UNIWERSYTET PRZYRODNICZY w LUBLINIE

Wydział Biologii Środowiskowej
Dyscyplina naukowa: Zootechnika i Rybactwo

Patrycja Skowronek

*Rozprawa doktorska*

# Wpływ ekstraktu z konopi oraz olejku CBD na parametry fizjologiczno - biochemiczne związane z odpornością u robotnic pszczół miodnych (*Apis mellifera*)

# *The effect of hemp extract and CBD oil on physiological and biochemical parameters related to immunity in honey bee workers (Apis mellifera)*

Rozprawa doktorska wykonana w Katedrze Ekofizjologii Bezkręgowców
i Biologii Eksperymentalnej

Promotor: prof. dr hab. Aneta Strachecka

Lublin, 2023

*Najszczersze podziękowania dla najlepszej Pani Promotor*

*Prof. dr hab. Anety Stracheckiej za nieocenioną pomoc naukową,*

*merytoryczną, ale i zaangażowanie emocjonalne*

*w ukształtowaniu mnie jako człowieka i młodego naukowca.*

*Dziękuję również całej mojej rodzinie za bycie ze mną*

*podczas tej przygody, to dzięki Wam jestem tu gdzie jestem.*

Oświadczenie promotora rozprawy doktorskiej

Oświadczam, że niniejsza rozprawa doktorska została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego.

Data ..19.06.2023 r..... Podpis promotora .....Grochecka Aneta

Oświadczenie autora rozprawy doktorskiej

Świadoma odpowiedzialności prawnej oświadczam, że:

☐ niniejsza rozprawa doktorska została przygotowana przez mnie samodzielnie pod kierunkiem Promotora/~~Promotorów/Promotora pomocniczego*~~ i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

☐ przedstawiona rozprawa doktorska nie była wcześniej przedmiotem procedur związanych z uzyskaniem stopnia naukowego.

☐ niniejsza wersja rozprawy doktorskiej jest tożsama z załączoną na płycie CD wersją elektroniczną.

Data ..19.06.2023 r..... Podpis autora .....Katarzyna Połczyńska

*niepotrzebne skreślić

WYKAZ PRAC NAUKOWYCH WCHODZĄCYCH W SKŁAD CYKLU

(1) **Patrycja Skowronek**, Łukasz Wójcik, Aneta Strachecka; Cannabis extract has a positive-immunostimulating effect through proteolytic system and metabolic compounds of honey bee (*Apis mellifera*) workers; 2021; Animals; 2021 T.11, Nr 8, s. 2190, DOI: 10.3390/ani11082190;

Liczba punktów MEiN: 100, IF: 3,231

**Wkład polegał na**: pomysłodawca badań, inicjatywa badań, twórca hipotezy badawczej, opracowanie metodyki; realizacja doświadczenia (założenie doświadczenia, kontrola poszczególnych etapów doświadczenia, pobranie materiału, wykonanie analiz laboratoryjnych); analiza i opracowanie wyników, sformułowanie wniosków; napisanie pierwszej wersji manuskryptu; redakcja publikacji, korespondencja z redakcją. Mój udział procentowy szacuję na 85%.

(2) **Patrycja Skowronek**, Łukasz Wójcik, Aneta Strachecka; Impressive impact of hemp extract on antioxidant system in honey bee (*Apis mellifera*) organism; 2022; Antioxidants; T.11, Nr 4, s. 707, DOI: 10.3390/antiox11040707,

Liczba punktów MEiN: 100, IF: 3,231

**Wkład polegał na**: pomysłodawca badań, inicjatywa badań, twórca hipotezy badawczej, opracowanie metodyki; realizacja doświadczenia (założenie doświadczenia, kontrola poszczególnych etapów doświadczenia, pobranie materiału, wykonanie analiz laboratoryjnych); analiza i opracowanie wyników, sformułowanie wniosków; napisanie pierwszej wersji manuskryptu; redakcja publikacji, korespondencja z redakcją. Mój udział procentowy szacuję na 85%.

(3) **Patrycja Skowronek**, Łukasz Wójcik, Aneta Strachecka; CBD supplementation has a positive effect on the activity of the proteolytic system and biochemical markers of honey bees (*Apis mellifera*) in the apiary; 2022; Animals; T.12, Nr 18, s. 2313, DOI: 10.3390/ani12182313,

Liczba punktów MEiN: 100, IF: 3,231

**Wkład polegał na:** pomysłodawca badań, inicjatywa badań, twórca hipotezy badawczej, opracowanie metodyki; realizacja doświadczenia (założenie doświadczenia, kontrola poszczególnych etapów doświadczenia, pobranie materiału, wykonanie analiz

laboratoryjnych); analiza i opracowanie wyników, sformułowanie wniosków; napisanie pierwszej wersji manuskryptu; redakcja publikacji, korespondencja z redakcją. Mój udział procentowy szacuję na 85%.

(4) **Patrycja Skowronek**, Aneta Strachecka; Cannabidiol (CBD) supports the honeybee worker organism by activating the antioxidant system; 2023; Antioxidants; 2023; T. 12, Nr 2, s. 279, DOI: 10.3390/antiox12020279
Liczba punktów MEiN: 100, IF: 7,675
**Wkład polegał na:** pomysłodawca badań, inicjatywa badań, twórca hipotezy badawczej, opracowanie metodyki; realizacja doświadczenia (założenie doświadczenia, kontrola poszczególnych etapów doświadczenia, pobranie materiału, wykonanie analiz laboratoryjnych); analiza i opracowanie wyników, sformułowanie wniosków; napisanie pierwszej wersji manuskryptu; redakcja publikacji, korespondencja z redakcją. Mój udział procentowy szacuję na 85%.

SPIS TREŚCI:

# 1. Streszczenie

W dzisiejszych czasach odnotowuję się spadki w populacjach pszczół miodnych, co ma odzwierciadlenie dla całego środowiska naturalnego. Czynniki takie jak: chemizacja, monokultury rolnicze oraz naturalne patogeny i pasożyty osłabiają układ odpornościowy pszczół powodując wyższą śmiertelność rodzin pszczelich. Pomocne mogą w tym przypadku okazać się substancje immunostymulujące. W związku z tym, celem rozprawy doktorskiej było przetestowanie wpływu ekstraktu z konopi i olejku CBD na biochemiczne parametry odporności w hemolimfie robotnic pszczół miodnych, w doświadczeniu klatkowym i pasiecznym. W doświadczeniu klatkowym użyto ekstraktu z konopi, a w pasiecznym komercyjnego olejku CBD. Dwa doświadczenia podzielono na analogiczne grupy: (1) suplement w syropie cukrowym, (2) suplement na materiałowym pasku oraz (3) czysty syrop cukrowy (kontrola). Doświadczenie pasieczne wykonano przy użyciu 6 ulików weselnych (2 uliki/grupa), w których stworzono mini-kolonie. W doświadczeniu klatkowym użyto 30 klatek (10 klatek/grupa). Podczas trwania doświadczeń raz na tydzień pobierano pszczoły na pozyskanie hemolimfy (10 pszczół/grupa/tydzień), z której następnie wykonano analizy biochemiczne - oznaczono: stężenie białka całkowitego, aktywność systemu proteolitycznego, biomarkerów enzymatycznych (aminotransferaza alaninowa, aminotransferaza asparaginianowa, fosfataza zasadowa), stężenie biomarkerów nieenzymatycznych (glukoza, triacyloglicerol, cholesterol, kwas moczowy, mocznik, kreatynina, albuminy, jony wapnia, magnezu oraz fosforu) i aktywność systemu antyoksydacyjnego (całkowity potencjał antyoksydacyjny, dysmutaza ponadtlenkowa, peroksydaza glutationowa, katalaza, glutation). Wyniki z dwóch doświadczeń charakteryzowały się podobnymi trendami. Podczas doświadczeń wykazano: w większości wyższe aktywności proteaz i inhibitorów proteaz, wyższe aktywności wszystkich enzymów antyoksydacyjnych i biomarkerów enzymatycznych oraz wyższe stężenia biomarkerów nieenzymatycznych w grupach doświadczalnych w stosunku do grupy kontrolnej. We wszystkich przypadkach większe efekty w aktywnościach/stężeniach charakterystyk biochemicznych zaobserwowano w grupach otrzymujących substancję czynną w syropie w porównaniu z tymi na paskach. Pszczoły w doświadczeniu klatkowym po suplementacji żyły najdłużej - 56 dni (ekstrakt w syropie), 49 dni (ekstrakt na paskach). Suplementacja „konopna” okazała się wykazywać pozytywny wpływ na odporność pszczół miodnych w środowisku klatkowym i pasiecznym.

**Słowa kluczowe**: hemolimfa, odporność humoralna, cannabidiol, antyoksydanty

## 2. Summary

Nowadays, we observe declines in honey bee populations, which is reflected in the entire natural environment. Factors such as: chemicalization, agricultural monocultures and natural pathogens and parasites weaken the immune system of bees, causing higher mortality of bee colonies. Immunostimulants may be helpful in this case. Therefore, the aim of the doctoral dissertation was to test the effect of hemp extract and CBD oil on the biochemical parameters of immunity in the hemolymph of honey bee workers in the cage and apiary experiment. Hemp extract was used in the cage experiment, and commercial CBD oil was used in the beekeeping experiment. Two experiments were divided into analogous groups: (1) sugar syrup with supplement, (2) supplement on textile strip, and (3) pure sugar syrup (control). The apiary experiment was carried out using 6 mating hives (2 hives/group) in which mini-colonies were obtained. 30 cages (10 cages/group) were used in the cage experiment. During the experiments, bees were collected once a week for hemolymph (10 bees/group/week), from which biochemical analyzes were then performed: total protein concentration, activity of the proteolytic system, enzyme biomarkers (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), concentration of non-enzymatic biomarkers (glucose, triacylglycerol, cholesterol, uric acid, urea, creatinine, albumin, calcium, magnesium and phosphorus ions) and the activity of the antioxidant system (total antioxidant capacity, superoxide dismutase, glutathione peroxidase, catalase, glutathione). The results obtained from the two experiments were mostly identical. The experiments showed: higher activity of protease inhibitors, lower activity of proteases, higher activity of all antioxidant enzymes, enzymatic and non-enzymatic biomarkers in the experimental groups in relation to the control group. In all cases, we noticed higher activities and concentrations in the group with the supplementation in sugar syrup. Bees in the cage experiment lived the longest: 56 days (extract in syrup), 49 days (extract on strips) and 35 days in the beekeeping experiment compared to the control (28 days). "Hemp" supplementations turned out to have a positive effect on the immunity of honeybees in the cage and apiary environment.



**Key words**: hemolymph, humoral immunity, cannabidiol, antioxidants

# 3. Wprowadzenie teoretyczne

Naukowcy jednoznacznie określają trwający wiek jako czas VI wielkiego wymierania gatunków. Oszacowano, że podczas tego procesu rocznie wymrze do 30 000 gatunków fauny na Ziemi. Głównymi przyczynami tego zjawiska jest silna antropopresja i zbyt duże tempo postępujących zmian klimatycznych/środowiskowych, do których większość organizmów nie potrafi się dostosować w tak krótkim czasie (Lacetera, 2019; O'Connor i in., 2020). Entomofauna jest najliczniejszą grupą organizmów, najbardziej narażonych na te zmiany. Działalność tych gatunków w naturalnym środowisku jest niezbędna do utrzymania bioróżnorodności, tak ważnej dla prawidłowego funkcjonowania ekosystemu. Zmniejszenie populacji tych bezkręgowców pogłębia problemy w środowisku i narusza jego równowagę (Conte i Navajas, 2008).

Owadami, które istotnie wpływają na kondycję środowiska, są zapylacze, a szczególnie pszczoły tj. *Apis mellifera*. Pszczoła miodna (*A. mellifera*) znalazła szczególne miejsce w ludzkiej historii. Towarzyszy człowiekowi już od czasów starożytnego Egiptu, gdzie była uznawana za boskie zwierzę dostarczające ludziom drogocennych produktów (np. miód, propolis, wosk itd.) („The Tears of Re: Beekeeping in Ancient Egypt - Gene Kritsky). Oprócz produkcji tych substancji, pszczoła miodna odpowiada za zapylanie ok. 70% roślin entomofilnych, które stanowią podstawową cześć diety człowieka oraz zwierząt w gospodarstwach rolniczych (Mizrahi i Lensky, 2013). Przykładem takiej rośliny może być rzepak (Polska jest jednym z największych producentów oleju rzepakowego w Unii Europejskiej). Wartość zapylania upraw jest wyceniana nawet na kilkaset miliardów dolarów amerykańskich rocznie. Ekonomiczna wartość zapylania przez pszczołowate wykracza ponad produkcję rolniczą i pszczelarstwo. Dlatego, spadki w populacjach wiążą się także ze stratami ekonomicznymi w sektorze rolniczym i są jedną z przyczyn głodu na świecie (Jankielsohn i Jankielsohn, 2018). To wszystko sprawia, że pszczelarze, naukowcy, ekonomiści i inni, zwracają szczególną uwagę na obniżanie odporności pszczół, ich dużą śmiertelność i konsekwencje (ekonomiczne, ekologiczne i społeczne) tych zjawisk. Pierwsze problemy związane ze zwiększoną śmiertelnością pszczoły miodnej odnotowano w latach 80-90 XX wieku. Wówczas przyczyną tego zjawiska było zastosowanie pestycydu DDT. Aktualnie, oprócz pestycydów, przyczynami upadków rodzin pszczelich są: chemizacja i zanieczyszczenia środowiska, celowe tworzenie monokultur rolniczych, zmiany klimatyczne, brak różnorodności flory pożytkowej, przesunięcia w terminach kwitnienia roślin, nieprawidłowo prowadzona

gospodarka pasieczna (brak dbałości o higienę w rodzinach, nieodpowiednie zakarmianie, nieprawidłowe warunki zimowania, zastosowanie środków leczniczych i wspomagających). Czynnikami zagrażającymi pszczołom są patogeny i pasożyty tj. mikrosporydia *Nosema* spp. (nosemoza), roztocza *Varroa destructor* (warroza), bakterie *Paenibacillus larvae* (zgnilec amerykański), wirus paraliżu skrzydeł oraz inne grzybicze, bakteryjne i wirusowe patogeny. Czynniki te są przyczyną zjawiska określonego terminem: masowe ginięcie pszczół miodnych - CCD (eng. collony colapse disorder). O CCD mówimy, kiedy obserwujemy wymieranie pszczół w rodzinach na poziomie powyżej 30%. To zjawisko opisywane jest głównie na terenie Ameryki, natomiast w Europie określane jest jako zjawisko depopulacji rodzin pszczelich (Higes i in., 2009; Strachecka i in., 2016; Hristov i in., 2020). Ww. czynniki (razem i osobno) osłabiają odporność pszczół i tym samym stwarzają środowisko do rozwoju/wpływu kolejnych negatywnych czynników uruchamiając w ten sposób tzw. zamknięty krąg/błędne koło.

Pszczoły miodne charakteryzują się dwoma rodzajami odporności: 1) społeczną (zwaną też rodzinną lub zbiorową) - na poziomie rodziny oraz 2) indywidualną/osobniczą - związaną z mechanizmami na poziomie pojedynczych osobników (Strachecka i in., 2018). W odporności rodzinnej wyróżniamy odporność: wydzielniczą (tzw. sekrecyjną) oraz behawioralną. Odporność wydzielnicza związana jest z obecnością w produktach pszczelich i wydzielinach pszczół związków o aktywności przeciwdrobnoustrojowej. Substancje te produkowane są w większości w gruczołach pszczół, a ich zadaniem jest ochrona przed rozprzestrzenianiem się patogenów wewnątrz ula i uszczelnianie gniazda (również ochrona przed warunkami atmosferycznymi). Zaliczamy do nich: propolis, wosk, mleczko pszczele, miód i jad. Mleczko pszczele produkowane w gruczołach gardzielowych pszczół w wieku 5-14 dni, bogate w witaminy i białko, jest podstawowym składnikiem diety matki (przez całe jej życie) oraz młodych larw (1-3 dniowych). Wpływa na regulację wielu procesów metabolicznych, również tych związanych z odpornością i długością życia matek pszczelich. Jad, produkowany w gruczołach jadowych, chroni gniazdo przed intruzami. Jego głównym związkiem jest kwas mrówkowy, a pH wydzieliny wynosi 5,0-5,5. Wosk pszczeli, dzięki obecności ponad 300 związków chemicznych (głównie monoestrów, węglowodorów, diestrów, triestrów, hydroksymonoestrów, hydropolyestrów, wolnych kwasów tłuszczowych, estrów kwasów, wolnych kwasów tłuszczowych, estrów kwasów, poliestrów kwasów, wolnych kwasów tłuszczowych, wolnych alkoholi i innych związków), jest materiałem wykorzystywanym do budowy plastrów pszczelich, które charakteryzują się właściwościami antyseptycznymi. Kit

pszczeli, wytwarzany z wydzielin oraz żywic drzewnych i kwiatowych, wyścielając wnętrze ula, uszczelnia i wzmacnia jego konstrukcję, chroniąc przed patogenami. Odporność behawioralna to zachowania higieniczne i pielęgnacyjne, które mają na celu ograniczenie rozprzestrzenianie się chorób i pasożytów. Do zachowań higienicznych zaliczamy np.: wykrywanie i eliminowanie patogenów, pasożytów, zapachów obcych; zasklepianie komórek z czerwiem; czyszczenie powłok ciała (np. z *V. destructor*; usuwanie pasożytów przez wyczesywanie ich ze swojej powierzchni ciała - *autogrooming* lub z powierzchni ciała innych osobników - *allogrooming*); wykrywanie i usuwanie chorego, martwego czerwiu i dorosłych pszczół poza gniazdo; zachowania altruistyczne dorosłych pszczół (chora pszczoła umiera na zewnątrz ula, zmniejszając tym samym ryzyko zarażenia pozostałych pszczół w gnieździe). Wszystkie te zachowania społeczne obniżają presję na indywidualny układ odpornościowy pszczół – ochrona wynikająca z życia w społeczności sprawia, że liczba genów u pszczół miodnych, odpowiedzialnych za syntezę białek ukierunkowanych na obronę przed patogenami, jest mniejsza niż np. u muchówek, które nie tworzą struktur społecznych (Larsen i in., 2019).

Odporność indywidualna/osobnicza opiera się na zewnętrznych (bariery anatomiczno-fizjologiczne układu oddechowego, pokarmowego i kutikuli) i wewnętrznych barierach obronnych. W obrębie barier wewnętrznych wyróżnia się dwa typy odporności: wrodzoną (nieswoistą, fizjologiczną) i nabytą (swoistą). Wrodzona odporność składa się z: odporności komórkowej oraz humoralnej. Odporność komórkowa opiera się na wytworzeniu i efektywnym działaniu komórek żernych, takich jak hemocyty, dzięki którym zachodzą następujące procesy: fagocytoza, nodulacja i inkapsulacja. Odporność humoralna polega na wyprodukowaniu białek zabezpieczających organizm przed wnikaniem i rozwojem patogenów, i składają się na nią: 1) związki systemu proteolitycznego, antyoksydacyjnego i markery biochemiczne, które zaliczane są do odporności biochemicznej; 2) białka odpornościowe funkcjonujące w hemolimfie (np. lizozym, fenolooksydaza, lektyny) oraz w innych tkankach (np. rojalizyna, melityna, ceratotoksyna). Po przełamaniu barier anatomiczno-fizjologicznych pszczoły przez patogen, zaczyna on penetrować tkanki i wydzielać enzymy niszczące komórki gospodarza. Wówczas uruchamiane są jednocześnie mechanizmy odporności komórkowej i biochemicznej (zaliczane do odporności humoralnej). Enzymy systemu proteolitycznego (tj. proteazy asparaginowe, serynowe, cysteinowe, metaloproteazy i ich inhibitory) hydrolizują białka patogenu na małe jednostki. Pozostałości po tych reakcjach oraz inne toksyczne metabolity i reaktywne formy tlenu (ROS) są neutralizowane i usuwane przez

system antyoksydacyjny. Oba systemy wspomagane są przez markery biochemiczne (biomarkery), które są wskaźnikami zdrowotności pszczół i zalicza się do nich aminotransferazę asparaginową (AST) i alaninową (ALT) oraz fosfatazę alkaliczną (ALP). W tym samym czasie, patogen otaczany jest przez hemocyty i uruchamiany jest proces fagocytozy. Powstały fagosom łączy się z lizosomem, tworząc fagolizosom, który uwalnia enzymy (fosfatazy, lizozym itp.) degradujące białka, lipidy i kwasu nukleinowe patogenu. Struktury takie otaczane są przez kolejne hemocyty (ziarniste) oraz melaniny i jednocześnie uwalniają hemokiny, które pobudzają kolejne hemocyty do reakcji oraz ciało tłuszczowe do produkcji białek odpornościowych, tj. lizozym, fenolooksydaza, antymikrobiologiczne peptydy (AMP: apidecyny, abecyny, hymenoptecyny, defensyny) i in., które wykazują gotowość do działania dopiero po kilkunastu godzinach od zakażenia. Podczas ich aktywacji uruchamiany jest szlak chinonowy, w którym syntetyzowana jest sklerotyna i melanina, uczestniczące w tworzeniu guzków melanotycznych, czyli w inkapsulacji. Takie guzki z uwięzionym patogenem są transportowane przez hemolimfę w kierunku kutikuli, która u pszczół ciemnieje po kolejnych kontaktach owada z patogenami, wraz z procesami starzenia.

Ponieważ systemy: proteolityczny i antyoksydacyjny oraz markery biochemiczne stanowią pierwszą i najistotniejszą linię obrony tuż po wniknięciu patogenu do organizmu pszczoły, postanowiłam skupić się w mojej dysertacji właśnie na nich.

Proteoliza jest procesem, polegającym na enzymatycznej hydrolizie wiązań peptydowych (a także estrowych) między aminokwasami w łańcuch białkowym w wyniku czego ulegają rozkładowi całe łańcuchy lub usuwane są określone sekwencje aminokwasowe (Stryer, 2003). Proteoliza zaangażowana jest w obróbkę potranslacyjną białek, trawienie wewnątrzkomórkowe, aktywację prekursorów enzymów, hydrolizę niepotrzebnych białek, transport błonowy, przekształcanie (zmiany konformacji) białek, strukturyzację tkanek, ale także do utrzymania homeostazy i zwalczania patogenów. Proteazy, oprócz bezpośredniego działania na patogeny, uczestniczą także u pszczół w aktywacji kaskady układu oksydazy polifenolowej (PO) (Gliński i in., 2011). Głównymi składnikami systemu proteolitycznego są: proteazy dzielone na: 1) endopeptydazy - odpowiedzialne za hydrolizę wiązań peptydowych na środku białka i 2) egzopeptydazy hydrolizujące wiązania na końcu białka; oraz inhibitory proteaz (Strachecka i in., 2018, 2021). Aktywacja proteaz jest procesem nieodwracalnym, dlatego jest ściśle regulowana poprzez działanie inhibitorów proteaz (Berg i in., 2020).

Proteazy wykazują wysoką specyficzność działania w pH, w którym osiągają optimum swojej aktywności, stąd można je podzielić na kwaśne, obojętne i zasadowe. Ze względu na obecność określonych grup funkcyjnych w centrach aktywnych, proteazy dzieli się na proteazy: aspartylowe, serynowe, cysteinowe, treoninowe, glutaminowe oraz metaloproteazy (Pushpam i in., 2011). System proteolityczny został opisany na kutikuli pszczół oraz wewnątrz ich ciała, w tkankach takich jak: hemolimfa, ciało tłuszczowe, przewód pokarmowy oraz gruczoły kieszonkowe (Strachecka i in., 2008; Strachecka i in., 2018; Skowronek i in., 2021a), a ponadto oznaczono je w jadzie (LIMA i in., 2000) Dotychczas, u pszczół miodnych wykazano obecność metaloproteaz (EC 3.4.24.), proteaz asparaginowych (EC 3.4.23), serynowych (EC 3.4.21), cysteinowych (EC 3.4.22) (Strachecka i Demetraki-Paleolog, 2011). Aktywności tych enzymów zmieniają się wraz z porą roku, stopniem zanieczyszczenia środowiska, rozwojem osobniczym, kastą (matka/robotnia/truteń), stanem fizjologicznym organizmu, a także pod wpływem różnych związków, tj. insektycydy, akarycydy, biostymulatory (Grzywnowicz i in., 2009; Strachecka i in., 2010, 2014, 2015, 2016; Paleolog i in., 2020).

Odporność pszczół (jak wspomniano wyżej) jest nierozerwalnie zależna od reakcji utleniania i redukcji zachodzących w ich tkankach. Stres oksydacyjny doprowadza do uszkodzeń komórek/tkanek i przyspiesza starzenie się owadów oraz zaburza homeostazę w ich organizmach (Puzanowska-Tarasiewicz i in., 2010; Kramer i in., 2021). Aby przeciwdziałać szkodliwym procesom oksydacji, organizmy wykształciły szereg systemów obrony antyoksydacyjnej. Homeostaza na poziomie komórek, jak i całego organizmu, zależy od prawidłowego balansu między reaktywnymi formami tlenu (ROS) i azotu (RNS) a działaniem antyoksydantów (Bartosz, 2003). Głównymi związkami systemu antyoksydacyjnego są antyoksydanty enzymatyczne, takie jak: dysmutazy ponadtlenkowe (SOD), katalazy (CAT), peroksydazy glutationowe (GPx), reduktazy glutationowe (GR) i S-transferazy glutationowe (GST). Ponadto, w skład systemu antyoksydacyjnego wchodzą związki nieenzymatyczne, takie jak np. koenzym Q10, melatonina, kwas moczowy i in. U pszczół miodnych, podstawowymi antyoksydantami są dysmutaza ponadtlenkowa (SOD), katalaza (CAT), peroksydaza gluationowa (GPx) oraz glutation (GST/GSSH). Związki te tworzą tzw. ”*primary internal antioxidants complete the defence system*” i chronią owada przed biotycznymi, jak i abiotycznymi stresorami oksydacyjnymi (Weirich i in., 2002; Orčić i in., 2017; Li i in., 2020). Proces redukcji reaktywnych form tlenu w mniej szkodliwe cząsteczki rozpoczyna SOD. W wyniku jego działania anionorodnik ponadtlenkowy zostaje przekształcony w nadtlenek wodoru i tlen cząsteczkowy. Następnie

CAT redukuje nadtlenku wodoru do tlenu cząsteczkowego i wody. Redukcję nadtlenku wodoru przeprowadza również enzym GPx (z przetworzeniem zredukowanego glutationu do formy utlenionej – GSH/GSSH). Potencjał systemu antyoksydacyjnego można określić w postaci „całkowitej zdolności antyoksydacyjnej organizmu" (TAC, eng. *Total Antioxidant Capacity*), która jest sumą aktywności związków (enzymatycznych i nieenzymatycznych) w danej próbce/komórce/tkance/organizmie (Skowronek i in., 2022b; Strachecka i in., 2021). Podobnie, jak w przypadku systemu proteolitycznego, aktywność systemu antyoksydacyjnego u pszczół zależy od wielu czynników, tj. wiek, kasta, stan fizjologiczny, czynniki antropogeniczne i in. (Strachecka i in., 2022).

Hemolimfatyczny układ proteolityczny i antyoksydacyjny (jak wspomniano wyżej) wzajemnie się uzupełniają. Białka mogą ulegać reakcjom rozszczepienia z pewnymi rodnikami/utleniaczami, co prowadzi do bezpośredniego powstania potencjalnie toksycznych fragmentów peptydów (Davies, 1986). Różne międzykomórkowe enzymy proteolityczne mogą rozpoznawać i rozkładać białka uszkodzone oksydacyjnie do aminokwasów (Bode i in., 1999; Strachecka i in., 2008). Tym samym związki te chronią przed przedwczesnym starzeniem się organizmu. Ich działanie jest wspomagane przez markery biochemiczne, głównie enzymatyczne. ALT (aminotransferaza alaninowa, EC 2.6.1.2) jest enzymem, który katalizuje przeniesienia grupy aminowej (pochodzącej z alaniny) na α-ketoglutaran. Jednocześnie podczas tej odwracalnej reakcji powstaje pirogronian i glutaminian (Berg i in., 2020). Z kolei, ALP (fosfataza zasadowa, EC 3.1.3.1) katalizuje defosforylację estrów fosforanowych w środowisku zasadowym (Berg i in., 2020). Ponadto, enzym ten jest odpowiedzialny za utrzymanie stałego zaopatrzenia energii poprzez uwalnianie jej z wysokoenergetycznych wiązań fosforanowych (Zhu i in., 2014). AST (aminotransferaza asparaginianowa, EC 2.6.1.1) katalizuje przeniesienie grupy aminowej z asparaginianu na α-ketoglutaran (Berg i in., 2020). Enzym ten wraz z ALT tworzy u owadów swoisty łącznik między metabolizmem węglowodanów a białek (Zhu i in., 2014). Aktywności tych enzymów są uważane za wskaźnik stanu fizjologicznego/metabolicznego ciała tłuszczowego pszczół miodnych (Ma i in., 2016; Skowronek i in., 2021a), a co za tym idzie są one wykorzystywane do oznaczenia stanu odporności owada. Aktywności ALT, ALP i AST wzrastają w hemolimfie tych pożytecznych owadów wraz z ich starzeniem się oraz pod wpływem egzogennych antyoksydantów/biostymulatorów dostarczanych w diecie (Şapcaliu i in., 2010; Strachecka i in., 2018).

Hemolimfa to niemal przezroczysty (nie zawiera hemu,) płyn złożony z wody, nieorganicznych soli, białek, hormonów, wolnych aminokwasów, węglowodanów, lipidów i komórek odpornościowych (hemocytów), który pełni funkcję „krwi" u owadów. Stanowi ona 16-20% masy ciała pszczoły, a jej pH waha się od 6 do 8. Skład hemolimfy i jej objętość zmieniają się w zależności od diety i stanu fizjologicznego pszczoły oraz od ilości wody w środowisku. Synteza składników hemolimfy zachodzi w ciele tłuszczowym. Podstawowymi funkcjami hemolimfy są: przenoszenie składników pokarmowych i związków chemicznych (np. hormonów), utrzymanie równego ciśnienia płynów w hemocelu oraz jest miejscem zachodzenia procesów odpornościowych. Zważywszy na tą trzecią funkcję hemolimfy to właśnie ona była tkanką, którą wykorzystałam w swoich badaniach (Gliński i in., 2011). W hemolimfie krążą (przekazane z ciała tłuszczowego) produkty metabolizmu oraz podstawowe składniki/związki, podobnie jak w przypadku krwi u kręgowców. Dzięki temu w hemolimfie oznaczać możemy stężenia różnych związków, np. biomarkerów nieenzymatycznych takich jak: glukoza, triacyloglicerol, cholesterol (substancje wysokoenergetyczne – zapasowe), fosfor, jony wapnia i magnezu (niezbędne w odpowiednim funkcjonowaniu błon komórkowych) (Strachecka i in., 2014; Skowronek i in., 2021b; Skowronek i in., 2022a).

Jednym z podstawowych problemów pszczelarstwa jest nieprawidłowa, słabo zbilansowana dieta (wynikająca z bioróżnorodności pożytków), której konsekwencją jest obniżanie odporności pszczół. Kolejnym aspektem są leki podawane koloniom, które są coraz mniej skuteczne z racji wykształcania u patogenów oporności na nie. Dlatego jednym z rozwiązań wydaje się wprowadzenie do gospodarki pasiecznej środków dodatkowych w postaci substancji/związków biostymulujących (biostymulatorów/suplementacji). Ważnym elementem jednak podczas wyboru suplementów jest: pochodzenie (brak efektu toksycznego i przechodzenia pozostałości do produktów pszczelich, co mogłoby powodować straty ekonomiczne), skład, możliwość zastosowania w różnych formach (syrop, ciasto, materiałowe paski stosowane z lekami na warrozę), możliwość łatwego podania dużej liczbie rodzin, cena oraz efekty. W ostatnim czasie coraz częściej testuje się suplementy o naturalnym pochodzeniu (często roślinnym), których substancje czynne/metabolity mają już częściowe potwierdzenie pozytywnego wpływu w testach na innych organizmach (również na ludziach). Do zastosowanych suplementacji o pochodzeniu naturalnym zaliczyć możemy związki/substancje/produkty tj.: resweratrol (substancja czynna z winogron), piperyna (z pieprzu), kurkumina (kurkuma), kofeina (obecna w ziarnach kawy, ziarnach kakaowca i herbacie), witamina C

(pietruszka, cytryna) oraz mieszanki roślin strączkowych (soja, kukurydza, groch) i mączek. Popularnymi suplementami są również kiszonki bogate w Lactobacillus, fitochemikalia stymulujące różnorodność mikroflory jelitowej pszczół (również Lactobacillus), koenzym Q10 (surowiec farmaceutyczny), propolis (nalewka propolisowa), spirulina, drożdże, odtłuszczone mleko w proszku. Związki pochodzenia roślinnego (resweratrol, spirulina, kofeina) oraz wskazane dodatki dietetyczne wykazały pozytywny efekt na organizm pszczół, głównie poprzez wydłużenie ich życia (Rascón i in., 2012; Strachecka i in., 2014; Ricigliano i Simone-Finstrom, 2020) nawet o 33–38% (resweratrol, kofeina) (Rascón i in., 2012; Strachecka i in., 2014). Ponadto, niektóre biostymulatory mają udowodniony pozytywny wpływ na układ odpornościowy poprzez zwiększenie stężeń białek odpornościowych oraz aktywności układu proteolitycznego (konopie, kurkumina, koenzym Q10) (Strachecka i in., 2014, 2015; Skowronek i in., 2022b) i antyoksydacyjnego (SOD, GPx, CAT, GST; dla testu z kurkuminą, koenzymem Q10, kofeiną, piperyną) (Strachecka i in., 2014; Strachecka i in., 2014, 2015; Schulz i in., 2019). Witamina C zwiększa aktywność systemu antyoksydacyjnego, zmniejszając straty po zimie nawet o 33% w porównaniu z grupami kontrolnymi. Dodatkowo suplementacja tej witaminy wpływa pozytywnie na ilość odchowywanego czerwiu przez pszczoły (Farjan i in., 2012). Koenzym Q10 zwiększa stężenie lipidów oraz jonów takich jak magnez i wapń w hemolimfie robotnic (Strachecka i in., 2014). Wyższe stężenia lipidów i białek oraz wzrost masy ciała odnotowano także w przypadku żywienia pszczół pokarmem z dodatkiem spiruliny (Ricigliano i Simone-Finstrom, 2020). Kofeina, oprócz wydłużenia życia, dodatkowo ogranicza rozwój nosemozy, a także zwiększa stężenia białek, kwasu moczowego, trójglicerydów, cholesterolu, glukozy, wapnia, kreatyniny, magnezu i proteaz w hemolimfie owadów (Strachecka i in., 2014). Piperyna zwiększa aktywności enzymów wątrobowych (aminotransferaz) u pszczół. Potwierdzono także pozytywny wpływ na pszczoły pyłku eukaliptusa *Corymbia calophylla* (Manning i in., 2007), mieszanin z kwasem linolowym, kwasem oleinowym, śrutą sojową i śrutą łubinową (Manning i in., 2007; Geldert i in., 2021).

Jednym z potencjalnie prozdrowotnych biostymulatorów jest konopia i jej różnorodne formy (rodzaje ekstraktów). Ekstrakty z konopi zawdzięczają swoje właściwości dzięki obecności substancji czynnych należących do grupy kannabinoidów: kannabidiol, kannabichromen, kannabigerol, Δ9-tetrahydrokannabinol (THC) oraz kannabinol (Hazekamp i in., 2004; Amin i Ali, 2019). Najczęściej jednak w publikacjach

omawia się dwa z nich: THC (substancja o działaniu psychoaktywnym) oraz CBD (substancja działająca prozdrowotnie). Większość dobroczynnych właściwości, również przeciwzapalnych, tej rośliny potwierdzono w badaniach na szczurach i myszach, a nośnikami związków czynnych/aktywnych są głównie SMB i EDTA (Obonga i in., 2019). Związki te zmniejszają produkcję prostaglandyny E2, jak również zwiększają aktywność systemu antyoksydacyjnego, co potwierdzono w badania na szczurach i psach. Ekstrakt z konopi zmniejsza zmiany histopatologiczne, takie jak uszkodzenia neuronów w korze mózgowej, zwyrodnienia niektórych komórek Purkinjego w móżdżku, zwyrodnienia wakuolowe w wątrobie oraz powiększenie i przekrwienie żyły wrotnej, wywołane środkiem degenerującym tzw. malationianem (Abdel-Salam i in., 2018). Konopie zmniejszają stężenie cholesterolu, peroksydację lipidów, zwiększają wrażliwość kanałów potasowych zależnych od ATP i wapnia (Majewski i in. 2021; Majewski and Jurgoński 2021). Zastosowanie kannabinoidów jako środków terapeutycznych może mieć szczególne znaczenie w kontekście zaburzeń neuro-nadpobudliwości ruchowej, takich jak padaczka. Wpływ kannabinoidów na pobudliwość nerwową potwierdzają również inne publikacje dot. testów na pacjentach z chorobą Parkinsona oraz osób cierpiących na zespół Draveta oraz Lennoxa-Gastauta (Yokota i in., 1995; Gray i Whalley, 2020). Kannabidiol (CBD) zmniejsza prędkość i dystanse pływania u Danio pręgowanego, jako organizmu modelowego (Samarut i in., 2019). Daje to szansę na zmniejszenie częstości napadów padaczkowych u osób cierpiących na padaczkę lekooporną lub zespół Draveta (Reithmeier i in., 2018). Okazuję się, że substancje zawarte w konopi oprócz opisanego wyżej działania w przypadku padaczki (kurczliwość), mogą również wspomagać regenerację mięśni i ich wydolność. W badaniach prowadzonych przez Boldaji i in. (2022), CBD przyczynił się do efektywnej rehabilitacji mięśnia sercowego po zawale (testy na szczurach) (Safian Boldaji i in., 2022). Ponadto, CBD wpływa na regenerację mięśni szkieletowych (osiągi sportowe) po treningu oporowym (Isenmann i in., 2021). Natomiast, u barciaka zakażonego patogenem *Listeria monocytogenes* zaobserwowano wyższe wskaźniki przeżywalności i zahamowanie rozwoju patogenu po użyciu olejku eterycznego z konopi (Marini i in., 2018). Wyżej opisane właściwości konopi stały się punktem wyjścia do podjęcia badań na pszczołach. Założyłam, iż ekstrakt z konopi i olejek CBD zwiększają odporność i witalność pszczół poprzez zwiększenie aktywności systemu proteolitycznego i antyoksydacyjnego oraz markerów biochemicznych (zarówno tych enzymatycznych, jak i nieenzymatycznych).

# 4. Hipotezy i cele badawcze

W związku z wyżej opisanym problemem badawczym, sformułowane zostały następujące hipotezy badawcze:

Ekstrakt z konopi (publikacje 1 i 2; strona 4-5) i olejek CBD (publikacje 3 i 4; strona 4-5) mają pozytywny wpływ na odporność pszczół miodnych przez:

– wzrost aktywności proteaz oraz ich inhibitorów w hemolimfie robotnic;

– wzrost aktywności enzymów systemu antyoksydacyjnego: SOD, CAT, GPx, GST oraz poziomu GSH i TAC w hemolimfie robotnic;

– wzrost aktywności biomarkerów enzymatycznych, tj.: aminotransferaza alaninowa, aminotransferaza asparginianowa oraz fosfataza zasadowa w hemolimfie robotnic;

– wzrost stężeń biomarkerów nieenzymatycznych: glukozy, triacylogliceroli, cholesterolu, kreatyniny i kwasu moczowego oraz zmniejszenie stężeń mocznika i albuminy; a także wzrost stężeń jonów fosforu, wapnia oraz magnezu w hemolimfie robotnic.

Hipotezy dodatkowe:

- Zastosowanie różnych metod suplementacji wpływa na efekty stymulacji układu odpornościowego w hemolimfie robotnic pszczół miodnych.

Aby zweryfikować hipotezy sformułowano następujące cele:

1. Określenie aktywności proteaz i ich inhibitorów w hemolimfie robotnic pszczół miodnych suplementowanych ekstraktem z konopi lub olejkiem CBD i porównanie ich do tych z grupy kontrolnej (bez dodatku ekstraktu lub CBD).
2. Określenie aktywności enzymów antyoksydacyjnych w hemolimfie robotnic pszczół miodnych suplementowanych ekstraktem z konopi lub olejkiem CBD i porównanie ich do tych z grupy kontrolnej (bez dodatku ekstraktu lub CBD).
3. Określenie aktywności biomarkerów enzymatycznych: aminotransferazy alaninowej, aminotransferazy asparginianowej oraz fosfatazy zasadowej w hemolimfie robotnic pszczół miodnych suplementowanych ekstraktem z konopi lub olejkiem CDB i porównanie ich do tych z grupy kontrolnej (bez dodatku ekstraktu lub CBD).
4. Określenie stężenia biomarkerów nieenzymatycznych: glukozy, triacylogliceroli, cholesterolu i mocznika w hemolimfie robotnic pszczół miodnych suplementowanych

ekstraktem z konopi i porównanie ich do tych z grupy kontrolnej (bez dodatku ekstraktu).

5. Określenie stężenia biomarkerów nieenzymatycznych: glukozy, triacylogliceroli, cholesterolu, kwasu moczowego, kreatyniny, albuminy, fosforu, wapnia oraz magnezu w hemolimfie robotnic pszczół miodnych suplementowanych olejkiem CBD i porównanie ich do tych z grupy kontrolnej (bez dodatku olejku CBD).

Cele dodatkowe:

6. Określenie wpływu różnych metod suplementacji (w syropie *vs* na pasku) substancji czynnych z konopi na stymulację układu odpornościowego w hemolimfie robotnic pszczół miodnych.

# 5. Materiały i metody badawcze

Dysertacja obejmuje doświadczenia pasieczne oraz klatkowe, które prowadzono w latach 2020-2022. Doświadczenia pasieczne wykonywano w Stacji Dydaktyczno-Badawczej Zwierząt Drobnych im. Laury Kaufman (51°13′31″ N, 22°38′07″ E), należącej do Instytutu Biologicznych Podstaw Produkcji Zwierzęcej Uniwersytetu Przyrodniczego w Lublinie. Doświadczenia klatkowe wykonano w Katedrze Ekofizjologii Bezkręgowców i Biologii Eksperymentalnej. Analizy biochemiczne do tych dwóch doświadczeń wykonano w laboratorium ww. Katedry. Szczegółowe metodyki (przygotowane zgodnie z wymogami czasopism) oraz wyniki doświadczenia klatkowego przedstawiono w publikacjach (1 i 3, strona 4-5): „*Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (Apis mellifera) Workers*" i „*Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (Apis mellifera) Organism*", a z doświadczenia pasiecznego – w publikacjach (2 i 4, strona 4-5): „*CBD Supplementation Has a Positive Effect on the Activity of the Proteolytic System and Biochemical Markers of Honey Bees (Apis mellifera) in the Apiary*" i „*Cannabidiol (CBD) Supports the Honeybee Worker Organism by Activating the Antioxidant System*".

## 5.1 Doświadczenie klatkowe

Doświadczenie rozpoczęto od uzyskania 1-dniowych robotnic pszczół miodnych (*A. mellifera carnica*) zgodnie z metodą Stracheckiej et al. (2014).

### 5.1.1 Przygotowanie doświadczenia klatkowego

Trzy sztucznie inseminowane matki (matki-siostry; trutnie: *A. mellifera carnica*; sztuczna inseminacja metodą (Cobey i in., 2015)) ograniczono indywidualnie w izolatorach zawierających po jednym plastrze, w celu pozyskania jaj w zbliżonym wieku. Po 12 godzinach, od chwili umieszczenia w izolatorze, matki uwalniano, natomiast zaczerwione przez nie plastry pozostawały w izolatorach do momentu wygryzienia się larw. Następnie, plastry z trzech rodzin oglądano i do dalszych etapów wybrano ten, który zawierał najwięcej larw. Dwudziestego dnia (licząc od momentu złożenia jaj przez matkę), plaster z czerwiem umieszczono w inkubatorze/cieplarce (35$^{o}$C) aż do momentu wygryzienia się robotnic. 1200 świeżo wygryzionych pszczół umieszczono w 30 wystandaryzowanych drewnianych klatkach (40 pszczół/klatka; objętość klatki: 576 cm$^3$; wymiary klatki: 12 × 12 × 4 cm). Optymalne warunki zapewniono w klimatyzowanej

komorze (tzw. pakamerze) – stała temperatura = 35°C i wilgotność względna = 65% (Dziechciarz i in., 2019).

Klatki podzielono na trzy grupy (na grupę przypadało 10 losowo wybranych klatek):

(1) grupa kontrolna - karmiona mieszaniną cukru i roztworu woda-gliceryna w stosunku 1:1 *ad libitum*

(2) grupa doświadczalna - karmiona czystym syropem cukrowym w stosunku 1:1 *ad libitum* i wewnątrz każdej klatki znajdował się bawełniany pasek nasączony 3 ml ekstraktu z konopi (0,25 g czystego ekstraktu z pasty konopnej + 3 ml roztworu wody z gliceryną)

(3) grupa doświadczalna - karmiona mieszaniną syropu cukrowego w stosunku 1:1 z dodatkiem ekstraku z konopi *ad libitum* (500 ml roztworu woda-gliceryna z 4,38 g ekstraktu z pasty konopnej)

### 5.1.2 Charakterystyka składników wchodzących w skład suplementu

Roztwór wodno-glicerynowy użyty w tym badaniu pochodził z firmy Chempur, nr. normy: BN-76/6193-12. Ekstrakt z konopi pochodził od producenta - firmy Melissa.

### 5.1.3 Kolekcjonowanie materiału biologicznego do badań/schemat doświadczenia

Materiałem do dalszych analiz biochemicznych była hemolimfa. Raz w tygodniu (rozpoczynając od 2 dnia trwania doświadczenia aż do śmierci wszystkich pszczół w klatkach) pobierano świeżą hemolimfę od 10 pszczół z danej grupy (10 pszczół x 3 grupy = 30 pszczół/tydzień). Świeżą hemolimfę pobrano z zatoki żylnej w odwłoku owada według metody Łoś i Strachecka (2018). Hemolimfę każdej pszczoły umieszczono oddzielnie w sterylnej probówce typu Eppendorf z 0,6% NaCl. Wszystkie próbki hemolimfy natychmiast mrożono w temperaturze −25°C do dalszych analiz biochemicznych. Liczba pobrań była uzależniona od obecności żywych pszczół w danej grupie.

## 5.2 Doświadczenie pasieczne

### 5.2.1 Przygotowanie doświadczenia pasiecznego

Procedura pozyskania matek

Matki wychowywano z larw wygryzionych z jaj złożonych przez matkę reprodukcyjną sztucznie inseminowaną zgodnie z metodą Cobey i in. (2013). Cztery doby przed planowanym wychowem, matkę ograniczano w izolatorze na jednym plastrze, celem pozyskania larw w podobnym wieku. Po 12 godz. od chwili umieszczenia w izolatorze, matkę uwalniano, natomiast plaster z jajami pozostawiono w izolatorze do momentu wygryzienia się larw. Następnie, plaster ten wyjmowano i przekładano z niego larwy do miseczek matecznikowych z tworzywa sztucznego, na mleczko pszczele rozcieńczone wodą. Ramki hodowlane z przełożonymi larwami umieszczano w trzech bezmatecznych, rodzinach wychowujących, utrzymywanych w ulach Dadanta, w których znajdowało się po 10 plastrów obsiadanych „na czarno" przez pszczoły. Siódmego dnia, licząc od przełożenia larw, izolowano mateczniki przy pomocy izolatorów i do momentu wygryzienia matek przetrzymywano je w cieplarce (35°C). Podczas izolacji usuwano małe i zniekształcone mateczniki. Wygryzione matki umieszczono w bezmatecznych rodzinach do momentu inseminacji. Do dalszej części doświadczenia wybrano 9 inseminowanych sztucznie matek-sióstr, które rozpoczęły czerwienie. Sześć z tych matek-sióstr umieszczono w nowo przygotowanych rodzinach w ulikach weselnych, a trzy pozostałe matki-siostry pozostawiono w ich rodzinach (ang. *source-colonies*) w celu pozyskania pszczół 1-dniowych (tak jak w doświadczeniu klatkowym).

Procedura utworzenia rodzin w ulikach weselnych

Do doświadczenia wykorzystano uliki weselne (cztero-ramkowe). Z rodzin w pełnowymiarowych ulach pobrano fragmenty plastrów, w których obserwowano różne stadia preimaginalne robotnic. Plastry z tych rodzin cięto tak, aby pasowały do ramek ulików weselnych. Następnie, w każdej tak przygotowanej mini-rodzinie w ulikach weselnych umieszczono po 200-300 ml robotnic w różnym wieku (stadia imago). Po dwóch dobach, do każdej mini-rodziny w uliku weselnym poddano unasiennioną sztucznie matkę (6 matek-sióstr w 6 ulikach). W ten sposób, każdy ulik weselny stanowił oddzielną, kompletną rodzinę. Przez miesiąc sprawdzano: przyjęcie matki, zdolność matek do czerwienia i zachowania robotnic względem siebie i matek. Po miesiącu, gdy matki rozpoczęły składanie jaj, do każdego ulika dodano po 200 pszczół 1-dniowe, które tuż po wygryzieniu zostały zaznakowane (6 kolorów = 6 ulików) (Siuda i in., 2014; Skowronek i in., 2022b). Sześć mini-rodzin podzielono na następujące grupy:

(1) grupa doświadczalna - pszczoły spożywające olejek CBD w syropie cukrowym *ad libitum* (2 uliki)
(2) grupa doświadczalna – uliki w którym umieszczono olejek CBD na materiałowych paskach (2 uliki)
(3) grupa kontrolna – pszczoły spożywające czysty syrop cukrowy *ad libitum* (2 uliki).

5.2.2 Przygotowanie suplementu

W części pasiecznej wykorzystany został komercyjny olejek CBD o stężeniu 30% (3g w 10ml oleju firmy HempOil). Olejek otrzymano metodą ekstrakcji przy użyciu $CO_2$. Ekstrakt CBD dla grupy (1) podawano do podkarmiaczki przez pierwszy tydzień, uzupełniając jego niedobory co drugi dzień. Olejek podawano w mieszaninie z syropem cukrowym (1:1 woda z cukrem) i gliceryną w stosunku 0,01:0,5:0,5 (ekstrakt: woda destylowana: gliceryna). Dla grupy (2) ekstrakt podawano w mieszaninie z wodą i gliceryną w stosunku 0,8:1,5:1,5 (ekstrakt: woda destylowana: gliceryna). Paski materiałowe o wymiarach 2 x 10 cm równomiernie zwilżono mieszaniną i umieszczono w ulikach weselnych. Pasek zwilżano w dniach, w których uzupełniano suplement/syrop w pozostałych grupach doświadczenia.

5.2.3 Kolekcjonowanie materiału biologicznego do badań

Schemat pobierania hemolimfy dla doświadczenia pasiecznego był tożsamy z doświadczeniem klatkowym. Hemolimfę pobierano od drugiego dnia podania CBD, a zakończono gdy w grupach zabrakło zaznakowanych robotnic. Hemolimfę pobierano co tydzień od każdej z 10 takich robotnic (według metodyki Łoś i Strachecka, 2018) i umieszczano oddzielnie w probówkach typu Eppendorf, a następnie zamrożono aż do momentu analiz biochemicznych (Łoś i Strachecka, 2018).

## 5.3 Analizy biochemiczne

W próbkach hemolimfy zgromadzonych w obydwu doświadczeniach (klatkowym i pasiecznym) oznaczono:

a.) Stężenie białka całkowitego metodą Lowry'ego zmodyfikowaną przez Schacterle i Pollack (1973) (Schacterle i in., 1973; Waterborg i Matthews, 2003);
b.) Aktywność systemu proteolitycznego:
- aktywności proteaz kwaśnych, obojętnych i zasadowych wg metody Ansona (1938) zmodyfikowanej przez Strachecką i in. (2011);

- aktywności naturalnych inhibitorów proteaz kwaśnych, obojętnych i zasadowych wg metody Lee i Lina (1995);

c.) Aktywność biomarkerów enzymatycznych (oznaczane według metodyki zawartej w komercyjnym zestawie odczynników firmy Cormay):

- aminotransferaza alaninowa (ALT)
- aminotransfereza asparginianowa (AST)
- fosfataza zasadowa (ALP)

d.) Stężenia biomarkerów nieenzymatycznych (oznaczane według metodyki zawartej w komercyjnym zestawie odczynników):

- glukozy (Cormay)
- triacyloglicerolu (Cormay)
- cholesterol (Cormay)
- kreatyniny (Alpha Diagnostics)
- albuminy (Alpha Diagnostics)
- mocznik (Alpha Diagnostics)
- kwasu moczowego (Alpha Diagnostics)
- fosforu (Alpha Diagnostics, phosphomolybdate)
- wapnia (Alpha Diagnostics, arsenazo III)
- magnezu (Alpha Diagnostics, Magnesium Xylidyl Blue)

e.) Aktywność systemy antyoksydacyjnego

- całkowity potencjał antyoksydacyjny:
    - w doświadczeniu klatkowym: wg metody zawartej w komercyjnym zestawie odczynników (TAC – Sigma Aldrich, Schnelldorf, Germany),
    - w doświadczeniu pasiecznym; wg metody zawartej w komercyjnym zestawie odczynników (TAC - OxiSelectTM Total Antioxidant Capacity Assay Kit (Cell BioLabs, Inc., Upper Heyford, UK, no. STA-360)
- dysmutazy ponadtlenkowej:
    - w doświadczeniu klatkowym: wg metody Podczasy i Wei (1988),
    - w doświadczeniu pasiecznym; wg metody zawartej w komercyjnym zestawie odczynników (SOD Sigma Aldrich, Schnelldorf, Germany, no. 1916-1KT-F)
- katalazy:
    - w doświadczeniu klatkowym: wg metody Aebi (1983),

- w doświadczeniu pasiecznym; wg metody zawartej w komercyjnym zestawie odczynników (CAT Assay Kit Sigma Aldrich, Schnelldorf, Germany no. CAT100-1KT)

- peroksydazy glutationowej:
  - w doświadczeniu klatkowym: wg metody wg metody Chance i Maehly (1955) (Chance i Maehly, 1955)
  - w doświadczeniu pasiecznym; wg metody zawartej w komercyjnym zestawie odczynników (GPx Assay Kit - Sigma Aldrich, Schnelldorf, Germany, no. MAK437)
- glutationu:
  - w doświadczeniu pasiecznym; wg metody zawartej w komercyjnym zestawie odczynników (EnzyChromTM GSH/GSSG Assay Kit (Bio Assay Systems, Hayward, CA, USA, no. EGTT-100)
- S-transferazy glutationowej:
  - w doświadczeniu klatkowym: wg metody Warholm i in. (1985) (Warholm i in., 1985)

Wszystkie aktywności enzymów przeliczono na 1 mg białka.

## 5.4 Analiza statystyczna

Wyniki analizowano za pomocą oprogramowania Statistica wersja 13.3 (2017) dla Windows, StatSoft Inc., Tusla, OK, USA. Zastosowano test ANOVA (dwuczynnikowy) i test hoc Tukey HSD ($p=0,05$), który zastosowano do porównania wyników dla każdego analizowanego parametru układu odpornościowego (całkowite stężenie białka, aktywność proteaz, aktywność inhibitorów proteaz, aktywność biomarkerów enzymatycznych i nieenzymatycznych, aktywność enzymów antyoksydacyjnych) robotnic pszczół miodnych w zależności od sposobu podania (pasek i strzykawka) oraz dnia (tożsame z terminem pobrania hemolimfy w doświadczeniach) suplementacji ekstraktem z konopi oraz olejkiem CBD.

# 6. Omówienie wyników i dyskusja

Wyniki uzyskane w doświadczeniu klatkowym i pasiecznym potwierdziły hipotezy badawcze i zostały przedstawione w powiązanych tematycznie czterech publikacjach (str. 4-5) z listy *Journal Citation Reports* o łącznej liczbie punktów 400 wg punktacji MEiN. Sumaryczny *Impact Factor* zgodny z rokiem wydania artykułów wynosi 21,812. Publikacje skupiają się na określeniu wpływu ekstraktu konopnego i substancji czynnej cannabidiolu (CBD) zawartej w konopi na aktywności/stężenia podstawowych parametrów odporności robotnic pszczół miodnych (*A. mellifera*). Schemat publikacji wygląda następująco: dwie publikacje z doświadczenia klatkowego oraz dwie publikacje z doświadczenia pasiecznego.

Wskutek rosnącej antropopresji i wymierania owadów lądowych, szczególnie pszczół, wpływ substancji protekcyjnych na ich organizmy jest jednym z zagadnień poruszanych w światowym dyskursie naukowym. Pszczoły będąc elementem stabilizującym funkcjonowanie systemu bioróżnorodności, zgodnie z polityką UE „w dziedzinie ochrony różnorodności biologicznej wg. dyrektywy 2009/147/WE i dyrektywy Rady 92/43/EWG", stają się jednocześnie składnikiem jej ochrony. Aby przeciwdziałać depopulacji rodzin pszczelich (np. w wyniku inwazji pasożyta *V. destructor*) zaczęto stosować na całym świecie leki/akarycydy. Szybko okazało się, że patogeny nabyły lekooporności, a człowiek ingeruje w znaczący sposób w ten „wyścig zbrojeń" i interakcje patogen-gospodarz. Dlatego najrozsądniejszym rozwiązaniem wydaje się wykorzystanie takich substancji, które będą przeciwdziałały tej szkodliwej, obniżającej odporność, presji środowiska. Wybór ekstraktu z konopi i CBD był podyktowany ich właściwościami przeciwzapalnymi, przeciwbólowymi, przeciwrakowymi, bakteriobójczymi i przeciwgrzybiczymi, udowodnionymi na kręgowcach (Yokota i in., 1995; Amin i Ali, 2019; Gray i Whalley, 2020) oraz na innych owadach (Barciak większy) .

W badaniach realizowanych w niniejszej dysertacji zastosowano dwie metody suplementacji, zarówno ekstraktu z konopi, jak i olejku CBD: w syropie cukrowym (standardowa metoda przedstawiana w publikacjach naukowych) oraz na materiałowych paskach nasączanych substancjami czynnymi (paski wykorzystywane są w praktyce pasiecznej do podawania akarycydów np. na *V. destructor*). W dotychczasowych pracach z zakresu apidologii, autorka nie znalazła próby wykorzystania pasków do podawania biostymulatorów. Dlatego to metodyczne opracowanie zaprezentowane w moich

badaniach można uznać za pionierskie i umożliwia szersze wykorzystanie substancji czynnych/aktywnych w gospodarce pasiecznej.

***Wpływ ekstraktu konopi i olejku CBD na aktywność systemu proteolitycznego w hemolimfie robotnic***

W pierwszej z cyklu publikacji (str. 4, poz. 1) wykazano, że ekstrakt konopny zmniejszał aktywność proteaz kwaśnych i obojętnych niezależnie od sposobu jego podania oraz podwyższał aktywność proteaz zasadowych. Nasze wyniki są zgodne z tymi uzyskanymi przez Strachecką i in. (2014, 2015), w których pszczoły suplementowano kurkuminą i kofeiną (*ad libitum*). Autorzy tych prac sugerują, że zmniejszenie aktywności ww. enzymów jest efektem pozytywnym i łączą go ze zwiększoną witalnością i przeżywalnością pszczół w suplementowanych grupach (Strachecka i in., 2014; Strachecka i in., 2015). Odwrotnie, w porównaniu z biostymulatorami/suplementami diety, na aktywność systemu proteolitycznego wpływają czynniki negatywne - szkodliwe dla organizmu pszczoły, tj. pole E (elektromagnetycznego) o częstotliwości 50 Hz (Migdał i in., 2021), bromfenwinfos (Aneta Strachecka i in., 2016) i pestycydy (np. imidakloprid) (Paleolog i in., 2020). Warto w tym miejscu podkreślić, iż wyżej przytoczone wyniki uzyskane przez różnych autorów i w niniejszej dysertacji, przeprowadzano w doświadczeniach klatkowych, w których pszczoły są przetrzymywane w wystandaryzowanych, stałych warunkach. Ponadto, klatka jest środowiskiem nienaturalnym (bez matki) dla pszczół i ogranicza funkcjonowanie zgodne z ich fizjologią. Środowisko ula – rodziny jest naturalnym, w którym pszczoły narażone są na dodatkowe czynniki (dostęp do wszystkich produktów pszczelich, w tym diety z pyłkami/białkami; narażenie na stres biotyczny lub abiotyczny). Może to być jedna z przyczyn uzyskania różnic w aktywnościach proteaz kwaśnych i obojętnych w hemolimfie robotnic w doświadczeniu pasiecznym (str. 4, poz. 3) w porównaniu z doświadczeniem klatkowym (str. 4, poz. 1). Drugą przyczyną takich zróżnicowanych wyników, którą należy uwzględnić, jest skład podawanych pokarmów: w przypadku doświadczenia klatkowego podawano ekstrakt z konopi zawierający wiele związków czynnych; natomiast w doświadczeniu pasiecznym podawano pszczołom w pokarmie czysty olejek CBD. Można przypuszczać, że bogatszy w związki chemiczne ekstrakt odmiennie wpływa na aktywację proteaz w środowisku kwaśnym i obojętnym. W przypadku aktywności proteaz zasadowych, które w przeważającej części są proteazami serynowymi, zaobserwowano wzrost ich aktywności w hemolimfie robotnic po podaniu im ekstraktu z konopi i CBD

zarówno w doświadczeniu klatkowym, jak i pasiecznym (str. 4, poz. 1 i 3). Proteazy te biorą udział w funkcjonowaniu układu odpornościowego oraz w jelitowych mechanizmach adsorpcji i transporcie (Vlahović i in., 2009; Chen i in., 2011). Zaburzenia w ich funkcjonowaniu w organizmie pszczoły są przyczyną nieprawidłowości w aktywowaniu fenolooksydazy, szlaku Dopa, szlaku tyrozynowym i in., których skutkiem jest rozchwianie metabolizmu melanin i sklerotyn niezbędnych w usztywnianiu i zabezpieczaniu (uszczelnieniu) kutikuli przed patogenami (Strachecka i in., 2018). Można więc przypuszczać, iż ekstrakt z konopi i sam CBD zwiększając aktywności tych proteaz, stabilizują szlaki metaboliczne różnych białek i związków niezbędnych w kształtowaniu odporności pszczół. Ponadto, wzrost aktywności proteaz jest pożądany w stanach chorobowych/stresowych i umożliwia szybką hydrolizę białek patogenu (Malone i Gatehouse, 1998). Potwierdzają to m.in. badania Holta i in. (2013) oraz Peghaire i in. (2020), którzy zauważyli, że *N. ceranae* zmienia aktywność genów kodujących białka odpornościowe. W wyniku tych zaburzeń dochodzi do degeneracji tkanek oraz destabilizacji enzymów, w tym proteaz (Holt i in., 2013; Peghaire i in., 2020).

Aktywność inhibitorów proteaz kwaśnych, obojętnych i zasadowych była wyższa w grupach suplementowanych ekstraktem z konopi lub olejkiem CBD w porównaniu z grupą karmioną samym syropem cukrowym (bez dodatku ekstraktu/CBD) zarówno w doświadczeniu klatkowym, jak i pasiecznym (str. 4, poz. 1 i 3). Inhibitory proteaz działają bezpośrednio na proteazy wydzielane przez patogeny, uniemożliwiając im wnikanie do jam ciała i hamując ich rozwój (Gliński i in, 2011). Inhibitory proteaz kwaśnych są skierowane przede wszystkim przeciwko chorobotwórczym grzybom, inhibitory proteaz zasadowych - przeciwko bakteriom i wirusom, a inhibitory proteaz obojętnych - przeciwko innym czynnikom. Ponadto, związki te są „strażnikami" nadczynności swoistych proteaz w organizmie pszczoły, regulują ich stężenia i aktywności, i tym samym są niezbędne aby utrzymać homeostazę w pszczelich tkankach (Gliński i in. 2011; Strachecka i in., 2018). Odwrotne tendencje w aktywnościach inhibitorów proteaz zaobserwowano w hemolimfie robotnic, które miały kontakt z ekstraktem konopnym / olejkiem CBD na paskach: w doświadczeniu klatkowym aktywności inhibitorów proteaz były niższe, a w doświadczeniu pasiecznym – wyższe u pszczół, które miały styczność z ekstraktem/CBD w porównaniu z grupą kontrolną. Przyczynami takiej sytuacji mogą być: 1) różnice w składzie chemicznym pomiędzy ekstraktem z konopi (podawany w doświadczeniu klatkowym), a olejkiem CBD

(podawanym w doświadczeniu pasiecznym); 2) warunki jakie panują w rodzinie – ulu oraz w klatce (omówiono je wyżej przy interpretacji proteaz) włącznie z ewentualnymi reakcjami zachodzącymi pomiędzy związkami, którymi nasączone były paski a tymi w organizmie pszczoły (środowisko rodziny umożliwia prawidłowe wydzielanie feromonów i innych substancji, których nie obserwuje się u pszczół przebywających w klatce).

Dodatek ekstraktu z konopi i olejku CBD do syropy cukrowego, jak również na paskach, wpłynął na zwiększenie stężenia białka całkowitego w porównaniu z grupą kontrolną (str. 4, poz. 1 i 3). Porównując metody podania ww. substancji, wyższe wartości obserwowano w przypadku podania ich w syropie cukrowym niż na paskach. Różnice te mogą być efektem koncentracji substancji czynnych jakie dostają się do pszczelego organizmu w jednostce czasu (w przypadku pasków pszczoły muszą zlizać substancje z paska lub/i otrzeć się o pasek i zlizać je z powierzchni kutikuli). Niezależnie od metody podania (syrop *vs* pasek), ekstrakt z konopi i CBD stymulują syntezę białek w organizmie pszczoły. Najprawdopodobniej, substancje czynne szybko (efekty obserwowano już po siedmiu dniach suplementacji) są wchłaniane przez układ pokarmowy i przetransportowywane do hemolimfy i tkanek tj. ciało tłuszczowe. To właśnie w tej tkance dochodzi do syntezy większości białek (również tych odpornościowych) krążących w pszczelim organizmie (Skowronek i in., 2021). W badaniach z roku 2011 wykazano, że niektóre substancje czynne obecne w konopiach (kannabidiol) wpływają na kształtowanie środowiska wewnątrz komórek np. poprzez regulacje stężenia jonów wapnia (De Petrocellis i in., 2011). W tkankach immunogennych tj. ciało tłuszczowe obecne są fosfolipazy komórkowe (cPLA2 − Ca + 2-zależna cytozolowa fosfolipaza A2), które będąc zależnymi od jonów wapnia, regulują procesy odpornościowe u owadów np. pośrednicząc w produkcji eikozanoidów (udział w odporności humoralnej oraz komórkowej). Zwielokrotniona translacja białek w ciele tłuszczowym przekłada się na zwiększenie ich stężeń w hemolimfie pszczół (Tunaz i in., 2003; Park i in., 2015; Sadekuzzaman i Kim, 2017; Vatanparast i in., 2018; Xia i in., 2018; Watkins, 2019). Można wnioskować, że jest to spowodowane obecnością, zarówno w ekstrakcie jak i w olejku, kannabidiolu (CBD), który jest substancją lipofilową o analogicznym działaniu jak koenzym Q10 w doświadczeniu opublikowanym przez Strachecką i in. (2014). Wzrost stężeń białek, również tych enzymatycznych, świadczy o aktywnym metabolizmie w komórkach ciała pszczoły, a także uruchomieniu procesów odpowiedzialnych za wytworzenie energii

(Haydak, 1970) niezbędnej do odbycia lotów po pyłek i nektar. Podobnie jak w niniejszej dysertacji, Słowińska i in. (2016) zauważyli, że stężenia białek w hemolimfie wzrastają wraz z wiekiem pszczół robotnic do 30 dnia ich życia i są wtedy nawet 4-krotnie wyższe niż w 1 dniu (Słowińska i in., 2016). Podobne tendencje zaobserwowali Strachecka i in. (2014; 2015), którzy pokazali, iż stężenie białka w hemolimfie u robotnic wzrasta wraz z wiekiem do ok 2-3 tygodnia życia, po czym maleje. Spadek ich stężeń związany jest ze starzeniem się organizmu (Cremonez i in., 1998; Amdam i in., 2003). Warto zauważyć, że pszczoły karmione syropem z dodatkiem olejku CBD (str. 4, poz. 3) charakteryzowały się bardzo wysokimi stężeniami białek nawet w 35 dniu życia. Można przypuszczać, iż CBD stabilizuje wielopoziomowo metabolizm robotnic, analogicznie jak u ssaków (Da Silva i in., 2013; Porter i in., 2021; Guard i in., 2022), a tym samym wydłuża ich życie.

***Wpływ ekstraktu konopi i olejku CBD na aktywności/stężenia markerów biochemicznych (biomarkerów) w hemolimfie robotnic***

Aktywności biomarkerów enzymatycznych, tj.: aminotransferaza alaninowa (ALT), aminotransferaza asparignianowa (AST) oraz fosfataza zasadowa (ALP), zwiększały się wraz z wiekiem robotnic we wszystkich grupach w doświadczeniu klatkowym i pasiecznym (str. 4, poz. 1 i 3). Podobne tendencje zaobserwowali Strachecka i in. (2014, 2015). Aktywności tych enzymów były wyższe w hemolimfie pszczół poddanych działaniu CBD w porównaniu z tymi z grupy kontrolnej. Strachecka i in. (2015) oraz Łoś i Strachecka (2018) wykazali, że odwrotnie jak u ssaków, wzrost aktywności tych markerów jest zjawiskiem pozytywnym, wzmacniającym odporność pszczół; z kolei obniżenie ich aktywności jest zjawiskiem patologicznym. Zmniejszenie aktywności AST, ALT i ALP zaobserwowali: Sokół (1996) - u pszczół miodnych porażonych *Varroa destructor*, Strachecka i in. (2016) – u pszczół potraktowanych bromfenwinfosem; Paleolog i in. (2020) – po imidaklopridzie; a także Migdał i in. (2021) – po działaniu fal elektromagnetycznych. Takie fluktuacje tych parametrów po kontakcie z czynnikiem szkodliwym, mogą świadczyć o degradacji ciała tłuszczowego, będącego funkcjonalnym analogiem wątroby kręgowców (Bojarski, 2016; Strachecka i in., 2018). Strachecka i in. (2016) wykazali, że AST, ALP i ALT wspierają działanie systemu antyoksydacyjnego i proteolitycznego, jako pierwszej linii obrony przed patogenami. Utrzymanie homeostazy tych dwóch systemów reguluje aktywności biomarkerów u pszczół, co wpływa na prawidłowość procesów metabolicznych/biochemiczych/fizjologicznych, np. cyklu Krebsa, łańcucha oddechowego, itp. (Berg i in., 2020). Wiąże się to bezpośrednio

z odpowiednimi ilościami energii i znajduje odzwierciedlenie w zaobserwowanych przez autorkę zwiększonych stężeniach glukozy i triglicerydów, szczególnie po zastosowaniu CBD (str. 4, poz. 3). Ponadto, odnotowano wyższą aktywność ww. biomarkerów (głównie AST i ALT) u pszczół, które dostawały substancję czynną w syropie w porównaniu z tymi dostającymi ją na paskach.

Stężenia biomarkerów nieenzymatycznych, tj. glukoza, triglicerydy, cholesterol, kreatynina i mocznik zwiększały się, a kwasu moczowego zmniejszały się wraz z wiekiem/postępującymi procesami starzenia pszczół we wszystkich grupach (str. 4, poz. 1 i 3). Stężenia ww. związków były wyższe w hemolimfie pszczół, które dostawały ekstrakt z konopi/CBD w syropie lub na pasku w porównaniu z tymi z grupy kontrolnej. Ponadto, pszczoły dostające substancje czynne w syropie miały zazwyczaj wyższe stężenia tych markerów niż te, które dostawały je na paskach. W przypadku stężenia albuminy zaobserwowano odwrotne tendencje: zmniejszanie stężenia z wiekiem pszczół; niższe wartości w grupach z CBD w porównaniu z kontrolą. Podobne tendencje w stężeniach tych wszystkich biomarkerów zaobserwowano w przypadku innych biostymulatorów, tj. kofeina, kurkumina, koenzym Q10, czy piperyna (Strachecka i in., 2014, 2015; Schulz i in., 2019). Glukoza i triglicerydy (triacyloglicerole) są podstawowymi związkami energetycznymi u pszczół (Gmeinbauer i Crailsheim, 1993; Brouwers, 2015). W wyniku wielu reakcji kaskadowych podczas glikolizy, cyklu Krebsa, a następnie łańcucha oddechowego, są one wykorzystywane do syntezy ATP (Solomon i in., 2020). Niedobory glukozy i triglicerydów są przyczyną silnego stresu energetycznego i oksydacyjnego, zachwiania metabolizmu i homeostazy, a następnie doprowadzają do zaburzeń w termoregulacji organizmu pszczoły (Mayack i Naug, 2009; Alaux i in., 2011). Spostrzeżenia te potwierdzili Grupe i Quandt (2020) wskazując, iż niedobory tych związków (szczególnie cukrów) w organizmach pszczół doprowadzają do tzw. „ospałości" owada i w efekcie końcowym do jego śmierci (Grupe i Alisha Quandt, 2020). Dodatkowo, warto wspomnieć, iż Mattes i in. (2021) potwierdzili korzystny wpływ olejku CBD w leczeniu cukrzycy i insulinooporności u ludzi. Układy endokannabinoidowe ssaków i owadów funkcjonują w analogiczny sposób i regulują stężenia związków odpowiedzialnych za produkcję energii. Tym samym, u pszczół przyczyniają się do wydłużenia ich życia. Ponadto, cholesterol oraz triacyloglicerol są podstawowymi związkami lipidowymi w ciele tłuszczowym gromadzonymi w postaci kropli tłuszczu w trofocytach. Rezerwy triacyloglicerolu, których stężenia zwiększają się u pszczół

suplementowanych CBD, mogą w każdym momencie zostać przekształcone w diglicerydy i kwasy tłuszczowe, i przetransportowane do określonych tkanek (Skowronek i in., 2021a). Właściwa gospodarka triacylogliceroli (poprzez np. lipolizę, lipogenezę) jest ściśle regulowana przez potencjał jonowy, a szczególnie jony $Ca^{2+}$. Stężenie tych jonów zwiększa się u pszczół suplementowanych CBD (str. 5, poz. 4). Ponadto jony wapnia regulując gospodarkę lipidową wpływają również na rozwój i starzenie się organizmu (diapauza, metamorfoza) (Furtado i in., 2013; Sadekuzzaman i in., 2017; Toprak i in., 2020). Stężenie tych jonów jest regulowane obecnością jonów magnezu i fosforu, których stężenia były wyższe w grupach suplementowanych CBD.

Wyższe stężenie mocznika u pszczół suplementowanych ekstraktem z konopi/CBD (str. 4, poz. 1) mogło być spowodowane wyższą podażą tłuszczy jako nośników substancji czynnej (zazwyczaj dieta pszczół bazuje na dużej ilości węglowodanów i białka, w mniejszym stopniu tłuszczy) (Skowronek i in., 2021b; Skowronek i in., 2022b). Z kolei, wyższe stężenie kwasu moczowego, zarówno po suplementacji olejkiem CBD (str. 4, poz. 3), jak również kurkuminą i koenzymem Q10, może wynikać z obecności grup ketonowych (Gilbert, 1967; Strachecka i in., 2014, 2015).

***<u>Wpływ ekstraktu konopi i olejku CBD na aktywność systemu antyoksydacyjnego w hemolimfie robotnic</u>***

Ekstrakt z konopi w doświadczeniu klatkowym oraz olejek CBD w doświadczeniu pasiecznym zwiększały aktywności enzymów antyoksydacyjnych, tj. dysmutaza ponadtlenkowa (SOD), peroksydaza glutationowa (GPx), S-transferaza glutationowa (GST), katalaza (CAT) oraz poziom glutationu (GSH) i całkowitego potencjału antyoksydacyjnego (TAC); w porównaniu z grupą kontrolną (str. 4-5, poz. 2 i 4). Większe wartości tych poszczególnych związków systemu antyoksydacyjnego obserwowano u pszczół, którym podawano substancje czynne w syropie niż na paskach. Ponadto, aktywność/poziomy poszczególnych elementów systemu antyoksydacyjnego we wszystkich trzech grupach zwiększały się wraz z wiekiem robotnic.

Związki pochodzące z konopi są silnymi antyoksydantami, które szybko i sprawnie wychwytują wolne rodniki i neutralizują szkodliwe produkty przemiany materii oraz inne związki egzogenne. Tym samym wzmacniają działanie układu immunologicznego pszczoły (str. 4-5, poz. 2 i 4), a w konsekwencji wydłużają jej życie, co zostało zaobserwowane w doświadczeniach realizowanych w ramach niniejszej dysertacji

(w doświadczeniu klatkowym robotnice żyły maks. 56 dni, a w doświadczeniu pasiecznym – 35 dni). Warto zwrócić uwagę, iż długość życia po suplementacji pszczół olejkiem CBD znacząco wpływa na ich długowieczność w porównaniu z innymi biostymulatorami, tj. kurkuma – długość życia pszczół wynosiła 48 dni, piperyna - 41 dni i koenzymu Q10 - 38 dni. Interesującym spostrzeżeniem jest fakt, że CBD charakteryzuje się silniejszą zdolnością wychwytywania rodników 2,2-difenylo-1-pikrylhydrazylowych niż THC, również izolowany z konopi (Samarut i in., 2019; Gruschow, 2020). Ten silny efekt antyoksydacyjny CBD wynika z budowy chemicznej i obecności dwóch grup fenolowych. Synergistyczne, skoordynowane działanie różnych antyoksydantów w hemolimfie zwielokrotnia/intensyfikuje działanie grup fenolowych w CBD, w wyniku czego wzrasta immunokompetencja pszczół i, jak wspomniano wyżej, wydłuża się ich życie. Analogiczne działanie do kannabidolu zaobserwowano w przypadku resweratrolu (Hacke i in., 2019), koenzymu Q10, kurkuminy, kofeiny i piperyny (Strachecka i in., 2014; Strachecka i in., 2014; Schulz i in., 2019). Jena i in. (2015) zasugerowali, że zwiększona aktywność poszczególnych związków systemu antyoksydacyjnego zabezpiecza przez przypadkową produkcją rodników tlenowych (np. podczas infekcji), które są neutralizowane i usuwane z organizmu.

W każdym z doświadczeń zaobserwowano wpływ metody suplementacji na wartość charakterystyk biochemicznych. Szybsze efekty i wyższe wartości tych charakterystyk uzyskano w przypadku podania ekstraktu z konopi/olejku CBD w syropie cukrowym. Podanie substancji czynnych w syropie sprawia, iż bezpośrednio trafiają one do przewodu pokarmowego pszczoły, skąd są rozprowadzane po jej organizmie. W przypadku pasków nasączonych ekstraktem prawdopodobnie efekt jest przesunięty w czasie przez fakt, że najpierw ekstrakt osadza się na ich kutikuli, a następnie jest zlizywany np. podczas czyszczenia. Wyniki te są istotną informacją z punktu widzenia praktycznego i zastosowania suplementacji w pasiekach (biorąc pod uwagę nie tylko ekstrakt konopny, ale i inne substancje).

# 7. Stwierdzenia i wnioski

Na podstawie uzyskanych wyników sformułowane zostały następujące wnioski:

1. Wyższe stężenia białek i aktywności systemu proteolitycznego w hemolimfie pszczół stymulowanych ekstraktem z konopi i olejkiem CBD świadczą o wzmożonych, wielokierunkowych kaskadach enzymatycznych związanych z wytworzeniem energii w postaci ATP oraz podniesieniu odporności tych owadów.
2. Wyższe aktywności markerów biochemicznych (aminotransferazy alaninowej, aminotransferazy asparginianowej oraz fosfatazy zasadowej) w hemolimfie pszczół, które suplementowano ekstraktem z konopi lub olejkiem CBD świadczą o pozytywnej stymulacji organizmu i wzmocnieniu jego barier ochronnych.
3. Zwiększenie stężeń nieenzymatycznych markerów biochemicznych w hemolimfie pszczół stymulowanych ekstraktem z konopi i olejkiem CBD uzupełnia i wzmacnia siłę, a także przyspiesza reakcję pozostałych elementów układu odpornościowego.
4. Wyższe aktywności enzymów systemu antyoksydacyjnego w hemolimfie pszczół suplementowanych ekstraktem z konopi lub olejkiem CBD zabezpieczają organizm przed reaktywnymi formami tlenu, a także wzmacniają biochemiczne mechanizmy odporności umożliwiające szybką neutralizację pozostałości po patogenach.
5. Suplementacja pszczół ekstraktem z konopi lub olejkiem CBD w syropie cukrowym jest efektywniejsza i mniej czasochłonna niż na paskach.

Ekstrakt z konopi, podobnie jak i olejek CBD, pozytywnie wpływały na parametry biochemiczne układu odpornościowego w hemolimfie robotnic pszczół miodnych poprzez stymulacje aktywności systemów proteolitycznego i antyoksydacyjnego oraz biomarkerów enzymatycznych i nieenzymatycznych w doświadczeniu klatkowym oraz pasiecznym. Wyniki doświadczenia pasiecznego, w którym wykorzystano olejek CBD, potwierdzają efekty uzyskane w doświadczeniu klatkowym przy użyciu ekstraktu konopnego. W obydwy przypadkach suplementacje wydłużały życie pszczół w porównaniu do kontroli. Ekstrakt z konopi i olejek CBD wywoływały większy efekt w biochemii/fizjologii pszczół podczas podania substancji czynnych w syropie.

# 7. Bibliografia


Abdel-Salam O., Sleem A., Youness E., Morsy F., 2018, *Preventive effects of cannabis on neurotoxic and hepatotoxic activities of malathion in rat*, „Asian Pacific Journal of Tropical Medicine” 11, nr 4: 272-279, https://www.apjtm.org/article.asp?issn=1995-7645;year=2018;volume=11;issue=4;spage=272;epage=279;aulast=Abdel-Salam (dostęp: 1 lipca 2021).

Alaux C., Folschweiller M., McDonnell C., Beslay D., Cousin M., Dussaubat C., Conte Y. Le, 2011, *Pathological effects of the microsporidium Nosema ceranae on honey bee queen physiology (Apis mellifera)*, „Journal of Invertebrate Pathology” 106, nr 3: 380-385.

Amdam G. V., Norberg K., Hagen A., Omholt S. W., 2003, *Social exploitation of vitellogenin*, „Proceedings of the National Academy of Sciences” 100, nr 4: 1799-1802, https://www.pnas.org/doi/abs/10.1073/pnas.0333979100 (dostęp: 19 czerwca 2023).

Amin M. R., Ali D. W., 2019, *Pharmacology of Medical Cannabis*, "Advances in Experimental Medicine and Biology", t. 1162, 151-165, https://doi.org/10.1007/978-3-030-21737-2_8 (dostęp: 4 lipca 2021).

Anson M. L., 1938, *The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin*, "The Journal of general physiology", 22.1: 79.http://rupress.org/jgp/article-pdf/22/1/79/1238867/79.pdf (dostęp: 1 lipca 2021).

Bartosz G., 2003, *Total antioxidant capacity*, „Advances in Clinical Chemistry” 37: 219-292.

Berg J.M., Stryer L., Tymoczko J.L. G. J. G., 2020, *Biochemia*, Warszawa.

Bode W., Fernandez-Catalan C., Tschesche H., Grams F., Nagase H., Maskos K., 1999, *Structural properties of matrix metalloproteinases*, „Cellular and Molecular Life Sciences”.

Bojarski B., 2016, *Oddziaływanie herbicydów na aktywność wybranych enzymów oraz ultrastrukturę skrzeli i wątroby ryb*, "dysertacja doktorska", http://rep.up.krakow.pl/xmlui/handle/11716/1238 (dostęp: 19 czerwca 2023).

Brouwers E. V. M., 2015, *Glucose/Fructose Ratio in the Food of Honeybee Larvae During Caste Differentiation*, " Journal of Apicultural Research", 23(2), 94-101. „http://dx.doi.org/10.1080/00218839.1984.11100616”, https://www.tandfonline.com/doi/abs/10.1080/00218839.1984.11100616 (dostęp: 19 czerwca 2023).

Chance B., Maehly A. C., 1955, *[136] Assay of catalases and peroxidases*, „Methods in Enzymology” 2, nr C: 764-775.

Chen K. T., Malo M. S., Beasley-Topliffe L. K., Poelstra K., Millan J. L., Mostafa G., Hodin R. A., 2011, *A role for intestinal alkaline phosphatase in the maintenance of local gut immunity*, „Digestive Diseases and Sciences” 56, nr 4: 1020-1027, https://link.springer.com/article/10.1007/s10620-010-1396-x (dostęp: 19 czerwca 2023).

Cobey S. W., Tarpy D. R., Woyke J., 2015, *Standard methods for instrumental insemination of Apis mellifera queens*, " Journal of Apicultural Research" „https://doi.org/10.3896/IBRA.1.52.4.09” 52, nr 4, https://www.tandfonline.com/doi/abs/10.3896/IBRA.1.52.4.09 (dostęp: 18 czerwca 2023).

Conte Y. Le, Navajas M., 2008, *Climate change: Impact on honey bee populations and diseases*, „OIE Revue Scientifique et Technique” 27, nr 2: 485-510.

Cremonez T. M., De Jong D., Bitondi M. M. G., 1998, *Quantification of Hemolymph Proteins as a Fast Method for Testing Protein Diets for Honey Bees (Hymenoptera: Apidae)*, „Journal of Economic Entomology” 91, nr 6: 1284-1289, https://dx.doi.org/10.1093/jee/91.6.1284 (dostęp: 19 czerwca 2023).

Da Silva V. K., De Freitas B. S., Da Silva Dornelles A., Nery L. R., Falavigna L., Ferreira R. D. P., Schröder N., 2013, *Cannabidiol Normalizes Caspase 3, Synaptophysin, and Mitochondrial Fission Protein DNM1L Expression Levels in Rats with Brain Iron Overload: Implications for Neuroprotection*, „Molecular Neurobiology 2013 49:1” 49, nr 1: 222-233, https://link.springer.com/article/10.1007/s12035-013-8514-7 (dostęp: 28 sierpnia 2022).

Davies K. J. A., 1986, *Intracellular proteolytic systems may function as secondary antioxidant defenses: An hypothesis*, „Journal of Free Radicals in Biology &

Medicine” 2, nr 3: 155-173.

De Petrocellis L., Ligresti A., Moriello A. S., Allarà M., Bisogno T., Petrosino S., Di Marzo V., 2011, *Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes*, „British Journal of Pharmacology” 163, nr 7: 1479-1494, http://dx.doi.org/10.1111/bph.2011.163.issue-7 (dostęp: 1 lipca 2021).

Dziechciarz P., Borsuk G., Olszewski K., 2019, *Prospects and Validity of Laboratory Cage Tests Conducted in Honeybee Research Part one: Main Directions of Use of Laboratory Cage Tests in Honeybee Research*, „Journal of Apicultural Science” 63, nr 2: 201-207.

Farjan M., Dmitryjuk M., LipiŃski Z., Biernat-Łopieńska E., Zółtowska K., 2012, *Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of Apis mellifera carnica brood at different stages*, „Journal of Apicultural Research” 51, nr 3: 263-270, https://www.tandfonline.com/action/journalInformation?journalCode=tjar20 (dostęp: 3 lipca 2021).

Furtado W. C. A., Azevedo D. O., Martins G. F., Zanuncio J. C., Serrão J. E., 2013, *Histochemistry and Ultrastructure of Urocytes in the Pupae of the Stingless Bee Melipona quadrifasciata (Hymenoptera: Meliponini)*, „Microscopy and Microanalysis” 19, nr 6: 1502-1510, https://www.cambridge.org/core/journals/microscopy-and-microanalysis/article/abs/histochemistry-and-ultrastructure-of-urocytes-in-the-pupae-of-the-stingless-bee-melipona-quadrifasciata-hymenoptera-meliponini/A79DA76FBB1B997C7251F7DAE1963A9F (dostęp: 30 października 2022).

Geldert C., Abdo Z., Stewart J. E., Arathi H. S., 2021, *Dietary supplementation with phytochemicals improves diversity and abundance of honey bee gut microbiota*, „Journal of Applied Microbiology” 130, nr 5: 1705-1720, https://onlinelibrary.wiley.com/doi/full/10.1111/jam.14897 (dostęp: 9 listopada 2022).

Gilbert L. I., 1967, *Lipid Metabolism and Function in Insects*, „Advances in Insect Physiology” 4, nr C: 69-211.

Gliński, Z., Buczek, K., & Marć M., 2011, *Zjawiska i mechanizmy odporności przeciwzakaźnej pszczoły miodnej - nowe osiągnięcia*, „Życie weterynaryjne" 86, nr 9: 684-693.

Gmeinbauer R., Crailsheim K., 1993, *Glucose utilization during flight of honeybee (Apis mellifera) workers, drones and queens*, „Journal of Insect Physiology" 39, nr 11: 959-967.

Gray R. A., Whalley B. J., 2020, *The proposed mechanisms of action of CBD in epilepsy,* „Epileptic Disorders" 22, nr S1: S10-S15, https://onlinelibrary.wiley.com/doi/full/10.1684/epd.2020.1135 (dostęp: 15 czerwca 2023).

Grupe A. C., Alisha Quandt C., 2020, *A growing pandemic: A review of Nosema parasites in globally distributed domesticated and native bees*, „PLOS Pathogens" 16, nr 6: e1008580, https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1008580 (dostęp: 19 czerwca 2023).

Gruschow A., 2020, *A Comparison of Antioxidant Potential, Total Phenolic Content, and Cannabidiol (CBD) Content of Cannabis Infused Hemp, MCT, and Olive Oils,* „Theses, Dissertations and Culminating Projects", https://digitalcommons.montclair.edu/etd/472 (dostęp: 28 września 2021).

Grzywnowicz K., Ciołek A., Tabor A., Jaszek M., 2009, *Profiles of the body-surface proteolytic system of honey bee queens, workers and drones: Ontogenetic and seasonal changes in proteases and their natural inhibitors*, „Apidologie" 40, nr 1: 4-19, http://dx.doi.org/10.1051/apido:2008057 (dostęp: 15 czerwca 2023).

Guard S. E., Chapnick D. A., Poss Z. C., Ebmeier C. C., Jacobsen J., Nemkov T., Old W. M., 2022, *Multiomic Analysis Reveals Disruption of Cholesterol Homeostasis by Cannabidiol in Human Cell Lines*, „Molecular and Cellular Proteomics" 21, nr 10, http://www.mcponline.org/article/S1535947622000706/fulltext (dostęp: 19 czerwca 2023).

Hacke A. C. M., Lima D., Costa F. de, Deshmukh K., Li N., Chow A. M., Kerman K., 2019, *Probing the antioxidant activity of Δ9-tetrahydrocannabinol and cannabidiol in Cannabis sativa extracts*, „Analyst" 144, nr 16: 4952-4961,

https://pubs.rsc.org/en/content/articlehtml/2019/an/c9an00890j (dostęp: 28 września 2021).

Haydak H., 1970, *Honey bee nutrition*, „Annual Review of Entomology" 15: 143-156.

Hazekamp A., Simons R., Peltenburg-Looman A., Sengers M., Van Zweden R., Verpoorte R., 2004, *Preparative isolation of cannabinoids from Cannabis sativa by centrifugal partition chromatography*, „Journal of Liquid Chromatography and Related Technologies" 27, nr 15: 2421-2439, https://www.tandfonline.com/doi/abs/10.1081/JLC-200028170 (dostęp: 2 lipca 2021).

Aebi H., 1983, *Catalase*, „Methods of Enzymatic Analysis": 273-286, https://cir.nii.ac.jp/crid/1574231875426901504 (dostęp: 18 czerwca 2023).

Higes M., Martín-Hernández R., Garrido-Bailón E., González-Porto A. V., García-Palencia P., Meana A., Bernal J. L., 2009, *Honeybee colony collapse due to Nosema ceranae in professional apiaries*, „Environmental Microbiology Reports" 1, nr 2: 110-113, https://onlinelibrary.wiley.com/doi/full/10.1111/j.1758-2229.2009.00014.x (dostęp: 12 października 2021).

Holt H. L., Aronstein K. A., Grozinger C. M., 2013, *Chronic parasitization by Nosema microsporidia causes global expression changes in core nutritional, metabolic and behavioral pathways in honey bee workers (Apis mellifera)*, „BMC Genomics" 14, nr 1: 1-16, https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-14-799 (dostęp: 19 czerwca 2023).

Hristov P., Shumkova R., Palova N., Neov B., 2020, *Factors Associated with Honey Bee Colony Losses: A Mini-Review*, „Veterinary Sciences 2020, Vol. 7, Page 166" 7, nr 4: 166, https://www.mdpi.com/2306-7381/7/4/166/htm (dostęp: 28 marca 2022).

Isenmann E., Veit S., Starke L., Flenker U., Diel P., 2021, *Effects of Cannabidiol Supplementation on Skeletal Muscle Regeneration after Intensive Resistance Training*, „Nutrients 2021, Vol. 13, Page 3028" 13, nr 9: 3028, https://www.mdpi.com/2072-6643/13/9/3028/htm (dostęp: 9 listopada 2022).

J.Strachecka A., Paleolog J., Grzywnowicz K., 2008, *The surface proteolytic activity in Apis mellifera*, „Journal of Apicultural Science" 52, nr 1.

Jankielsohn A., Jankielsohn A., 2018, *The Importance of Insects in Agricultural*

*Ecosystems*, „Advances in Entomology” 6, nr 2: 62-73, http://www.scirp.org/journal/PaperInformation.aspx?PaperID=83726 (dostęp: 26 lipca 2022).

Jena S., 2015, *Effect of persistent and transient hypothyroidism on histoarchitecture and antioxidant defence system in rat brain*, „Neurological Sciences” 36, nr 6: 953-959, https://link.springer.com/article/10.1007/s10072-015-2199-9 (dostęp: 19 czerwca 2023).

Kramer B. H., Nehring V., Buttstedt A., Heinze J., Korb J., Libbrecht R., Bernadou A., 2021, *Oxidative stress and senescence in social insects: a significant but inconsistent link?*, „Philosophical Transactions of the Royal Society B” 376, nr 1823, https://royalsocietypublishing.org/doi/10.1098/rstb.2019.0732 (dostęp: 18 czerwca 2023).

Lacetera N., 2019, *Impact of climate change on animal health and welfare*, „Animal Frontiers” 9, nr 1: 26-31, https://academic.oup.com/af/article/9/1/26/5168813 (dostęp: 28 marca 2022).

Larsen A., Reynaldi F. J., Guzmán-Novoa E., Larsen A., Reynaldi F. J., Guzmán-Novoa E., 2019, *Fundaments of the honey bee (Apis mellifera) immune system. Review*, „Revista mexicana de ciencias pecuarias” 10, nr 3: 705-728, http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S2007-11242019000300705&lng=es&nrm=iso&tlng=en (dostęp: 18 czerwca 2023).

Li Z., Hou M., Qiu Y., Zhao B., Nie H., Su S., 2020, *Changes in Antioxidant Enzymes Activity and Metabolomic Profiles in the Guts of Honey Bee (Apis mellifera) Larvae Infected with Ascosphaera apis*, „Insects 2020, Vol. 11, Page 419” 11, nr 7: 419, https://www.mdpi.com/2075-4450/11/7/419/htm (dostęp: 18 czerwca 2023).

Lima P. R. M. De, Brochetto-Braga M. R., Chaud-Netto J., 2000, *Proteolytic activity of africanized honeybee (Apis mellifera: hymenoptera, apidae) venom*, „Journal of Venomous Animals and Toxins” 6, nr 1: 64-76, https://www.scielo.br/j/jvat/a/QRdLwS7DjqdDSTN8tHrpzDP/?format=html (dostęp: 18 czerwca 2023).

Lin J., In Soo Lee, Frey J., Slonczewski J. L., Foster J. W., 1995, *Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia*

*coli*, „Journal of Bacteriology" 177, nr 14: 4097-4104, /pmc/articles/PMC177142/?report=abstract (dostęp: 2 lipca 2021).

Łoś A., Strachecka A., 2018, *Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution*, „Sensors (Switzerland)", 18(5), 1494.

Ma L., Wang Y., Zhang W., Wang H., Liu Z., Xu B., 2016, *Alterations in protein and amino acid metabolism in honeybees (Apis mellifera) fed different l-leucine diets during the larval stage*, „Journal of Asia-Pacific Entomology" 19, nr 3: 769-774.

Majewski M., Jurgoński A., 2021, *The Effect of Hemp (Cannabis sativa L.) Seeds and Hemp Seed Oil on Vascular Dysfunction in Obese Male Zucker Rats*, „Nutrients 2021, Vol. 13, Page 2575" 13, nr 8: 2575, https://www.mdpi.com/2072-6643/13/8/2575/htm (dostęp: 9 listopada 2022).

Malone L. A., Gatehouse H. S., 1998, *Effects ofNosema apisInfection on Honey Bee (Apis mellifera) Digestive Proteolytic Enzyme Activity*, „Journal of Invertebrate Pathology" 71, nr 2: 169-174.

Manning R., Rutkay A., Eaton L., Dell B., 2007, *Lipid-enhanced pollen and lipid-reduced flour diets and their effect on the longevity of honey bees (Apis mellifera L.)*, „Australian Journal of Entomology" 46, nr 3: 251-257, https://onlinelibrary.wiley.com/doi/full/10.1111/j.1440-6055.2007.00598.x (dostęp: 23 stycznia 2023).

Marini E., Magi G., Ferretti G., Bacchetti T., Giuliani A., Pugnaloni A., Facinelli B., 2018, *Attenuation of Listeria monocytogenes virulence by Cannabis sativa L. Essential oil*, „Frontiers in Cellular and Infection Microbiology" 8, nr AUG, https://pubmed.ncbi.nlm.nih.gov/30186775/ (dostęp: 2 lipca 2021).

Mattes R. G., Espinosa M. L., Oh S. S., Anatrella E. M., Urteaga E. M., 2021, *Cannabidiol (CBD) Use in Type 2 Diabetes: A Case Report*, „Diabetes Spectrum" 34, nr 2: 198-201, https://dx.doi.org/10.2337/ds20-0023 (dostęp: 19 czerwca 2023).

Mayack C., Naug D., 2009, *Energetic stress in the honeybee Apis mellifera from Nosema ceranae infection*, „Journal of Invertebrate Pathology" 100, nr 3: 185-188.

Migdał P., Murawska A., Strachecka A., Bieńkowski P., Roman A., 2021, *Honey bee*

*proteolytic system and behavior parameters under the influence of an electric field at 50 hz and variable intensities for a long exposure time*, „Animals" 11, nr 3: 1-12, https://doi.org/10.3390/ani11030863 (dostęp: 2 lipca 2021).

Mizrahi A., Lensky Y., 2013, *Bee Products, Bee Products*, .

O'Connor B., Bojinski S., Röösli C., Schaepman M. E., 2020, *Monitoring global changes in biodiversity and climate essential as ecological crisis intensifies*, „Ecological Informatics" 55: 101033.

Obonga W. O., Nnadi C. O., Okafor S. N., Omeje E. O., Igbinnaduwa P., Okoli C. O., Osadebe P. O., 2019, *Anti-inflammatory activity and accelerated stability studies of crude extract syrup of* Cannabis sativa, „Tropical Journal of Pharmaceutical Research" 18, nr 2: 295-301, https://www.ajol.info/index.php/tjpr/article/view/184521 (dostęp: 15 czerwca 2023).

Orčić S., Nikolić T., Purać J., Šikoparija B., Blagojević D. P., Vukašinović E., Kojić D., 2017, *Seasonal variation in the activity of selected antioxidant enzymes and malondialdehyde level in worker honey bees*, „Entomologia Experimentalis et Applicata" 165, nr 2-3: 120-128, https://onlinelibrary.wiley.com/doi/full/10.1111/eea.12633 (dostęp: 18 czerwca 2023).

Paleolog J., Wilde J., Siuda M., Bąk B., Wójcik Ł., Strachecka A., 2020, *Imidacloprid markedly affects hemolymph proteolysis, biomarkers, DNA global methylation, and the cuticle proteolytic layer in western honeybees*, „Apidologie" 51, nr 4: 620-630, https://link.springer.com/article/10.1007/s13592-020-00747-4 (dostęp: 18 czerwca 2023).

Park Y., Kumar S., Kanumuri R., Stanley D., Kim Y., 2015, *A novel calcium-independent cellular PLA2 acts in insect immunity and larval growth*, „Insect Biochemistry and Molecular Biology" 66: 13-23, https://pubmed.ncbi.nlm.nih.gov/26429672/ (dostęp: 2 lipca 2021).

Peghaire E., Moné A., Delbac F., Debroas D., Chaucheyras-Durand F., El Alaoui H., 2020, *A Pediococcus strain to rescue honeybees by decreasing Nosema ceranae- and pesticide-induced adverse effects*, „Pesticide Biochemistry and Physiology" 163: 138-146.

Podczasy J. J., Wei R., 1988, *Reduction of iodonitrotetrazolium violet by superoxide*

*radicals*, „Biochemical and Biophysical Research Communications” 150, nr 3: 1294-1301.

Porter B., St. Marie B., Milavetz G., Herr K., 2021, *Cannabidiol (CBD) use by older adults for acute and chronic pain*, „Journal of Gerontological Nursing” 47, nr 7: 6-15.

Pushpam P. L., Rajesh T., Gunasekaran P., 2011, *Identification and characterization of alkaline serine protease from goat skin surface metagenome*, „AMB Express” 1, nr 1: 1-10, https://amb-express.springeropen.com/articles/10.1186/2191-0855-1-3 (dostęp: 18 czerwca 2023).

Puzanowska-Tarasiewicz H., Kuźmicka L., Tarasiewicz M., 2010, *Antyoksydanty A Reaktywne Formy Tlenu,* "Bromat. Chem. Toksykol" 43.2010.

Rascón B., Hubbard B. P., Sinclair D. A., Amdam G. V., 2012, *The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction*, „Aging” 4, nr 7: 499-508, /pmc/articles/PMC3433935/ (dostęp: 4 lipca 2021).

Reithmeier D., Tang-Wai R., Seifert B., Lyon A. W., Alcorn J., Acton B., Huntsman R. J., 2018, *The protocol for the Cannabidiol in children with refractory epileptic encephalopathy (CARE-E) study: A phase 1 dosage escalation study*, „BMC Pediatrics” 18, nr 1, https://pubmed.ncbi.nlm.nih.gov/29981580/ (dostęp: 2 lipca 2021).

Ricigliano V. A., Simone-Finstrom M., 2020, *Nutritional and prebiotic efficacy of the microalga Arthrospira platensis (spirulina) in honey bees*, „Apidologie” 51, nr 5: 898-910, https://doi.org/10.1007/s13592-020-00770-5 (dostęp: 4 lipca 2021).

Sadekuzzaman M., Gautam N., Kim Y., 2017, *A novel calcium-independent phospholipase A2 and its physiological roles in development and immunity of a lepidopteran insect, Spodoptera exigua*, „Developmental & Comparative Immunology” 77: 210-220.

Sadekuzzaman M., Kim Y., 2017, *Specific inhibition of Xenorhabdus hominickii, an entomopathogenic bacterium, against different types of host insect phospholipase A2,* „Journal of Invertebrate Pathology” 149: 97-105, http://dx.doi.org/10.1016/j.jip.2017.08.009.

Safian Boldaji M., Jalali Dehkordi K., Taghian F., Author C., 2022, *Effect of eight weeks of*

*swimming training and CBD oil consumption on PI3K and ERK gene expression in the heart tissue of rats with myocardial infarction*, „Journal of Shahrekord University of Medical Sciences” 24, nr 2: 54-59, http://j.skums.ac.ir/Article/jskums-582 (dostęp: 9 listopada 2022).

Samarut É., Nixon J., Kundap U. P., Drapeau P., Ellis L. D., 2019, *Single and synergistic effects of cannabidiol and δ-9-tetrahydrocannabinol on zebrafish models of neuro-hyperactivity*, „Frontiers in Pharmacology” 10, nr MAR: 226, www.frontiersin.org (dostęp: 2 lipca 2021).

Şapcaliu A., Pavel C., Savu V., Cãuia E., 2010, *Biochemical and cytological investigations on haemolymph of Apis mellifera carpathica Bee in stressful conditions*, „Bull UASVM Anim Sci Biotechnol" , 67, 313-320, https://core.ac.uk/download/pdf/276533754.pdf (dostęp: 18 czerwca 2023).

Schacterle G., Gp S., Rl P., 1973, *A Simplified Method For The Quantitative Assay Of Small Amounts Of Proteins In Biologic Material*, „Analyt Biochem", 51, 654-655A.

Schulz M., Łoś A., Grzybek M., Ścibior R., Strachecka A., 2019, *Piperine as a new natural supplement with beneficial effects on the life-span and defence system of honeybees*, „Journal of Agricultural Science” 157, nr 2: 140-149, https://www.cambridge.org/core/journals/journal-of-agricultural-science/article/abs/piperine-as-a-new-natural-supplement-with-beneficial-effects-on-the-lifespan-and-defence-system-of-honeybees/2AECEA7288D95E7A281D0F8D6233BEC8 (dostęp: 2 lipca 2021).

Siuda M., Wilde J., Woyke J., Jasiński Z., Madras-Majewska B., 2014, *Wintering reserve queens in mini-plus and 3-comb nuclei*, „Journal of Apicultural Science” 58, nr 1: 61-68.

Skowronek P., Wójcik Ł., Strachecka A., 2021a, *Fat Body—Multifunctional Insect Tissue*, „Insects 2021, 12, 547,12, https://www.mdpi.com/2075-4450/12/6/547/htm (dostęp: 30 października 2022).

Skowronek P., Wójcik Ł., Strachecka A., 2021b, *Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (Apis mellifera) Workers*, „Animals", 11, 2190, 11, https://www.mdpi.com/2076-2615/11/8/2190/htm (dostęp: 30 stycznia 2022).

Skowronek P., Wójcik Ł., Strachecka A., 2022a, *Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (Apis mellifera) Organism*, "Antioxidants", 11(4), 707, https://doi.org/10.3390/antiox11040707.

Skowronek P., Wójcik Ł., Strachecka A., 2022b, *CBD Supplementation Has a Positive Effect on the Activity of the Proteolytic System and Biochemical Markers of Honey Bees (Apis mellifera) in the Apiary*, „Animals" 18: 2313, https://www.mdpi.com/2076-2615/12/18/2313/htm (dostęp: 9 listopada 2022).

Skowronek P., Wójcik Ł., Strachecka A., Brivio F., 2021, *Fat Body-Multifunctional Insect Tissue*, "Insects", 12(6), 547, https://doi.org/10.3390/insects12060547.

Słowińska M., Nynca J., Wilde J., Bąk B., Siuda M., Ciereszko A., 2016, *Total antioxidant capacity of honeybee haemolymph in relation to age and exposure to pesticide, and comparison to antioxidant capacity of seminal plasma*, „Apidologie" 47, nr 2: 227-236, https://link.springer.com/article/10.1007/s13592-015-0391-9 (dostęp: 19 czerwca 2023).

Sokol R., 1996, *Wybrane wskazniki biochemiczne hemolimfy w przebiegu inwazji Varroa jacobsoni u pszczol. II. Aktywnosc transaminazy asparaginianowej i alaninowej w hemolimfie czerwia, pszczol i trutni*, „Acta Academiae Agriculturae ac Technicae Olstenensis. Veterinaria" 24: 113-125.

Solomon E. P., Berg L., Martin D., 2020, *Biologia*.

Strachecka A. J., Paleolog J., Borsuk G., Olszewski K., 2015, *The influence of formic acid on the body surface proteolytic system at different developmental stages in Apis mellifera L. workers*, "Journal of Apicultural Research", 51(3), 252-262 „http://dx.doi.org/10.3896/IBRA.1.51.3.06" 51, nr 3: 252-262, https://www.tandfonline.com/doi/abs/10.3896/IBRA.1.51.3.06 (dostęp: 18 czerwca 2023).

Strachecka A., Krauze M., Olszewski K., Borsuk G., Paleolog J., Merska M., Grzywnowicz K., 2014, *Unexpectedly strong effect of caffeine on the vitality of western honeybees (Apis mellifera)*, „Biochemistry (Moscow)" 79, nr 11: 1192-1201, https://pubmed.ncbi.nlm.nih.gov/25540004/ (dostęp: 2 lipca 2021).

Strachecka A., Demetraki-Paleolog J., 2011, *System proteolityczny powierzchni ciała apis mellifera w zachowaniu zdrowotności rodzin pszczelich*, „Kosmos" 60, nr 1-2: 43-51.

Strachecka A. G. M. M., 2010, *Influence of Environmental Pollution on the Protective Proteolytic Barrier of the Honey Bee Apis mellifera mellifera*, „Polish Journal of Environmental Studies" 19, nr 4: 855-859, http://www.pjoes.com/Influence-of-Environmental-Pollution-on-the-Protective-Proteolytic-Barrier-of-the,88457,0,2.html (dostęp: 4 lipca 2021).

Strachecka A., Kuszewska K., Olszewski K., Skowronek P., Grzybek M., Grabowski M., Woyciechowski M., 1121, *Activities of Antioxidant and Proteolytic Systems and Biomarkers in the Fat Body and Hemolymph of Young Apis mellifera Females*, „Animals 2022" 12: 1121.

Strachecka A., Łoś A., Filipczuk J., Schulz M., 2018, *Individual and social immune mechanisms of the honey bee*, „Medycyna Weterynaryjna".

Strachecka A., Olszewski K., Paleolog J., 2015, *Curcumin stimulates biochemical mechanisms of Apis mellifera resistance and extends the apian life-span*, „Journal of Apicultural Science" 59, nr 1: 129-141.

Strachecka A., Olszewski K., Paleolog J., 2016, *Varroa treatment with bromfenvinphos markedly suppresses honeybee biochemical defence levels*, „Entomologia Experimentalis et Applicata" 160, nr 1: 57-71, https://onlinelibrary.wiley.com/doi/full/10.1111/eea.12451 (dostęp: 4 lipca 2021).

Strachecka A., Olszewski K., Paleolog J., Borsuk G., Bajda M., Krauze M., Chobotow J., 2014, *Coenzyme Q10 treatments influence the lifespan and key biochemical resistance systems in the honeybee, apis mellifera*, „Archives of Insect Biochemistry and Physiology" 86, nr 3: 165-179, https://pubmed.ncbi.nlm.nih.gov/24659567/ (dostęp: 2 lipca 2021).

Stryer L., 2003, *Biochemia*, PWN.

Toprak U., Hegedus D., Doğan C., Güney G., 2020, *A journey into the world of insect lipid metabolism*, „Archives of Insect Biochemistry and Physiology" 104, nr 2: 1-67.

Tunaz H., Park Y., Büyükgüzel K., Bedick J. G., Aliza A. R. N., Stanley D. W., 2003, *Eicosanoids in insect immunity: Bacterial infection stimulates hemocytic phospholipase A2 activity in tobacco hornworms*, „Archives of Insect Biochemistry and Physiology" 52, nr 1: 1-6, https://onlinelibrary.wiley.com/doi/full/10.1002/arch.10056 (dostęp: 30 października

2022).

Vatanparast M., Ahmed S., Herrero S., Kim Y., 2018, *A non-venomous sPLA2 of a lepidopteran insect: Its physiological functions in development and immunity*, „Developmental and Comparative Immunology" 89: 83-92, https://europepmc.org/article/med/30107251 (dostęp: 2 lipca 2021).

Vlahović M., Lazarević J., Perić-Mataruga V., Ilijin L., Mrdaković M., 2009, *Plastic responses of larval mass and alkaline phosphatase to cadmium in the gypsy moth larvae*, „Ecotoxicology and Environmental Safety" 72, nr 4: 1148-1155.

Warholm M., Guthenberg C., von Bahr C., Mannervik B., 1985, *[62] Glutathione transferases from human liver*, „Methods in Enzymology" 113, nr C: 499-504.

Waterborg J. H., Matthews H. R., 2003, *The Lowry Method for Protein Quantitation*, *Proteins*, The protein protocols handbook, 7-10, https://pubmed.ncbi.nlm.nih.gov/20512668/ (dostęp: 2 lipca 2021).

Watkins A. R., 2019, *Cannabinoid interactions with ion channels and receptors*, "Channels" 13.1: 162-167.https://doi.org/10.1080/19336950.2019.1615824.

Weirich G. F., Collins A. M., Williams V. P., 2002, *Antioxidant enzymes in the honey bee, Apis mellifera*, „Apidologie" 33, nr 1: 3-14, http://dx.doi.org/10.1051/apido:2001001 (dostęp: 18 czerwca 2023).

Xia X., You M., Rao X. J., Yu X. Q., 2018, *Insect C-type lectins in innate immunity*, „Developmental and Comparative Immunology" 83: 70-79, https://doi.org/10.1016/j.dci.2017.11.020.

Yokota T., Saito Y., Shimizu Y., 1995, *Increased corticomotoneuronal excitability after peripheral nerve stimulation in dopa-nonresponsive hemiparkinsonism*, „Journal of the Neurological Sciences" 129, nr 1: 34-39.

Zhu, F., Cui, Y., Lavine, L. C., Walsh, D. B. , Chandrasekar, R., Tyagi, B. K., & Reeck G. R., 2014, *Short Views on Insect Biochemistry and Molecular Biology*, .

*The Tears of Re: Beekeeping in Ancient Egypt - Gene Kritsky - Google Książki*, https://books.google.pl/books?hl=pl&lr=&id=BfaACgAAQBAJ&oi=fnd&pg=PP1&dq=bee+history+egypt&ots=sejPT_BGu4&sig=pRa9D9RgmJ9nIMidfJX7Q0fgg5w&redir_esc=y#v=onepage&q=bee history egypt&f=false (dostęp: 15 czerwca 2023).

Gliński, Z., Jarosz J., 1995, *Immunobiologia pszczoły miodnej,* https://vetbooks.pl/p/7/421/immunobiologia-pszczoly-miodnej-pszczelarstwo-hodowla-zwierzat.html (dostęp: 2 lipca 2021).

*Insect Physiology and Biochemistry - James L. Nation, Sr.*, https://books.google.pl/books?hl=pl&lr=&id=IS5sEAAAQBAJ&oi=fnd&pg=PP1&dq=Nation,+J.+Insect+Physiology+and+Biochemistry%3B+CRC+Press:+London,+UK,+2008&ots=w06FSFSL7p&sig=3WE3v9C5u0yczdHBxjwMbrM3w_I&redir_esc=y#v=onepage&q=Nation%2C J. Insect Physiology and Biochemistry%3B CRC Press%3A London%2C UK%2C 2008&f=false (dostęp: 19 czerwca 2023).

Lublin, 19.06.2023r.

mgr inż. Patrycja Skowronek
ul. Porzeczkowa 10
608315787
patrycja.skowronek
@up.lublin.pl

**Rada Dyscypliny**
**Zootechnika i Rybactwo**
**Uniwersytetu Przyrodniczego**
**w Lublinie**

## Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracach:

(1) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2021, Cannabis extract has a positive-immunostimulating effect through proteolytic system and metabolic compounds of honey bee (Apis mellifera) workers, Animals, Tom 11 Numer 6 s. 2190.

(2) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, Impressive impact of hemp extract on antioxidant system in honey bee (Apis mellifera) organism, Antioxidants, Tom 11 Numer 4 s. 707.

(3) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, CBD supplementation has a positive effect on the activity of the proteolytic system and biochemical markers of honey bees (Apis mellifera) in the apiary, Animals, Tom 12 Numer 18 s. 2313.

(4) Patrycja Skowronek, Aneta Strachecka, 2023, Cannabidiol (CBD) supports the honeybee worker organism by activating the antioxidant system, Antioxidants, Tom 12 Nr 2 s. 279.

Mój udział polegał na: pomysłodawca badań, inicjatywa badań, twórca hipotezy badawczej, opracowanie metodyki; realizacja doświadczenia (założenie doświadczenia, kontrola poszczególnych etapów doświadczenia, pobranie materiału, wykonanie analiz laboratoryjnych); analiza i opracowanie wyników, sformułowanie wniosków; napisanie pierwszej wersji manuskryptu; redakcja publikacji, korespondencja z redakcją.
Wkład w każdą z prac wynosi: 85%.

Podpis

Lublin, 19.06.2023r.

dr Łukasz Wójcik
ul. Wrońska 1B/58
20-327, Lublin
512315288
lukasz7wojcik@gmail.com

**Rada Dyscypliny**
**Zootechnika i Rybactwo**
**Uniwersytetu Przyrodniczego**
**w Lublinie**

## Oświadczenie o współautorstwie

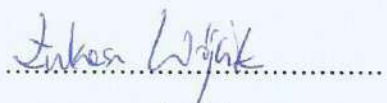
Niniejszym oświadczam, że w pracach:

(1) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2021, Cannabis extract has a positive-immunostimulating effect through proteolytic system and metabolic compounds of honey bee (Apis mellifera) workers, Animals, Tom 11 Numer 6 s. 2190.

(2) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, Impressive impact of hemp extract on antioxidant system in honey bee (Apis mellifera) organism, Antioxidants, Tom 11 Numer 4 s. 707.

(3) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, CBD supplementation has a positive effect on the activity of the proteolytic system and biochemical markers of honey bees (Apis mellifera) in the apiary, Animals, Tom 12 Numer 18 s. 2313.

Mój udział polegał na pomocy w uzyskaniu robotnic 1-dniowych do doświadczeń oraz pomocy technicznej podczas pobierania hemolimfy.
Wkład w każdą z prac (1)-(3): 5%



Podpis

Lublin, 19.06.2023r.

prof. dr hab. Aneta Strachecka
ul. Prawiedniki 158A
20-515, Lublin
505991615
aneta.strachecka@up.lublin.pl

**Rada Dyscypliny**
**Zootechnika i Rybactwo**
**Uniwersytetu Przyrodniczego**
**w Lublinie**

## Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracach:

(1) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2021, Cannabis extract has a positive-immunostimulating effect through proteolytic system and metabolic compounds of honey bee (Apis mellifera) workers, Animals, Tom 11 Numer 6 s. 2190.

(2) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, Impressive impact of hemp extract on antioxidant system in honey bee (Apis mellifera) organism, Antioxidants, Tom 11 Numer 4 s. 707.

(3) Patrycja Skowronek, Łukasz Wójcik, Aneta Strachecka, 2022, CBD supplementation has a positive effect on the activity of the proteolytic system and biochemical markers of honey bees (Apis mellifera) in the apiary, Animals, Tom 12 Numer 18 s. 2313.

(4) Patrycja Skowronek, Aneta Strachecka, 2023, Cannabidiol (CBD) supports the honeybee worker organism by activating the antioxidant system, Antioxidants, Tom 12 Nr 2 s. 279.

Mój udział polegał na pomocy w opracowaniu wstępnych hipotez badawczych, pomocy naukowej i merytorycznej podczas prowadzenia doświadczenia, opracowanie metodyki, wkładzie intelektualnym w końcową analizę wyników i współredagowaniu manuskryptu. Wkład w artykuły (1)-(3) wynosi: 10%, w artykuł (4) 15%.

Strachecka Aneta

.....

Podpis

*animals*

MDPI

*Article*

# Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (*Apis mellifera*) Workers

**Patrycja Skowronek *, Łukasz Wójcik and Aneta Strachecka**

Department of Zoology and Animal Ecology, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; l.wojcik@up.lublin.pl (Ł.W.); aneta.strachecka@up.lublin.pl (A.S.)
* Correspondence: patrycja.skowronek@up.lublin.pl

**Simple Summary:** The aim of our study was to test the immunostimulating effect of a diet with hemp extract on the resistance of the honey bee (*Apis mellifera*). The experiment compared the effect of supplementation between the bees receiving the extract in the form of a mixture with sugar syrup and on the strip with the extract, compared to the bees that had no contact with substance. In order to determine this effect, the biochemical indicators were analyzed: the proteolytic system (proteases, protease inhibitors, total protein concentration) responsible for the fight against pathogens/parasites, biomarkers (ALT, AST, ALP), and the basic components of metabolism (glucose and urea concentrations). Parameters were determined in the hemolymph of 2- and 7-day-old workers. Hemp extracts caused an increase in the protein concentrations. Regardless of the method of administration, proteases decreased. Protease inhibitors increased, except supplementation on strips where the activity decreased. The biomarker activities increased in the control group and workers feeding extract in syrup and decreased in workers supplemented with the extract on strips. The results of the metabolic component were as follows: glucose and urea concentrations indicate that the extract will not adversely affect metabolic changes in the insect's organism. Hemp extract improves the natural immunity of bees.

**Abstract:** In the study, we assessed the effect of hemp extract on activities of resistance parameters and the metabolic compound concentration in adult workers' hemolymph. Bees were divided into the following groups: (1) control group fed with mixture of sugar and water-glycerine solution, (2) experimental group with pure sugar syrup and inside with cotton strips soaked with hemp extract, (3) experimental group with a mixture of sugar syrup with hemp extract. Hemp extracts caused an increase in the protein concentrations and reduced the protease activities regardless of the administration method. The protease inhibitor activities were decreased only in the group that received hemp extract on the strips. The biomarker activities (ALP, ALT, AST) increased from the control group and workers feeding extract in syrup and decreased in workers supplemented with the extract on strips. In young, 2-day-old workers, the glucose concentration was higher in the groups feeding with the extract than in the control. Hemp extract influenced an increase in urea concentrations in workers' hemolymph in comparison with the control. The hemp supplementation positively influences the immune system of workers, and the appropriate method of administration may be adapted to the health problems of bees.

**Keywords:** hemolymph; bees' resistance; immunity of insect; hemp extract; supplementation of bees; glucose; urea; proteolytic enzymes; biochemical pathway; pollinators; biochemistry



**Citation:** Skowronek, P.; Wójcik, Ł.; Strachecka, A. Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (*Apis mellifera*) Workers. *Animals* **2021**, *11*, 2190. https://doi.org/10.3390/ani11082190

Academic Editor: Alessia Giannetto

Received: 6 June 2021
Accepted: 20 July 2021
Published: 23 July 2021

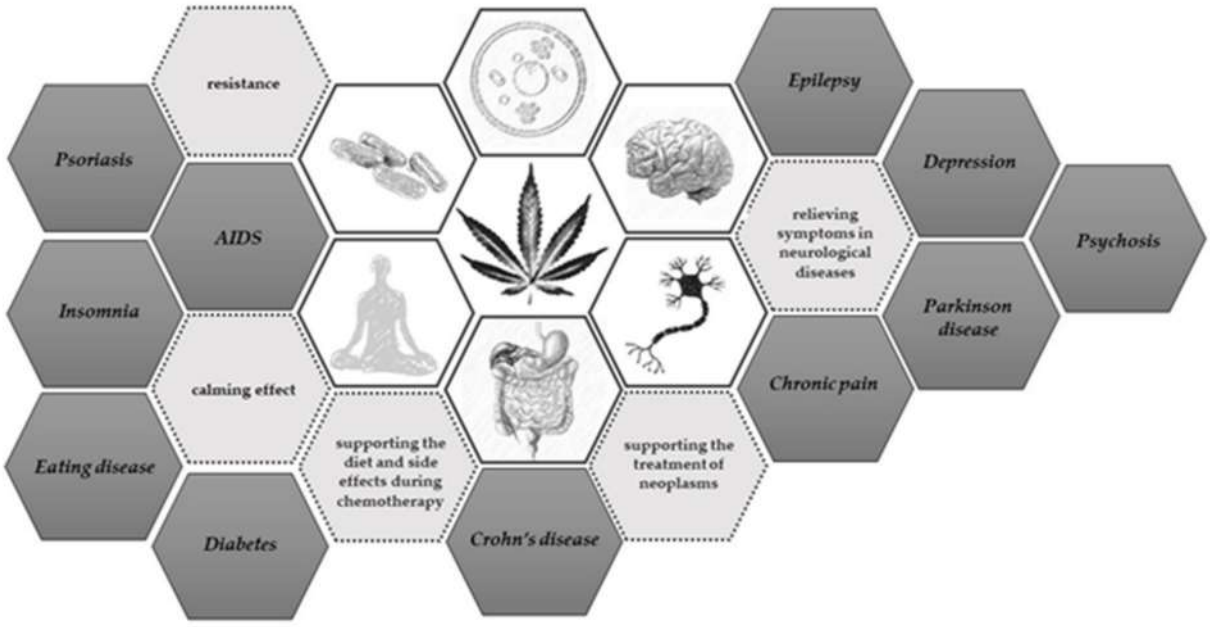


## 1. Introduction

Entomofauna is one of the most represented groups of living organisms on Earth, and the same organisms are the most endangered. Some insect species have been in close

relationships with humans for many centuries. One of these species is the honey bee (*Apis mellifera*) [1]. It is estimated that insects contribute to the pollination of as much as 80% of plants that are food sources. Nowadays, this ecological service plays a key role in providing enough food for the constantly growing human population [2]. For over a dozen years, significant declines in the bee population have been recorded throughout the world. In order to minimize these effects, beekeepers decide to use medicinal or preventive substances to stimulate the bees' organism against possible dangers. Therefore, the search for supplements/biostimulants/compounds that will strengthen bees' immunity began. Many substances and compounds with a unique composition have been tested, such as resveratrol, spirulina, vitamin C, caffeine, propolis, and dietary additives such as yeast, soybean meal, and pollen substitutes [3–9]. Compounds of plant origin (resveratrol, spirulina, caffeine) and the indicated dietary additives showed a positive effect on the organism of bees, mainly by extending lifespan or in the case of studies conducted by Strachecka [6], additionally stimulation of the activity of the proteolytic system and/or stimulation of the activity of antioxidant enzymes which counteract the aging of the organism was noted [3,4]. In the case of bees fed with spirulina, there was an increase in physical values, i.e., weight or an increase in abdominal lipids and proteins e.g., in the head part [4]. Supplementation with vitamin C increased the activity of the antioxidant system, reducing losses after winter by up to 33% compared to control groups [5]. Other dietary supplements usually led to an increase in protein concentration or extending the lifespan [7,8]. One of such substances may be hemp extract, which, thanks to the content of active substances from the cannabinoid group, is widely used in medicine as an agent that affects the nervous system (epilepsy, multiple sclerosis) and has a high antioxidant potential (supporting the regeneration of the body after exposure to degrading factors) [10–12]. Hemp (*Cannabis sativa*) is the most known and widespread species of hemp cultivated for various purposes and used in many industries and environmental protection. The main active compounds include: cannabidiol, cannabichromene, cannabigerol, Δ9-tetrahydrocannabinol, and cannabinol [12,13].

The extract is mainly used in cases of multiple sclerosis; drug-resistant epilepsy; pain during cancer; phantom, post-traumatic, and chronic pains; and side effects of treatment with chemotherapy/radiotherapy and Alzheimer's diseases (Scheme 1) [10,11,14].




**Scheme 1.** Use of cannabis extract. Hemp in various forms has been tested in the treatment of many neurological, psychological, cancer, viral, and metabolic diseases [11,14–17].

Its beneficial properties have been tested on other insects, e.g., *Galleria mellonella* [18]. Due to the above-mentioned properties, the extract may prove to be a good solution in solving problems with individual factors of the phenomenon of colony collapse disease—CCD. Therefore, the aim of our research was to determine the influence of hemp extract on the activities of the proteolytic system and enzymatic biomarkers as an element of bee resistance and also their vitality [19–21]. The proteolytic system composed of proteases and their inhibitors in the bee's organism is responsible for inactivating pathogens by destroying their proteins (proteases) or by inhibiting the activity of their proteolytic enzymes (protease inhibitors) acting on immune proteins in insects. Their activity supports bees in their fight against factors that have a negative impact on their health [22,23]. The biomarkers selected for this experiment are intended to help characterize the supplement's effect by providing information on the level of damage and the severity of apoptosis in key immune tissue cells such as the fat body and hemolymph. The severity of damage is especially visible in older bees, whose tissues are subject to degradation and general inflammatory changes [24,25]. Damage causes the activation and release of specific enzymes to the external bee's body environment, which is reflected in changes in the biochemical levels of the hemolymph [25,26]. The tested glucose and urea concentrations provide with information on the effect of the supplement on the metabolic changes of sugars (glucose concentration) and proteins (urea concentration) [13]. Additionally, the evolution of beekeeping presents scientists with new challenges. The expanding offer of medicinal substances and methods of their administration forces scientists to carry out more extensive analyses. Therefore, in the experiment we used two methods of administering the extract; the first method consisted of fabric strips, often used in the treatment of varroosis disease, and the other one using a 1:1 sugar syrup mixture with the test substance (hemp extract).

## 2. Materials and Methods

### *2.1. Cages Part*

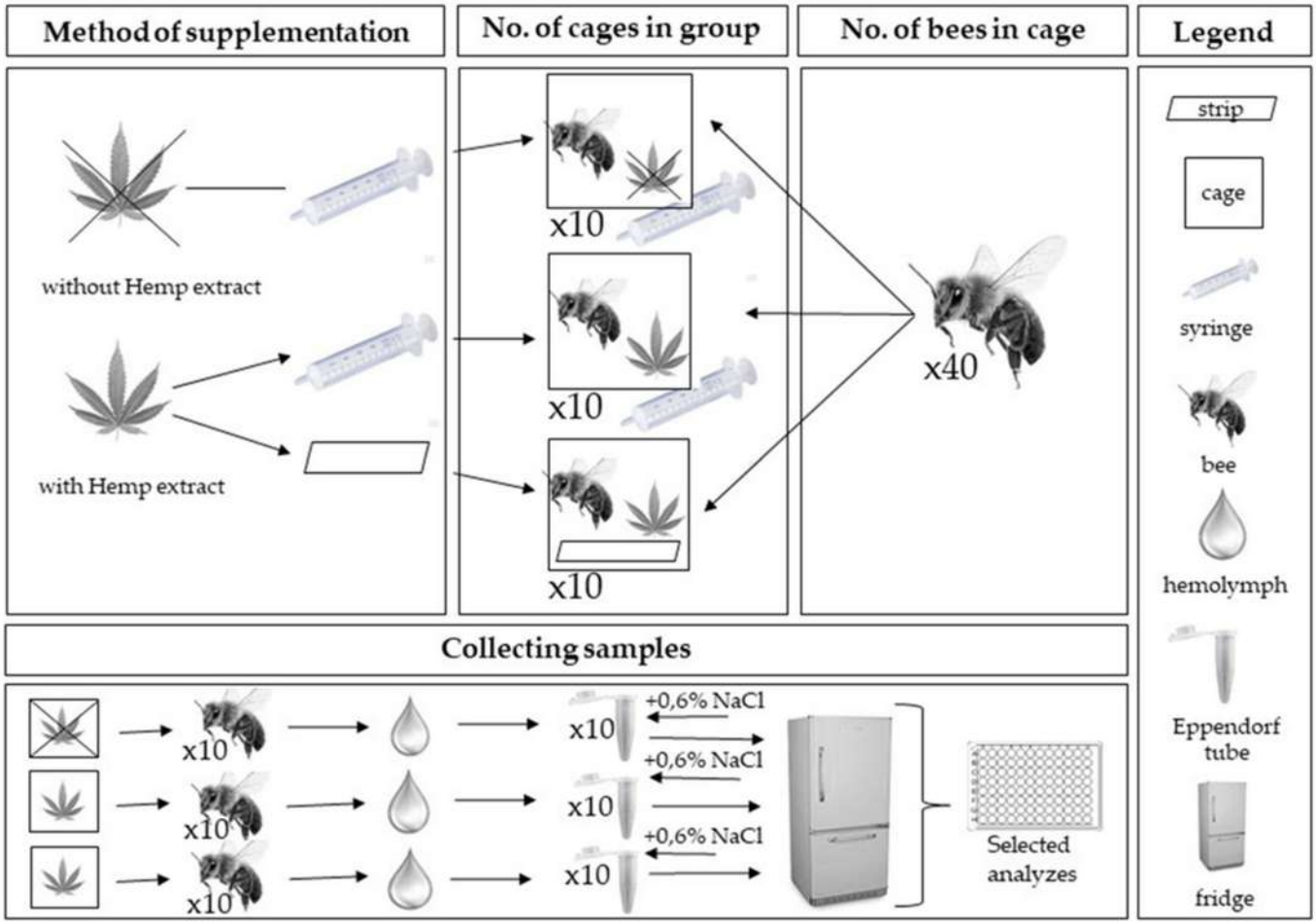
The experiment was carried out with 1-day-old worker bees (*A. mellifera carnica*; collected from an apiary belonging to the University of Life Sciences in Lublin–51°13′31″ N, 22°38′07″ E), which were prepared according to the method of Strachecka [27]. Bees were settled into 30 wooden cages (40 bees/cage) with a volume of 576 $cm^3$ each (12 × 12 × 4 $cm^3$, length/height/width), with a sliding front window, air vents located on the side, and a feeder (a syringe with a modified adaptor) on the top. Optimal conditions were guaranteed in an air-conditioned chamber-constant temperature at 35 °C and 65% relative humidity.

The cages were divided into the following groups:

(1) control group fed with mixture of sugar and water-glycerine solution in a 1:1 ratio ad libidum
(2) experimental group with 1:1 pure sugar syrup ad libidum and inside with cotton strips soaked with 3 mL hemp extract (0.25 g pure hemp paste extract +3 mL water-glycerine solution)
(3) experimental group with a mixture of sugar syrup 1:1 with hemp extract ad libidum (500 mL water-glycerine solution with 4.38 g hemp paste extract)

The water-glycerine solution used in this examination is from Chempur, no. of standards: BN-76/6193-12. The hemp extract was sourced from the manufacturer Melissa.

For each group, 10 randomly selected cages were allocated. In each of the three groups, workers from cages were used for biochemical analyses (Scheme 2). The procedures described below were started 2 days after start of worker feeding (1-day-old bees).




**Scheme 2.** Scheme of collecting samples in experiment.

### 2.2. Analytical Part

In each of the groups, fresh hemolymph was taken (puncturing the venous sinus in the insect abdomen into a glass capillary) twice from 10 living workers at age 2- and 7-day [25]. Hemolymph from each bee was separately placed in a sterile Eppendorf tube (hemolymph from one bee = one Eppendorf tube) containing 200 µL of ice-cooled 0.6% NaCl. The samples (3 groups × 2 sampling × 10 workers) were immediately refrigerated at −25 °C for further biochemical analyses. For more details, refer to the Strachecka method [6,28].

The following biochemical analyses were performed in the hemolymph from individual samples:

(1) Total protein concentration was determined using the Lowry method, as modified by Schacterle [29,30];
(2) Proteolytic system activity was determined as follows:
   - activities of acidic, neutral, and alkaline proteases according to the Anson method modified by Strachecka and in Łoś [23,25,31,32];
   - activities of natural inhibitors of acidic, neutral, and alkaline proteases according to the Lin method [33];
(3) Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) using monotests from Cormay (Lublin, Poland) according to the manufacturer's procedure (methodological details in: File S1);
(4) Concentration of urea and glucose using monotests from Cormay (Lublin, Poland) according to the manufacturer's procedure (methodological details in: File S1).

### 3. Statistical Analysis

The results were analyzed using Statistica formulas, version 13.3 (2017) for Windows, StatSoft Inc., Tusla, OK, USA. The mixed-model two-way ANOVA followed by post hoc Tukey HSD tests ($p = 0.05$) were used to compare the results for each basic immunity system parameter (total protein concentration, protease activities, inhibitor protease activities, biomarker activities, urea and glucose concentration) of honey bee workers depending on the method of administration (strip and syringe) and the day (2 day and 7 day) of supplementation with hemp extract.

### 4. Results

Protein concentrations increased with age of workers in all groups (Figure 1). Hemp extracts caused an increase in the protein concentrations in the hemolymph of workers. Higher values were always observed in the group that was administered it in the syrup.

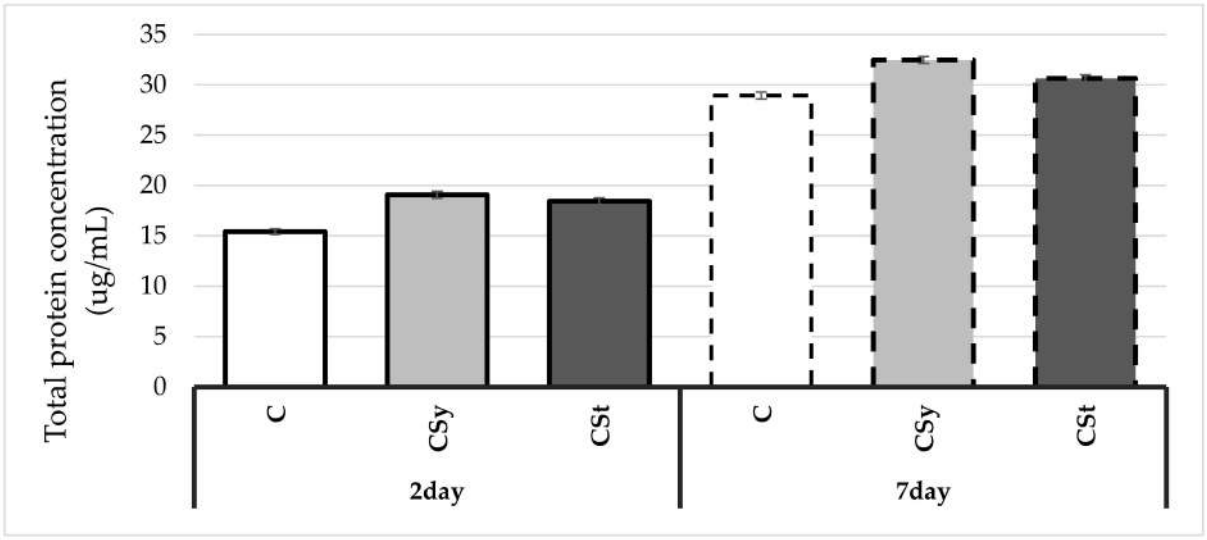



**Figure 1.** Protein concentrations in the 2- and 7-day-old workers' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips (Two-Way ANOVA: supplementation method: $F_{(2,59)} = 62.085$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 2266.3$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 2.2833$, $p = 0.11089$).

The protease activities as well as acidic and alkaline protease inhibitor activities increased with age of workers (Figures 2 and 3). The extract reduced the protease activities regardless of the administration method. The protease inhibitor activities were decreased only in the group that received hemp extract on the strips. A greater effect of the extract was observed after administration in syrup than in strips compared to the control.

The biomarker activities increased with age of workers in the control and in those supplemented with the extract in syrup and decreased in workers supplemented with the extract on strips (Figure 4). Activities of biomarkers were lower in the group with cannabis on strips than other groups.

The glucose concentrations increased with age of workers in the control group, in opposite to the two groups treated with the extract (Figure 5). In young, 2-day-old workers, the glucose concentration was higher in the groups supplemented with the extract than in the control. On the other hand, in 7-day-old workers, the opposite tendency was observed, and the extract decreased the glucose concentration.

Urea concentration increased with age of workers in all groups (Figure 6). Hemp extract influenced an increase in urea concentrations in workers' hemolymph in comparison with the control group. The highest values were observed in workers supplemented with hemp in syrup.

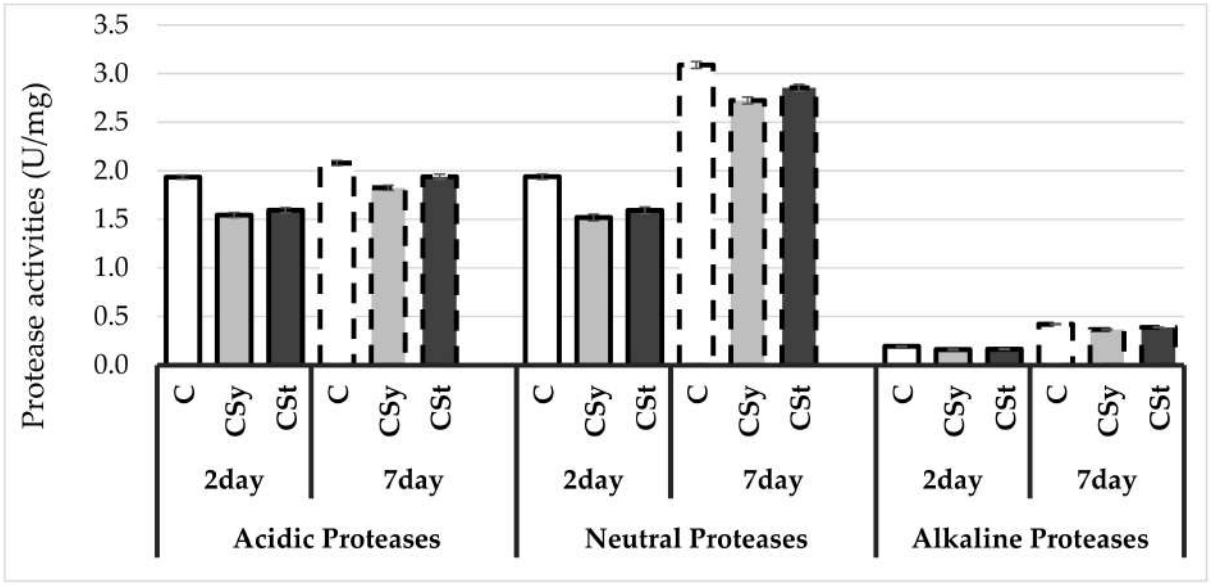



**Figure 2.** Protease activities in the 2- and 7-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips (Two-Way ANOVA: Acidic Proteases: supplementation method: $F_{(2,59)} = 87.226$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 145.78$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 7.8591$, $p = 0.00094$, Neutral Proteases: supplementation method: $F_{(2,59)} = 74.315$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 1853.4$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 1.3401$, $p = 0.26966$, Alkaline Proteases: supplementation method: $F_{(2,59)} = 59{,}780$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 4008.1$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 3.3549$, $p = 0.04169$).

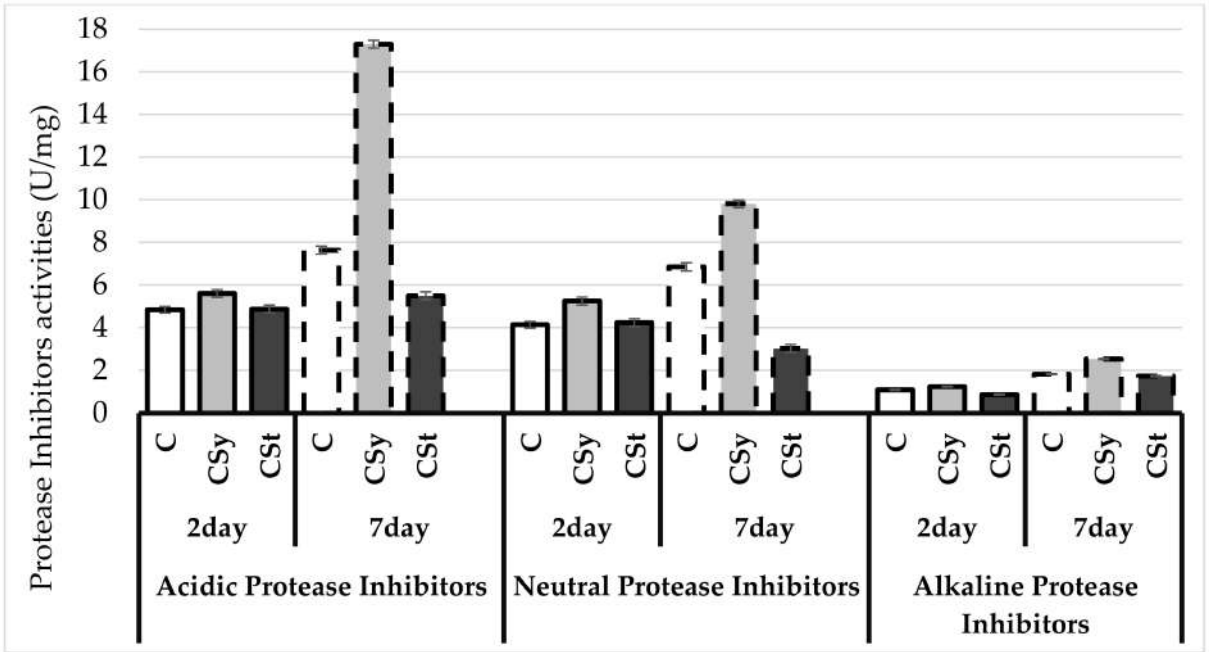



**Figure 3.** Protease inhibitors activities in the 2- and 7-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips. (Two-Way ANOVA: Acidic Protease Inhibitors: supplementation method: $F_{(2,59)} = 683.41$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 1203.9$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 521.54$, $p = 0.000$, Neutral Protease Inhibitors: supplementation method: $F_{(2,59)} = 215.50$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 184.30$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 124.00$, $p = 0.000$, Alkaline Protease Inhibitors: supplementation method: $F_{(2,59)} = 404.61$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 3251.9$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 98.237$, $p = 0.000$).

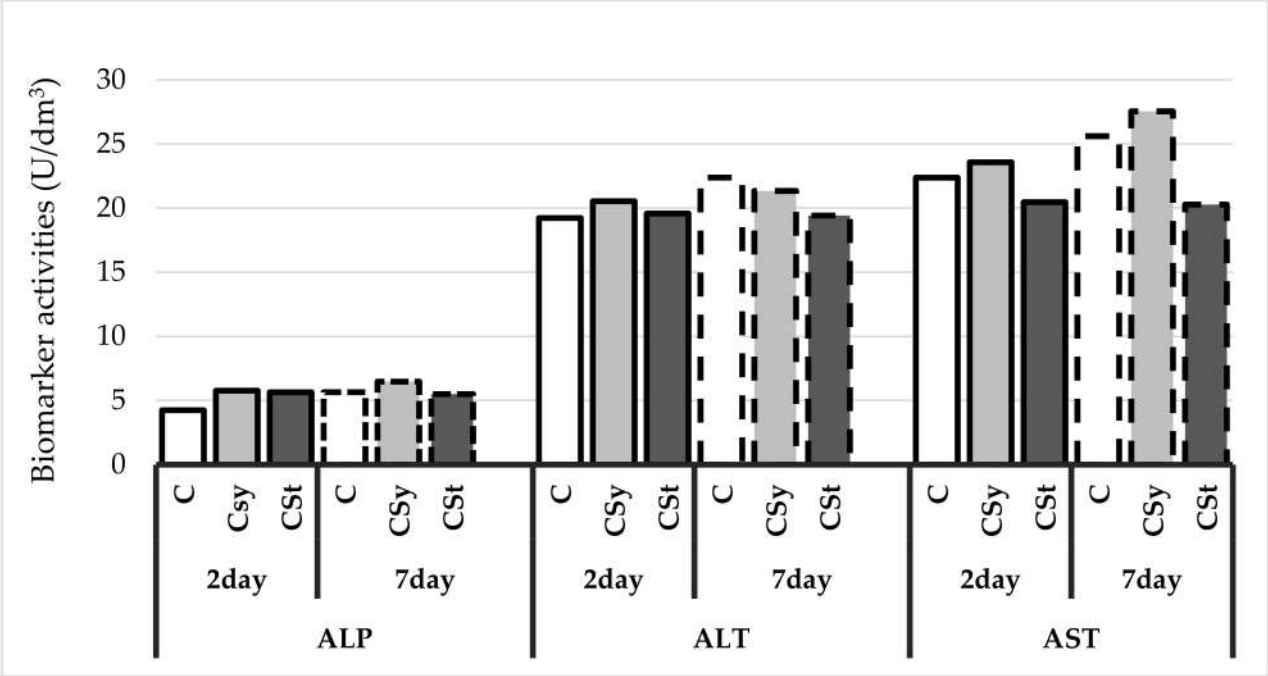



**Figure 4.** Biomarker activities in the 2- and 7-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips. (Two-Way ANOVA: ALT (alanine aminotransferase): supplementation method: $F_{(2,59)} = 163.68$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 321.14$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 205.18$, $p = 0{,}11089$, AST (aspartate aminotransferase): supplementation method: $F_{(2,59)} = 1180.7$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 719.49$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 206.94$, $p = 0.000$, ALP (alkaline phosphatase): supplementation method: $F_{(2,59)} = 204.58$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 185.72$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 87.550$, $p = 0.000$).

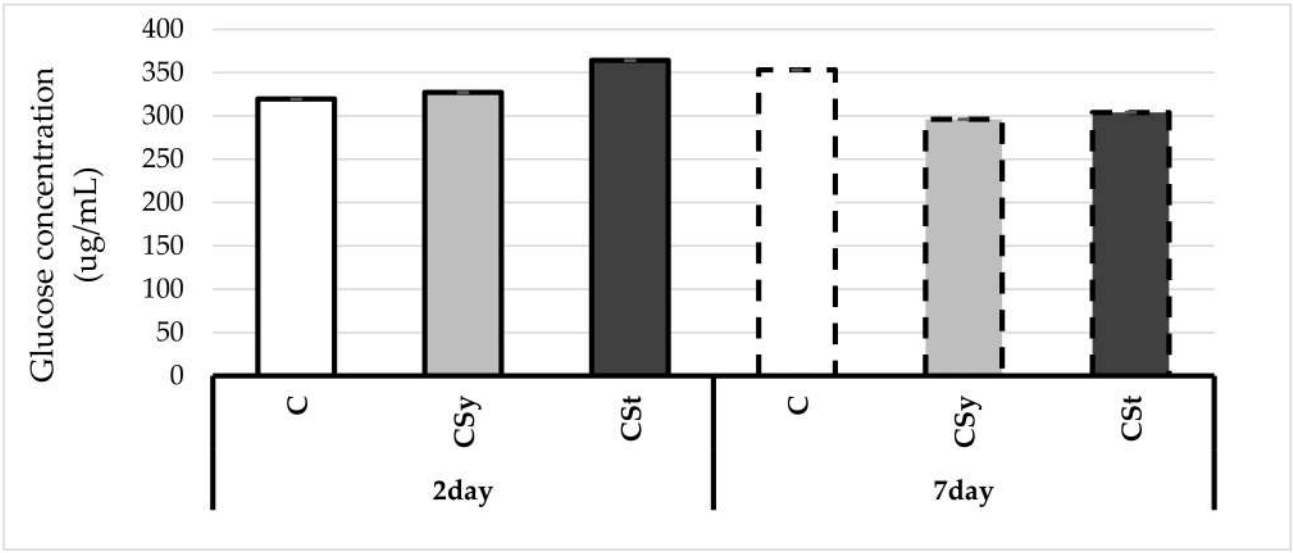



**Figure 5.** Glucose concentrations in the 2- and 7-day-old workers' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,59)} = 494.15$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 748.37$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 1639.0$, $p = 0.000$).

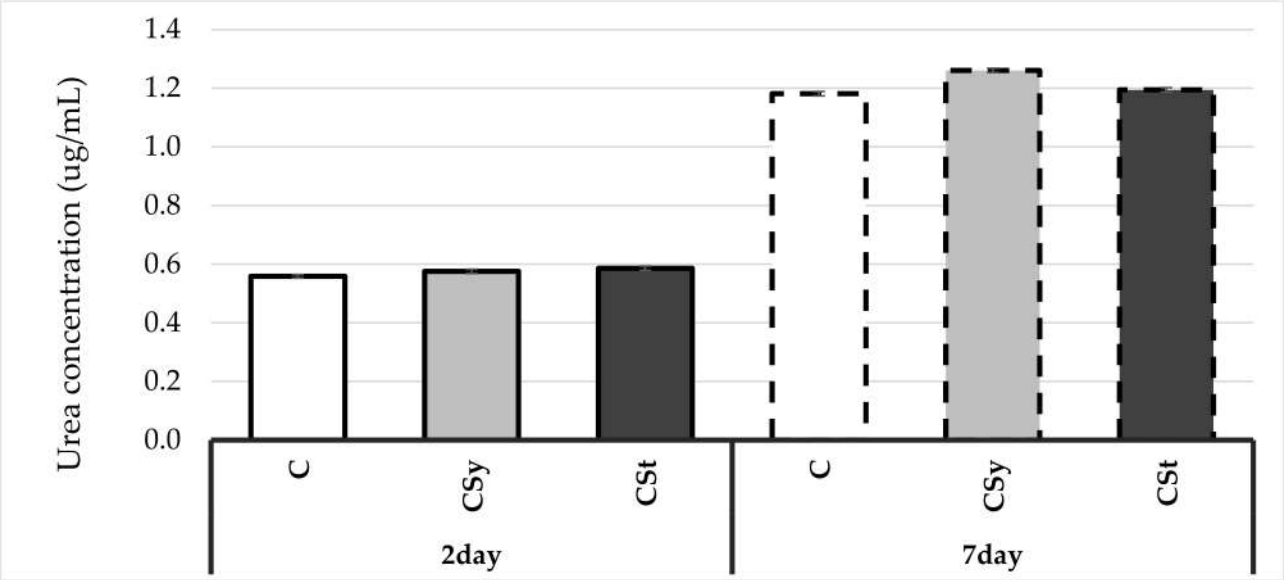



**Figure 6.** Urea concentrations in the 2- and 7-day-old workers' hemolymph after two methods of supplementation with hemp extract (Two-Way ANOVA: supplementation method: $F_{(2,59)} = 23.004$, $p = 0.000$; days of supplementation: $F_{(1,59)} = 11912.0$, $p = 0.000$; supplementation method*days of supplementation $F_{(2,59)} = 15.560$, $p = 0.000$). C–control (pure sugar syrup), Csy–hemp extract in syrup, Cst–hemp extract on strips.

## 5. Discussion

The positive effect and wide use of hemp extract has been demonstrated in many publications which touched upon neurological, behavioral, and cancer issues [11,34]. Thanks to the scientifically proven properties, we used an extract for bee supplementation in this study. Earlier studies on greater wax moths suggested that giving the supplement to insects living in the close environment of the honey bee will probably be safe also for the pollinators themselves and, as in the experiment with moths, it will have an equally satisfactory/positive effect.

We used two types of supplementation of hemp extracts in the experiment: (1) in sugar syrup (given in syringes), which is the main method in many publications (we can compare the effect of the type of supplementation with others already tested); and (2) with the use of material strips, commonly used in beekeeping practice in the treatment of diseases, e.g., caused by *Varroa destructor* mites. We have decided on these two methods because of their practical aspect in future use as well as in future beekeeping tests. Determining the effect of the supplement depending on the method of administration is important in order to adapt the best methods in the future to the problem of bee colonies. Statistical analysis showed significant changes in the activity of immune parameters depending on the method of administration, which will have an impact on the application of a given method and the activity shown depending on the threats and needs of beekeepers.

The high concentration of total protein for groups fed with hemp extract may indicate their increased production in the fat body, where most of the immune proteins are synthesized. When the extract was fed, the active substances could be absorbed in the digestive system and passed into the hemolymph and surrounding tissues such as the fat body. Some of the active substances in cannabis have been shown to affect the environment inside cells. For example, the aforementioned action, a positive effect of the active substance CBD on the increase in the concentration and homeostasis of Ca2+ ions was noted [35,36]. In immunogenic tissues (fat body, hemolymph), calcium-dependent cellular phospholipases (cPLA2 − Ca + 2-dependent cytosolic phospholipase A2) play a key role in the cellular immunity of insects (e.g., Lepidoptera) [37,38]. Calcium-dependent phospholipases mediate the production of eicosanoids present in the fat body, which are involved in both cellular and humoral immune responses [37]. The effect on humoral

immunity may result in an increased production of immune proteins, which translates into a higher total protein concentration reported in our experience. CBD can therefore have an immunostimulating effect. A similar effect was obtained in the studies of Strachecka concerning supplementation with coenzyme Q10 [28], which, similar to CBD, is defined by the authors as a lipophilic antioxidant. In the work of the authors, the concentration of calcium ions was measured, which confirms the potential process indicated by us. In order to confirm this thesis, additional tests should be performed to confirm the concentration of calcium ions in the hemolymph and the fat body of bees.

In addition, hemp extract on the strip could act as an external defensive shield, tightly covering the surfaces of the bees' body, making it difficult for pathogens to contact with these cuticles. Thus, the substances contained in cannabis could stimulate protein synthesis in the fat body and help seal the surfaces of the insect's body, in addition to the action of the proteolytic system (proteases and inhibitors presented on a cuticle) [39].

It turned out that the activity of proteases and their inhibitors decreased in the hemolymph of workers who were subjected to hemp extract in experimental groups as compared to the control group.

Alkaline proteases are mostly serine proteases that are involved in many physiological processes in the bee organism; the most important of them is the activation of phenoloxidase, which activates the chinones and tyrosine pathways, and through a number of indirect processes, including the formation of Dopa leads to the activation of sclerotization and melanization mechanisms, as a result of which the bee's body surface is sealed. Moreover, as a result of melanization, pathogens are coated and locked in melanotic nodules where they are degraded [40].

Our results are in line with the results of Strachecka [6,26], who observed a reduction in the activities of acidic and alkaline proteases after administering curcumin and caffeine to bees ad libitum. The bees, in the experiment with curcumin and caffeine, with similar parameters, showed significantly higher survival than the bees not subjected to supplementation [6,26]. This suggests that the obtained parameters reflect the positive effect on the bee's organism. A similar result was also obtained in the results of Strachecka on the decrease in the activity of proteases after the use of coenzyme Q10 [28], which, according to the authors' assumption, also showed a positive effect on the organism of bees. The positive effect of these supplementations can be compared to studies in which potentially negative factors were used. The results of the study by Migdał [41] showed, that the E-field at 50 Hz for 12 h caused an increase in the level of proteases compared to the control group. The authors assumed that the effect of the E-field was negative, suggesting other studies in which the effect of the pesticide bromfenvinphos was tested, after which increased activity of proteases in the hemolymph was observed. In an experiment, Strachecka found that bromfenvinphos significantly lowers the level of biochemical defense of honey bees [27].

The positive effect of the supplementation is confirmed by the above-mentioned studies, in which the activity levels of the parameters in our research are the same as in other tests with factors considered positive (curcumine, coenzyme Q10, caffeine), and different compared to tests with negative factors (E- field, bromfenvimphos).

Lowering the activity of this type of proteases in our results may indicate the previously mentioned sealing of the insect cuticle, reducing the susceptibility to damage, and, as a final result, the alkaline proteases did not need to be activated.

The activities of protease inhibitors in the extract-fed bees were significantly higher than in the control group and the group that received the extract on the strips. This proves the activation of proteolytic reactions, thus strengthening the bee's organism, which makes it better protected against pathogens. It is worth noting that the activities of the proteolytic system increase with the age of workers, which is consistent with the results obtained by Strachecka [26,28]. Łoś [25] also showed that the activities of proteases and their inhibitors usually increase with the age of workers until about 20–25 days of their life, and then decrease. Bees treated with biostimulants have lower protease inhibitor activities. Protease inhibitors act directly on proteases secreted by pathogens, preventing them from entering

body cavities and inhibiting their development. Acid protease inhibitors are directed primarily against pathogenic fungi, basic against bacteria and viruses, and neutral against other agents [19,42]. Based on these studies, it can be concluded that the extract on the strips actually sealed the first immune barriers and limited the penetration of pathogenic factors through these barriers; therefore, the body had no need to respond to the appearance of foreign proteins by producing appropriate enzymes. In the case of the extract in syrup, the bee's organism strengthened the internal immune mechanisms after being absorbed from the intestine and distributed by the hemolymph throughout the body.

Activities of biomarkers: ALT, AST, ASP were in most cases higher in the bees fed with the hemp extract in the syrup and lower for the bees in group with extract on the strip, compared to the activity in the control bees. Biomarkers determine the functioning, viability, and susceptibility to damage of fat body cells, which is called the "invertebrate liver". One of the most important biomarkers is the level of ALT. ALT is an intracellular enzyme. Increasing its concentration is often caused by damage to cells that play the role of the liver in insects, i.e., the fat body cells. The lowering of the ALT value on the 7th day of supplementation in both experimental groups proves the potential protection of cells against damage in relation to the body without supplementation. In the case of supplementation on the strips, we observe a reduction in the value of other biomarkers, such as AST, ASP, due to the previously mentioned sealing of the insect cuticle and the creation of an external protective barrier. In the case of supplementation of the extract in syrup, the increased parameters of biomarkers may result from the consumption of a non-standard substance to which the enzymes are not originally adapted and may cause deviations from the norm. In order to determine the further influence of hemp extract on these parameters, their height should be examined in the future in bees older than 7 days. Additionally, the increased activity in relation to the control group should not be negatively considered. The research of Sokol indicates that biomarkers in bees may show the opposite tendency towards mammalian organisms, e.g., have decreased activity during infection with *V. destructor* [43].

CBD (cannabidiol), a substance responsible for sedative effects, has the ability to act intracellularly on mitochondria and nuclear receptors due to cannabidiol lipophilic nature. Hemp extract with syrup may turn out to be more energetic thanks to the influence of active substances on cellular organelles, i.e., mitochondria (the aforementioned effect of CBD), which would result in lower consumption of the syrup and next in lower glucose concentration [35]. In addition, hemp extract, depending on the base (e.g., fat in oils and pastes), may cause changes and a slowdown of sugar metabolism due to the content of fats that digest slower, and as a result the supplied sugar could be metabolized by the body for a longer time [44]. This may be a factor in reducing the glucose concentration in our study. Confirmation of the positive effect of a lower consumption of enriched syrup may be the fact that higher food intake usually occurs in the case of infections, e.g., with microsporidia of *Nosema* spp., where the energy economy is disturbed, and oxidative stress and thermoregulation disorders are undoubtedly a negative effect. Bees suffering from nosemosis consume huge amounts of food [45,46]. This symptom is the opposite reaction to the results obtained in our supplementation.

The increase in urea concentrates in the case of the administration of cannabis in syrup may be related to the increased supply of protein and lipids in the diet of experimental bees compared to control bees, whose main food was sugar. Therefore, we can also observe a slight increase in urea concentration in relation to the control for supplementation with strips due to less direct exposure of bees to the extract compared to administration in syrup. Our results in this case are opposite to the results obtained by Strachecka [26,28]. Coenzyme and curcumin had a lower effect on the level of urea in the bees' hemolymph, causing an increase in uric acid during the tests. Both substances contain ketone groups which may suggest that the indicated trends are specific for these compounds. Digestion of hemp extract having a different chemical structure compared to the supplementation above may take place in a different way, the effect of which was visible in the increased concentration

of urea, and maybe lowered in the case of uric acid. To confirm this, studies should be carried out on the concentration of uric acid in supplementation with hemp extract.

## 6. Conclusions

The hemp supplementation positively influences the immune system of the honey bee, and the appropriate method of administration may be adapted to the health problems of bees. Sealing physiological and anatomical barriers may help in the future with large parasites, such as *V. destructor*, and stimulating the organism from the inside may be helpful in the case of pathogens easily penetrating into organisms such as *Nosema* spp. or also microorganisms, such as bacteria, viruses, and fungi.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/ani11082190/s1, File S1: Supplement to the methodology of monotests.

**Author Contributions:** Idea, implementation of analyses, the scheme of conducting the experiment, conceptualization, methodology, writing—first draft preparation, P.S.; idea, methodology, review, and editing, A.S.; assistance in carrying out research, Ł.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was financed from the funds allocated to the implementation of research within the Doctoral School on University of Life Sciences in Lublin.

**Institutional Review Board Statement:** Ethical review and approval were waived for this study, due to that the studies included invertebrates.

**Data Availability Statement:** The datasets and materials for this study results that have been used, analyzed and presented in this manuscript are not publicly available. Available on University of Life Sciences in Lublin. At the justified request of the interested party, they may be made available by the respective author.

**Acknowledgments:** We thank Aleksandra Splitt (The National Institute of Horticultural Research, Apiculture Division in Puławy, Institute of Nature Conservation, Polish Academy of Sciences) for help in preparing the experiment.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Mizrahi, A.; Lensky, Y. *Bee Products*; Springer: New York, NY, USA, 2013.
2. Aizen, M.A.; Harder, L.D. The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Curr. Biol.* **2009**, *19*, 915–918. [CrossRef] [PubMed]
3. Rascón, B.; Hubbard, B.P.; Sinclair, D.A.; Amdam, G.V. The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging* **2012**, *4*, 499–508. [CrossRef]
4. Ricigliano, V.A.; Simone-Finstrom, M. Nutritional and prebiotic efficacy of the microalga *Arthrospira platensis* (spirulina) in honey bees. *Apidologie* **2020**, *51*, 898–910. [CrossRef]
5. Farjan, M.; Dmitryjuk, M.; Lipiński, Z.; Biernat-Łopieńska, E.; Zółtowska, K. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera carnica* brood at different stages. *J. Apic. Res.* **2012**, *51*, 263–270. [CrossRef]
6. Strachecka, A.; Krauze, M.; Olszewski, K.; Borsuk, G.; Paleolog, J.; Merska, M.; Chobotow, J.; Bajda, M.; Grzywnowicz, K. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochem* **2014**, *79*, 1192–1201. [CrossRef] [PubMed]
7. Van der Steen, J. Effect of a home-made pollen substitute on honey bee colony development. *J. Apic. Res.* **2007**, *46*, 114–119. [CrossRef]
8. Kaznowski, A.; Szymas, B.; Jazdzinska, E.; Kazimierczak, M.; Paetz, H.; Mokracka, J. The effects of probiotic supplementation on the content of intestinal microflora and chemical composition of worker honey bees (*Apis mellifera*). *J. Apic. Res.* **2005**, *44*, 10–14. [CrossRef]
9. Suwannapong, G.; Maksong, S.; Phainchajoen, M.; Benbow, M.E.; Mayack, C. Survival and health improvement of *Nosema* infected *Apis florea* (Hymenoptera: Apidae) bees after treatment with propolis extract. *J. Asia. Pac. Entomol.* **2018**, *21*, 437–444. [CrossRef]
10. Benson, M.J.; Abelev, S.V.; Connor, S.J.; Corte, C.J.; Martin, L.J.; Gold, L.K.; Suraev, A.S.; McGregor, I.S. Medicinal cannabis for inflammatory bowel disease: A survey of perspectives, experiences, and current use in australian patients. *Crohn's Colitis 360* **2020**, *2*. [CrossRef]
11. Reithmeier, D.; Tang-Wai, R.; Seifert, B.; Lyon, A.W.; Alcorn, J.; Acton, B.; Corley, S.; Prosser-Loose, E.; Mousseau, D.D.; Lim, H.J. The protocol for the Cannabidiol in children with refractory epileptic encephalopathy (CARE-E) study: A phase 1 dosage escalation study. *BMC Pediatrics* **2018**, *18*, 221. [CrossRef]

12. Samarut, É.; Nixon, J.; Kundap, U.P.; Drapeau, P.; Ellis, L.D. Single and synergistic effects of cannabidiol and δ-9-tetrahydrocannabinol on zebrafish models of neuro-hyperactivity. *Front. Pharmacol.* **2019**, *10*, 226. [CrossRef]
13. Hazekamp, A.; Simons, R.; Peltenburg-Looman, A.; Sengers, M.; Van Zweden, R.; Verpoorte, R. Preparative isolation of cannabinoids from *Cannabis sativa* by centrifugal partition chromatography. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 2421–2439. [CrossRef]
14. Amin, M.R.; Ali, D.W. Pharmacology of Medical Cannabis. In *Advances in Experimental Medicine and Biology*; Springer: New York, NY, USA, 2019; Volume 1162, pp. 151–165.
15. Lopez, H.L.; Cesareo, K.R.; Raub, B.; Kedia, A.W.; Sandrock, J.E.; Kerksick, C.M.; Ziegenfuss, T.N. Effects of Hemp Extract on Markers of Wellness, Stress Resilience, Recovery and Clinical Biomarkers of Safety in Overweight, But Otherwise Healthy Subjects. *J. Diet. Suppl.* **2020**, *17*, 561–586. [CrossRef] [PubMed]
16. Di Giacomo, V.; Recinella, L.; Chiavaroli, A.; Orlando, G.; Cataldi, A.; Rapino, M.; Di Valerio, V.; Politi, M.; Antolini, M.D.; Acquaviva, A. Metabolomic profile and antioxidant/anti-inflammatory effects of industrial hemp water extract in fibroblasts, keratinocytes and isolated mouse skin specimens. *Antioxidants* **2021**, *10*, 44. [CrossRef] [PubMed]
17. Mabou Tagne, A.; Fotio, Y.; Lin, L.; Squire, E.; Ahmed, F.; Rashid, T.I.; Karimian Azari, E.; Piomelli, D. Palmitoylethanolamide and hemp oil extract exert synergistic anti-nociceptive effects in mouse models of acute and chronic pain. *Pharmacol. Res.* **2021**, *167*, 105545. [CrossRef] [PubMed]
18. Marini, E.; Magi, G.; Ferretti, G.; Bacchetti, T.; Giuliani, A.; Pugnaloni, A.; Rippo, M.R.; Facinelli, B. Attenuation of Listeria monocytogenes virulence by *Cannabis sativa* L. Essential oil. *Front. Cell. Infect. Microbiol.* **2018**, *8*. [CrossRef] [PubMed]
19. Evans, J.D.; Aronstein, K.; Chen, Y.P.; Hetru, C.; Imler, J.L.; Jiang, H.; Kanost, M.; Thompson, G.J.; Zou, Z.; Hultmark, D. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.* **2006**, *15*, 645–656. [CrossRef]
20. Strachecka, A.; Łoś, A.; Filipczuk, J.; Schulz, M. Individual and social immune mechanisms of the honey bee. *Med. Weter.* **2018**, *74*, 426–433.
21. Wilde, J.; Frączek, R.J.; Siuda, M.; Bąk, B.; Hatjina, F.; Miszczak, A. The influence of sublethal doses of imidacloprid on protein content and proteolytic activity in honey bees (*Apis mellifera* L.). *J. Apic. Res.* **2016**, *55*, 212–220. [CrossRef]
22. Immunobiologia Pszczoły Miodnej, Zdzisław Gliński, Jan Jarosz. Available online: https://vetbooks.pl/p/7/421/immunobiologia-pszczoly-miodnej-pszczelarstwo-hodowla-zwierzat.html (accessed on 2 July 2021).
23. Strachecka, A.; Demetraki-Paleolog, J. System proteolityczny powierzchni ciała apis mellifera w zachowaniu zdrowotności rodzin pszczelich. *Kosmos* **2011**, *60*, 43–51.
24. Aw, D.; Silva, A.B.; Palmer, D.B. Immunosenescence: Emerging challenges for an ageing population. *Immunology* **2007**, *120*, 435–446. [CrossRef] [PubMed]
25. Łoś, A.; Strachecka, A. Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution. *Sensors* **2018**, *18*, 1494. [CrossRef] [PubMed]
26. Strachecka, A.; Olszewski, K.; Paleolog, J. Curcumin stimulates biochemical mechanisms of *Apis mellifera* resistance and extends the apian life-span. *J. Apic. Sci.* **2015**, *59*, 129–141. [CrossRef]
27. Strachecka, A.; Olszewski, K.; Paleolog, J. *Varroa* treatment with bromfenvinphos markedly suppresses honeybee biochemical defence levels. *Entomol. Exp. Appl.* **2016**, *160*, 57–71. [CrossRef]
28. Strachecka, A.; Olszewski, K.; Paleolog, J.; Borsuk, G.; Bajda, M.; Krauze, M.; Merska, M.; Chobotow, J. Coenzyme Q10 treatments influence the lifespan and key biochemical resistance systems in the honeybee, *Apis mellifera*. *Arch. Insect Biochem. Physiol.* **2014**, *86*, 165–179. [CrossRef]
29. Schacterle, G.; Pollack, R. A Simplified Method for the Quantitative Assay of Small Amounts of Proteins in Biologic Material. *Anal. Biochem.* **1973**, *51*, 654–655. [CrossRef]
30. Waterborg, J.H. The Lowry Method for Protein Quantitation. In *The Protein Protocols Handbook*; Humana Press: Totowa, NJ, USA, 2009; pp. 7–10.
31. Anson, M.L. The Estimation Of Pepsin, Trypsin, Papain, And Cathepsin With Hemoglobin. *J. Gen. Physiol.* **1938**, *22*, 79–89. [CrossRef]
32. Strachecka Aneta, G.M.M. Influence of Environmental Pollution on the Protective Proteolytic Barrier of the Honey Bee *Apis mellifera mellifera*. *Polish J. Environ. Stud.* **2010**, *19*, 855–859.
33. Lin, J.; Lee, I.S.; Frey, J.; Slonczewski, J.L.; Foster, J.W. Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia coli. *J. Bacteriol.* **1995**, *177*, 4097–4104. [CrossRef]
34. Abdel-Salam, O.; Sleem, A.; Youness, E.; Morsy, F. Preventive effects of cannabis on neurotoxic and hepatotoxic activities of malathion in rat. *Asian Pac. J. Trop. Med.* **2018**, *11*, 272–279. [CrossRef]
35. De Petrocellis, L.; Ligresti, A.; Moriello, A.S.; Allarà, M.; Bisogno, T.; Petrosino, S.; Stott, C.G.; Di Marzo, V. Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br. J. Pharmacol.* **2011**, *163*, 1479–1494. [CrossRef]
36. Dube, K.A.; McDonald, D.G.; O'Donnell, M.J. Calcium homeostasis in larval and adult *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* **2000**, *44*, 27–39. [CrossRef]
37. Park, Y.; Kumar, S.; Kanumuri, R.; Stanley, D.; Kim, Y. A novel calcium-independent cellular PLA2 acts in insect immunity and larval growth. *Insect Biochem. Mol. Biol.* **2015**, *66*, 13–23. [CrossRef] [PubMed]
38. Underwood, R.M.; Traver, B.E.; López-Uribe, M.M. Beekeeping management practices are associated with operation size and beekeepers' philosophy towards in-hive chemicals. *Insects.* **2019**, *10*, 10. [CrossRef]
39. Strachecka, A.; Paleolog, J.; Grzywnowicz, K. The surface proteolytic activity in *Apis mellifera*. *J. Apic. Sci.* **2008**, *52*, 49–56.

40. Dhule, S.S.; Penfornis, P.; Frazier, T.; Walker, R.; Feldman, J.; Tan, G.; He, J.; Alb, A.; John, V.; Pochampally, R. Curcumin-loaded γ-cyclodextrin liposomal nanoparticles as delivery vehicles for osteosarcoma. *Nanomed. Nanotechnol. Biol. Med.* **2012**, *8*, 440–451. [CrossRef] [PubMed]
41. Migdał, P.; Murawska, A.; Strachecka, A.; Bieńkowski, P.; Roman, A. Honey bee proteolytic system and behavior parameters under the influence of an electric field at 50 hz and variable intensities for a long exposure time. *Animals* **2021**, *11*, 863. [CrossRef] [PubMed]
42. Bode, W.; Fernandez-Catalan, C.; Tschesche, H.; Grams, F.; Nagase, H.; Maskos, K. Structural properties of matrix metalloproteinases. *Cell. Mol. Life Sci.* **1999**, *55*, 639–652. [CrossRef]
43. Sokol, R. Wybrane wskazniki biochemiczne hemolimfy w przebiegu inwazji *Varroa jacobsoni* u pszczol. II. Aktywnosc transaminazy asparaginianowej i alaninowej w hemolimfie czerwia, pszczol i trutni. *Acta Acad. Agric. Tech. Olstenensis. Vet.* **1996**, *24*, 113–125.
44. Gilbert, L.I. Lipid Metabolism and Function in Insects. *Adv. Insect Phys.* **1967**, *4*, 69–211. [CrossRef]
45. Fries, I.; Chauzat, M.-P.; Chen, Y.P.; Doublet, V.; Genersch, E.; Gisder, S.; Higes, M.; McMahon, D.P.; Martín-Hernández, R.; Natsopoulou, M. Standard methods for *Nosema* research. *J. Apic. Res.* **2013**, *52*, 1–28. [CrossRef]
46. Schulz, M.; Skowronek, P.; Tyszczuk, J.; Los, A.; Strachecka, A. *Nosema apis* i *Nosema ceranae*—Porównanie morfologii, metod identyfikacji oraz przebiegu mikrosporydiozy u pszczół miodnych. *Przegląd Hod.* **2018**, *86*, 21–24.

*antioxidants*

MDPI

*Article*

# Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (*Apis mellifera*) Organism

**Patrycja Skowronek *, Łukasz Wójcik and Aneta Strachecka**

Department of Invertebrate Ecophysiology and Experimental Biology, University of Life Sciences in Lublin, Doświadczalna 50a, 20-280 Lublin, Poland; lukasz7wojcik@gmail.com (Ł.W.); aneta.strachecka@up.lublin.pl (A.S.)
* Correspondence: patrycja.skowronek@up.lublin.pl

**Abstract:** We examined the effect of hemp extract on the activity of the antioxidant system (catalase, peroxidase, glutathione, superoxide dismutase, and total antioxidant capacity) in the hemolymph of adult honey bees (*Apis mellifera*). The bees were divided into three groups: (1) an experimental group fed with pure sugar syrup with cotton strips soaked with hemp extract put inside the cage; (2) an experimental group fed with a mixture of sugar syrup with hemp extract; and (3) a control group fed with a mixture of sugar and a water–glycerine solution. Hemolymph samples were collected on the 1st day of this study and then every week, until all bees in the group died. The activities of all antioxidant enzymes were higher for the experimental groups, compared to those for the control group. The highest antioxidant activities were noted in the group supplemented with cannabis with the use of syringes. Supplementation with hemp also increased the lifespan of bees in this group compared to that of the bees consuming only sugar syrup (control: 35 days), with 49 and 52 days for groups of cannabis on strips and in syrup, respectively. Hemp extract, thanks to its antioxidant properties, increased the activities of key antioxidant enzymes that protect the bee's organisms against free radicals and thus delay the aging processes.

**Keywords:** bees' resistance; cannabis; supplementation of bees; antioxidants



**Citation:** Skowronek, P.; Wójcik, Ł.; Strachecka, A. Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (*Apis mellifera*) Organism. *Antioxidants* **2022**, *11*, 707. https://doi.org/10.3390/antiox11040707

Academic Editor: Antonio Rescigno

Received: 24 February 2022
Accepted: 31 March 2022
Published: 2 April 2022



## 1. Introduction

In recent years, it has been observed the intensification of changes in the environment caused by progressive pollution, which has a negative impact on all living organisms. Despite many activities aimed at protecting the environment, the effects of these changes are observed in many animals, including beneficial insects, such as honey bees [1,2]. Nowadays, high declines in populations of this pollinator and increased susceptibility to infection by specific parasites and pathogens are being recorded [3]. Chemical compounds, i.e., pesticides, are main pollutants that also affect the health of bees [4]. In addition, a general phenomenon of weakening the immune system of bees may be the reason for higher mortality among pollinators [5,6].

Factors negatively affecting the organism (including its immunity) cause strong reactions, often through the increased production of free radicals responsible for chronic inflammation and premature aging of the body [7,8]. The antioxidant system is responsible for protecting against these molecules and, thanks to the activity of its enzymes, neutralizes reactive oxygen species (ROS) produced by organisms. However, due to the increase in the amount of negative factors, a delay in the response of defense systems or too much excitability is observed in a short time, which can lead to autoimmune diseases [9–11].

In order to additionally strengthen the organism, medicine commonly tests new sources of antioxidants to support animal organisms. Such sources include spices (e.g., curcumin), vegetables (e.g., pumpkin rich in beta-carotene), fruits such as currants (vitamin C), grapes (resveratrol), or dietary additives such as spirulina [12–15]. These substances/products affect not only the human body, but have also been used as supplementation supporting

the health of honey bees [12–17]. The addition of curcumin to sugar syrup results in the change of key parameters of immunity in bees fed with this formula, including an increase in the activities of antioxidant enzymes (superoxide dismutase (SOD), peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST), and total antioxidant capacity (TAC)) [13]. The administration of resveratrol extends the life of bees by approximately 33–38%, despite keeping bees under hyperoxic stress conditions [14]. Another product tested on bees is spirulina, which influences physiological parameters (weight) and increases the contents of proteins and lipids in individual parts of the body [12].

In this publication, we checked hemp extract which, according to the reports from the fields of human and animal medicine, has a proven antioxidant effect and a number of other properties thanks to which it is widely used in the treatment of many diseases such as epilepsy, Crohn's disease, depression, and Alzheimer's disease [18–21]. Hemp is also used as a supplement in treating cancer or in counteracting the effects of chronic stress/trauma [22]. Its positive effect is attributed to the active substances from the group of cannabinoids (cannabidiol (CBD), cannabichromene, cannabigerol, Δ9-tetrahydrocannabinol (THC), and cannabinol) and the content of phytochemicals [21,23]. During in vitro studies with human cell lines, the aqueous cannabis extract showed a protective effect against the cytotoxicity and apoptosis of the tested fibroblasts and keratinocytes [24]. The other researches involved also other animals. The usage of isolated rat skin confirmed the anti-inflammatory effect of cannabis by reducing the production of prostaglandin E2 and positively changed the values of tryptophan and kynurenine [24]. The latest research also confirms the effect of the extracts on neurological ailments, i.e., chronic pain, thanks to enriching the diet of rodents with hemp oil and palmitoylethanolamide, which together creates antinociceptive effects [18]. The effect on the increase of interleukin transcription (the amount of mRNA), which takes part in numerous processes of the immune system and mediates information in the body, was also noticed [18]. Moreover, cannabis essential oils prolong the life of *Galleria mallonela* by limiting the invasion of *Listeria monocytogenes*. This confirms that the extract, at appropriate concentrations, is not dangerous to insects [25]. Like humans, bees are also exposed to ROS in their bodies. Therefore, the enzymes present in humans can also be tested in bees. There are four main enzymes that make up the basic first lines of defense against oxidants: SOD, CAT, GPx, GST, and TAC [9,26–29]. The task of antioxidant enzymes is to trap oxygen radicals and transform them into less reactive forms. SOD participates in the first reactions with radicals, converting them into hydrogen peroxide and molecular oxygen. Then, the hydrogen peroxide is reduced to molecular oxygen and water by CAT or by GPx while reducing glutathione to its oxidized form. GST participates in the changes that detoxify oxygen (the second line of defense). TAC tells us about the overall capacity of the antioxidants present in each organism. Due to the confirmed antioxidant properties of cannabis, we assumed that the extract would have a positive effect on the activity of the enzymes of the antioxidant system: SOD, CAT, GPx, GST, and TAC [9].

The aim of our research was to determine the effect of hemp extract on the activities of enzymes in the antioxidant system in bees' hemolymph, along with the aging processes.

## 2. Materials and Methods

### *2.1. Sampling*

The experiment began with 1-day-old worker bees (*A. mellifera carnica*), collected from the apiary belonging to the University of Life Sciences in Lublin (51°13′31″ N, 22°38′07″ E). The colonies were not treated against *Varroa destructor* (*V. destructor*). The treatment was not applied, because the substances used against *V. destructor* could be an additional factor influencing the level of the parameters activities of the immune system. The introduction of this factor would not allow a clear assessment of the effect of the hemp extract. We did not record any spores of *Nosema* spp. in the bees of the colony. The bees in the colonies were in good health. Five mother queens were raised from larvae hatches of eggs laid by one purebred reproductive queen *A. mellifera carnica*. This queen was earlier artificially

inseminated. The queen was restricted on one comb in a queen-excluder comb-cage containing one empty comb for 12 h, where laid eggs. After that, the queens were released to the colony, and 4 days after the release, we grafted the larvae from the comb in artificial queen cells. The grafted larvae were placed on a drop of a royal jelly solution (*v*:*v*, 1:1) in the center of the queen cell. The frames with the grafted cells were placed in a 10-frame-strong, queenless colony. After 7 days, the queen cells were isolated and kept in an incubator (35 °C) until emergence. Five of these mother queens were subjected, in Eppendorf tubes (1.5 mL) with cut ends and sealed with wax, to previously prepared five queenless colonies kept in Dadant hives. Queens on the 8th day of their life were artificially inseminated with the semen of *A. mellifera carnica* drones. These males, at 18–20 days of age, were collected from other colonies of the experimental apiary. They were not brothers of the queens. After about a month, the queens were confined to a queen-excluder comb-cage containing one empty comb for 12 h to lay eggs. After 20 days, from which time the queens laid their eggs, the combs were transferred to an incubator, where the workers (1-day-old workers) were emerged. These workers were randomly placed into 30 wooden cages (40 bees per cage) and divided into three groups (10 cages each). The cages were divided into three groups according to Table 1 [4,30].

**Table 1.** Descriptions of supplementation and administration methods for the group in the experiment.

| Type of Group | Feeding | Method of Supplementation |
|---|---|---|
| (1) CSt | sugar syrup (*v*:*v*, 1:1) ad libitum and inside with cotton strips soaked with 3 mL hemp extract (0.25 g hemp paste extract + 3 mL water–glycerine solution) | extract on a cotton strip |
| (2) CSy | a mixture of sugar syrup (*v*:*v*, 1:1) with hemp extract ad libitum (500 mL water–glycerine solution with 4.38 g hemp paste extract) | extract in a syringe |
| (3) C | mixture of sugar and a water–glycerine solution (*v*:*v*, 1:1) | syrup in a syringe |

The cages were kept in optimal conditions of 35 °C and a 65% relative humidity [30].

Water–glycerine solutions were brought/delivered from Chempur (standard: BN-76/6193-12; Piekary Śląskie, Poland). The hemp extract used in this test/research was sourced from the manufacturer Melisa (Brzeziny, Poland).

In each group, food was administered ad libitum and replenished every other day during the experiment.

Ten randomly selected cages were allocated to each group. The analytical procedure, described below, began two days after the first feeding.

### *2.2. Hemolymph Extraction*

In each group, fresh hemolymph was taken from 10 bees at the age of 1, 7, 14, 21, 28, 35, 42, 49, and 56 days (by puncturing the venous sinus in the insect's abdomen), according to Łoś and Strachecka method [10]. The hemolymph from each bee was placed separately in a sterile Eppendorf tube with 0.6% NaCl. All samples (from the control group: 6 sampling × 10 workers; from the experimental group with a strip: 8 sampling × 10 workers; from the experimental group with a syringe: 9 sampling × 10 workers) were immediately refrigerated at −25 °C for further biochemical analyses. The number of sampling depended on the presence of living bees in a given group [31].

### *2.3. Determination of Antioxiant Activities*

– SOD according to Podczasy and Wei (1988)

The analyzed biological material was hemolymph. We added 0.5 mM xanthine, 0.3 mM EDTA, 49 mM p-iodonitrotetrazolium, and 0.92 mM $Na_2CO_3$ to an Eppendorf tubes. Then, 5 µL of hemolymph were added to the mixture. Reactions were started by

adding a maximum of 0.25 U/mL xanthine oxidase. Eppendorf tubes were subjected to a 15-min incubation at 25 °C and then cooled. In the next step, the absorbance was measured at 505 nm.

– GPx according to the methods described by Chance and Maehly (1955)

The analyzed biological material was hemolymph. The Eppendorf tube contained 25 µL assay mixture of 125 µM phosphate buffer (pH 6.8), 50 µM pyrogallol, 50 µM $H_2O_2$, and 5 µL of the biological material sample. The samples were incubated for 5 min at 25 °C. In the next step, the reaction was stopped by adding 5 µL of 5% $H_2SO_4$ to the mixture. Absorbance was measured at a wavelength of 420 nm.

– CAT according to Aebi (1983)

The biological material used in the analyses was hemolymph. Three-hundred and thirty-five microliters of 50 mM phosphate buffer (pH 7.0) were mixed with 165 µL of 54 mM $H_2O_2$ at 25 °C. The reaction was initiated by adding 5 µL of the biological material. One unit of CAT is the amount of the enzyme that decomposes 1 µmol of $H_2O_2$ per minute at 25 °C. The $H_2O_2$ decomposition was measured at a wavelength of 240 nm.

– GST according to Warholm et al. (1985)

The biological material used in the analysis was hemolymph. The following mixture was added to a cuvette: 215 µL 0.1 M sodium phosphate buffer (pH 6.5), 13 µL 20 mM GSH, and 13 µL 20 mM 1-chloro-2,4-dinitrobenzene in 95% ethanol. The reaction was initiated by supplementing the mixture with 12 µL of the biological material. The reaction was carried out at a temperature of 30 °C. Absorbances were measured at 340 nm.

– TAC according to the protocol included in the Assay Kit produced by Sigma Aldrich. The reaction was carried out at a temperature of 30 °C. Absorbances were measured at 570 nm.

All antioxidant enzyme activities were calculated per 1 mg of protein.

### *2.4. Statistical Analysis*

The results were analyzed using Statistica formulas version 13.3 (2017) for Windows (StatSoft Inc., Tusla, OK, USA). The mixed-model two-way ANOVA followed by Tukey HSD post hoc tests ($p = 0.05$) was used to compare the results for each antioxidant enzymes (SOD, GST, CAT, GPx, and TAC) of honey bee workers, depending on the method of administration (strip and syringe) and the day (1st, 7th, 14th, 21st, 28th, 35th, 42nd, 49th, and 56th) of the supplementation with hemp extract.

## 3. Results

In the experimental groups, we noticed a continuous increase in activity up to the 42nd day of the life of bees. For comparison, the bees that did not receive supplementation only survived for 35 days (Figures 1–5).

In the group where the extract was administered in a syringe, we observed the highest values of the enzyme activity, compared to in the other groups. The bees that had the extract strip in the cages survived longer (49 days) than those in the control group (35 days) but shorter than the bees in the syrup extract group (56 days) (Figures 1–5).

In the control group, the activities of antioxidant enzymes increased by day 28 for SOD (Figure 1) and GST (Figure 2) and by day 21 for CAT (Figure 3) and GPx (Figure 4). Then, these values decreased. In the groups supplemented with hemp extract, all tested enzymes achieved higher activities compared to those in the control groups.

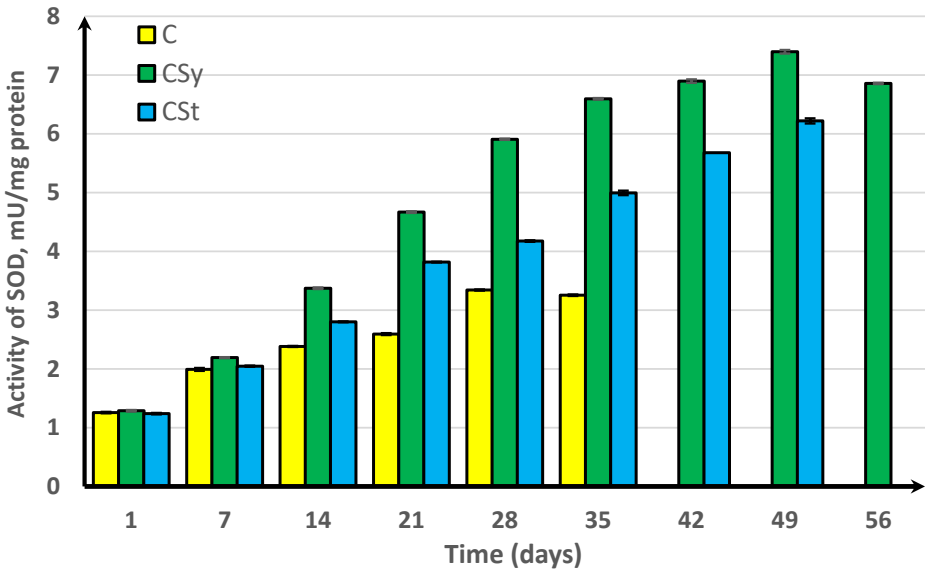



**Figure 1.** Superoxide dismutase (SOD) activities in workers' hemolymph along with the aging processes, after using two methods of supplementation with hemp extract. C, control (pure sugar syrup); CSy, hemp extract in syrup; CSt, hemp extract on strips (two-way ANOVA; days of supplementation: $F_{(5,1078)}$ = 17,089, $p$ = 0.0000, se ± 1.26111 (1st day), se ± 2.07672 (7th day), se ± 2.85162 (14th day), se ± 3.69264 (21st day), se ± 4.47489 (28th day), se ± 4.94804 (35th day), se ± 6.89712 (42nd day), se ± 6.80810 (59th day), and se ± 6.85724 (56th day); supplementation method: se ± 0.00767 (C), se ± 0.00627 (CSy), and se ± 0.00710 (CSt); supplementation method × days of supplementation: $F_{(11,1078)}$ = 1205.8, $p$ = 0.000, and se ± 0.01880). The chart shows statistical averages. The standard deviations for the means in this plot ranged from 0.04136 to 0.30656.

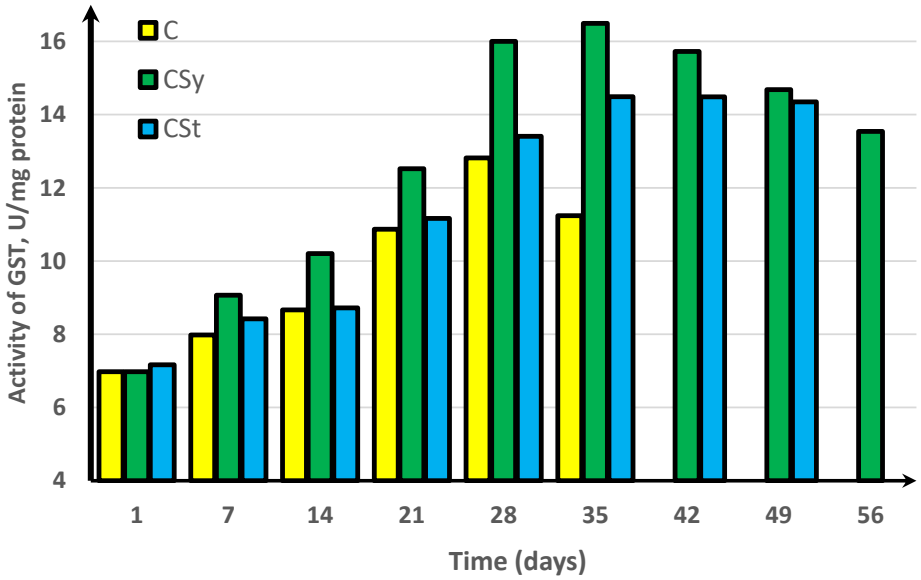



**Figure 2.** Glutathione S-transferase (GST) activities in workers' hemolymph along with the aging processes, after using two methods of supplementation with hemp extract. C, control (pure sugar syrup); CSy, hemp extract in syrup; CSt, hemp extract on strips (two-way ANOVA: supplementation method: $F_{(5,1078)}$ = 40,402, $p$ = 0.0000, se ± 0.010431 (C), se ± 0.008517 (CSy), and se ± 0.009657 (CSt); days of supplementation: $F_{(5,1078)}$ = 40,402, $p$ = 0.0000, se ± 0.014751 (1st–35th days), se ± 0.025550 (42nd day and 56th day), and se ± 0.018066 (49th day); supplementation method × days of supplementation $F_{(11,1078)}$ = 1416.0, $p$ = 0.0000, and se ± 0.025550). The chart shows statistical averages. The standard deviations for the means in this plot ranged from 0.04158 to 0.31201.

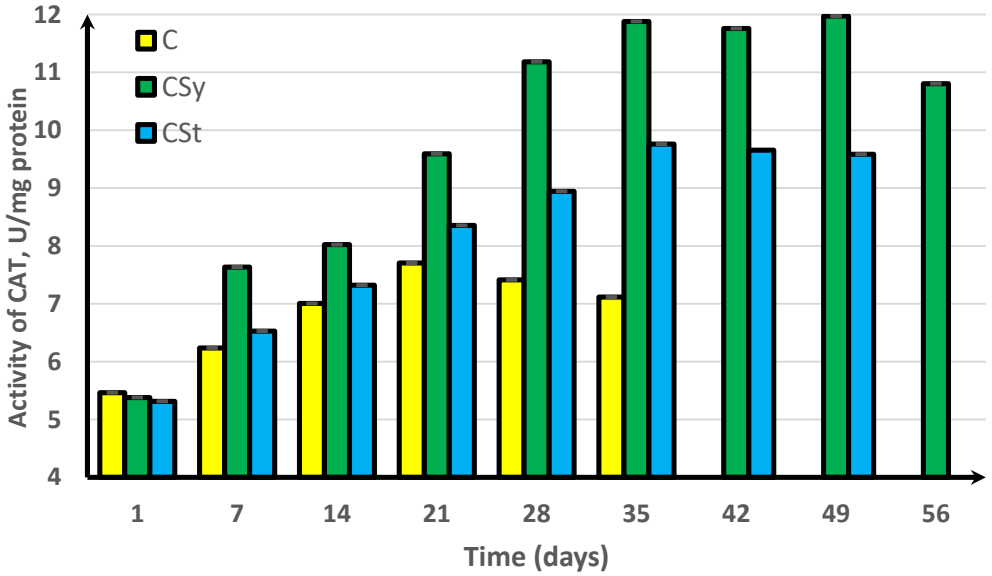



**Figure 3.** Catalase (CAT) activities in workers' hemolymph along with the aging processes, after using two methods of supplementation with hemp extract. C, control (pure sugar syrup); CSy, hemp extract in syrup; CSt, hemp extract on strips (two-way ANOVA: days of supplementation: $F_{(5,1078)}$ = 25,133, $p$ = 0.0000, se ± 0.010013 (1st–35th days), se ± 0.017343 (42nd day and 56th day), se ± 0.012263 (49th day); supplementation method: se ± 0.00708 (C), se ± 0.00578 (CSy), and se ± 0.00656 (CSt); supplementation method × days of supplementation $F_{(11,1078)}$ = 2701.1, $p$ = 0.0000, and se ± 0.017343). The chart shows statistical averages. The standard deviations for the means in this plot ranged from 0.05238 to 0.21342.

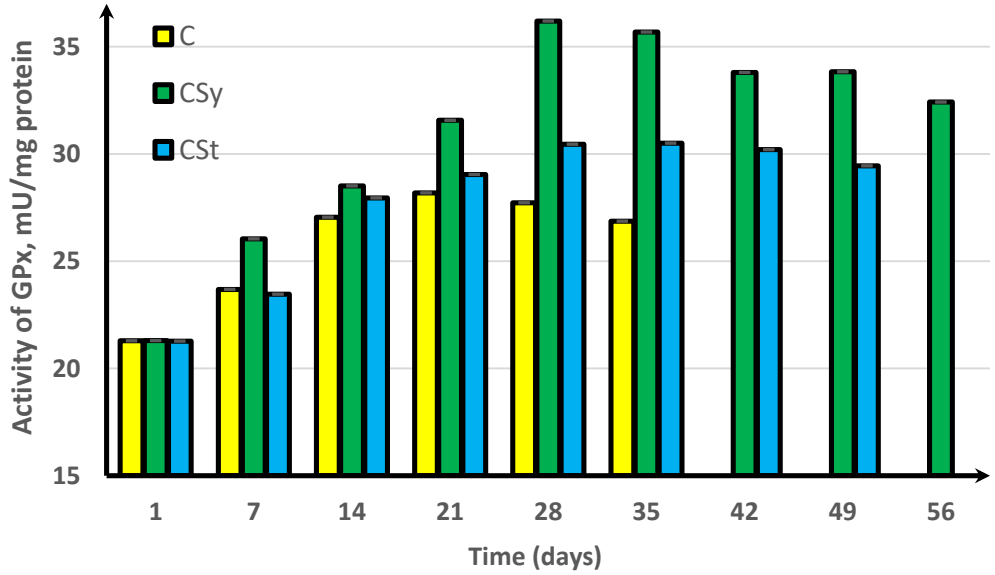



**Figure 4.** Peroxidase (GPx) activities in workers' hemolymph along with the aging processes, after using two methods of supplementation with hemp extract. C, control (pure sugar syrup); CSy, hemp extract in syrup; CSt, hemp extract on strips (two-way ANOVA: supplementation method: $F_{(5,1078)}$ = 41,905, $p$ = 0.0000, se ± 0.013879 (C), se ± 0.011332 (CSy), and se ± 0.012849 (CSt); days of supplementation: $F_{(5,1078)}$ = 41,905, $p$ = 0.0000, se ± 0.019628 (1st–35th days), se ± 0.033996 (42nd day and 56th day), se ± 0.024039 (49th day); supplementation method × days of supplementation $F_{(11,1078)}$ = 2910.4, $p$ = 0.0000, and se ± 0.033996). The chart shows statistical averages. The standard deviations for the means in this plot ranged from 0.06757 to 0.47376.

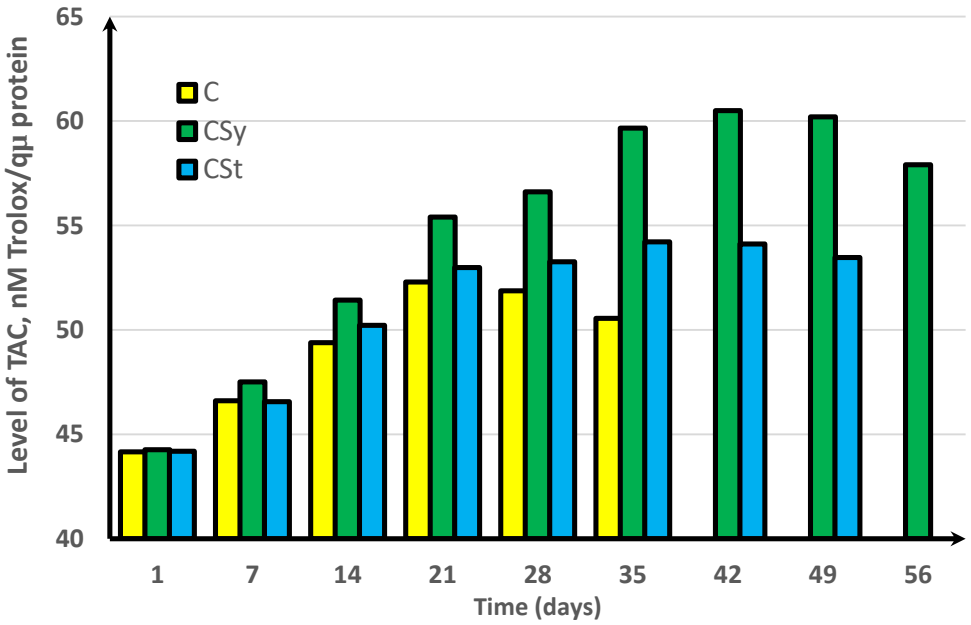



**Figure 5.** Levels of total antioxidant capacity (TAC) in workers' hemolymph along with the aging processes, after using two methods of supplementation with hemp extract. C, control (pure sugar syrup); CSy, hemp extract in syrup; CSt, hemp extract on strips (two-way ANOVA: supplementation method: $F_{(5,1078)}$ = 50,034, $p$ = 0.0000, se ± 0.011082 (CSy), se ± 0.012565 (CSt), and se ± 0.013572 (C); days of supplementation: $F_{(5,1078)}$ = 50,034, $p$ = 0.0000, se ± 0.019194 (1st–35th days), se ± 0.033245 (42nd day and 56th day), and se ± 0.023508 (49th day); supplementation method × days of supplementation: $F_{(11,1078)}$ = 2952.4, $p$ = 0.0000, and se ± 0.033245). The chart shows statistical averages. The standard deviations for the means in this plot ranged from 0.10133 to 0.35040.

## 4. Discussion

Hemp extract visibly increased the activity of antioxidant enzymes in the bees that were fed with this additive, compared to in the bees in the control group.

The enzymes indicated in our work, i.e., SOD, GST, CAT, GPx, and TAC, are key to the defense of the organism of invertebrates against ROS, and as a consequence of the action of free radicals, they combat oxidative stress. Significant differences between both experimental groups and the control group in activities were demonstrated in the SOD, CAT, and GPx enzymes, which constitute the primary internal antioxidants that complete the defense system. SOD is responsible for the first transformation of free radicals into hydrogen peroxide and oxygen molecules. The hydrogen peroxide formed in the reaction is then converted by CAT into molecular oxygen and water. The enzyme GPx, which has the ability to reduce both organic and inorganic peroxides, also takes part in the reduction of hydrogen peroxide. GST is involved in detoxification by catalyzing the fusion reaction of glutathione with other endogenous and exogenous compounds (e.g., xenobiotics) [13]. TAC gives information about the total nonenzymatic antioxidant capacity of the capability to counteract ROS.

The increase in the activities of antioxidant enzymes could be caused by the influence of CBD on the permeability of ion channels, i.e., potassium, sodium, and calcium, and therefore change in the cell membrane environment. Changes in ion concentration may induce an increase in antioxidants and thus also induce an increase in catalase, which is synthesized in the liver of mammals, depending on the amount of $H_2O_2$, the level of which relies on the amount of calcium in the cytosol [32,33]. High concentrations of antioxidants in insects are usually observed in metabolically active tissues, i.e., the fat body that functions as the liver. This may indicate that the synthesis of antioxidants may be dependent on the processes that take place in vertebrates in the organs equivalent to those of invertebrates [9].

On the other hand, many studies suggest that an increased mRNA expression of genes corresponding to the antioxidant activity in insects depends on external factors (including stress factors) that cause ROS formation. We suggest that the activities of antioxidants are modified and depend on the number of oxidizing agents with which the organisms of insects meet. This may suggest that by administering a foreign substance (cannabis) to the bees, we caused a temporary increase in oxidative stress. This enabled us to stimulate higher mRNA expression in the early stages of life, which in the later age of the bees allowed for a better/more intense reaction to standard ROS (higher enzyme activities in the early stage of life) [34,35].

The antioxidant activity of cannabis has so far been described in studies that used seeds and oils. The studies on the hydrolyzate of proteins from hemp seeds (obtained during in vitro digestion) showed significant antioxidant properties (up to 67% of the radical scavenging activity, i.e., 2,2-diphenyl-1-picrylhydrazyl), metal chelating (activity up to 94%), and $Fe^{3+}$ reduction. It owes its properties to the peptide fractions: Trp-Val-Tyr-Tyr (tetrapeptide) and Pro-Ser-Leu-Pro-Ala (pentapeptide). Additionally, fractionated peptides showed higher chelating activity than glutathione in studies [36,37]. Such a strong radical scavenging process may be related to the extension of the life of bees in our experiment (given the great antioxidant effects for various forms of the processed hemp raw materials). Similar properties have been demonstrated for the essential oil in relation to 2,2-diphenyl-1-picrylhydrazyl, and β-carotene/linoleic acid tests and $Fe^{3+}$-reducing properties have been reported [38]. Hemp owes much of its properties to the active substances from the cannabinoid group. The antioxidant potential of the oils has been confirmed and measured in tests in which the main active substances were CBD, THC, or compounds from the group of flavonoids and terpenes [39]. Due to the use of an oil consisting mainly of CBD in our research, we looked for experimental results of this compound in the literature. Hacke et al. [16] showed a remarkable antioxidant activity for CBD during spectrophotometric and electrochemical analysis, which we also showed in our experiment with the bees consuming hemp extract [16]. During the research, the activity of this active compound was compared with the properties of other antioxidant compounds, i.e., resveratrol. Resveratrol has also been tested in bee supplementation and has also shown a positive effect on antioxidant activity. Interestingly, according to the research, CBD has a stronger scavenging capacity for 2,2-diphenyl-1-picrylhydrazyl radicals than pure THC. CBD owes its strong action to its chemical structure: it has two phenolic groups responsible for the antiradical function [16]. Phenolic antioxidants (group of polyphenols) create nonradical products as a result of their transformation from superoxide radicals [40]. Such strong activities of antioxidants in the hemolymph of the bees fed with the supplement can multiply/intensify the beneficial effects of phenolic groups and thus extend the life of these pollinators.

The antioxidant activities of compounds belonging to polyphenols were also demonstrated by other compounds used in bee supplementation, i.e., the previously mentioned curcumin, coenzyme Q10, caffeine, resveratrol, and piperine [13,14,41,42]. All publications have reported a positive effect of plant metabolites on increasing the activities of the antioxidant system enzymes and/or significantly extending the life of bees in the groups fed with the supplement. The trends observed in the cannabis extract tests are similar to the rest of the tests conducted by Strachecka on curcumin, coenzyme Q10, caffeine, and piperine [11,13,41,42]. The addition of hemp extract stood out from other studies as it extended life up to day 56, while other publications report 48 days for turmeric, 41 days for piperine, and 38 days for coenzyme Q10. Additionally, in our study, we tested two key supplementation methods used in laboratories (syrup in a syringe) and in hives on an apiary (strips). The purpose of using two methods was to determine whether the method of administration had an effect on the performance of the biostimulator. As it was found, the syringe method of supplementation had better results compared to the strip method. We suspect that this is related to the longer time it takes for the extract from the strip to enter the bees' organism. The positive effect was visible in insects consuming cannabis directly through the syrup, which allowed for the maximum use of its potential and direct

distribution in the body. The extract placed on the strips probably made its way into the bees' digestive system and then to other tissues by steaming and sticking to the particles of the bodies of bees that then were licked by other workers during trophallaxis.

## 5. Conclusions

Hemp extract significantly increased the activity of antioxidant enzymes, extending the life of bees to 49 days (for the strip method) and 56 days (for the syringe method). In addition, we showed that a faster and stronger effect was obtained during supplementation in syrup in syringes, where the activities for the enzymes SOD, CAT, GPx, GST, and TAC were the highest. Thanks to this, we believe that hemp extract can in the future contribute to the improvement of the natural immunity of honey bees and help them with the fight against environmental pollution and the increase of oxidative stress.

**Author Contributions:** P.S., creation of the idea, implementation of analyses, the scheme of conducting the experiment, conceptualization, methodology, writing of the first draft preparation; A.S., creation of the idea, selection of the methodology, review, and editing; Ł.W., assistance in carrying out research. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was financed from the funds allocated to the implementation of the research within the Doctoral School on University of Life Sciences in Lublin: SD/25/ZiR/2021.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets and materials that have been used, analyzed and presented in this manuscript are not publicly available but available at the University of Life Sciences in Lublin. At a justified request of the interested party, they may be made available by the corresponding author.

**Acknowledgments:** We thank Aleksandra Splitt (the National Institute of Horticultural Research, Apiculture Division in Puławy, Institute of Nature Conservation, Polish Academy of Sciences) for help in preparing the experiment.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Le Conte, Y.; Navajas, M. Climate change: Impact on honey bee populations and diseases. *OIE Rev. Sci. Tech.* **2008**, *27*, 485–510. [CrossRef]
2. Lacetera, N. Impact of climate change on animal health and welfare. *Anim. Front.* **2019**, *9*, 26–31. [CrossRef] [PubMed]
3. Hristov, P.; Shumkova, R.; Palova, N.; Neov, B. Factors Associated with Honey Bee Colony Losses: A Mini-Review. *Vet. Sci.* **2020**, *7*, 166. [CrossRef] [PubMed]
4. Strachecka, A.; Olszewski, K.; Paleolog, J. *Varroa* treatment with bromfenvinphos markedly suppresses honeybee biochemical defence levels. *Entomol. Exp. Appl.* **2016**, *160*, 57–71. [CrossRef]
5. Higes, M.; Martín-Hernández, R.; Garrido-Bailón, E.; González-Porto, A.V.; García-Palencia, P.; Meana, A.; Del Nozal, M.J.; Mayo, R.; Bernal, J.L. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. *Environ. Microbiol. Rep.* **2009**, *1*, 110–113. [CrossRef] [PubMed]
6. Aizen, M.A.; Harder, L.D. The Global Stock of Domesticated Honey Bees Is Growing Slower than Agricultural Demand for Pollination. *Curr. Biol.* **2009**, *19*, 915–918. [CrossRef] [PubMed]
7. Araujo, J.A. Particulate air pollution, systemic oxidative stress, inflammation, and atherosclerosis. *Air Qual. Atmos. Health* **2011**, *4*, 79–93. [CrossRef] [PubMed]
8. Birch-Machin, M.A.; Bowman, A. Oxidative stress and ageing. *Br. J. Dermatol.* **2016**, *175*, 26–29. [CrossRef] [PubMed]
9. Felton, G.W.; Summers, C.B. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.* **1995**, *29*, 187–197. [CrossRef] [PubMed]
10. Strachecka, A.; Łoś, A.; Filipczuk, J.; Schulz, M. Individual and social immune mechanisms of the honey bee. *Med. Weter.* **2018**, *74*, 426–433.
11. Strachecka, A.; Olszewski, K.; Paleolog, J.; Borsuk, G.; Bajda, M.; Krauze, M.; Merska, M.; Chobotow, J. Coenzyme Q10 treatments influence the lifespan and key biochemical resistance systems in the honeybee, *Apis mellifera*. *Arch. Insect Biochem. Physiol.* **2014**, *86*, 165–179. [CrossRef]
12. Ricigliano, V.A.; Simone-Finstrom, M. Nutritional and prebiotic efficacy of the microalga *Arthrospira platensis* (spirulina) in honey bees. *Apidologie* **2020**, *51*, 898–910. [CrossRef]
13. Strachecka, A.; Olszewski, K.; Paleolog, J. Curcumin stimulates biochemical mechanisms of *Apis mellifera* resistance and extends the apian life-span. *J. Apic. Sci.* **2015**, *59*, 129–141. [CrossRef]

14. Rascón, B.; Hubbard, B.P.; Sinclair, D.A.; Amdam, G.V. The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging* **2012**, *4*, 499–508. [CrossRef]
15. Farjan, M.; Dmitryjuk, M.; LipiŃski, Z.; Biernat-ŁOpieŃska, E.; Zółtowska, K. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera carnica* brood at different stages. *J. Apic. Res.* **2012**, *51*, 263–270. [CrossRef]
16. Hacke, A.C.M.; Lima, D.; de Costa, F.; Deshmukh, K.; Li, N.; Chow, A.M.; Marques, J.A.; Pereira, R.P.; Kerman, K. Probing the antioxidant activity of Δ9-tetrahydrocannabinol and cannabidiol in *Cannabis sativa* extracts. *Analyst* **2019**, *144*, 4952–4961. [CrossRef]
17. Yadav, M.; Jain, S.; Tomar, R.; Prasad, G.B.K.S.; Yadav, H. Medicinal and biological potential of pumpkin: An updated review. *Nutr. Res. Rev.* **2010**, *23*, 184–190. [CrossRef] [PubMed]
18. Tagne, A.M.; Fotio, Y.; Lin, L.; Squire, E.; Ahmed, F.; Rashid, T.I.; Azari, E.K.; Piomelli, D. Palmitoylethanolamide and hemp oil extract exert synergistic anti-nociceptive effects in mouse models of acute and chronic pain. *Pharmacol. Res.* **2021**, *167*, 105545. [CrossRef] [PubMed]
19. Benson, M.J.; Abelev, S.V.; Connor, S.J.; Corte, C.J.; Martin, L.J.; Gold, L.K.; Suraev, A.S.; McGregor, I.S. Medicinal *Cannabis* for inflammatory bowel disease: A survey of perspectives, experiences, and current use in australian patients. *Crohn's Colitis 360* **2020**, *2*, otaa015. [CrossRef]
20. Reithmeier, D.; Tang-Wai, R.; Seifert, B.; Lyon, A.W.; Alcorn, J.; Acton, B.; Corley, S.; Prosser-Loose, E.; Mousseau, D.D.; Lim, H.J.; et al. The protocol for the Cannabidiol in children with refractory epileptic encephalopathy (CARE-E) study: A phase 1 dosage escalation study. *BMC Pediatr.* **2018**, *18*, 221. [CrossRef] [PubMed]
21. Amin, M.R.; Ali, D.W. Pharmacology of Medical *Cannabis*. In *Advances in Experimental Medicine and Biology*; Springer: New York, NY, USA, 2019; Volume 1162, pp. 151–165.
22. Blessing, E.M.; Steenkamp, M.M.; Manzanares, J.; Marmar, C.R. Cannabidiol as a Potential Treatment for Anxiety Disorders. *Neurotherapeutics* **2015**, *12*, 825–836. [CrossRef] [PubMed]
23. Ashton, C.H. Pharmacology and effects of cannabis: A brief review. *Br. J. Psychiatry* **2001**, *178*, 101–106. [CrossRef] [PubMed]
24. Di Giacomo, V.; Recinella, L.; Chiavaroli, A.; Orlando, G.; Cataldi, A.; Rapino, M.; Di Valerio, V.; Politi, M.; Antolini, M.D.; Acquaviva, A.; et al. Metabolomic profile and antioxidant/anti-inflammatory effects of industrial hemp water extract in fibroblasts, keratinocytes and isolated mouse skin specimens. *Antioxidants* **2021**, *10*, 44. [CrossRef] [PubMed]
25. Marini, E.; Magi, G.; Ferretti, G.; Bacchetti, T.; Giuliani, A.; Pugnaloni, A.; Rippo, M.R.; Facinelli, B. Attenuation of *Listeria monocytogenes* virulence by *Cannabis sativa* L. Essential oil. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 293. [CrossRef] [PubMed]
26. Wilson, J.L. Biochemistry; Third edition (Stryer, Lubert). *J. Chem. Educ.* **1988**, *65*, A337. [CrossRef]
27. Karihtala, P.; Soini, Y. Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. *APMIS* **2007**, *115*, 81–103. [CrossRef] [PubMed]
28. Nunes, B.; Simões, M.I.; Navarro, J.C.; Castro, B.B. First ecotoxicological characterization of paraffin microparticles: A biomarker approach in a marine suspension-feeder, *Mytilus* sp. *Environ. Sci. Pollut. Res.* **2020**, *27*, 41946–41960. [CrossRef]
29. Nakagawa, Y. Role of Mitochondrial Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) as a Antiapoptotic Factor. *Biol. Pharm. Bull.* **2004**, *27*, 956–960. [CrossRef]
30. Skowronek, P.; Wójcik, Ł.; Strachecka, A. Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (*Apis mellifera*) Workers. *Animals* **2021**, *11*, 2190. [CrossRef]
31. Łoś, A.; Strachecka, A. Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution. *Sensors* **2018**, *18*, 1494. [CrossRef]
32. Yang, T.; Poovaiah, B.W.; Ryan, C.A. Hydrogen peroxide homeostasis: Activation of plant catalase by calciumcalmodulin. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4097–4102. [CrossRef] [PubMed]
33. Watkins, A.R. Cannabinoid interactions with ion channels and receptors. *Channels* **2019**, *13*, 162–167. [CrossRef] [PubMed]
34. Corona, M.; Hughes, K.A.; Weaver, D.B.; Robinson, G.E. Gene expression patterns associated with queen honey bee longevity. *Mech. Ageing Dev.* **2005**, *126*, 1230–1238. [CrossRef] [PubMed]
35. Mittapalli, O.; Neal, J.J.; Shukle, R.H. Antioxidant defense response in a galling insect. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1889–1894. [CrossRef] [PubMed]
36. Girgih, A.T.; He, R.; Malomo, S.; Offengenden, M.; Wu, J.; Aluko, R.E. Structural and functional characterization of hemp seed (*Cannabis sativa* L.) protein-derived antioxidant and antihypertensive peptides. *J. Funct. Foods* **2014**, *6*, 384–394. [CrossRef]
37. Girgih, A.T.; Udenigwe, C.C.; Aluko, R.E. In Vitro Antioxidant Properties of Hemp Seed (*Cannabis sativa* L.) Protein Hydrolysate Fractions. *J. Am. Oil Chem. Soc.* **2011**, *88*, 381–389. [CrossRef]
38. Nafis, A.; Kasrati, A.; Jamali, C.A.; Mezrioui, N.; Setzer, W.; Abbad, A.; Hassani, L. Antioxidant activity and evidence for synergism of *Cannabis sativa* (L.) essential oil with antimicrobial standards. *Ind. Crops Prod.* **2019**, *137*, 396–400. [CrossRef]
39. Gruschow, A. A Comparison of Antioxidant Potential, Total Phenolic Content, and Cannabidiol (CBD) Content of *Cannabis* Infused Hemp, MCT, and Olive Oils. Master's Thesis, Montclair State University, Montclair, NJ, USA, 2020.
40. Ziaja, P. Aktywność Antyoksydacyjna Kaliks[4]rezorcynarenów i Kaliks[4]pirogallolarenów. *UW Repository*. Available online: http://www.depotuw.ceon.pl/handle/item/419 (accessed on 3 February 2022).

41. Strachecka, A.; Krauze, M.; Olszewski, K.; Borsuk, G.; Paleolog, J.; Merska, M.; Chobotow, J.; Bajda, M.; Grzywnowicz, K. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochemistry* **2014**, *79*, 1192–1201. [CrossRef]
42. Schulz, M.; Łoś, A.; Grzybek, M.; Ścibior, R.; Strachecka, A. Piperine as a new natural supplement with beneficial effects on the life-span and defence system of honeybees. *J. Agric. Sci.* **2019**, *157*, 140–149. [CrossRef]

*animals*

MDPI

*Article*

# CBD Supplementation Has a Positive Effect on the Activity of the Proteolytic System and Biochemical Markers of Honey Bees (*Apis mellifera*) in the Apiary

**Patrycja Skowronek *, Łukasz Wójcik and Aneta Strachecka**

Department of Invertebrate Ecophysiology and Experimental Biology, University of Life Sciences in Lublin, Akademicka 13, 20950 Lublin, Poland

* Correspondence: patrycja.skowronek@up.lublin.pl

**Simple Summary:** The purpose of our study was to determine how CBD extract influences resistance in the hemolymph (insect blood) of honey bees in the hive test. The bees were divided into 3 groups: (CSy) bees fed with CBD in sugar syrup; (CSt) cotton strip with CBD placed in hive, (C) control bees fed sugar syrup. To determine the state of immunity, we used the analysis of the activity of the proteolytic system and biochemical markers, such as "liver tests", and the concentration of selected ions and key compounds for the functioning of the organism. CBD extract increased the total protein concentration, proteases and their inhibitor activities in each age (except for acidic protease activities in the 21st and 28th day and alkaline protease inhibitor activities in the 28th day in the CSt group), increased concentrations of markers: ALP, AST, ALT; and glucose; triglycerides; cholesterol and creatinine. A decrease in concentration in experimental groups was noticed for urea acid and albumin compared to group C. Higher activities/concentrations of most of parameters were obtained in the CSy compared to the CSt and C. The CBD supplementation can positively influence bees' resistance.

**Abstract:** We examined how CBD extract influences the activity of the immune system in the hemolymph of honey bees in the hive test. The bees were divided into 3 groups: (CSy) bees fed with CBD in sugar syrup with glycerin; (CSt) cotton strip with CBD placed in hive bees fed pure sugar syrup, (C) control bees fed sugar syrup with glycerin. CBD extract increased the total protein concentrations, proteases and their inhibitor activities in each age (the except for acidic protease activities in the 21st and 28th day and alkaline protease inhibitor activities in the 28th day in CSt group) in comparison with group C. In the groups with the extract there was also an increase in the enzymatic marker activities: ALP, AST (decrease on day 28 for CSt), ALT; and non-enzymatic marker concentrations: glucose; triglycerides; cholesterol and creatinine. The urea acid and albumin concentrations were lower in CSy and CSt groups compared to the C group (higher concentration of albumin was displayed by control bees). Higher activities/concentrations of most of biochemical parameters were obtained in the CSy compared to the CSt and C. CBD supplementation can positively influence workers' immune system.

**Keywords:** hemp extract; honey bee resistance; immunology; pollinators; cannabidiol; hemp oil; biochemistry; metabolism; biochemical pathway



**Citation:** Skowronek, P.; Wójcik, Ł.; Strachecka, A. CBD Supplementation Has a Positive Effect on the Activity of the Proteolytic System and Biochemical Markers of Honey Bees (*Apis mellifera*) in the Apiary. *Animals* **2022**, *12*, 2313. https://doi.org/10.3390/ani12182313

Academic Editors: Miroslava Kačániová and Klaus Eder

Received: 26 July 2022
Accepted: 1 September 2022
Published: 6 September 2022



## 1. Introduction

Nowadays, scientists report that we are witnessing a global ecological crisis [1]. This crisis concerns changes in the environment that affect not only people, but other living organisms, including those that, thanks to their unique biology, act as pillars stabilizing the ecosystem. An important biotic element is insects, which constitute one of the most diverse and numerous groups of animals. Among them we distinguish very useful insects, which additionally remain in close relationship with people. One of such insects is honey bees.

Bees are responsible for pollination of approx. 70–80% of plants, the fruit/crops of which are part of the diet of humans and livestock [2]. Bees that have been kept in breeding since ancient times also provide products widely used in diet, pharmaceuticals and cosmetology, such as: honey, propolis, wax and royal jelly [3]. This confirms their inter-disciplinary role in the life cycle on Earth.

Recently, more and more scientific reports confirm the high decline in the population of honeybees as a result of climate change, environmental chemisation (pesticides), homogenization of agriculture (creation of monocultures, reduction of the diversity of bee food), human errors during bee breeding (treatment with bad agents, poor hygiene, adulteration wax) and other natural CCD (colony collapse disorder) etiological factors [4–6]. These above-mentioned factors caused a weakened immune system of pollinators and more susceptibility to the ingress of unwanted guests especially like: Nosema spp., *Varroa destructor* and other diseases such as rot (*Paenibacillus larvae*), viruses (deformed wings virus), etc. [4,7,8].

One of the key immune barriers responsible for dealing with pathogens is the proteolytic system, which consists of proteolytic enzymes (proteases) and protease inhibitors. Proteases are responsible for cutting the proteins of pathogens entering the bee's organism. They are also responsible for digestion in lysosomes, activate proenzymes and prohormones, proliferative, fertile and apoptotic (cysteine proteases) and are involved in apoptosis, necrosis, virulence of microorganisms and digestion in lysosomes (metalloproteases) [9,10]. Protease inhibitors inhibit the action/protease activation of unwanted organisms [11,12]. Proteases and their inhibitors are found in one of the key tissues of bees: the hemolymph, which acts as the 'blood' of insects. The second key tissue related to immunity is the fat body, where most immune proteins are synthesized and the metabolic processes responsible for the proper functioning and development of insects take place. The fat body is the liver of insects. Proteins and other metabolites/substances/compounds from the fat body are transferred to the hemolymph where they are distributed throughout the body [13,14]. Therefore, laboratory 'imaging' of the hemolymph is one of the key indicators in determining the health of bees. In addition to the proteolytic system, in the hemolymph, we can examine the activity, concentrations of, e.g., enzymes such as alanine aminotransferase (ALT), aspartate (AST) and alkaline phosphatase (ALP), which reflect the functioning and damage of liver cells in bees of the fat body.

Due to reports of weakened bee immunity, many studies have focused on looking for agents that will naturally support these systems [15]. By enhancing the health of bees, they are expected to be able to deal with many factors at once. Recently, substances of natural (usually plant) origin, which can act as an immune biostimulator, have become very popular. Usually, the selected substances are characterized by a proven health-promoting effect, thanks to the content of active substances. In the case of bees, substances/compounds/products were tested, i.e., curcumin, vitamin C, caffeine, piperine, spirulina, coenzyme Q10, resveratrol, pollen substitutes, antiseptic herbal mixtures, yeast and even natural silage (as a source of *Lactobacillus*) [12,15–22]. Recently, we can also include hemp extracts (research carried out by our team) among the tested substances. Most of the mentioned biostimulants prolonged the life of bees by up to 33–38% (resveratrol, caffeine) [17,20]. In addition, some of the tested substances had a proven positive effect on the stimulation of the immune system by increasing the activity of the proteolytic system and the content of immune proteins (hemp, curcumin, coenzyme Q10) and antioxidant enzymes (coenzyme Q10, hemp, piperine, vitamin c) [12,15,16,18,23]. Coenzyme Q10 additionally increased the concentration of lipids in the body and of ions such as magnesium and calcium. Higher lipid levels were noted also after feeding bees with spirulina [19]. Caffeine, in addition, had a positive effect in the case of bees infected with *Nosema* spp. [17].

Despite the large range of tested substances, it has not been possible to find one effective agent that meets the needs of modern bee breeding and meets the strict requirements for the safety of its use. The gap can be filled with hemp, which has a strong antioxidant effect due to the content of active substances from the cannabinoid group [24–26]. The

positive effects of hemp extracts have been described many times in relation to diseases such as depression, epilepsy, Alzheimer's, appetite disorders, as an aid in the treatment of cancer and in multiple sclerosis [27–31]. There are also interesting studies in which the hemp extract helped to regenerate damaged brain tissues in rats, damaged as a result of the action of chemical agents [32]. Rats that took the extract showed faster regeneration than those that did not take the preparation.

In the case of tests on invertebrates, in our previous studies we have shown that bees fed with hemp extract in a cage experiment showed a positive increase in immunity by stimulating the proteolytic and antioxidant system (growth recorded for all tested antioxidants) [23,26]. Taking into account our previous research and the results obtained showing the main effects of cannabidiol (CBD), for further testing we chose a commercial preparation containing the active ingredient CBD with a known concentration, which is one of the most described and tested active substances of cannabis along with THC (tetrahydrocannabinol) (THC has not been tested due to its psychoactive effect and legal conditions). We also added selected biochemical markers to the research in this paper, the results of which may help determine the effect of CBD on changes in the directions of metabolism in bees. The selected markers include: lipid compounds, i.e., cholesterol, triglycerides (the main compounds of the fat body), sugar: glucose (carbohydrate pathways and transformations take place in the fat body and affect the level of energy and hunger in bees), protein: albumin (transport of substances, buffer properties and anti-inflammatory effect), and metabolites of the end pathways, i.e., uric acid, creatinine (the concentration of metabolites gives information about the way the body is dealing with-metabolize the supplied supplements) [14,16,17]. Additionally, to get real results from the natural habitat of bees, we transferred our experiment from cage conditions to the beekeeping environment.

We assumed in the research that CBD extract has a positive effect on the immunity of honeybees by stimulating the proteolytic system and has a positive effect on the parameters of "liver" enzymes.

The aim of our study was to determine the activities of the proteolytic system, "liver" enzymes and additional metabolic biomarkers in bees supplemented with CBD extract under apiary conditions.

## 2. Materials and Methods

The research was carried out using 4-frame mating hives. Bees and queens were obtained from colonies of similar strength and age. Selected colonies were not treated preventively against *Varroa destructor* in order not to disturb the natural activity of the immune system. No *Nosema* spp. infection was detected in the colonies.

### *2.1. Preparatory Activities*

#### 2.1.1. Getting Queens to Experience

Nine queens participated in the experiment (6 queens for the hive test + 3 queens to obtain 1-day-old workers). All 9 queens were sisters obtained from one source-queen. The young source-queen was caged within a queen-excluder comb-cage containing one empty comb in source-colony for egg laying. After 12 h, the source-queen was released. After 96 h, the larvae were transferred from the comb to the queen cup with the addition of mixed royal jelly in water. The future queens were placed in the queen-less colony for 7 days. Next, these queen pupae were transferred to the incubator (35 °C) for 7 days until emerged queens. Queen-sisters were placed in queen-less colonies until insemination. Nine queen-sisters were inseminated and 3 of them were placed in 3 colonies of similar strength (hives-Dadant Blatt; 20 frames; 435 × 150 mm), and 6 of them in mating hives.

#### 2.1.2. Preparation of Mating Hives

From the source-colony, fragments of combs containing different stages of larvae development were cut out and fit to the frames from the mating hives and placed in this hives. Additionally, worker bees of different ages (imago stages) were collected from the

source-colony. Workers were divided, per 200 individuals to each of the mating hives. The queen was subjected to such constructed hives (containing all stages of development) (6 queens = 6 hives). During the week we checked whether the queen had been admitted, and within a month whether she had started lay eggs After a month of the colony's functioning, 1-day-old bees were added to each of them, acquired from the 3 remaining queen-sisters in the colonies [33].

### *2.2. Obtaining and Marking of 1-Day-Old Bees*

The each of three queen-sisters were caged within a queen excluder comb-cage containing one empty comb for egg laying for 12 h, in each of the three colonies, populating one-box hives (Dadant Blatt from Łysoń Beekeeping Company, Klecza Górna, Poland; frames: 435 × 150 $mm^2$) [23]. The combs were marked and placed in their native colonies. After 20 days, these combs with broods were placed in an incubator (35 °C) that the 1-day-old bees emerged [23].

These 1-day-old workers were randomly marked with a colored oil marker depending on the assignment to the group. A total of 200 such workers were placed in each of the six mating hives. There were 2 mating hives per group (6 hives in total). The following groups were created: (1) CSy-experimental group, CBD in sugar syrup; (2) CSt-experimental group, CBD on a cotton strip; (3) C-control bees (supplemented with pure sugar syrup) [23,34].

### *2.3. Preparation and Administration of CBD Extract*

We purchased a commercial hemp extract (HempOil) in the form of an oil with a concentration of 30% (3 g in 10 mL). The oil was obtained by means of $CO_2$ extraction. CBD extract for group CSy was administered to the frameless chamber ad libitum in the 2nd, 4th and 6th days of the experiment. The oil was administered in a mixture with sugar syrup (1: 1 water with sugar) and glycerin in the ratio of 0.01:0.5:0.5 (extract: distilled water: glycerin). For the CSt group, the extract was given in a mixture with water and glycerin in a ratio of 0.8:1.5:1.5 (extract: distilled water: glycerin). Cotton strips measuring 2 by 10 cm were evenly moistened with the mixture and placed in the hives. The strip was wetted with the mixture in the 2nd, 4th and 6th days of the experiment.

### *2.4. Bees Sampling*

From each of 6 colonies, we collected 10 marked bees once for week (10 bees × 6 colonies). Workers were collected on the following experiment days: 2, 7, 14, 21, 28 (CSt, CSy, C) and 35 (CSy). In the experiment, a total of 320 bees were collected (CSt-10 bees × 2 colonies × 5 samplings; CSy-10 bees × 2 colonies × 6 samplings; C-10 bees × 2 colonies × 5 samplings).

### *2.5. Collection of Hemolymph*

The hemolymph was collected from each worker according to the methodology of Łoś and Strachecka (2018) [35]. A capillary (20 µL; 'end to end' type; without anticoagulant; Medlab Products, Raszyn, Poland) was individually inserted between the third and fourth tergite of living workers to obtain fresh hemolymph. Capillaries with the hemolymph of individual bees were placed in separate Eppendorf tubes with a capacity of 1.5 mL with a solution of 200 µL of 0.6% NaCl (10 bees = 10 tubes). Then, the material was frozen at −25 °C until biochemical analysis [35].

### *2.6. Biochemical Analyzes*

#### 2.6.1. Proteolytic System and Total Protein Concentration

The proteolytic system activities and protein concentrations were determined in the hemolymph samples using the following methods:

Total protein concentration assay with the Lowry method modified by Schacterle and Pollack (1973) [36].

Acidic, neutral and alkaline protease activities according to the Anson method (1938) modified by Strachecka et al. (2011, 2012) [37].

Acidic, neutral and alkaline natural protease inhibitor activities according to the Lee and Lin method (1995) [38].

Details of these methods are provided in Łoś and Strachecka (2018) manuscript [35].

#### 2.6.2. Metabolic Markers

In the hemolymph samples, the following biomarkers were determined using a commercial kit with modified instructions by Łoś and Strachecka (2018) [35]:

1. Enzymatic biomarkers activities, i.e., alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT);
2. Energy reserves, i.e., glucose, triacyloglycerol and cholesterol concentrations;
3. Creatinine concentration;
4. Uric acid concentration; and
5. Albumin concentration

### *2.7. Statistical Analysis*

The results were analyzed using Statistica formulas, version 13.3 (2017) for Windows, StatSoft Inc., Tusla, OK, USA. The mixed-model two-way ANOVA followed by post hoc Tukey HSD tests ($p = 0.05$) was used to compare the results for each basic immunity system parameter (total protein concentration, protease activities, protease inhibitor activities, biomarker activities/concentrations) of honey bee workers depending on the method of administration (hemp on strip and hemp in sugar syrup) and the day (2, 7, 14, 21, 28, 35 day) of supplementation with hemp extract.

## 3. Results

In most cases, groups with bees supplemented with CBD extract had higher concentrations and activities of immune parameters.

### *3.1. The Total Protein Concentration*

The total protein concentrations increased with workers' age for all groups. The protein concentration was always higher in the CSt group compared to the CSy and C groups. The lowest protein concentrations were observed in C group in all samplings, except on the seventh day, when the lowest value was in the CSt group (Figure 1).

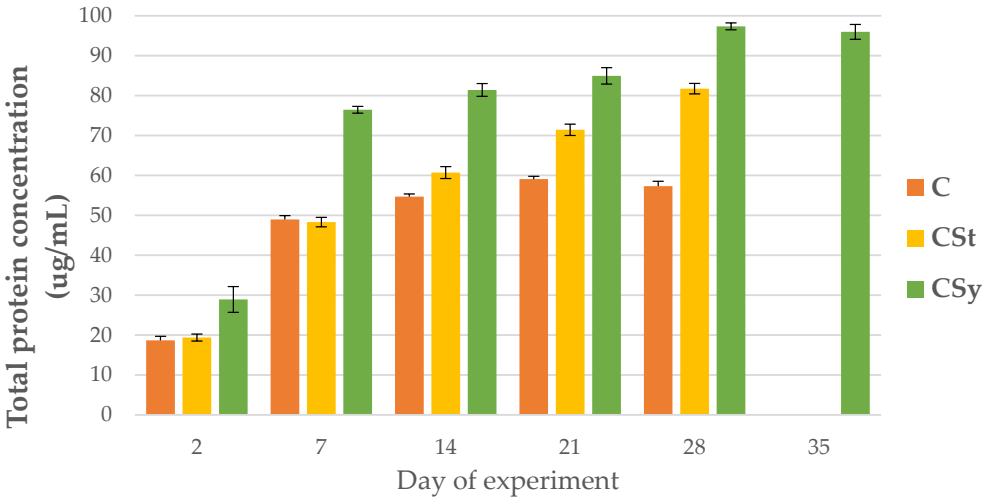



**Figure 1.** Total protein concentration in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method $F_{(2, 156)} = 31{,}397$; $p = 0.0000$, se ± 2.931476–2.649163; days of supplementation: $F_{(4, 143)} = 6677.0$, $p = 0.0000$, se ± 0.268945–0.465826; supplementation method × days of supplementation $F_{(8, 143)} = 203.83$, $p = 0.0000$, se ± 0.465826–0.491024.

### *3.2. The Proteolytic System Activities*

The addition of CBD to the syrup (CSy group) caused an unexpected increase in the activities of proteases and their inhibitors compared to the other two groups (CSt and C; Figures 2–7). These activities increased with the age of the workers in all groups, with the exception of acid proteases in C and CSt groups, in which decreased activities were noted in old bees (Figure 2).

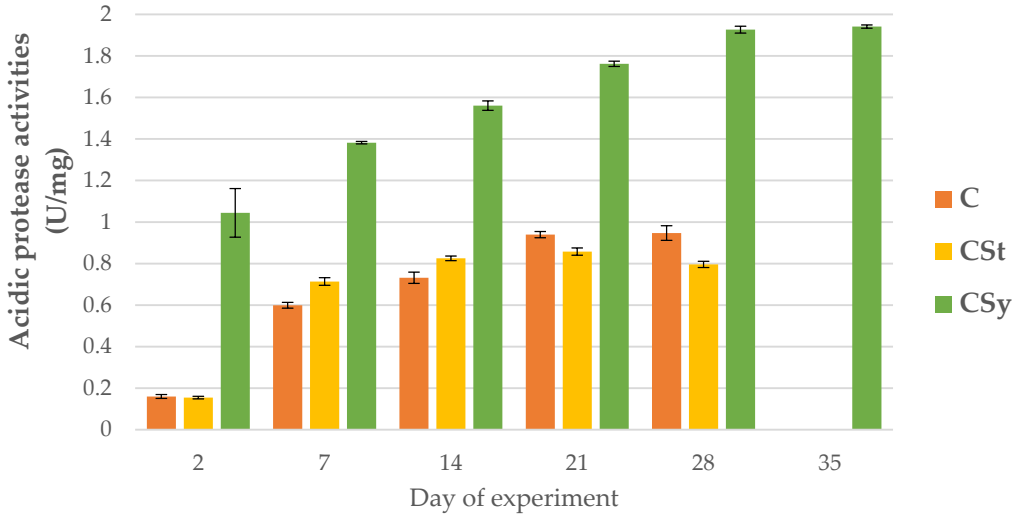



**Figure 2.** Acidic protease activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)}$ = 182,90, $p$ = 0.0000, se ± 0.042535–0.038439; days of supplementation: $F_{(4, 143)}$ = 2518.9, $p$ = 0.0000, se ± 0.006184–0.01071; supplementation method × days of supplementation: $F_{(8, 143)}$ = 71.420, $p$ = 0.0000, se ± 0.01071–0.01129.

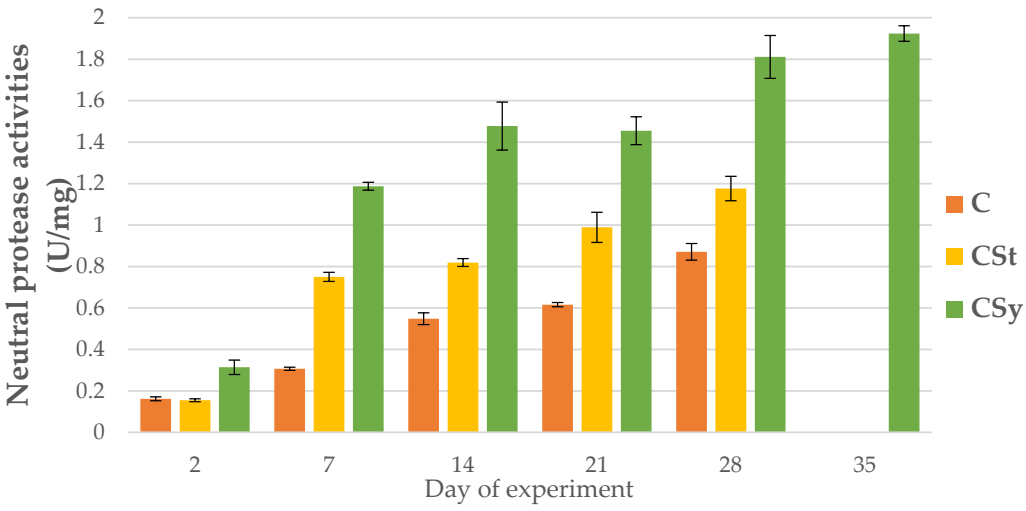



**Figure 3.** Neutral protease activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips (two-Way ANOVA) supplementation method: $F_{(2, 156)}$ = 64.957, $p$ = 0.0000, se ± 1.361555–0.500698 days of supplementation: $F_{(4, 143)}$ = 1754.7, $p$ = 0.0000, se ± 0.009558–0.016555; supplementation method × days of supplementation $F_{(8, 143)}$ = 110.82, $p$ = 0.0000, se ± 0.016555.

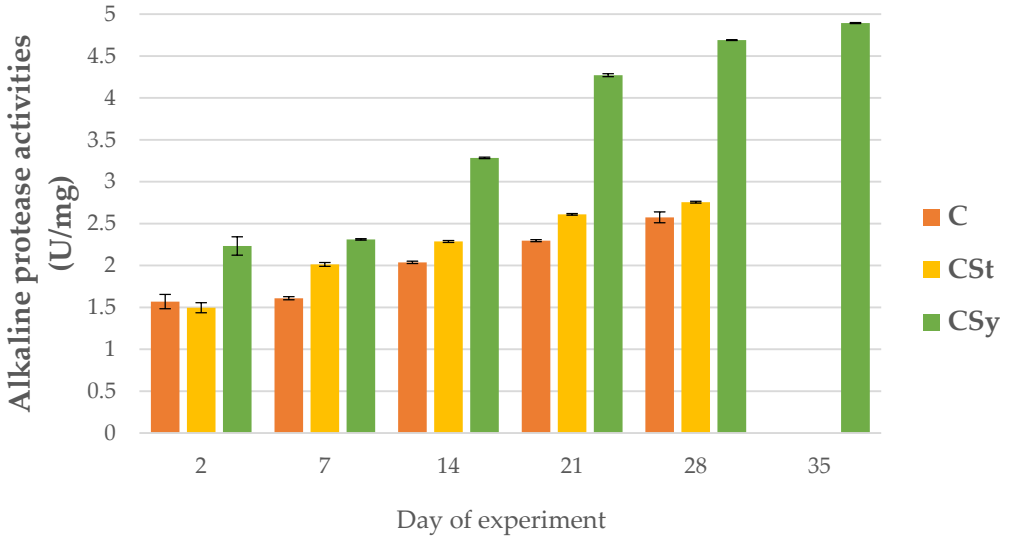



**Figure 4.** Alkaline protease activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips; two-Way ANOVA: supplementation method: $F_{(2, 156)} = 75.790$, $p = 0.0000$, se ± 0.105637–0.096433; days of supplementation: $F_{(4, 143)} = 7364.6$, $p = 0.0000$, se ± 0.007821–0.013546; supplementation method × days of supplementation $F_{(8, 143)} = 856.83$, $p = 0.00094$, se ± 0.013546.

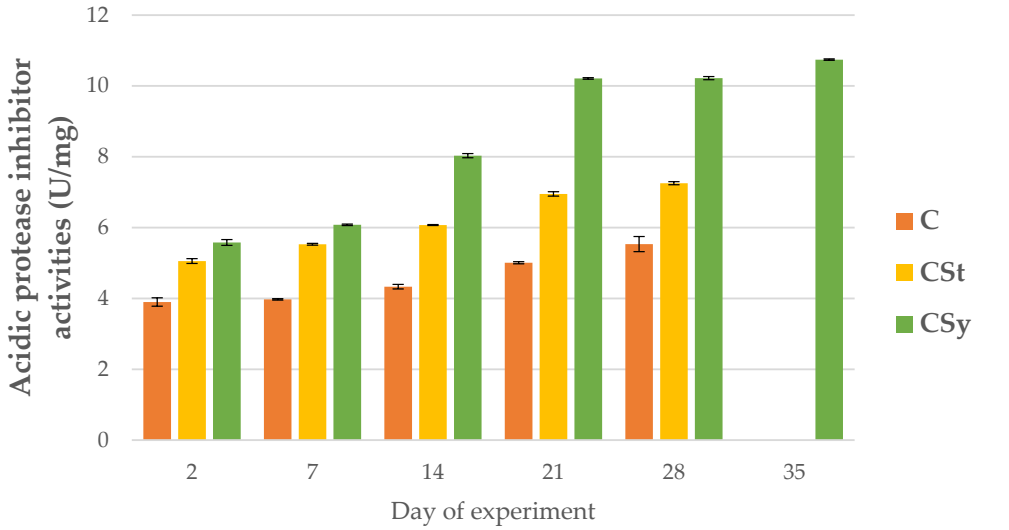



**Figure 5.** Acidic protease inhibitor activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 108.68$, $p = 0.0000$, se ± 0.201107–0.181740; days of supplementation: $F_{(4, 143)} = 8449.9$, $p = 0.0000$, se ± 0.013698–0.023726; supplementation method × days of supplementation $F_{(8, 143)} = 1230.1$, $p = 0.0000$, se ± 0.023726–0.025009.

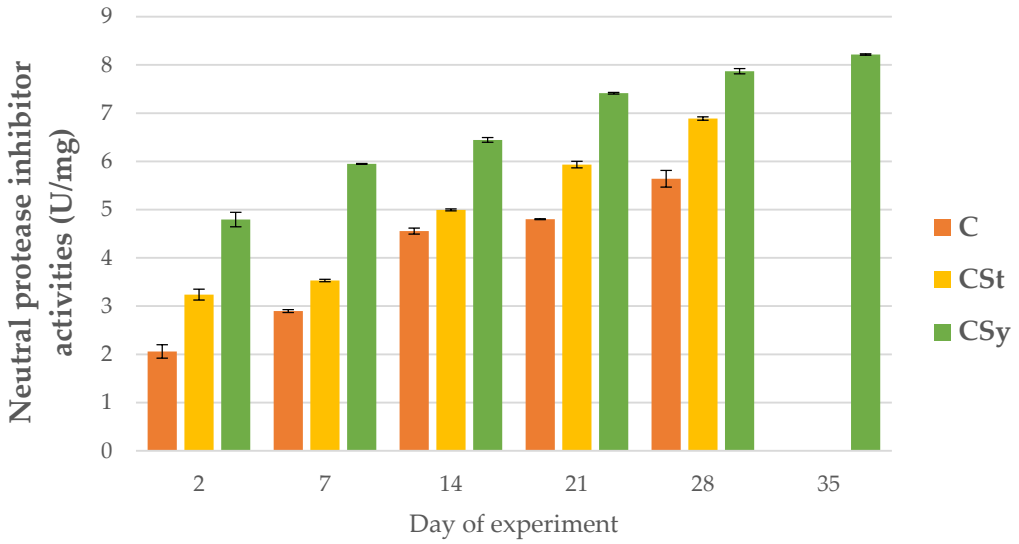



**Figure 6.** Neutral protease inhibitor activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 66.832$, $p = 0.0000$, se ± 0.185759–0.167870; days of supplementation: $F_{(4, 143)} = 8999.5$, $p = 0.0000$, se ± 0.014626–0.025333; supplementation method × days of supplementation $F_{(8, 143)} = 156.29$, $p = 0.0000$, se ± 0.025333.

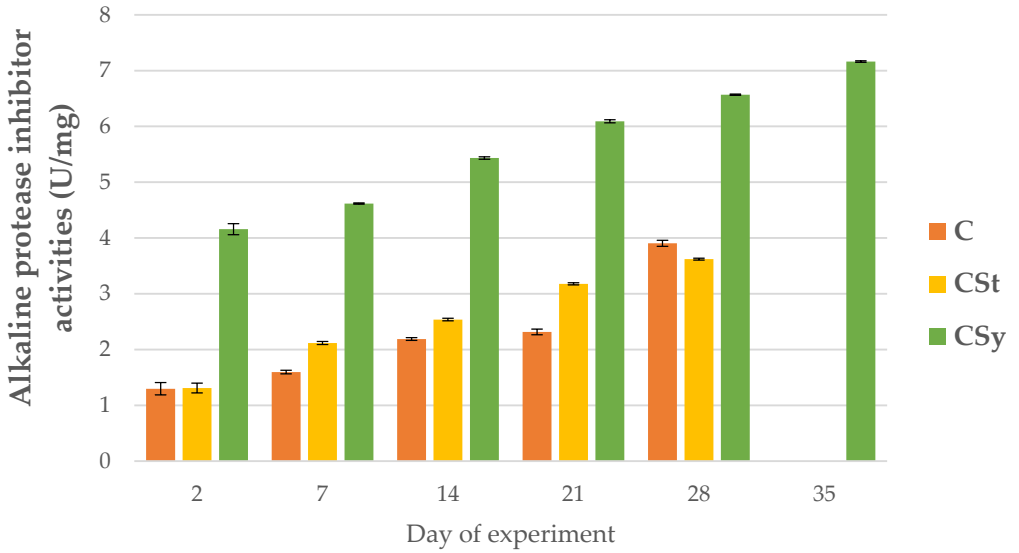



**Figure 7.** Alkaline protease inhibitor activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), CSy–hemp extract in syrup, CSt–hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 225.68$, $p = 0.0000$, se ± 0.121993–0.133636; days of supplementation: $F_{(4, 143)} = 10{,}359$, $p = 0.0000$, se ± 0.009407–0.016; supplementation method × days of supplementation $F_{(8, 143)} = 266.45$, $p = 0.0000$, se ± 0.016–0.016866.

### *3.3. The Enzymatic Biomarkers*

ALP, AST and ALT activities increased with age of the workers in all group (Figures 8–10).

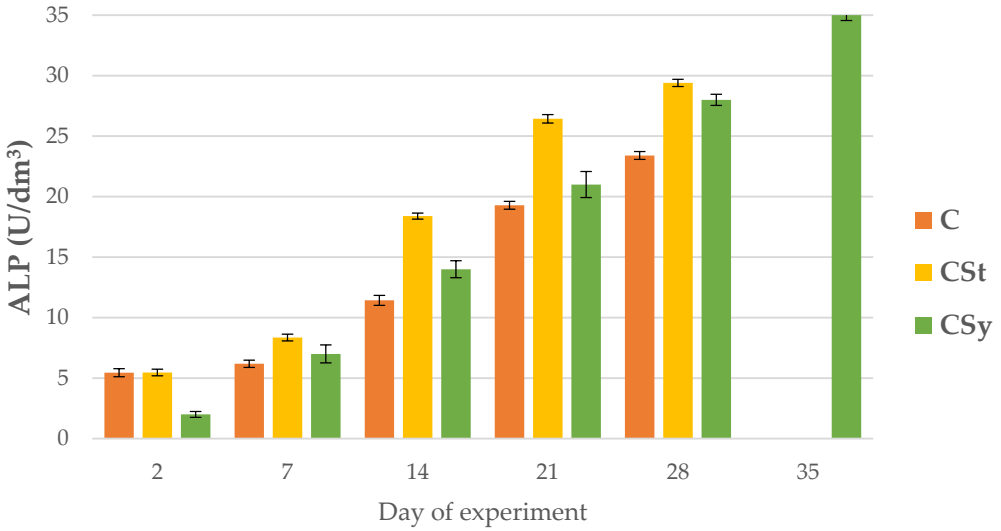



**Figure 8.** ALP activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 18.598$, $p = 0.0000$, se ± 1.275927–1.411898; days of supplementation: $F_{(4, 143)} = 13{,}672$, $p = 0.0000$, se ± 0.089307–0.151897; supplementation method × days of supplementation $F_{(8, 143)} = 300.67$, $p = 0.0000$, se ± 0.0151897.

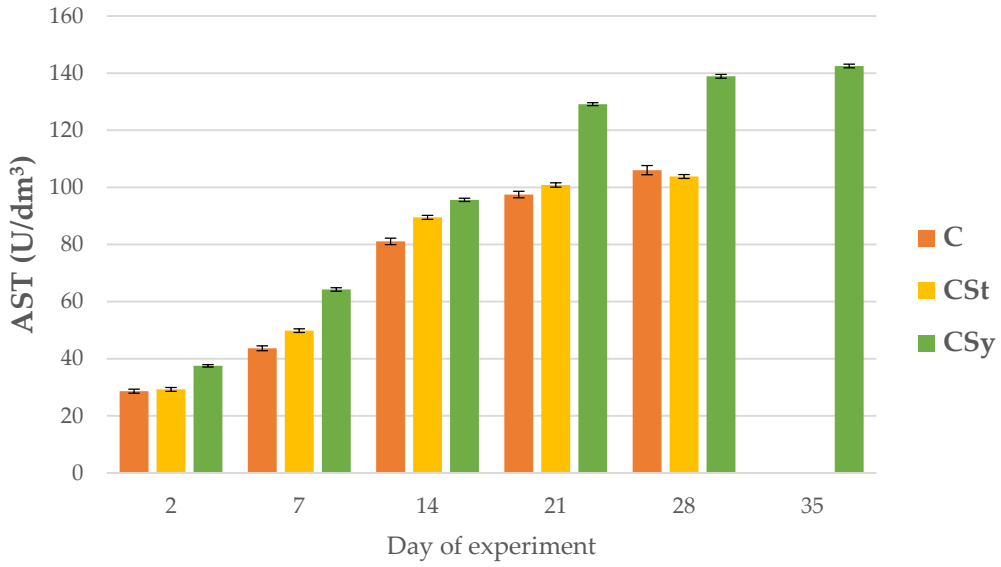



**Figure 9.** AST activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 13.130$, $p = 0.00001$, se ± 4.891108–4.420075; days of supplementation: $F_{(4, 143)} = 58{,}610$, $p = 0.0000$; se ± 0.149875–0.259592; supplementation method × days of supplementation $F_{(8, 143)} = 705.49$, $p = 0.0000$, se ± 0.2595952–0.273634.

Regardless of the method of administration (syrup or strips; CSy or CSt), CBD increased the ALP activities in the hemolymph of workers of all ages compared to the control (C) group. Between 7 and 28 days of workers' age, higher ALP activities were observed in the CSt group compared to CSy and C (Figure 8).

AST and ALT activities were higher in groups administrated with CBD compared to C group; with the highest values always being observed in the CSy group (Figures 9 and 10). AST activities in the 28-day-old workers from the CSt group were lower compared to the CSy and C groups (Figure 9).

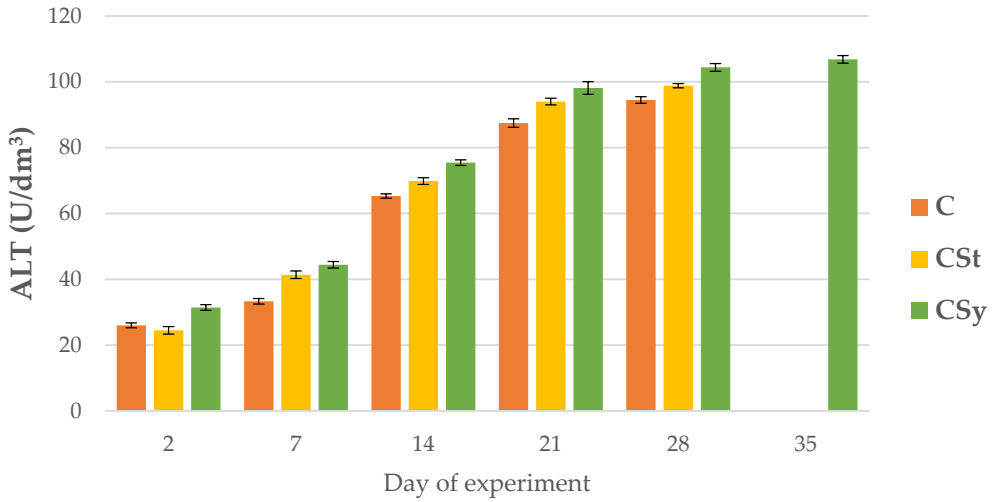



**Figure 10.** ALT activities in the 2, 7, 14, 21, 28, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 4.3070$, $p = 0.01511$, se ± 3.750347–4.150009; days of supplementation: $F_{(4, 143)} = 26349$, $p = 0.0000$, se ± 0.19505–0.337837; supplementation method × days of supplementation $F_{(8, 143)} = 30.235$, $p = 0.0000$, se ± 0.337837.

### *3.4. The Non-Enzymatic Biomarkers (Including Energy Reserves)*

The glucose concentrations in the hemolymph of the control bees decreased over the course of their lives, while in the groups supplemented with CBD they increased with the days. In the CSt group, a decrease in concentration was noted on day 28. The experimental groups showed higher glucose levels than the control. The highest glucose concentrations were in the CSy group (Figure 11).

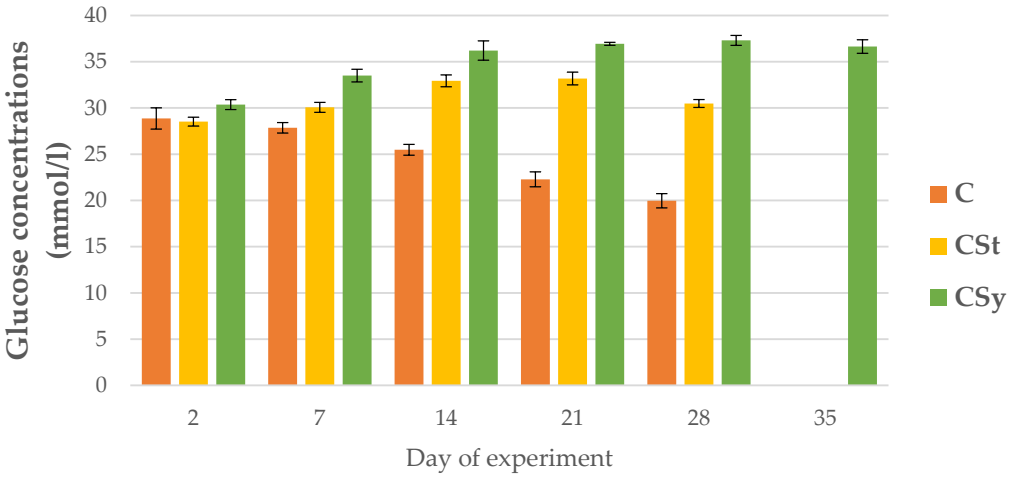



**Figure 11.** Glucose concentrations in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 195.30$, $p = 0.0000$, se ± 0.388458–0.351048; days of supplementation: $F_{(4, 143)} = 63.193$, $p = 0.0000$, se ± 0.217085–0.125334; supplementation method × days of supplementation $F_{(8, 143)} = 249.21$, $p = 0.0000$, se ± 0.217085–0.228828.

The triglyceride concentrations were higher in the groups supplemented with cannabis compared to the control group. The concentration increased with the age of the bees in all groups. The highest concentrations were recorded for the CSy group (Figure 12).

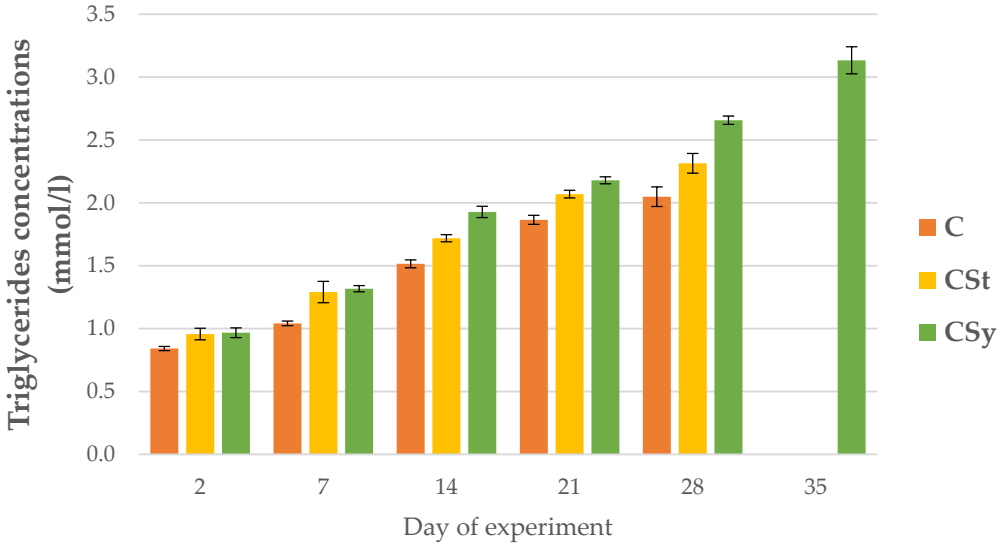



**Figure 12.** Triglyceride concentrations in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 12.927$, $p = 0.00001$, se ± 0.085425–0.077199; days of supplementation: $F_{(4, 143)} = 3683.5$, $p = 0.0000$, se ± 0.009509–0.016471; supplementation method × days of supplementation $F_{(8, 143)} = 33.426$, $p = 0.0000$, se ± 0.016471–0.017362.

A continuous increase in cholesterol concentrations was observed with the age of workers in the CSy group (Figure 13). In this group, the highest cholesterol concentrations were in relation to the other groups. For CSt and C groups, the increase in concentration continued until day 21, on day 28, the values decreased. The CSt group, despite a similar downward trend on day 28, had a higher cholesterol concentration than the C group (Figure 13).

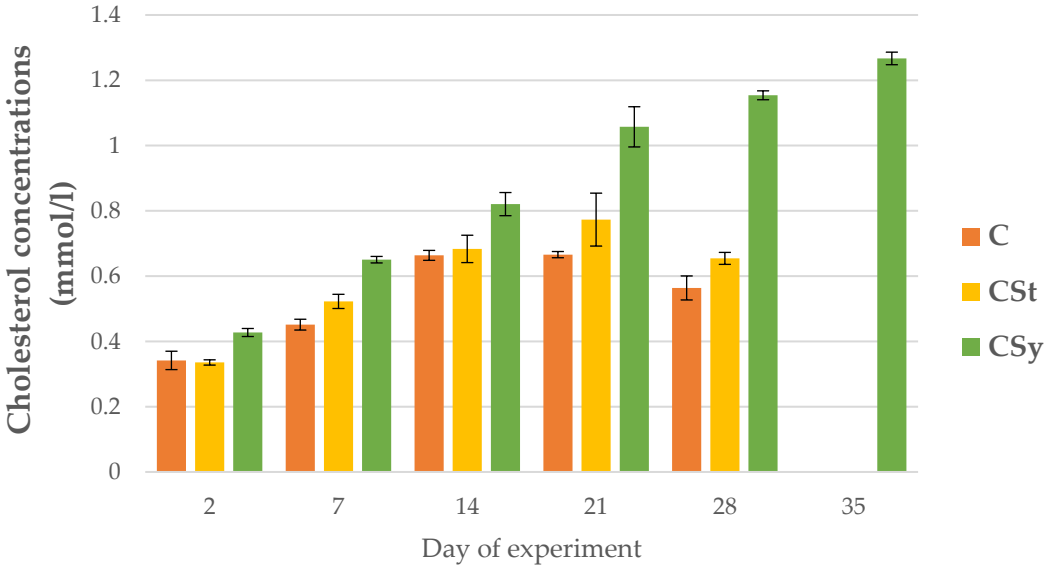



**Figure 13.** Cholesterol concentrations in 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), Csy-hemp extract in syrup, Cst-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 44.905$, $p = 0.0000$, se ± 0.027850–0.030509; days of supplementation: $F_{(4, 143)} = 997.34$, $p = 0.0000$, se ± 0.006106–0.010575; supplementation method × days of supplementation $F_{(8, 143)} = 107.76$, $p = 0.0000$, se ± 0.010575–0.11147.

The creatinine concentrations were always higher in groups administrated CBD than C group and these values were the highest in the hemolymph workers in the CSy group. The creatinine concentrations increased with the age of the bees for all groups (Figure 14).

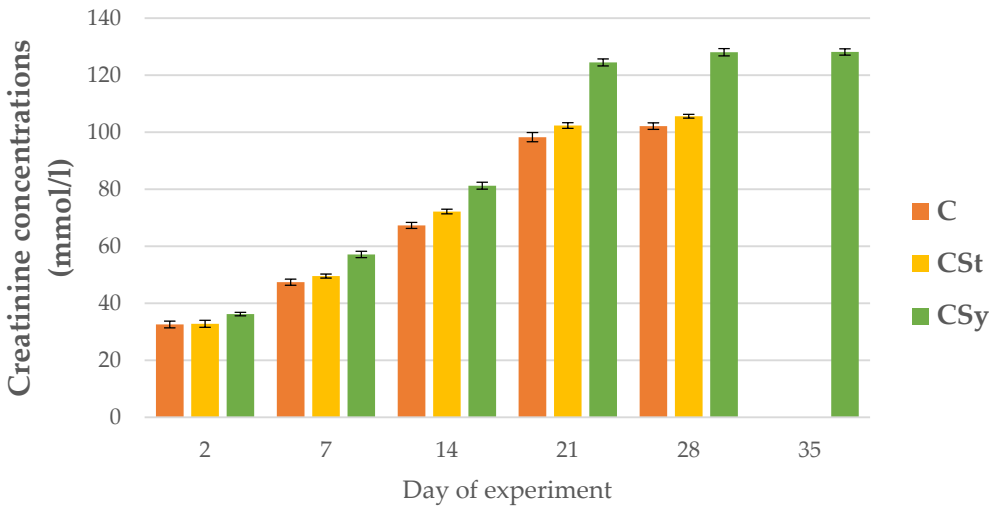



**Figure 14.** Creatinine concentrations in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 8.8603$, $p = 0.00023$, se ± 4.553192–4.114702; days of supplementation: $F_{(4, 143)} = 29{,}106$, $p = 0.0000$, se ± 0.200493–0.347264; supplementation method × days of supplementation $F_{(8, 143)} = 248.02$, $p = 0.0000$, se ± 0.347264–0.366048.

The uric acid and albumin concentrations decreased with the aging of bees for all groups (Figures 15 and 16).

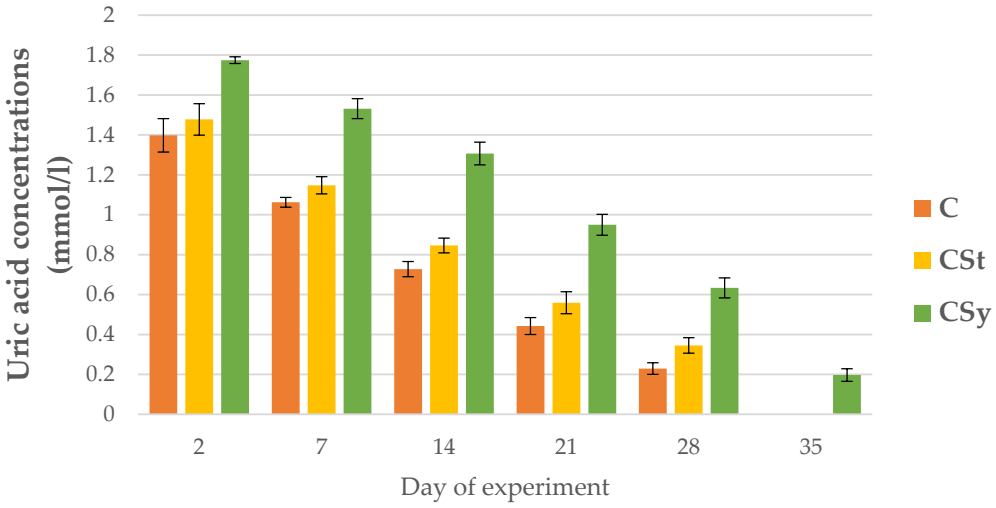



**Figure 15.** Uric acid concentrations in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 5.4955$, $p = 0.00494$, se ± 0.067071–0.060612; days of supplementation: $F_{(4, 143)} = 2605.7$, $p = 0.0000$, se ± 0.008902–0.015419; supplementation method × days of supplementation $F_{(8, 143)} = 8.4650$, $p = 0.0000$, se ± 0.015419–0.016253.

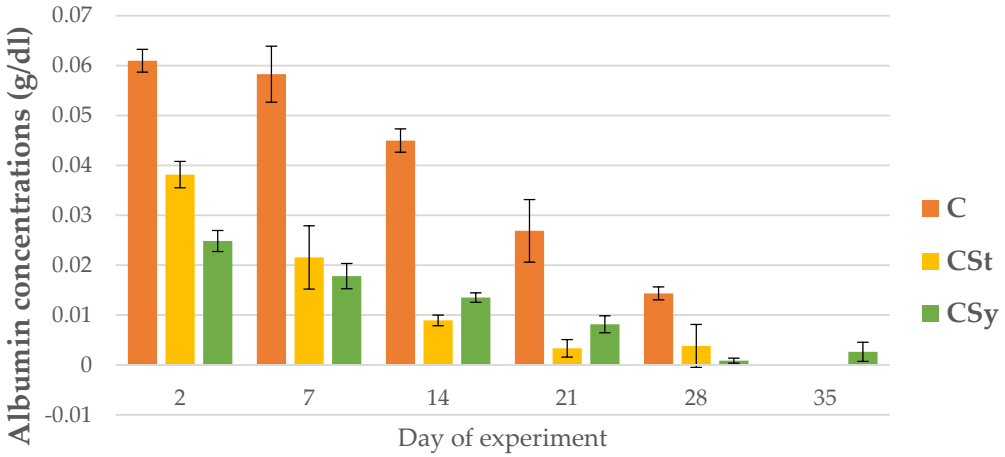



**Figure 16.** Albumin concentration in the 2, 7, 14, 21, 28, 35-day-old workers' hemolymph after two methods of supplementation with hemp extract: C-control (pure sugar syrup), CSy-hemp extract in syrup, CSt-hemp extract on strips. Two-Way ANOVA: supplementation method: $F_{(2, 156)} = 69.318$, $p = 0.0000$, se ± 0.002002–0.011302; days of supplementation: $F_{(4, 143)} = 561.33$, $p = 0.0000$, se 0.000596–0.001033; supplementation method × days of supplementation $F_{(8, 143)} = 47.417$, $p = 0.0000$, se 0.001033–0.001089.

The addition of CBD (regardless of administration-syrup or strips; CSy or CSt) caused an increase in the uric acid concentrations in each day of life of workers in comparison with group C (Figure 15). The CSy group had the highest uric acid concentrations on each analysis day.

The albumin concentrations were the highest in the bees' hemolymph from the C group on each sampling day. By day 7, the CSt group had higher the albumin concentrations, however, from day 14, the albumin concentrations were higher in CSy compared to CSt (Figure 16).

## 4. Discussion

Pure CBD extract as a potent active substance significantly influenced the changes in the parameters of the immune system in our study. During the experiment, two methods of administering the supplement were used. As this research shows, the method of administration has a significant impact on changes and their intensity in the activities of the proteolytic system and the concentrations of biochemical markers. The intensity of the effect is also reflected in the lifespan of the bees. The bees from the CSy group showed the highest parameters of activity and were the only ones to survive until the 35th day of the experiment. The CSt group, despite the higher activity than in the C group, lived until the 28th day.

The results of total protein concentration obtained in this experiment confirm the results obtained in the study by Skowronek et al. (2021) which contain results only from cage experiment on 7 and 14-days-old bees [23]. The higher concentration of total protein in the groups supplemented with CBD indicate a higher production of immune proteins in the bees' organism. Regardless of the method of administration, CBD entered the bees' digestive system, where from the gut it could be further absorbed by other surrounding tissues and affect the production of proteins in the fat body. The lower concentration of proteins in the CSt group could be caused by a delayed action due to the longer route of the extract entering the gastrointestinal tract (not direct administration). The same tendency is visible for other parameters characterizing the humoral immunity of bees. Supplementation could increase the concentration of $Ca^{2+}$ ions, which affect the functioning of many enzymes involved in the body's defense [39]. The activity of specific calcium phospholipases (cPLA2-$Ca^{+2}$-dependent cytosolic phospholipase (A2), mentioned in Skowronek et al. (2022)), depends on the calcium content [26]. They influence the production of eicosanoids, which

support immune processes at the level of the humoral response of the fat body [40]. The use of pure CBD and the achievement of a comparable effect with the studies carried out earlier in cages confirms that it is this substance that is responsible for the effects obtained in the previous experiment. This also confirms the similar effect of lipophilic compounds in supplementation on bees, i.e., CBD or coenzyme Q10 [12].

For all proteases and protease inhibitors, we noted higher activities of these parameters for the CSy group. In the CSt group, the exceptions were acid proteases and alkaline inhibitors. Contrary to the cage studies, proteases could be activated and increased because the bees kept in natural conditions were not exposed to the negative/stress factor like the cage. The cage is a foreign environment (without queen) for bees and limits the functioning in line with their physiology. Under natural conditions, bees may be exposed to biotic or abiotic stress, which is visible, for example, in the activity of serine proteases. In this case, the increase in the activity of the proteolytic system of the supplemented bees may be associated with a stronger and faster response to possible negative factors and thus their faster neutralization. In the case of the cages, the bees were isolated from these factors.

A positive effect on immunogenic tissues, in particular on the fat body, can be observed in higher activities of markers such as ALP, AST and ALT. As we have described before, and as confirmed by many other publications, the fat body functions as the liver of bees. However, as it turns out, in invertebrates, the increase in the concentration of these substances is not associated with damage to the cells of the fat body. The increase in ALP, AST and ALT was noted with other supplements defined as positive, i.e., caffeine, coenzyme Q10, curcumin [12,16,17]. After the collection of previously published articles, we conclude that these parameters can be used to determine the health level of bees like in humans. However, in the case of bees, the increase in these parameters is a positive effect (not inflammation as in the case of the research's interpretation of humans). In the case of ALP, we observed a higher concentration in the CSt group. This may be related to the sealing of physiological barriers and the deposition of evaporating CBD on the bee's cuticle, as well as its penetration and reaching the fat body. However, this effect only occurred with this one enzyme. In order to establish this relationship, more research and knowledge of the biochemical functioning of the fat body should be obtained. In the studies published so far, it turns out that the reduced values of the activities of these enzymes were noted with the negative impact of administering amphotericin B, bromfenvinfos and formic acid to bees, which had an immunosuppressive effect, which confirms that the decrease in these parameters proves the poor health of bees [41,42]. In addition to the increase in liver enzymes, all supplemented groups had a high concentration of triglycerides and cholesterol, which are the main fatty compounds in the fat body (over 50%) [14]. Triglycerides and cholesterol are the main equivalent of reserve substances and together form fat droplets embedded in the cells. When needed, triglycerides are converted by metabolic pathways into diglycerides or fatty acids and transported to tissues when needed. The increase in triglycerides suggests again the previously indicated effect of CBD on the fat body by increasing the concentration of $Ca^{2+}$ ions [43]. The level and homeostasis of these ions has a large impact on the activation and course of pathways (lipolysis, lipogenesis) and the regulation of the level of lipid reserves [13,14,44]. In addition, calcium ions by regulating lipid metabolism also affect the development and aging of the body (diapause, metamorphosis). Similar trends occurred in the case of using other supplements in the publications of the Strachecka team [12,16,17].

A higher level of glucose indicates a high availability of sugars in the diet (no hunger), thanks to which the bee can carry out all the necessary metabolic processes related to the use of high-energy organic compounds. Additionally, a high level of glucose in the organism of bees may have a positive effect on the level of energy needed for flights and for obtaining food. Glucose is a key carbohydrate that is both a basic substrate and a product of metabolic pathways. Furthermore, trehalose is synthesized from glucose, which affects the lipid metabolism by inactivating lipase enzymes [14,45]. The positive effect of increased glucose concentration in the hemolymph may also confirm that bees that are infected with

*Nosema* spp. or *Varroa destructor* parasites show a lower glucose concentration, as a result of which bees experience energy stress [46]. These results are inconsistent with the results obtained from the cage studies, but it is also due to a different energy balance in the bees remaining in the cages, restricting their movement, and in the hives where the bees have access to honeyflow and at the same time show different energy consumption (flights).

The increase in the concentration of creatinine, albumin and urea acid indicates that, despite a different chemical structure, CBD was metabolized similarly to curcumin and coenzyme Q10, which are referred to together with CBD as antioxidants or substances with antioxidant potential [12,16]. In the case of bees from the CSt group, they showed a lower concentration of creatinine and uric acid due to the fact that supplementation was not directly consumed in such amounts as in the CSy group, which could affect the natural metabolism. It should be noted that although the groups where the bees consumed the extract had higher values, the trend with the age of the bees was the same as for the control bees.

## 5. Conclusions

CBD extract may prove to be a good supplement and can have positive effect on the immune system of honeybees by stimulating the proteolytic system and other metabolic parameters. We observed a positive effect in our study with two methods of administration, but the results show that supplementation in sugar syrup allows for higher immunity parameters in relation to the administration of CBD on strips in our experiment. However, both methods can be used due to a possible increase in potential of humoral immunity and anatomical and physiological barriers in honey bees.

**Author Contributions:** Idea, implementation of analyses, the scheme of conducting the experiment, conceptualization, methodology, writing-first draft preparation, P.S.; idea, methodology, review, and editing, A.S.; assistance in carrying out research, Ł.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was financed from the funds allocated to the implementation of research within the Doctoral School on University of Life Sciences in Lublin, no. SD/25/ZiR/2021.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets and materials for this study results that have been used, analyzed and presented in this manuscript are not publicly available. Available on University of Life Sciences in Lublin. At the justified request of the interested party, they may be made available by the respective author.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. O'Connor, B.; Bojinski, S.; Röösli, C.; Schaepman, M.E. Monitoring global changes in biodiversity and climate essential as ecological crisis intensifies. *Ecol. Inform.* **2020**, *55*, 101033. [CrossRef]
2. Jankielsohn, A.; Jankielsohn, A. The Importance of Insects in Agricultural Ecosystems. *Adv. Entomol.* **2018**, *6*, 62–73. [CrossRef]
3. Mizrahi, A.; Lensky, Y. *Bee Products*; Springer: Greer, SC, USA, 2013.
4. Hristov, P.; Shumkova, R.; Palova, N.; Neov, B. Factors Associated with Honey Bee Colony Losses: A Mini-Review. *Vet. Sci.* **2020**, *7*, 166. [CrossRef] [PubMed]
5. Lupi, D.; Mesiano, M.P.; Adani, A.; Benocci, R.; Giacchini, R.; Parenti, P.; Zambon, G.; Lavazza, A.; Boniotti, M.B.; Bassi, S.; et al. Combined Effects of Pesticides and Electromagnetic-Fields on Honeybees: Multi-Stress Exposure. *Insects* **2021**, *12*, 716. [CrossRef]
6. Higes, M.; Martín-Hernández, R.; Garrido-Bailón, E.; González-Porto, A.V.; García-Palencia, P.; Meana, A.; Del Nozal, M.J.; Mayo, R.; Bernal, J.L. Honeybee colony collapse due to Nosema ceranae in professional apiaries. *Environ. Microbiol. Rep.* **2009**, *1*, 110–113. [CrossRef]
7. Underwood, R.M.; Traver, B.E.; López-Uribe, M.M. Beekeeping management practices are associated with operation size and beekeepers' philosophy towards in-hive chemicals. *Insects* **2019**, *10*, 10. [CrossRef]

8. Woodford, L.; Christie, C.R.; Campbell, E.M.; Budge, G.E.; Bowman, A.S.; Evans, D.J.; Huang, Y.-J.; Kenney, J.L.; Woodford, L.; Christie, C.R.; et al. Quantitative and Qualitative Changes in the Deformed Wing Virus Population in Honey Bees Associated with the Introduction or Removal of Varroa destructor. *Viruses* **2022**, *14*, 1597. [CrossRef] [PubMed]
9. Bode, W.; Fernandez-Catalan, C.; Tschesche, H.; Grams, F.; Nagase, H.; Maskos, K. Structural properties of matrix metalloproteinases. *Cell. Mol. Life Sci.* **1999**, *55*, 639–652. [CrossRef]
10. Strachecka, A.; Kuszewska, K.; Olszewski, K.; Skowronek, P.; Grzybek, M.; Grabowski, M.; Paleolog, J.; Woyciechowski, M. Activities of Antioxidant and Proteolytic Systems and Biomarkers in the Fat Body and Hemolymph of Young Apis mellifera Females. *Animals* **2022**, *12*, 1121. [CrossRef]
11. Strachecka, A.J.; Paleolog, J.; Grzywnowicz, K. The surface proteolytic activity in Apis mellifera. *J. Apic. Sci.* **2008**, *52*, 57–68.
12. Strachecka, A.; Olszewski, K.; Paleolog, J.; Borsuk, G.; Bajda, M.; Krauze, M.; Merska, M.; Chobotow, J. Coenzyme Q10 treatments influence the lifespan and key biochemical resistance systems in the honeybee, *Apis mellifera*. *Arch. Insect Biochem. Physiol.* **2014**, *86*, 165–179. [CrossRef] [PubMed]
13. Li, S.; Yu, X.; Feng, Q. Fat body biology in the last decade. *Annu. Rev. Entomol.* **2019**, *64*, 315–333. [CrossRef] [PubMed]
14. Skowronek, P.; Wójcik, Ł.; Strachecka, A.; Brivio, F. Fat Body-Multifunctional Insect Tissue. *Insects* **2021**, *12*, 547. [CrossRef] [PubMed]
15. Farjan, M.; Dmitryjuk, M.; Lipiński, Z.; Biernat-łopieńska, E.; Zółtowska, K. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera* carnica brood at different stages. *J. Apic. Res.* **2012**, *51*, 263–270. [CrossRef]
16. Strachecka, A.; Olszewski, K.; Paleolog, J. Curcumin stimulates biochemical mechanisms of Apis mellifera resistance and extends the apian life-span. *J. Apic. Sci.* **2015**, *59*, 129–141. [CrossRef]
17. Strachecka, A.; Krauze, M.; Olszewski, K.; Borsuk, G.; Paleolog, J.; Merska, M.; Chobotow, J.; Bajda, M.; Grzywnowicz, K. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochemistry* **2014**, *79*, 1192–1201. [CrossRef]
18. Schulz, M.; Łoś, A.; Grzybek, M.; Ścibior, R.; Strachecka, A. Piperine as a new natural supplement with beneficial effects on the life-span and defence system of honeybees. *J. Agric. Sci.* **2019**, *157*, 140–149. [CrossRef]
19. Ricigliano, V.A.; Simone-Finstrom, M. Nutritional and prebiotic efficacy of the microalga *Arthrospira platensis* (spirulina) in honey bees. *Apidologie* **2020**, *51*, 898–910. [CrossRef]
20. Rascón, B.; Hubbard, B.P.; Sinclair, D.A.; Amdam, G.V. The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging* **2012**, *4*, 499–508. [CrossRef]
21. van der Steen, J. Effect of a home-made pollen substitute on honey bee colony development. *J. Apic. Res.* **2007**, *46*, 114–119. [CrossRef]
22. Kaznowski, A.; Szymas, B.; Jazdzinska, E.; Kazimierczak, M.; Paetz, H.; Mokracka, J. The effects of probiotic supplementation on the content of intestinal microflora and chemical composition of worker honey bees (*Apis mellifera*). *J. Apic. Res.* **2005**, *44*, 10–14. [CrossRef]
23. Skowronek, P.; Wójcik, Ł.; Strachecka, A. Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (*Apis mellifera*) Workers. *Animals* **2021**, *11*, 2190. [CrossRef] [PubMed]
24. Gruschow, A. A Comparison of Antioxidant Potential, Total Phenolic Content, and Cannabidiol (CBD) Content of Cannabis Infused Hemp, MCT, and Olive Oils. Master's Theses, College of Education and Human Services, Logan, UT, USA, 2020.
25. Samarut, É.; Nixon, J.; Kundap, U.P.; Drapeau, P.; Ellis, L.D. Single and synergistic effects of cannabidiol and δ-9-tetrahydrocannabinol on zebrafish models of neuro-hyperactivity. *Front. Pharmacol.* **2019**, *10*, 226. [CrossRef] [PubMed]
26. Skowronek, P.; Wójcik, Ł.; Strachecka, A. Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (*Apis mellifera*) Organism. *Antioxidants* **2022**, *11*, 707. [CrossRef] [PubMed]
27. Lopez, H.L.; Cesareo, K.R.; Raub, B.; Kedia, A.W.; Sandrock, J.E.; Kerksick, C.M.; Ziegenfuss, T.N. Effects of Hemp Extract on Markers of Wellness, Stress Resilience, Recovery and Clinical Biomarkers of Safety in Overweight, But Otherwise Healthy Subjects. *J. Diet. Suppl.* **2020**, *17*, 561–586. [CrossRef] [PubMed]
28. Reithmeier, D.; Tang-Wai, R.; Seifert, B.; Lyon, A.W.; Alcorn, J.; Acton, B.; Corley, S.; Prosser-Loose, E.; Mousseau, D.D.; Lim, H.J.; et al. The protocol for the Cannabidiol in children with refractory epileptic encephalopathy (CARE-E) study: A phase 1 dosage escalation study. *BMC Pediatr.* **2018**, *18*, 221. [CrossRef]
29. Amin, M.R.; Ali, D.W. Pharmacology of Medical Cannabis. In *Advances in Experimental Medicine and Biology*; Springer: New York, NY, USA, 2019; Volume 1162, pp. 151–165.
30. Blessing, E.M.; Steenkamp, M.M.; Manzanares, J.; Marmar, C.R. Cannabidiol as a Potential Treatment for Anxiety Disorders. *Neurotherapeutics* **2015**, *12*, 825–836. [CrossRef]
31. Ashton, C.H. Pharmacology and effects of cannabis: A brief review. *Br. J. Psychiatry* **2001**, *178*, 101–106. [CrossRef]
32. Da Silva, V.K.; De Freitas, B.S.; Da Silva Dornelles, A.; Nery, L.R.; Falavigna, L.; Ferreira, R.D.P.; Bogo, M.R.; Hallak, J.E.C.; Zuardi, A.W.; Crippa, J.A.S.; et al. Cannabidiol Normalizes Caspase 3, Synaptophysin, and Mitochondrial Fission Protein DNM1L Expression Levels in Rats with Brain Iron Overload: Implications for Neuroprotection. *Mol. Neurobiol.* **2013**, *49*, 222–233. [CrossRef]
33. Siuda, M.; Wilde, J.; Woyke, J.; Jasiński, Z.; Madras-Majewska, B. Wintering reserve queens in mini-plus and 3-comb nuclei. *J. Apic. Sci.* **2014**, *58*, 61–68. [CrossRef]

34. Scheiner, R.; Abramson, C.I.; Brodschneider, R.; Crailsheim, K.; Farina, W.M.; Fuchs, S.; Grünewald, B.; Hahshold, S.; Karrer, M.; Koeniger, G.; et al. Standard methods for behavioural studies of Apis mellifera. *J. Apic. Res.* **2013**, *52*, 1–58. [CrossRef]
35. Łoś, A.; Strachecka, A. Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution. *Sensors* **2018**, *18*, 1494. [CrossRef] [PubMed]
36. Schacterle, G.; Pollack, R.L. A Simplified Method For The Quantitative Assay Of Small Amounts Of Proteins In Biologic Material. *Anals Biochem.* **1973**, *51*, 654–655. [CrossRef]
37. Anson, M.L. The Estimation Of Pepsin, Trypsin, Papain, And Cathepsin With Hemoglobin. *J. Gen. Physiol.* **1938**, *22*, 79. [CrossRef] [PubMed]
38. Lin, J.; Lee, I.S.; Frey, J.; Slonczewski, J.L.; Foster, J.W. Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia coli. *J. Bacteriol.* **1995**, *177*, 4097–4104. [CrossRef]
39. Watkins, A.R. Cannabinoid interactions with ion channels and receptors. *Channels* **2019**, *13*, 162–167. [CrossRef] [PubMed]
40. Park, Y.; Kumar, S.; Kanumuri, R.; Stanley, D.; Kim, Y. A novel calcium-independent cellular PLA2 acts in insect immunity and larval growth. *Insect Biochem. Mol. Biol.* **2015**, *66*, 13–23. [CrossRef]
41. Strachecka, A.; Olszewski, K.; Paleolog, J. Varroa treatment with bromfenvinphos markedly suppresses honeybee biochemical defence levels. *Entomol. Exp. Appl.* **2016**, *160*, 57–71. [CrossRef]
42. Strachecka, A. The Effect of Amphotericin B on the Lifespan, Body-surface Protein Concentrations, and DNA Methylation Levels of Honey Bees (*Apis mellifera*) Analysis of the relative importance in long-term studies of the factors regulating the seroprevalence of *Toxocara* spp. in bank voles (*Myodes glareolus*) in N.E. Poland View project. *J. Apic. Sci.* **2017**, *107*, 107–113. [CrossRef]
43. Dube, K.A.; McDonald, D.G.; O'Donnell, M.J. Calcium homeostasis in larval and adult Drosophila melanogaster. *Arch. Insect Biochem. Physiol.* **2000**, *44*, 27–39. [CrossRef]
44. Gilbert, L.I. Lipid Metabolism and Function in Insects. *Adv. Insect Phys.* **1967**, *4*, 69–211. [CrossRef]
45. Suwannapong, G.; Maksong, S.; Phainchajoen, M.; Benbow, M.E.; Mayack, C. Survival and health improvement of Nosema infected Apis florea (Hymenoptera: Apidae) bees after treatment with propolis extract. *J. Asia Pac. Entomol.* **2018**, *21*, 437–444. [CrossRef]
46. Schulz, M.; Skowronek, P.; Tyszczuk, J.; Los, A.; Strachecka, A. Nosema apis i Nosema ceranae-porównanie morfologii, metod identyfikacji oraz przebiegu mikrosporydiozy u pszczół miodnych. *Przegląd Hod.* **2018**, *86*, 21–24.

*antioxidants*

MDPI

*Article*

# Cannabidiol (CBD) Supports the Honeybee Worker Organism by Activating the Antioxidant System

**Patrycja Skowronek * and Aneta Strachecka**

Department of Invertebrate Ecophysiology and Experimental Biology, University of Life Sciences in Lublin, Doświadczalna 50a, 20-280 Lublin, Poland

* Correspondence: patrycja.skowronek@up.lublin.pl

**Abstract:** In the experiment, we tested the effect of 30% CBD oil on the activity of the antioxidant system (superoxide dismutase, catalase, glutathione peroxidase, glutathione), the level of total antioxidant capacity, and the concentrations of ions (calcium, magnesium, and phosphorus) in honeybee workers in the hive test. For this purpose, we prepared hives containing all stages of the development of honey bees and started the experiment by adding 200 marked, one-day old bees to each colony (intended for hemolymph collection). In the test, we created three groups (two colonies per group): (1) Experimental with CBD oil mixed with sugar syrup (CSy); (2) experimental with CBD oil on textile strips (CSt); and (3) control with pure sugar syrup only (C). Every week, we collected hemolymph from the marked bees. In the experiment, all antioxidant enzyme activities were higher for the experimental groups CSy and CSt compared to group C. The highest concentrations/levels were obtained for the CSy group. Concentrations of calcium, magnesium, and phosphorus ions were also higher for the experimental groups compared to the C group (the highest concentration for the CSy group). We conclude that CBD oil positively contributes to stimulating the antioxidant system of honeybees.

**Keywords:** hemp extract; CBD oil; antioxidant enzymes; biochemistry; resistance; *Apis mellifera*; calcium



**Citation:** Skowronek, P.; Strachecka, A. Cannabidiol (CBD) Supports the Honeybee Worker Organism by Activating the Antioxidant System. *Antioxidants* **2023**, *12*, 279. https://doi.org/10.3390/antiox12020279

Academic Editor: José Eduardo Serrão

Received: 1 December 2022
Revised: 23 January 2023
Accepted: 25 January 2023
Published: 27 January 2023



## 1. Introduction

Nowadays, the scientific world has been dealing with the problems of the decrease in the biodiversity of flora and fauna due to global environmental changes. Disruptions in the populations of living organisms cause wide-ranging changes that will affect all organisms on this planet. Among the frequently mentioned organisms, the populations of which are shrinking, are insects. As the most diverse group of organisms, it plays an important role in agriculture, from the decomposition of organic matter to pollination [1,2].

The beneficial insects that suffer from these negative effects are bees. As pollinators, bees are responsible for regulating the biodiversity of the natural environment. Their work includes the pollination of entomophilic plants, which include about 70% of plants that are part of the human diet and livestock and pets. In recent years, with the increase in the ecological crisis and problems with food production, it is extremely important to maintain the health and good condition of the populations of these insects. Due to the changing environment as a result of climate change, anthropopressure, and chemization, the natural resistance of bees decreases, and thus they are more susceptible to infections with parasites and pathogens (i.e., nosematosis, varroosis). All of these factors, acting together or separately, make a significant contribution to reducing the work efficiency and shortening the life of pollinators. The dying of colonies also causes far-reaching economic losses in the beekeeping, agricultural, and food industries [3–8]. In response to this crisis caused by the general weakening of the population of bee colonies, various supplements have been tested to both stimulate the health potential and meet a number of requirements (i.e., non-toxicity). Due to the keeping of honey bees in breeding farms from which bee

products are obtained, the substances administered to the hive should be safe in the event that residues of these stimulants pass into food products (e.g., honey) [9,10].

Therefore, the most commonly used substances come from natural sources (i.e., plants with documented properties and classified as safe for human and animal health). Thus far, the tested substances of this type include resveratrol (active substance from grapes), piperine (from pepper), curcumin (turmeric), caffeine (present in coffee beans, cocoa beans and tea), vitamin C (parsley, lemon), and mixtures of pulses (soybean, corn, pea) and meals. Popularly used supplements also include the recently popular silages rich in Lactobacillus, phytochemicals stimulating the diversity of the intestinal microflora of bees (also Lactobacillus), coenzyme Q10 (pharmaceutical raw material), propolis (propolis tincture), spirulina, yeast, powdered skim milk, and pollen [11–22]. Most of the mentioned biostimulants prolonged the life of bees. Some of the tested substances had a proven positive effect on the stimulation of the immune system by increasing the activity of the proteolytic system, antioxidant enzyme like: SOD (superoxide dismutase), GpX (peroxidase), CAT (catalase), GST (glutathione S-transferase), and the concentration of immune proteins (hemp extract, curcumin, coenzyme Q10, caffeine, piperine). The addition of coenzyme Q10 additionally increased the concentration of lipids and ions such as magnesium and calcium. Higher lipid levels were noted after feeding bees with spirulina. Caffeine, in addition, had a positive effect in the case of bees infected with Nosema spp. and the test reported higher concentrations of proteins, urea acid, triglycerides, cholesterol, glucose, calcium, creatinine, magnesium, and proteases. The greatest stimulating effect was observed in older bees. The activities of liver enzymes (transaminases) were also higher in the piperine-treated group. Piperine decreased the DNA methylation levels significantly in the older bees [12,15,16,20,22,23]. Natural substances used in recent years also include eucalyptus pollen from *Corymbia calophylla*. Mixtures with linoleic acid, oleic acid, soybean meal, and lupin meal were used. The life-prolonging effect has been reported with the exclusive use of pollen alone. Each addition to the pollen resulted in a shortened lifespan. Oleic acid showed the weakest effect on bees [24]. Despite the large number of tested substances, it has not been possible to find one effective agent that suits all the needs of modern beekeeping and the strict requirements for the safety of use.

We believe that a good supplementation can be the addition of hemp extract in the form of CBD oil to the diet of bees, which has many documented potentially health-promoting properties. In the latest research, more and more scientists are showing the positive effects of cannabis on animal organisms. According to Boldaji et al. (2022), cannabidiol contributed to better outcomes in myocardial rehabilitation after myocardial infarction in rat tissue tests [25]. The research carried out by Majewski et al. (2021), also conducted on rats, confirmed that cannabis had a positive effect on the body by changing the blood biochemical parameters (lowering cholesterol levels, reducing lipid peroxidation, increasing the sensitivity of ATP, and calcium ion-dependent potassium channels) [26]. Studies have also been carried out on the regeneration of other (skeletal) muscles for sports performance in humans. It was noted that the CBD consuming group showed little but significant differentiation in muscle recovery after resistance training [27]. In other animal studies, horses that consumed CBD showed lower reactivity compared to the control groups, which may contribute to their welfare in the future [28]. It has also shown a positive effect on the antioxidant system by improving superoxide dismutation in dogs. The addition of hemp oil to the diet of these dogs also improved the digestibility of nutrients [29].

Our previous studies have shown that CBD oil/hemp extracts added to the diet of bees prolonged the life of insects and contributed to a positive stimulation of the immune system by stimulating the activity of the proteolytic system, increasing the enzymatic concentrations (ALT, AST, ALP), and non-enzymatic biomarkers (e.g., glucose, cholesterol, triglycerides, calcium, and magnesium ions) tested from the hive test. Additionally, in previous studies, we showed that supplementation with hemp extract increased the activity of the antioxidant system in the cage experiment (all enzymes) and prolonged the life of bees in cage experiments. Therefore, we assumed that in the case of a beekeeping

experiment, we will obtain results confirming the positive effect of CBD oil obtained in the case of previous publications using a hemp extract. In addition, the results obtained in this publication will complement the information on CBD supplementation from previous studies on the proteolytic system [10,23,30].

The antioxidant system is a very important element of the immunity of living organisms. It is responsible for scavenging free radicals, which can cause the body to age faster, create inflammatory processes, and mediate in creating diseases and cancers. The main defense consists of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH). All of these enzymes interact with each other to neutralize reactive oxygen species (ROS) by transforming it into hydrogen peroxide and molecular oxygen (SOD), then into molecular oxygen and water (CAT, GPx, GSH) [31,32].

The aim of this study was to evaluate the effect of a commercial hemp extract in the form of CBD oil on the activity of the antioxidant system (TAC—total antioxidant capacity, SOD, CAT, GPx, GSH) and the basic ion concentrations important in cellular processes (calcium, magnesium, and phosphorus) in honey bees (*Apis mellifera*).

## 2. Materials and Methods

The research was carried out with the use of bees and queens from colonies of similar strength and age (number of brood, mother's age, number of bees, frame construction, amount of feed stock, varroosis free, Nosema free, etc.) that had not been subjected to prophylactic treatment against *Varroa destructor* (and thus obtained the natural activity of their immune systems without the healing agents affecting them). No spores of *Nosema* spp. were detected in the colonies.

### *2.1. Preparatory Activities*

#### 2.1.1. Obtaining Queens for the Experiment

Nine queens were prepared for the experiment (six queens for the colony test + three queens for day-old workers). All nine queens were sisters descended from one source mother (all mothers were inseminated). The methodology obtained mothers according to Skowronek et al. [23].

#### 2.1.2. Preparation of the Mating Hives

Four-frame hives were used for the experiment.

Fragments of various combs were collected from the source colony, in which we observed different stages of larvae development. Fragments were fitted to frames in the experimental hives. Additionally, worker bees of different ages (imago stages) were collected from the source colony as a complement to the colony, and 200 individuals were placed in each of the prepared hives. Each hive contained all development stages. The queen was put into each hive (six queens = six hives). We checked the queen abilities to lay eggs after 1 month and next we added 1 day old bees to the colonies, obtained from the three remaining queens—sisters [23,33].

### *2.2. Collection and Tagging of Day-Old Bees*

Three queen-sisters were caged for 12 h in a queen excluder comb-cage containing one empty comb for egg laying. When the queens laid eggs, we released them to the source colonies. The combs with eggs were marked and placed in their native colonies. After 20 days, these combs with broods were placed in an incubator (35 °C) where the 1-day-old bees emerged. More details about the collection and marked one-day-old bees from three mother-sisters are described in Skowronek et al. [23].

One-day workers were placed in each of the six mating hives. In the experiment, the hives were divided into three groups: (1) The experimental group CSy, CBD in sugar syrup; (2) experimental group CSt, CBD on a cotton strip; and (3) the C control (supplemented with pure sugar syrup). Each group consisted of two mating hives [23,34].

### *2.3. Preparation and Administration of CBD Extract*

Hemp extract in the form of 30% CBD oil (producer' name: HempOil, 3 g of CBD in 10 mL of oil) was used for the experiment. The oil was obtained by $CO_2$ extraction (producer information). Supplementation details for the experimental groups are as follows:

- CSy: ad libitum, on the second, fourth, and sixth days of the experiment, in a mixture with sugar syrup (1:1 water with sugar) and glycerin in the volume ratio of 0.01:0.5:0.5 (extract:distilled water:glycerin);
- CSt: mixture with water and glycerin in a volume ratio of 0.8:1.5:1.5 (extract:distilled water:glycerin), textile strips (2 × 10 cm) were evenly moistened with 10 mL of the mixture and placed in the hives, wetted with the mixture on the second, fourth, and sixth days of the experiment.

Details on the composition of supplementation according to Skowronek et al. [23].

### *2.4. Sampling of Bees*

From six colonies, we collected 10 marked bees once per week (10 bees × 6 colonies). Workers were collected on the following days of the experiment: 2, 7, 14, 21, 28 (CSt, CSy, C), and 35 (CSy) [23].

### *2.5. Collection of Hemolymph*

The hemolymph samples were collected from each worker according to the methodology of Łoś and Strachecka (2018) using capillary tubes (20 µL; end to end type; no anticoagulant; Medlab Products, Raszyn, Poland). The hemolymph capillaries of the individual bees were placed in separate 1.5 mL Eppendorf tubes with a 200 µL 0.6% NaCl solution (10 bees = 10 tubes). Then, the material was stored at −25 °C [35].

### *2.6. Biochemical Analyses*

#### 2.6.1. Antioxidant System

The analyses were carried out using the commercial assay kit:

- TAC—OxiSelectTM Total Antioxidant Capacity Assay Kit (Cell BioLabs, Inc., Upper Heyford, UK, no. STA-360);
- SOD Assay Kit (Sigma Aldrich, Schnelldorf, Germany, no. 1916-1KT-F);
- CAT Assay Kit (Sigma Aldrich, Schnelldorf, Germany, no. CAT100-1KT);
- GPx—Glutathione peroxidase Assay Kit (Sigma Aldrich, Schnelldorf, Germany, no. MAK437);
- GSH—EnzyChromTM GSH/GSSG Assay Kit (Bio Assay Systems, Hayward, CA, USA, no. EGTT-100).

All antioxidant enzyme activities were calculated per 1 mg of protein.

The original protocols owned by the companies are available in an electronic version on the manufacturers' website.

#### 2.6.2. Ions Concentration

The ion concentrations were analyzed using commercial one-component reagents:

- Calcium (Alpha Diagnostics, arsenazo III method, reagent composition: TRIS buffer pH 8.5; arsenazo III, 8-hydroxy-quinoline-5-sulfonic acid, inactive stabilizers and detergents 630–670 nm);
- Magnesium (Alpha Diagnostics Magnesium Xylidyl Blue, reagent composition: trioglycolic acid, DMSO, Xylidyl Blue, measurement at 550 nm);
- Phosphorus (Alpha Diagnostics, direct method with phosphomolybdate, reagent composition: sulfuric acid, ammonium molybdate, measurement at 340 nm).

### *2.7. Statistical Analysis*

The results were analyzed using Statistica formulas version 13.3 (2017) for Windows (StatSoft Inc., Tusla, OK, USA). The mixed-model two-way ANOVA followed by Tukey HSD

post hoc tests ($p = 0.05$) was used to compare the results for each antioxidant enzyme (SOD, GST, CAT, GPx, and TAC) and the ions (calcium, magnesium, phosphorus) of the honey bee workers, depending on the method of administration (strip—CSt and syringe—CSy) and the day (1st, 7th, 14th, 21st, 28th, 35th) of supplementation with CBD oil.

## 3. Results

### *3.1. Antioxidant Enzymes*

The total antioxidant capacity and all antioxidant enzyme activities (SOD, CAT, GPx, GSH) achieved higher values for the groups in which bees were exposed to CBD oil (CSy, CSt) compared to the control group (C). The highest activities were recorded for bees that directly consumed sugar syrup with CBD oil (CSy). The values of the activity in each parameter increased with the age of the bees (Figures 1–5, Table 1).

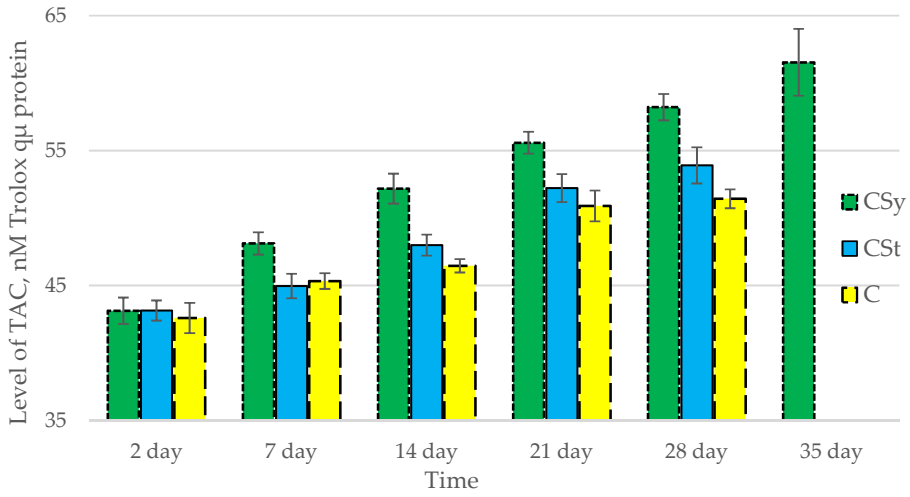



**Figure 1.** Total Antioxidant Capacity (TAC) levels in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C–control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 100,26$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 506,23$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 13,046$, $p = 0.0000$.

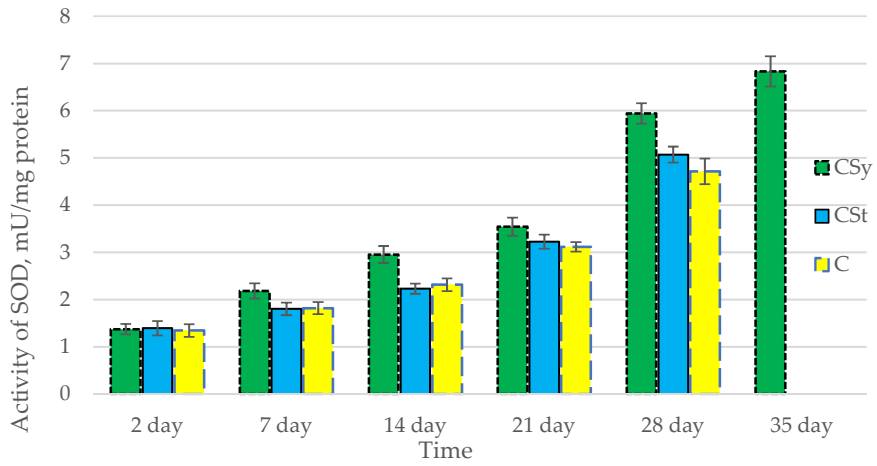



**Figure 2.** Activities of Superoxide Dismutase (SOD) in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 66,223$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 1888,5$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 16,781$, $p = 0.0000$.

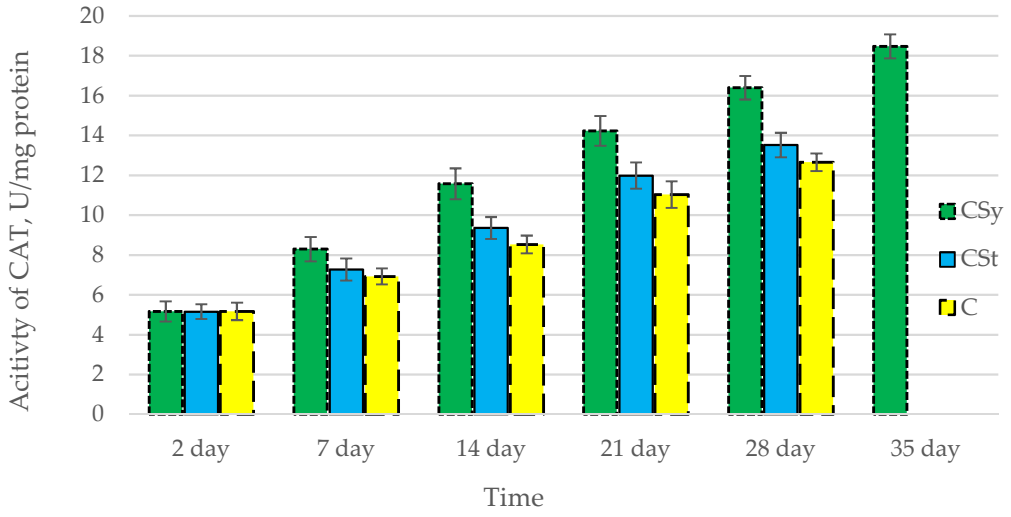



**Figure 3.** Activities of Catalase (CAT) in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 96{,}388$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 1070{,}6$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 18{,}549$, $p = 0.0000$.

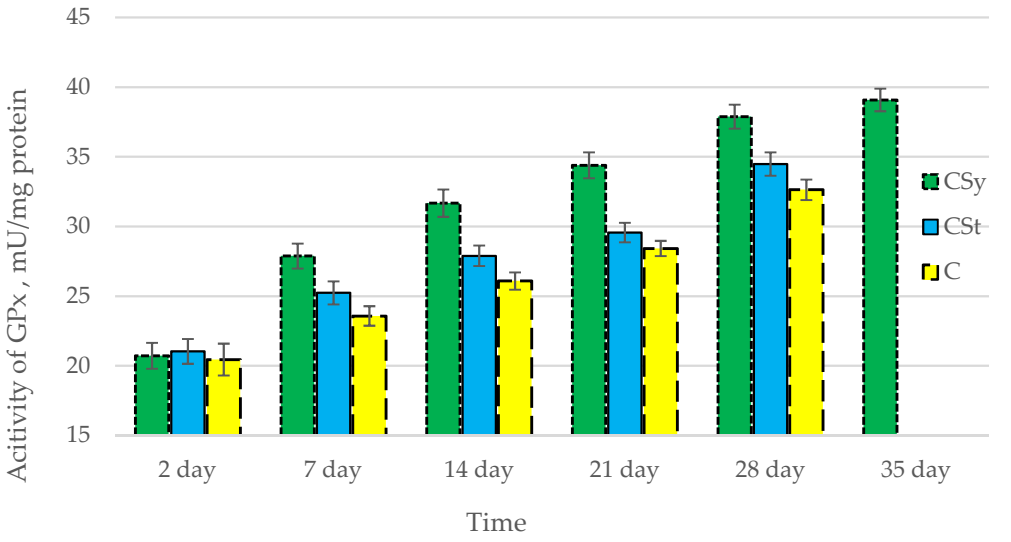



**Figure 4.** Activities of Glutathione Peroxidase (GPx) in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 145{,}31$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 1093{,}6$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 22{,}266$, $p = 0.0000$.

### *3.2. Ion Concentrations*

In all of the tested ions, we observed higher concentrations of calcium, magnesium, and phosphorus ions for the experimental groups with CBD oil (CSy, CSt) compared to the control group (C). The highest concentrations were achieved by bees consuming the addition of CBD oil in the sugar syrup. For all groups, the concentrations of ions increased until day 21, and on day 28, we recorded a downward trend for the concentrations of all of the tested ions (Figures 6–8, Table 2).

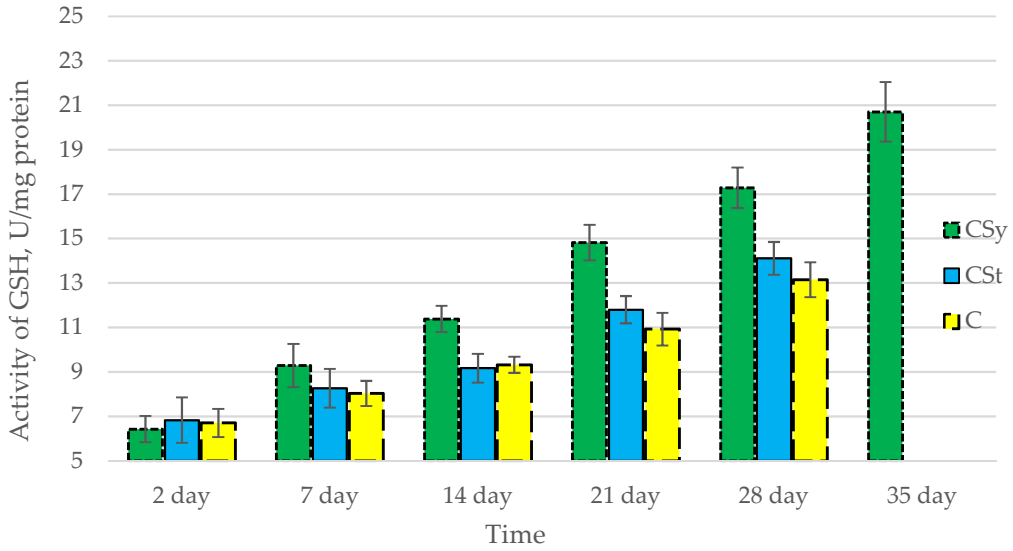



**Figure 5.** Activities of Glutathione (GSH) in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 56{,}662$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 435{,}40$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 14{,}742$, $p = 0.0000$.

**Table 1.** Standard error for activities for antioxidant enzymes (for all groups from 2–35 days during the experiment) in hemolymph samples after two methods of supplementation with the hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syr-up, Cst—hemp extract on strips, according to two-way ANOVA.

| Groups | Day of Supplementation | se± | | | | |
|---|---|---|---|---|---|---|
| | | TAC | SOD | CAT | GPx | GSH |
| CSt | 2 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 7 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 14 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 21 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 28 days | 0.367377 | 0.05739 | 0.192164 | 0.279045 | 0.264402 |
| | 35 days | - | - | - | - | - |
| CSy | 2 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 7 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 14 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 21 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 28 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 35 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| C | 2 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 7 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 14 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 21 days | 0.348525 | 0.054445 | 0.182303 | 0.264725 | 0.250834 |
| | 28 days | 0.492888 | 0.076997 | 0.257815 | 0.374378 | 0.354732 |
| | 35 days | - | - | - | - | - |

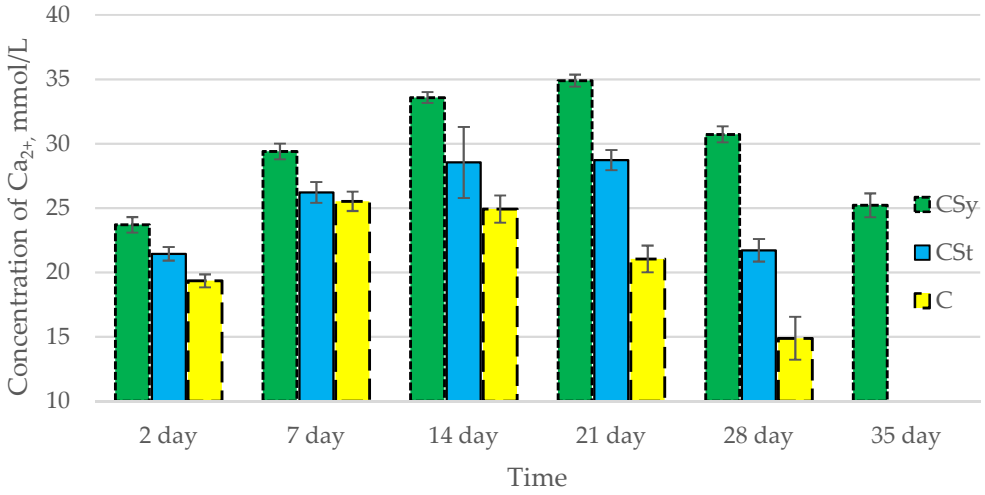



**Figure 6.** Calcium concentrations in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supple-mentation method: $F_{(2,146)} = 213{,}61$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 310{,}89$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 66{,}793$, $p = 0.0000$.

**Table 2.** Standard error for the calcium, magnesium, and phosphorus ions (for all groups from 2–35 days during the experiment) in hemolymph samples after two methods of supplementation with the hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips, according to two-way ANOVA.

| Groups | Day of Supplementation | se± | | |
|---|---|---|---|---|
| | | $Ca^{2+}$ | $Mg^{2+}$ | P |
| C | 2 days | 0.336983 | 0.016605 | 0.080677 |
| | 7 days | 0.336983 | 0.016605 | 0.080677 |
| | 14 days | 0.336983 | 0.016605 | 0.080677 |
| | 21 days | 0.336983 | 0.016605 | 0.080677 |
| | 28 days | 0.336983 | 0.016605 | 0.080677 |
| | 35 days | - | - | - |
| CSt | 2 days | 0.336983 | 0.016605 | 0.080677 |
| | 7 days | 0.336983 | 0.016605 | 0.080677 |
| | 14 days | 0.336983 | 0.016605 | 0.080677 |
| | 21 days | 0.336983 | 0.016605 | 0.080677 |
| | 28 days | 0.355211 | 0.017503 | 0.085041 |
| | 35 days | - | - | - |
| CSy | 2 days | 0.336983 | 0.016605 | 0.080677 |
| | 7 days | 0.336983 | 0.016605 | 0.080677 |
| | 14 days | 0.336983 | 0.016605 | 0.080677 |
| | 21 days | 0.336983 | 0.016605 | 0.080677 |
| | 28 days | 0.336983 | 0.016605 | 0.080677 |
| | 35 days | 0.336983 | 0.016605 | 0.080677 |

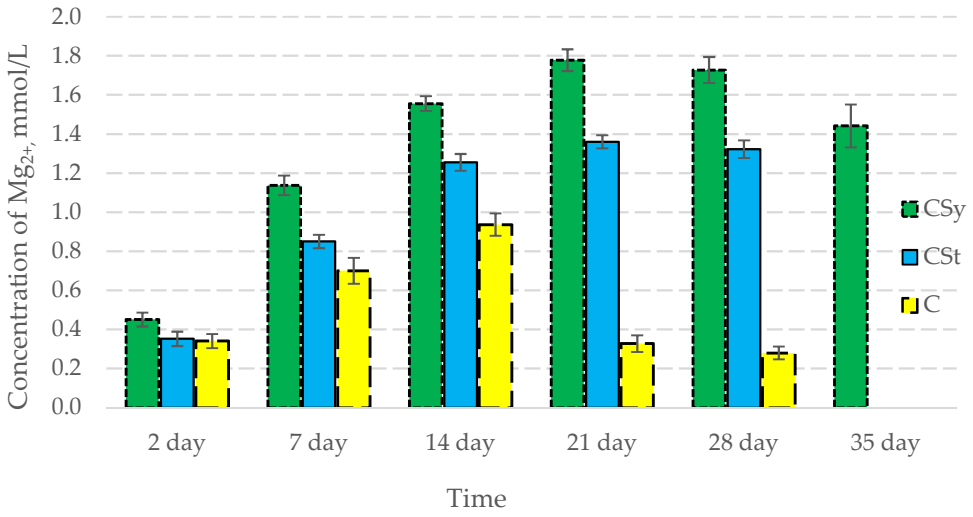



**Figure 7.** Magnesium concentrations in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 145,02$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 1312,0$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 379,63$, $p = 0.0000$.

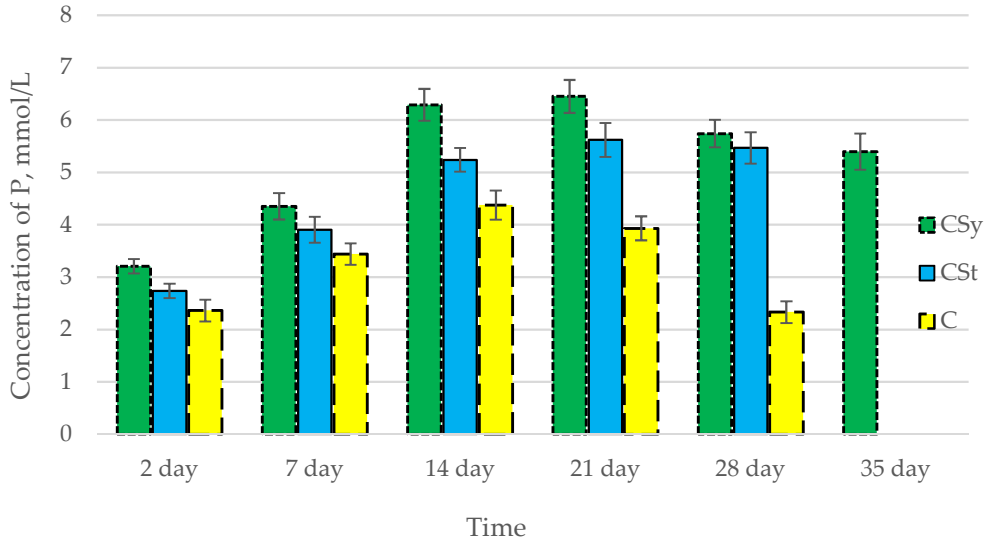



**Figure 8.** Phosphorus concentrations in the 2-, 7-, 14-, 21-, 28-, 35-day-old honey bees' hemolymph after two methods of supplementation with hemp extract: C—control (pure sugar syrup), Csy—hemp extract in syrup, Cst—hemp extract on strips. (Two-Way ANOVA: supplementation method: $F_{(2,146)} = 166,54$, $p = 0.0000$; days of supplementation: $F_{(4,138)} = 530,24$, $p = 0.0000$; supplementation method × days of supplementation $F_{(8,138)} = 65,556$, $p = 0.0000$.

## 4. Discussion

In our study, it turned out that all of the activities of the antioxidant enzymes tested by us were higher for the experimental groups with CBD oil, in particular, the best stimulation effect was obtained for the group consuming the addition of this oil. The results obtained in this article coincide with our previous publications on the antioxidant system in a cage experiment and the proteolytic system in apiary conditions [10,23,30]. In addition, by examining the concentrations of ions, in particular calcium ions, it turns out that the mechanism of CBD action assumed by us in bees is correct. The increase in the concentrations of calcium ions in the experiment suggests that it may have an effect on the ion-dependent immune enzymes involved in the body's defense (i.e., specific calcium phospholipases such

as cytosolic phospholipase (A2), dependent on cPLA2-$Ca^{2+}$). Phospholipase A2 (PLA2) is of key importance in the synthesis of eicosanoids, prostaglandins, and the production of lipoxygenase products (by the hydrolysis of polyunsaturated fatty acid, linoleic acid, and conversion to arachidonic acid), which can exert positive and negative effects on immunity depending on the age of insects (prostaglandin can cause inflammation in older individuals, but is a positive factor in the development of young individuals). PLA2 is activated in insects immediately after infection is detected, thanks to which the body quickly reacts to the threat of eicosanoid synthesis [30,36–38]. Eicosanoids are responsible for many mechanisms of cellular resistance to infection, invasions (phagocytosis and mediating the production of reactive oxygen species used to kill microbes) and wounding (melanization), influencing the production of nodules, signals of hemocyte migration. It is also responsible for the signal to release profenoloxidase (in Lepidoptera) [37]. Higher calcium levels, better phospholipase A2 performance, and eicosanoid synthesis may also be related to the antioxidant system in this study. According to the research by Büyükgüzel et al. (2010) and Büyükgüzel et al. (2017), the inhibition of eicosanoid synthesis increases oxidative stress in the insect organism and increases lipid peroxidation. The authors suggest that eicosanoids may be an intermediate in the action and activities of the antioxidant system in invertebrates by also mediating the production of reactive oxygen species during the phagocytosis process [38–41]. Thus, in a sense, the synthesis of eicosanoids and their activities in the body may contribute to the stimulation of antioxidant enzymes. In addition, the calcium and potassium ions (we noticed an increase in concentration) suggest the correctness of the assumption that CBD may affect the permeability of ion channels (sodium, potassium, and calcium) and their integrity (also suggested by the increase in magnesium concentration) in the cell membrane. Higher ion concentrations may contribute to higher activities and the production of antioxidants (e.g., catalase), the formation of which depends on the amount of calcium, which affects the level of $H_2O_2$ (catalase synthesis) [42–44]. In the case of the production of ROS by eicosanoids and the dependence of the production of antioxidant enzymes on its level, it is also possible that by giving CBD oil, it caused a temporary increase in ROS. Thanks to this, we stimulated an increase in the activity of the antioxidant system (stimulation of higher mRNA expression in the early stages of life), as a result of which we obtained higher concentrations in the later stages of the life of bees (higher level of enzymes in early bee life stages caused better resistance for ROS in later stages of bee life). We believe that the increased immunity at an early age increased the immunity of the bees collected, which upon leaving the hive were exposed to oxidative stress in the natural environment [44–46]. On the other hand, studies by Zhang et al. (2022) suggest that CBD in the *Caenorhabditis elegans* insect test produced a positive effect on the body without the additional overexpression of antioxidant genes. Regardless of the process, these studies suggest a significant effect of CBD on the possibility of inhibiting the progress of Alzheimer's disease and that CBD substances themselves (thanks to the presence of phenolic groups) are excellent at scavenging free radicals in vitro and in vivo [47]. As with previous articles, we observed the same trends between the bees that had been exposed to CBD on the strip and the bees that consumed the CBD supplement. We maintain the opinion that the better effect of stimulating the immune system of bees in the CSy group is due to the direct entry of supplementation into the digestive system of insects, thanks to which the active substances have a chance to act faster. In the case of the CSt groups, we suggest that the lower effect is due to the longer time that CBD takes to enter the body, probably through tropholaxy and hygienic behavior in bees (cleaning). The stimulation of the antioxidant system is a positive effect, which can be confirmed by other publications in the field of bee supplementation conducted by Strachecka et al. In studies with the use of other strong antioxidants (i.e., coenzyme Q10 and curcumin and other biostimulants like caffeine), the same increase in the activities of selected antioxidant enzymes was noted. The positive effect in the cases of the above-mentioned publications and in our article was confirmed by the extension of the life span of bees. In the case of all supplementation, an increase in the concentrations of calcium and magnesium ions were also noted [15,16,20].

## 5. Conclusions

After a series of studies in cages and in colony conditions, we found that supplementation with CBD will potentially support the immune system of honeybees through stimulating the antioxidant system (protection against oxidative stress affecting cells and their biochemistry). Depending on the need, the effects can be obtained regardless of the method of administration, but for the best results, we suggest using CBD in nutritional supplements (direct, faster action). In addition, research confirms that the active substance CBD may be responsible for the positive effect of the hemp extract.

**Author Contributions:** Idea, implementation of analyses, the scheme of conducting the experiment, conceptualization, methodology, writing-first draft preparation, P.S.; Idea, methodology, review, and editing, A.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was financed from the funds allocated to the implementation of research within the Doctoral School at the University of Life Sciences in Lublin, no. SD/25/ZiR/2021 and financed from the fund no. LKE/S/64/2022 (University of Life Sciences in Lublin, Department of Invertebrate Ecophysiology and Experimental Biology).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets and materials for the study results that were used, analyzed, and presented in this manuscript are not publicly available, but are available at the University of Life Sciences in Lublin. At the justified request of the interested party, they may be made available by the respective author.

**Acknowledgments:** We thank Łukasz Wójcik for their help in starting the experiment and for help with the beekeeping during the hive test.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. O'Connor, B.; Bojinski, S.; Röösli, C.; Schaepman, M.E. Monitoring global changes in biodiversity and climate essential as ecological crisis intensifies. *Ecol. Inform.* **2020**, *55*, 101033. [CrossRef]
2. Le Conte, Y.; Navajas, M. Climate change: Impact on honey bee populations and diseases. *OIE Rev. Sci. Tech.* **2008**, *27*, 485–510. [CrossRef]
3. Hristov, P.; Shumkova, R.; Palova, N.; Neov, B. Factors Associated with Honey Bee Colony Losses: A Mini-Review. *Vet. Sci.* **2020**, *7*, 166. [CrossRef] [PubMed]
4. Higes, M.; Martín-Hernández, R.; Garrido-Bailón, E.; González-Porto, A.V.; García-Palencia, P.; Meana, A.; Del Nozal, M.J.; Mayo, R.; Bernal, J.L. Honeybee colony collapse due to Nosema ceranae in professional apiaries. *Environ. Microbiol. Rep.* **2009**, *1*, 110–113. [CrossRef] [PubMed]
5. Lupi, D.; Mesiano, M.P.; Adani, A.; Benocci, R.; Giacchini, R.; Parenti, P.; Zambon, G.; Lavazza, A.; Boniotti, M.B.; Bassi, S.; et al. Combined Effects of Pesticides and Electromagnetic-Fields on Honeybees: Multi-Stress Exposure. *Insects* **2021**, *12*, 716. [CrossRef] [PubMed]
6. Aizen, M.A.; Harder, L.D. The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Curr. Biol.* **2009**, *19*, 915–918. [CrossRef] [PubMed]
7. Xiao, J.; He, Q.; Liu, Q.; Wang, Z.; Yin, F.; Chai, Y.; Yang, Q.; Jiang, X.; Liao, M.; Yu, L.; et al. Analysis of honey bee exposure to multiple pesticide residues in the hive environment. *Sci. Total Environ.* **2022**, *805*, 150292. [CrossRef]
8. Jankielsohn, A.; Jankielsohn, A. The Importance of Insects in Agricultural Ecosystems. *Adv. Entomol.* **2018**, *6*, 62–73. [CrossRef]
9. van der Steen, J. Effect of a home-made pollen substitute on honey bee colony development. *J. Apic. Res.* **2007**, *46*, 114–119. [CrossRef]
10. Skowronek, P.; Wójcik, Ł.; Strachecka, A. Cannabis Extract Has a Positive–Immunostimulating Effect through Proteolytic System and Metabolic Compounds of Honey Bee (*Apis mellifera*) Workers. *Animals* **2021**, *11*, 2190. [CrossRef]
11. Rascón, B.; Hubbard, B.P.; Sinclair, D.A.; Amdam, G.V. The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging* **2012**, *4*, 499–508. [CrossRef]
12. Schulz, M.; Łoś, A.; Grzybek, M.; Ścibior, R.; Strachecka, A. Piperine as a new natural supplement with beneficial effects on the life-span and defence system of honeybees. *J. Agric. Sci.* **2019**, *157*, 140–149. [CrossRef]
13. Suwannapong, G.; Maksong, S.; Phainchajoen, M.; Benbow, M.E.; Mayack, C. Survival and health improvement of Nosema infected Apis florea (Hymenoptera: Apidae) bees after treatment with propolis extract. *J. Asia. Pac. Entomol.* **2018**, *21*, 437–444. [CrossRef]

14. Malassigné, S.; Minard, G.; Vallon, L.; Martin, E.; Moro, C.V.; Luis, P. Diversity and Functions of Yeast Communities Associated with Insects. *Microorganisms* **2021**, *9*, 1552. [CrossRef] [PubMed]
15. Strachecka, A.; Olszewski, K.; Paleolog, J. Curcumin stimulates biochemical mechanisms of *Apis mellifera* resistance and extends the apian life-span. *J. Apic. Sci.* **2015**, *59*, 129–141. [CrossRef]
16. Strachecka, A.; Krauze, M.; Olszewski, K.; Borsuk, G.; Paleolog, J.; Merska, M.; Chobotow, J.; Bajda, M.; Grzywnowicz, K. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochem.* **2014**, *79*, 1192–1201. [CrossRef]
17. Farjan, M.; Dmitryjuk, M.; LipiŃski, Z.; Biernat-ŁOpieŃska, E.; Zółtowska, K. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera* carnica brood at different stages. *J. Apic. Res.* **2012**, *51*, 263–270. [CrossRef]
18. Ahmad, S.; Khan, K.A.; Khan, S.A.; Ghramh, H.A.; Gul, A. Comparative assessment of various supplementary diets on commercial honey bee (*Apis mellifera*) health and colony performance. *PLoS ONE* **2021**, *16*, e0258430. [CrossRef] [PubMed]
19. Stanimirović, Z.; Glavinić, U.; Ristanić, M.; Jelisić, S.; Vejnović, B.; Niketić, M.; Stevanović, J. Diet Supplementation Helps Honey Bee Colonies in Combat Infections by Enhancing their Hygienic Behaviour. *Acta Vet. Brno.* **2022**, *72*, 145–166. [CrossRef]
20. Strachecka, A.; Olszewski, K.; Paleolog, J.; Borsuk, G.; Bajda, M.; Krauze, M.; Merska, M.; Chobotow, J. Coenzyme Q10 treatments influence the lifespan and key biochemical resistance systems in the honeybee, apis mellifera. *Arch. Insect Biochem. Physiol.* **2014**, *86*, 165–179. [CrossRef]
21. Geldert, C.; Abdo, Z.; Stewart, J.E.; Arathi, H.S. Dietary supplementation with phytochemicals improves diversity and abundance of honey bee gut microbiota. *J. Appl. Microbiol.* **2021**, *130*, 1705–1720. [CrossRef] [PubMed]
22. Ricigliano, V.A.; Simone-Finstrom, M. Nutritional and prebiotic efficacy of the microalga Arthrospira platensis (spirulina) in honey bees. *Apidologie* **2020**, *51*, 898–910. [CrossRef]
23. Skowronek, P.; Wójcik, Ł.; Strachecka, A. CBD Supplementation Has a Positive Effect on the Activity of the Proteolytic System and Biochemical Markers of Honey Bees (*Apis mellifera*) in the Apiary. *Animals* **2022**, *12*, 2313. [CrossRef]
24. Manning, R.; Rutkay, A.; Eaton, L.; Dell, B. Lipid-enhanced pollen and lipid-reduced flour diets and their effect on the longevity of honey bees (*Apis mellifera* L.). *Aust. J. Entomol.* **2007**, *46*, 251–257. [CrossRef]
25. Safian Boldaji, M.; Jalali Dehkordi, K.; Taghian, F.; Author, C. Effect of eight weeks of swimming training and CBD oil consumption on PI3K and ERK gene expression in the heart tissue of rats with myocardial infarction. *J. Shahrekord Univ. Med. Sci.* **2022**, *24*, 54–59. [CrossRef]
26. Majewski, M.; Jurgoński, A. The Effect of Hemp (*Cannabis sativa* L.) Seeds and Hemp Seed Oil on Vascular Dysfunction in Obese Male Zucker Rats. *Nutrients* **2021**, *13*, 2575. [CrossRef]
27. Isenmann, E.; Veit, S.; Starke, L.; Flenker, U.; Diel, P. Effects of Cannabidiol Supplementation on Skeletal Muscle Regeneration after Intensive Resistance Training. *Nutrients* **2021**, *13*, 3028. [CrossRef] [PubMed]
28. Draeger, A.L.; Thomas, E.P.; Jones, K.A.; Davis, A.J.; Porr, C.A.S. The effects of pelleted cannabidiol supplementation on heart rate and reaction scores in horses. *J. Vet. Behav.* **2021**, *46*, 97–100. [CrossRef]
29. Xin, G.; Yang, J.; Li, R.; Gao, Q.; Li, R.; Wang, J.; Zhang, J.; Wang, J. Dietary supplementation of hemp oil in teddy dogs: Effect on apparent nutrient digestibility, blood biochemistry and metabolomics. *Bioengineered* **2022**, *13*, 6173–6187. [CrossRef]
30. Skowronek, P.; Wójcik, Ł.; Strachecka, A. Impressive Impact of Hemp Extract on Antioxidant System in Honey Bee (*Apis mellifera*) Organism. *Antioxidants* **2022**, *11*, 707. [CrossRef]
31. Birch-Machin, M.A.; Bowman, A. Oxidative stress and ageing. *Br. J. Dermatol.* **2016**, *175*, 26–29. [CrossRef] [PubMed]
32. Felton, G.W.; Summers, C.B. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.* **1995**, *29*, 187–197. [CrossRef] [PubMed]
33. Siuda, M.; Wilde, J.; Woyke, J.; Jasiński, Z.; Madras-Majewska, B. Wintering reserve queens in mini-plus and 3-comb nuclei. *J. Apic. Sci.* **2014**, *58*, 61–68. [CrossRef]
34. Scheiner, R.; Abramson, C.I.; Brodschneider, R.; Crailsheim, K.; Farina, W.M.; Fuchs, S.; Grünewald, B.; Hahshold, S.; Karrer, M.; Koeniger, G.; et al. Standard methods for behavioural studies of *Apis mellifera*. *J. Apic. Res.* **2013**, *52*, 1–58. [CrossRef]
35. Łoś, A.; Strachecka, A. Fast and cost-effective biochemical spectrophotometric analysis of solution of insect "blood" and body surface elution. *Sensors* **2018**, *18*, 1494. [CrossRef]
36. Hu, X.; Zhang, B.; Zheng, X.; Ji, H.; Feng, K.; Hu, X.; Gul, I.; Abbas, M.N.; Cui, H.; Zhu, Y. Molecular Characterization of Two Genes Encoding Novel $Ca^{2+}$-Independent Phospholipase A2s from the Silkworm, Bombyx mori. *Curr. Issues Mol. Biol.* **2022**, *44*, 777–790. [CrossRef]
37. Kim, Y.; Stanley, D. Eicosanoid Signaling in Insect Immunology: New Genes and Unresolved Issues. *Genes* **2021**, *12*, 211. [CrossRef]
38. Büyükgüzel, E.; Erdem, M.; Tunaz, H.; Küçük, C.; Atılgan, U.C.; Stanley, D.; Büyükgüzel, K. Inhibition of eicosanoid signaling leads to increased lipid peroxidation in a host/parasitoid system. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2017**, *204*, 121–128. [CrossRef]
39. Park, Y.; Stanley, D.W.; Kim, Y. Eicosanoids up-regulate production of reactive oxygen species by NADPH-dependent oxidase in Spodoptera exigua phagocytic hemocytes. *J. Insect Physiol.* **2015**, *79*, 63–72. [CrossRef]
40. Park, Y.; Kumar, S.; Kanumuri, R.; Stanley, D.; Kim, Y. A novel calcium-independent cellular PLA2 acts in insect immunity and larval growth. *Insect Biochem. Mol. Biol.* **2015**, *66*, 13–23. [CrossRef]

41. Büyükgüzel, E.; Hyršl, P.; Büyükgüzel, K. Eicosanoids mediate hemolymph oxidative and antioxidative response in larvae of *Galleria mellonella* L. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2010**, *156*, 176–183. [CrossRef] [PubMed]
42. Yang, T.; Poovaiah, B.W.; Ryan, C.A. Hydrogen peroxide homeostasis: Activation of plant catalase by calciumcalmodulin. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4097–4102. [CrossRef] [PubMed]
43. Watkins, A.R. Cannabinoid interactions with ion channels and receptors. *Channels* **2019**, *13*, 162–167. [CrossRef] [PubMed]
44. Corona, M.; Hughes, K.A.; Weaver, D.B.; Robinson, G.E. Gene expression patterns associated with queen honey bee longevity. *Mech. Ageing Dev.* **2005**, *126*, 1230–1238. [CrossRef]
45. Mittapalli, O.; Neal, J.J.; Shukle, R.H. Antioxidant defense response in a galling insect. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1889–1894. [CrossRef]
46. Tunaz, H.; Park, Y.; Büyükgüzel, K.; Bedick, J.G.; Aliza, A.R.N.; Stanley, D.W. Eicosanoids in insect immunity: Bacterial infection stimulates hemocytic phospholipase A2 activity in tobacco hornworms. *Arch. Insect Biochem. Physiol.* **2003**, *52*, 1–6. [CrossRef]
47. Zhang, Y.; Li, H.; Jin, S.; Lu, Y.; Peng, Y.; Zhao, L.; Wang, X. Cannabidiol protects against Alzheimer's disease in *C. elegans* via ROS scavenging activity of its phenolic hydroxyl groups. *Eur. J. Pharmacol.* **2022**, *919*, 174829. [CrossRef]