laboratory cage tests, (Dziechciarz et al. 2023a), which indicates that the results of laboratory experiments on honey bees should be verified in apiary conditions, where numerous environmental factors have an impact on the bee colony (Olszewski and Paleolog, 2005). Moreover, the different trends in the protein concentrations in the groups of the 1-, 21-, and 28-day-old workers in 2020 (Fig. 1, Table 3) indicate a possible effect of the season on the results of apiary experiments, which proves the necessity to conduct several-season studies to obtain reliable results.

The results of the determination of the proteolytic system activity (proteases and their inhibitors) on the cuticle surface in the 7-, 14-, 21-, and 28-day-old workers (Figs. 2-7, Table 3) are consistent with the results of analyses of the activity of the proteolytic system

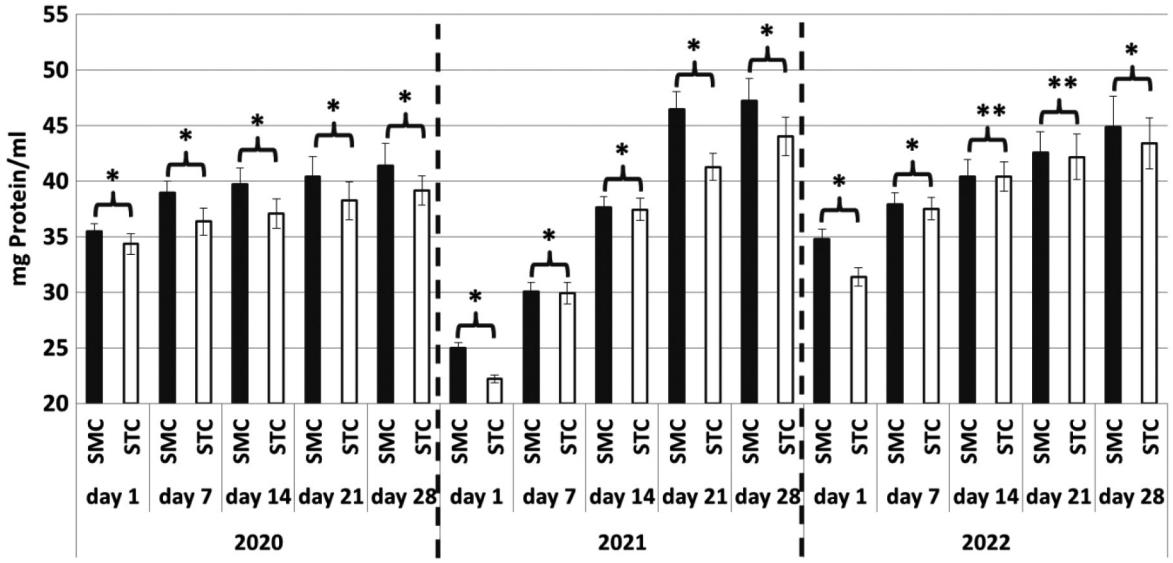

Fig. 4. Alkaline protease activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs; STC – workers reared in standard-cell combs.
* differences between the SMC and STC workers within the age group are significant at $p \leq 0.05$, ** differences between the SMC and STC workers within the age group are significant at $p \leq 0.01$; vertical bars indicate standard deviation.

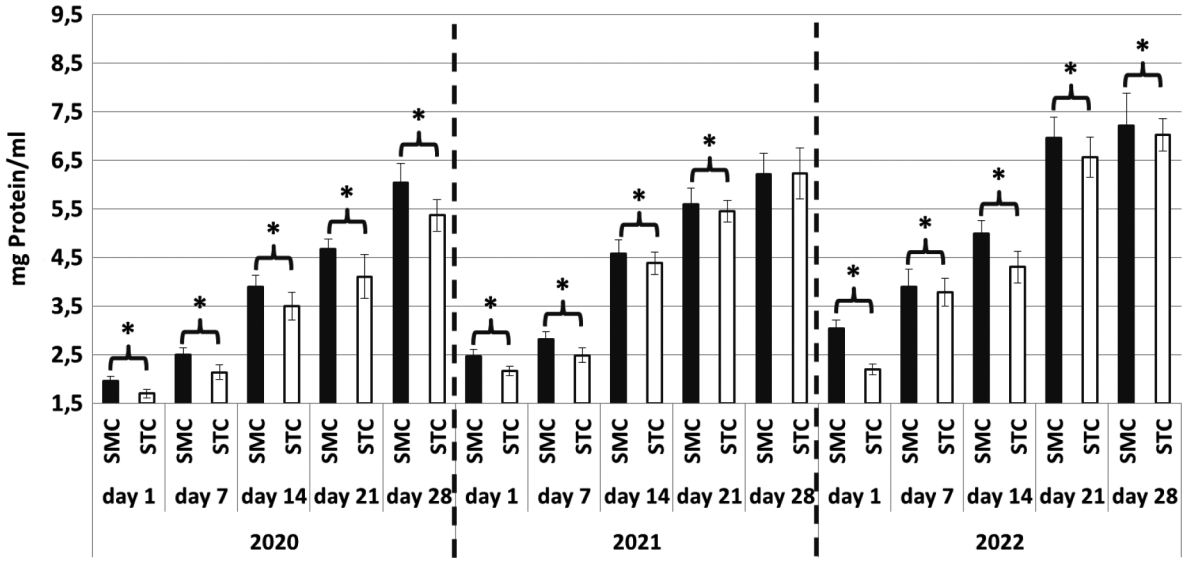

Fig. 5. Acidic protease inhibitor activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs, STC – workers reared in standard-cell combs. * differences between the SMC and STC workers within the age group are significant at $p \leq 0.01$, vertical bars indicate standard deviation.

in the hemolymph of worker bees conducted in previous apiary (Dziechciarz et al. 2022a) and laboratory experiments (Dziechciarz et al. 2023a). The group of the SMC workers exhibited higher activity, with differences noted only in the 1-day-old workers. At this age, the activities of proteases and their inhibitors in the hemolymph were always higher in the STC than SMC workers (Dziechciarz et al. 2022a, 2023a). Contrasting results were obtained in the analyses of the cuticle surface (Figs. 2-7, Table 3). These differences may indicate that, at the final preimaginary stage and on the first days of life, the activity of the proteolytic system on the cuticle surface in SMC workers differs from that operating in the hemolymph.

The trends in the age-related changes in the protein concentrations in the hemolymph (Dziechciarz et al. 2022a) and on the cuticle surface of the SMC and STC workers assessed in the apiary conditions indicated a decrease in the value of this parameter of the SMC workers and an increase in the STC group. The protein concentration on the cuticle surface increased in both groups of workers (Fig.1). The activity of the proteolytic system on the cuticle surface also increased in both groups of workers as well (Figs. 2-7, Table 3), whereas

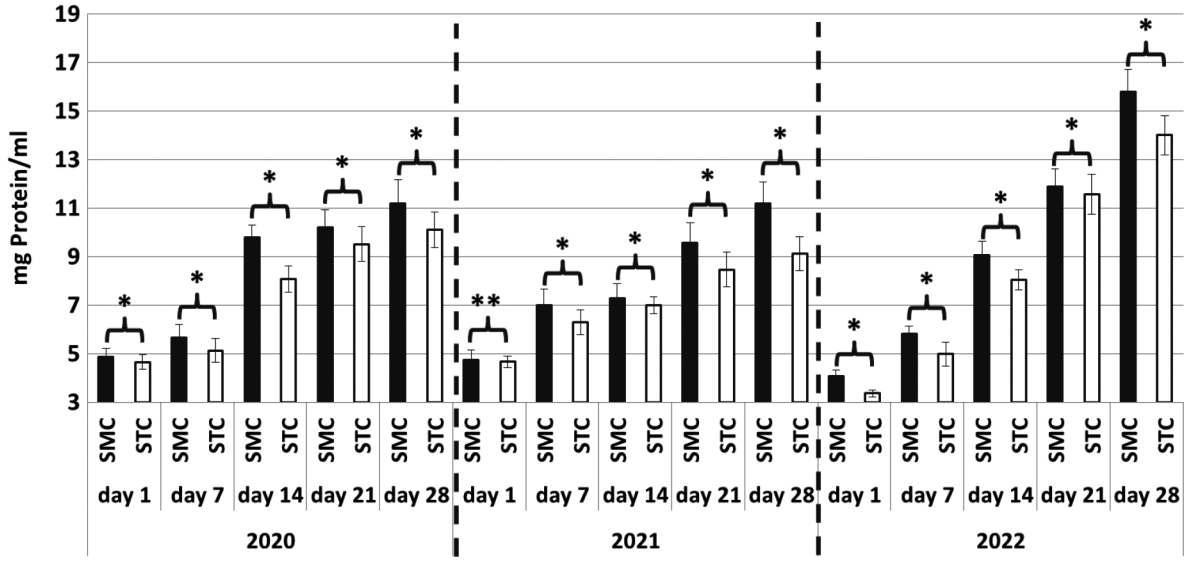

Fig. 6. Neutral protease inhibitor activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs; STC – workers reared in standard-cell combs. * differences between the SMC and STC workers within the age group are significant at $p \leq 0.05$, ** differences between the SMC and STC workers within the age group are significant at $p \leq 0.01$; vertical bars indicate standard deviation.

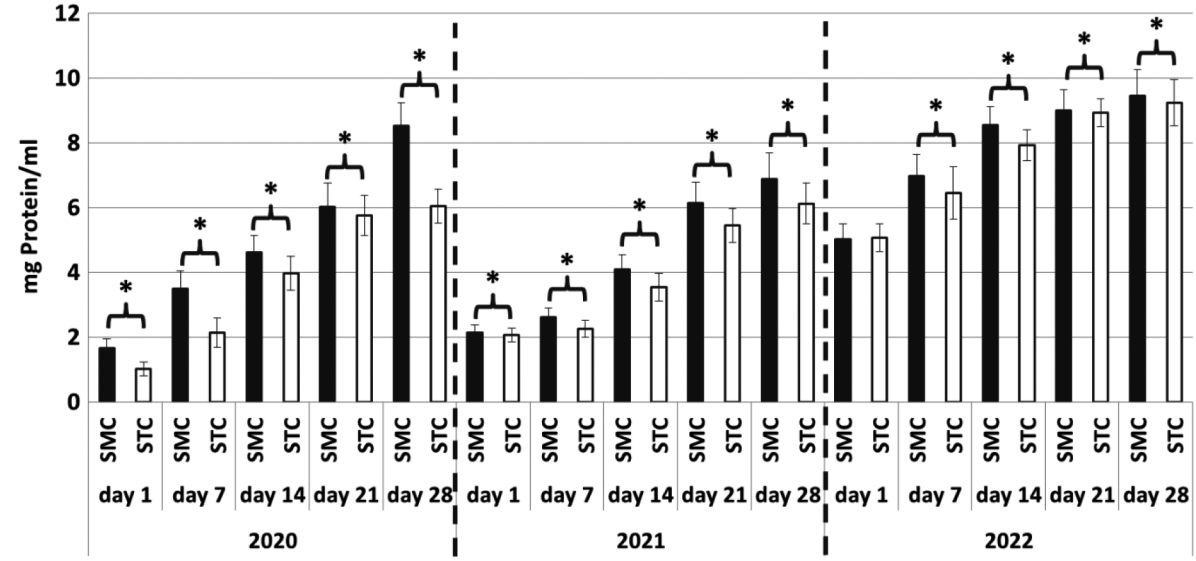

Fig. 7. Alkaline protease inhibitor activities on the cuticle surface of workers in three consecutive years.
SMC – workers reared in small-cell combs, STC – workers reared in standard-cell comb, * the differences between the SMC and STC workers within the age group are significant at $p \leq 0.01$, vertical bars indicate standard deviation.

the trends in the case of hemolymph were not as clear (Dziechciarz et al. 2022a). In comparison to the 1-day old workers, the activity of the proteolytic system in the older SMC workers usually increased with age, while its highest values in the STC workers were recorded in the first week of life and then remained at a constant level or declined. The trends in the age-related changes in the proteolytic system activity in the SMC worker group are consistent with results reported in previous studies (Strachecka et al. 2012, Strachecka et al. 2014, Łoś and Strachecka 2018) showing an increase in the activities of proteases and their inhibitors in workers until the 22nd day of life followed by a decline. In the present study an increasing trend has also been shown on day 28 of the experiment. This is probably the added value of rearing worker bees in combs with different cell diameters – increasing the variation of cells in the nest. Better nutrition of STC workers expressed by higher protein concentrations than SMC workers and a more efficient proteolytic system of SMC bees than STC bees mean that despite significant differences between these groups in the parameters studied, worker bees from both groups exhaust their proteolytic system capabilities much later than bees in studies where the factor of different widths of comb cells in the bee colony nest was not taken into consideration.

In our previous study, we associated the age-related decrease in protein concentrations in the hemolymph of SMC workers in apiary conditions with their transition from nurse/nest workers to foragers (Dziechciarz et al. 2022a). This conclusion was based on the observation of lower protein concentrations in the hemolymph of foragers than in the hemolymph of nurse workers (Fluri et al. 1982, Crailsheim 1986, Wilson et al. 2008, Eckholm et al. 2015). Higher protein levels were also determined in the bodies of nurse bees (Crailsheim et al. 1992). Therefore, we assumed that SMC workers serve as foragers earlier and more frequently, and STC workers fulfill their tasks as e.g. nurse bees in the nest, since the protein concentrations in their hemolymph increase with age (Dziechciarz et al. 2022a). The results of this study seem to confirm our hypothesis. The higher activities of proteases and their inhibitors in the SMC workers both in the hemolymph (Dziechciarz et al. 2022a, 2023a) and on the cuticle surface (Figs. 2-7, Table 3) may result from the greater exposure of these bees to numerous immunosuppressive factors outside the nest. The age regulation of tasks fulfilled at a particular stage of a honeybee's life is referred to as temporal polyethism. Typically, foraging behavior, as taking on more dangerous activities with decreasing life expectancy, occurs in workers aged around 23 days and older (Winston 1987). Subsequent to emergence, the organism's physical attributes remain relatively constant, whereas glandular systems undergo continuous remodeling in response to changing colony necessity and bee age (Seeley, 1995). The hypopharyngeal glands are for example highly enlarged and secretetive in workers performing nursing tasks inside the nest, while they are degraded in honeybees that have already foraged (Crailsheim and Stolberg 1989). Foragers then become more sensitive to sucrose (Scheiner et al., 2017) and more responsive to light (Thamm and Scheiner, 2014). Additionally, precocious foraging is linked with starvation during larval development (Scofield and Mattila 2015) and pressure from agricultural pesticides, herbicides and fungicides (Fisher et al., 2021). Such pest control agents lead to degeneration of cellular structures and morphological changes that may disturb the bioenergetic functions of mitochondria and lead to cell apoptosis (Faita et al. 2018).

The higher activities of proteases and their inhibitors on the cuticle surface in the SMC workers may indicate their innate adaptation to early exposure to various pathogens present in the environment outside the colony nest. It has been evidenced that immunity based on enzymatic reactions and antimicrobial peptides (AMP) is caste-specific and more efficient in foragers (la Luz et al. 2022). Moreover, the forager proteome is necessary for the synthesis of pheromones, which ensures defense for the colony and improves the ability to acquire food (Huo et al. 2016). Additionally, not the age but the foraging behavior stimulates the expression of genes related to secondary immune defense (e.g. AMPs) and the enzyme defense and immune gene expression (la Luz et al. 2022). Nevertheless, our hypothesis about the greater predispositions of SMC workers to work as foragers requires clear confirmation.

In addition to the higher activity of the proteolytic system, we also determined higher catalase and superoxide dismutase activities and total antioxidant capacity in the hemolymph in the SMC workers, compared to the STC group, in laboratory and apiary conditions (Dziechciarz et al. 2023b).

Conclusions

The width of comb cells where the workers are reared exerts a considerable effect on protein concentrations and proteolytic system activities on the cuticle surface reflected in the activities of proteases and their inhibitors. The protein concentration was significantly higher in workers reared in standard- than in small-cell combs. The activities of proteases and their inhibitors were significantly higher in workers reared in small-cell combs.

Given the proteolytic system activities on their cuticle surface, workers reared in small-cell combs may be predisposed to work as foragers outside the nest. However, this hypothesis has yet to be confirmed.

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