MASARYKOVA UNIVERZITA
Přírodovědecká fakulta
Ústav experimentální biologie
Oddělení fyziologie a imunologie živočichů

Habilitační práce

RNDr. Pavel Hyršl, Ph.D.

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Poděkování

Na tomto místě bych chtěl poděkovat všem, kteří umožnili, aby tato habilitační práce vznikla. Mezi nimi především mým kolegům a přátelům z Oddělení fyziologie a imunologie živočichů, Oddělení parazitologie Masarykovy univerzity, Biofyzikálního ústavu AV ČR, v.v.i., Entomologického ústavu AV ČR v.v.i. a Ústavu biologie obratlovců AV ČR, v.v.i. Také našim spolupracovníkům ze zahraničních institucí, zejména Stockholm University, University of Turku, Bülent Ecevit University, Univesity of Azores a University of Bari. Obzvláště bych chtěl poděkovat všem svým současným a bývalým studentům za jejich pracovitost a ochotu podílet se na týmové práci naší laboratoře, které umožnily vznik prezentovaných studií. Rád bych také poděkoval své rodině za jejich podporu.

Obsah

Abstract

Invertebrates and especially insect belong to the ecologically most successful organisms living on Earth. An adaptation to the antigen pressure of the environment (mainly to micro-organisms) depends on insect innate immunity. Invertebrates compensated the absence of complicated immune reactions by specific adaptations and functions of cellular and humoral parts of their immune system. Although an adaptive immunity in the form we know in vertebrates does not exist in invertebrates, there are advanced mechanisms modulating their immune response. Presented studies on fruit fly *Drosophila melanogaster* and wax moth *Galleria mellonella* described new modulation of immune response including the role of eicosanoids and antioxidants. Using natural infection model combining three organisms – bacteria *Photorhabdus luminescens*, nematode *Heterorhabditis bacteriophora* and fruit fly *Drosophila melanogaster*, new mechanisms of insect immune response to nematobacterial pathogens were identified. Among the genes significantly affected by the infection, mostly those related to immunity, cellular and developmental processes were demonstrated as crucial, e.g. genes coding members of coagulation cascade and recognition molecules.

Innate immunity is also basic for developing adaptive immunity in vertebrates including humans, which contains mainly coagulation and antibacterial mechanisms of body liquids and secrets. Published studies described seasonal dynamics of fish innate immunity, its relation to sex, parasite loads and environmental pollution. Novel important molecular mechanisms of vertebrate innate immunity were also reported in further studies on birds, bank voles and isolated human neutrophils.

Abstrakt

Bezobratlí živočichové a zejména hmyz patří mezi ekologicky nejúspěšnější skupiny organismů žijících na naší planetě. Antigennímu tlaku prostředí, především různým mikroorganismům, čelí bezobratlí reakcemi své přirozené (vrozené) imunity. Absenci komplikovaných imunitních reakcí bezobratlí nahrazují na jedné straně specifickými adaptacemi a na druhé straně také dobře fungujícím imunitním systémem. Adaptivní imunita založená na protilátkách u bezobratlých neexistuje, jsou ale přítomné pokročilé mechanismy, které umožňují modulaci imunitní odpovědi. Studie zejména na octomilce *Drosophila melanogaster* a zavíječi voskovém *Galleria mellonella* popsaly nové mechanismy modulace imunitní odpovědi včetně zapojení eikosanoidů a antioxidantů. Na infekčním modelu zahrnujícímu tři organismy - bakterie *Photorhabdus luminescens*, hlístici *Heterorhabditis bacteriophora* a octomilku *Drosophila melanogaster* - byly identifikovány nové mechanismy podílející se na reakci hmyzu proti nematobakteriálním patogenům. Mezi geny významně ovlivněnými infekcí byly zejména ty, které jsou zapojeny v imunitních reakcích, buněčných a vývojových procesech. Pomocí experimentálních nákaz bylo v našich studiích identifikováno několik imunitních genů, kódujících např. složky koagulační kaskády nebo rozpoznávací molekuly, jako klíčových pro zdolání entomopatogenní nákazy.

Přirozená imunita je také základem imunity u obratlovců včetně člověka, opět se jedná zejména o koagulaci a antibakteriální mechanismy tělních tekutin a sekretů během nespecifického rozpoznání patogenů. Publikované studie popsaly sezónní dynamiku přirozené imunity ryb, její vztah k pohlaví, ploidii, parazitaci a znečištění prostředí. Nové detaily mechanismů přirozené imunity přinesly také další studie na ptácích, nornících a izolovaných lidských neutrofilech.

Úvod

Imunitní reakce obratlovců včetně člověka zahrnují prvky, které se mění během života jedince, tj. adaptivní část imunitního systému, a prvky vrozené, které tvoří tzv. **přirozenou (vrozenou) imunitu**. Všichni živočichové počínaje skupinou živočišných hub (Porifera) disponují přirozenou imunitou, která je chrání před nejčastějšími patogeny v jejich životním prostředí, ale také jim umožňuje rozlišit vlastní struktury od cizích. Právě rozpoznání cizího a tolerance vlastního je klíčovou složkou pro udržení homeostázy organismu. Na základě přirozené imunity se rozvíjí **imunita adaptivní**, přesné rozhraní ale lze těžko stanovit, protože nové poznatky posouvají kořeny této pokročilejší imunity až k bezobratlým živočichům (členovci - Arthropoda, ostnokožci - Echinodermata), naopak primitivní obratlovci (např. sliznatky - Mixozoa) jsou svými reakcemi mnohem blíže k bezobratlým živočichům. Musíme brát také v úvahu odlišné životní strategie živočichů, kdy se setkáváme s vývojovými stádii, která jsou někdy přisedlá, jindy volně se pohybující a tím pádem pokročilejší i v jejich imunitním systému. Někdy také proběhl regresní vývoj k radiální symetrii (ostnokožci - Echinodermata). U nejjednodušších a zejména přisedlých forem převažuje význam termínu imunita ve smyslu nedotknutelnost než častěji používaný aktivní přístup k odstranění patogenů.

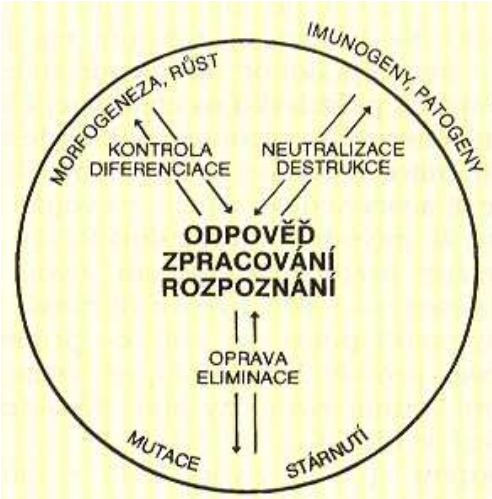
U většiny imunitních reakcí obratlovců se oba typy imunity navzájem doplňují, což ztěžuje studium jednotlivých mechanismů. Je tedy velmi výhodné používat modelové organismy, které mají pouze přirozenou imunitu a následně například zvolit vhodnou strategii terapie u obratlovců. Přirozené reakce mohou být cíleně ovlivněny, pouze pokud známe jejich detailní signální dráhy. V poslední době jsou proto intenzivně studovány imunitní systémy bezobratlých, zejména výzkum na octomilce *Drosophila melanogaster* výrazně rozšířil znalosti o přirozené imunitě obecně (Lemaitre & Hoffmann, 2007). Po zveřejnění genomu *Drosophily* bylo zjištěno, že 75% genů pro lidská onemocnění zde má svůj ekvivalent, proto se octomilka používá např. jako „prescreening“ médium pro selekci léčiv.

1. Přirozená imunita bezobratlých

Ačkoli bezobratlí živočichové tvoří naprostou většinu živočišných druhů, byl jejich imunitní systém dlouhodobě málo studován. Popsání vztahů hostitel-patogen je důležité zejména proto, že řada z nich parazituje obratlovce včetně člověka. Poznáním mechanismů můžeme získat biologickou kontrolu nad škůdci, nebo naopak zlepšit chovy komerčně využívaných bezobratlých. Další oblasti výzkumu jsou monitorování životního prostředí

nebo objasnění evolučních základů imunitního systému obratlovců. Bezobratlí se dostali také do popředí zájmu farmaceutických firem, kdy z nich bylo izolováno mnoho imunoreaktivních molekul pro humánní medicínu (Šíma & Trebichavský, 2001). Bezobratlí živočichové používají v imunitních reakcích jiné struktury než obratlovci, ale funkčně se mohou blížit pokročilým reakcím obratlovců. Nejdetailněji je studován imunitní systém hmyzu díky snadno dostupným modelovým organismům (bourec morušový *Bombyx mori*, zavíječ voskový *Galleria mellonella*, octomilka *Drosophila melanogaster*, martináč *Hyalophora cecropia*, potemník moučný *Tenebrio molitor*, šváb americký *Periplaneta americana*, ruměnice pospolná *Pyrrhocoris apterus* aj.), u jiných skupin bezobratlých jsou přítomné podobné mechanismy nebo se jejich přítomnost alespoň předpokládá. Díky obrovské variabilitě taxonů se setkáváme s výraznými rozdíly ve vnějších bariérách (sliz, ulita/lastura, kutikula), tak i jednotlivých imunitních reakcích, např. fenoloxidázová kaskáda byla popsána jen u měkkýšů (Mollusca) a členovců (Arthropoda). Liší se také zastoupení antibakteriálních peptidů, využití reaktivních kyslíkových metabolitů během fagocytózy apod.

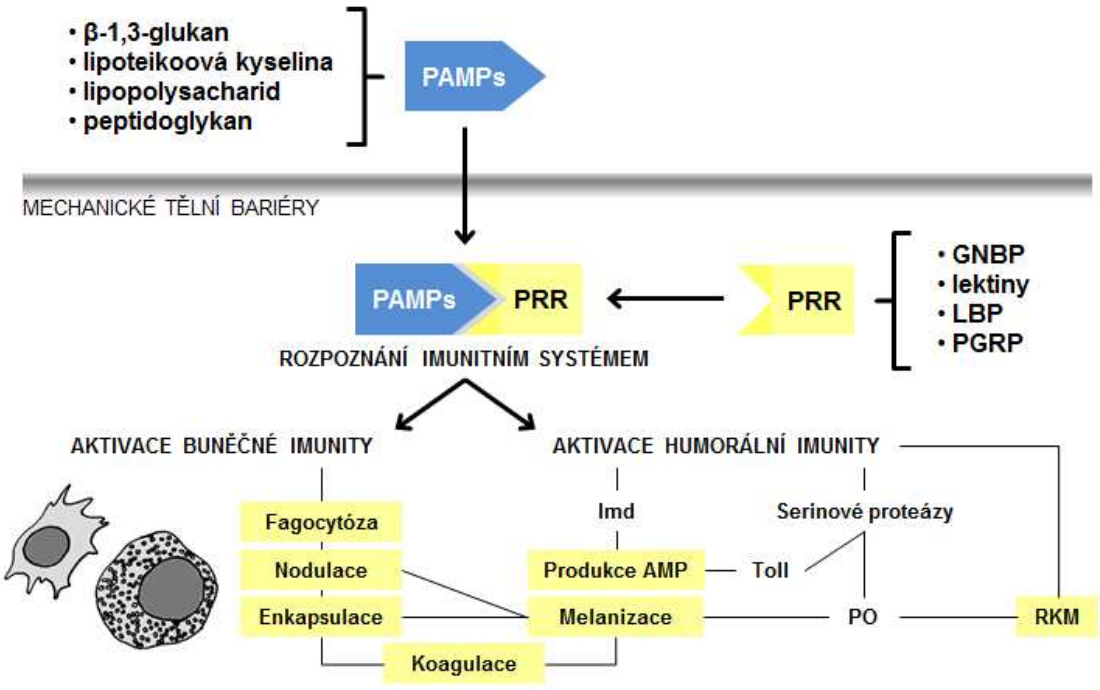
Rozpoznání mikroorganismů jako základ pro spuštění imunitních mechanismů (obr. 1) je u bezobratlých založeno na nespecifických fyzikálněchemických vlastnostech jako je povrchový náboj, hydrofobicita aj., nebo prostřednictvím specifických senzorů – lektinových molekul a složek fenoloxidázové kaskády, které se vážou na glycidy bakteriálních buněčných stěn (popsáno už u Protista). Rozpoznání probíhá jako tzv. rozlišení vzorů (**pattern recognition**) pomocí tzv. pattern recognition receptors (PRR). PRR se vyskytují volně v tělní tekutině i na povrchu cirkulujících buněk a jsou schopny rozpoznat charakteristické struktury na povrchu patogenů, příp. jimi sekretované látky, souhrnně nazývané jako „pathogen-associated molecular patterns“ (PAMPs), případně „microbe-associated molecular patterns“ (MAMPs). Mezi PAMPs patří široké spektrum molekul včetně oligosacharidů, proteinů, glykoproteinů, lipidů a cizích motivů nukleových kyselin, které jsou často pro samotné patogeny životně důležité (Brivio et al., 2005). Vazba PAMPs s příslušným PRR vede ke spuštění intracelulárních signálních drah a vzápětí ke zvýšení



Obr. 1: Obecné schéma úlohy imunitního systému, Šíma 1997.

exprese vysoce účinných efektorových molekul zprostředkujících látkovou a buněčnou imunitní odpověď (Yu et al., 2002; obr. 2). PRR se mohou podílet na odstranění cizích látek z organismu také přímo prostřednictvím fagocytózy, při které fungují jako opsoniny (Kim et al., 2010), nebo aktivací serinových proteáz, které jsou součástí fenoloxidázové kaskády (Cerenius & Söderhäll, 2004).

Ne všechny mikroorganismy jsou patogeny, řada z nich produkuje PAMPs, ale nepoškozují vlastní buňky hostitele. Jako nebezpečné jsou tedy rozpoznány až po uvolnění vzorů produkovaných poškozenými buňkami tzv. DAMPs – „damage associated molecular patterns“ (podle tzv. „danger hypothesis“, Matzinger, 1994). PAMPs a DAMPs následně aktivují intracelulární nebo povrchové receptory rozpoznávající tyto specifické struktury (PRRs) za účelem stabilizace homeostázy (oprava poškození, kontrola/potlačení infekce nebo udržení symbiontů, Lazzaro & Rolff, 2011). Dalším mechanismem rozpoznávající symbiotické bakterie může být produkce uracilu, kdy ztráta produkce uracilu odlišuje patogeny od střevních symbiontů (Ha et al., 2005; Lee et al., 2013). Podle lokalizace rozlišujeme u bezobratlých podobně jako u obratlovců slizniční imunitu, imunitu střeva, pohlavních orgánů, dýchacího systému apod. V úvahu také musíme vzít metamorfózu a stárnutí.



Obr. 2: Aktivace přirozeného systému bezobratlých živočichů. Struktury charakteristické pro patogenní organismy (PAMPs) jsou v organismu hostitele rozpoznávány příslušnými receptory (PRR) a poté dochází k aktivaci jednotlivých imunitních reakcí. U nižších taxonomických skupin bezobratlých nemusí být všechny reakce zastoupeny, upraveno podle Brivio et al., 2005).

Pro poznání mechanismů imunity jsou důležité stresové situace, ve kterých organismus reaguje na podnět (**stres** - porušení homeostázy organismu), který způsobí aktivaci jedné nebo více složek imunitního systému. Stres lze charakterizovat jako negativní působení chemických, fyzikálních či fyziologických vlivů na organismus. Tyto vlivy mohou vyvolat porušení homeostázy organismu a vést až k rozvratu fyziologických funkcí případně ke smrti organismu. Jako modelové situace se využívá experimentů s injikací (bakterie, inertní partikule, části bakteriálních stěn aj.), teplotní stres (vliv nízké a vysoké teploty), mechanický stres (poranění, ligatura), parazitace, změna potravy (popřípadě hladovění), působení hormonů, analog hormonů a jiných chemických látek a mnohé další (viz review Brey, 1994). Předchozí případy lze přiřadit k exogenním stresovým vlivům, existují ale také endogenní stresové situace vycházející z chování a vývoje – metamorfóza a migrace.
Zvláštním typem stresu je **oxidační stres**. Je způsoben tzv. **reaktivními kyslíkovými metabolity** (RKM), které vznikají jako produkt buněčného kyslíkového metabolismu. K oxidačnímu stresu dochází, když je nadprodukce RKM spojena s nedostatkem obranných antioxidačních systémů. Jinými slovy oxidační stres je výsledkem nahromadění RKM, které mohou porušit rovnováhu mezi prooxidanty a antioxidačními reakcemi organismu. Na takový stav organismus odpovídá aktivací obranného systému, který zahrnuje několik rovin se širokou škálou svého působení (viz dále). V zásadě však tyto systémy můžeme rozdělit na enzymatické, které mění RKM na méně škodlivé nebo neškodné metabolity aktivací řady příslušných enzymů, a neenzymatické, které působí jako vychytávače volných radikálů. O aktivaci těchto mechanismů existuje velké množství údajů, na druhou stranu však řada z nich není zcela objasněna.

Buněčná imunita:

Buněčná imunita je u bezobratlých zprostředkována volnými krevními buňkami – hemocyty cirkulující v hemolymfě nebo coelomocyty v coelomové tekutině (obecně je lze nazvat imunocyty). Kromě cirkulujících buněk bývají k dispozici fixní buňky lokalizované v tzv. „sessile compartments“, odkud mohou být uvolněny při aktivaci imunitní reakce. Taxonomie jednotlivých buněčných typů se velmi liší (nejčastěji jsou rozlišovány prohemocyty, granulocyty, plasmatocyty, koagulocyty, spherulocyty a oenocyty), proto se zjednodušeně používá dělení podle jejich funkce na progenitorové, fagocytující, hemostatické, pigmentové a nutritivní (Turner, 1994). Rozlišujeme čtyři základní imunitní aktivity: fagocytóza, enkapsulace, nodulace a koagulace. Počet a aktivita cirkulujících buněk jsou modulovány **humorálními faktory** a **neuroendokrinním systémem**. Imunitní

odpověď také ovlivňuje celkový fyziologický stav organismu a vnější fyzikální a chemické faktory.

Fagocytóza

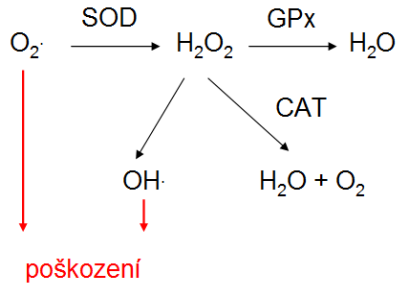
Fagocytóza je základní imunitní funkcí u všech bezobratlých živočichů i obratlovců, u hmyzu se na ní podílejí zejména plasmatocyty a granulocyty. Fagocytující hemocyty a coelomocyty jsou funkčně podobné fagocytům obratlovců a člověka; při likvidaci patogenů se uplatňují RKM, které vznikají u obratlovců z molekulárního kyslíku v procesu nazývaném **oxidativní (respirační) vzplanutí**. Zvýšená tvorba RKM byla nalezena v aktivovaných hemocytech a coelomocytech některých skupin bezobratlých jako jsou Bivalvia (např. Ordas et al., 2000), Clitellata (Valembois & Lassegues, 1995), Malacostraca (Bell & Smith, 1993), Arachnida (Pereira et al., 2001), Echinoidea (Ito et al., 1992) nebo Ascidiacea (Azumi et al., 2002). Proto se zdá být produkce RKM významným mikrobicidním faktorem v hemocytech a coelomocytech některých bezobratlých, podobně jako ve fagocytech obratlovců.

O respiračním vzplanutí u hmyzu je velmi málo informací, navíc s protichůdnými výsledky. Někteří autoři tvorbu RKM popírají jako např. Mazet et al. (1994). Stejně tak v našich experimentech (Hyršl et al., 2004; **příloha 1**) jsme neprokázali tvorbu RKM hemocyty *Bombyx mori* za použití luminometrických, spektrofotometrických ani fluorescenčních metod. Na druhou stranu se ale objevují pozitivní výsledky - Arakawa (1995) popsal významnou produkci superoxidu v supernatantu hemolymfy z larev *Pseudaletia separata* (Lepidoptera). Whitten & Ratcliffe (1999) přinesli důkaz existence imunitní odpovědi podobající se oxidačnímu vzplanutí v hemolymfě a hemocytech švába *Blaberus discoidalis* (Blattodea). Několik článků prokazujících produkci superoxidu u hmyzu se týká zavíječe voskového *G. mellonella*, např. Slepneva et al. (1999), Glupov et al. (2001) a Krishnan et al. (2008), nebo *D. melanogaster* (Nappi & Vass, 1993). Peroxid vodíku se nám podařilo detekovat u *G. mellonella* (Vašíček et al., 2011; **příloha 2**), ale jeho produkce je ve srovnání lidskými fagocyty velmi malá. Bergin et al. (2005) a Renwick et al. (2007) kromě produkce superoxidu uvádějí analogii s oxidativním vzplanutím savců, řada detailů ale stále není známa. Homologii obou procesů dokazuje například zapojení proteinů p47phox and p67phox (Geiszt et al., 2003).

Podobně jako RKM jsou rozlišovány **reaktivní dusíkové metabolity** (RDM). Nejstudovanější je oxid dusnatý (NO), který slouží také jako signální molekula a je součástí mnoha fyziologických funkcí (Foley & O'Farrell, 2003). Naše předchozí

experimenty (Krishnan et al., 2006; **příloha 3**) prokázaly produkci NO hemocyty *G. mellonella* a jeho indukci, např. bakteriálním lipopolysacharidem (LPS). NO je tedy součástí buněčné antibakteriální imunity, zatímco jeho vliv na fenoloxidázovou kaskádu (humorální imunita) nebyl prokázán. Zajímavé je, že nadprodukce NO může působit více cytotoxicky než cytoprotektivně.

Působení radikálů by mohlo být letální, pokud by nebyly odstraňovány účinným antioxidačními systémy. Zvýšené koncentrace volných radikálů vedou k oxidačnímu poškození proteinů, lipidů a nukleových kyselin (Halliwell & Gutteridge, 1999). Bezobratlí proto stejně jako obratlovci používají celou řadu **antioxidačních enzymů**, které ve vzájemně propojených reakcích čelí nárůstu endogenně vytvářených nebo s potravou přijímaných oxidantů. **Oxidační stres** se rozvíjí, pokud se zvyšuje produkce radikálů a vychytávací systém antioxidančních enzymů je poškozen. Antioxidační enzymy zahrnují superoxid dismutázu (SOD), katalázu (CAT), glutathion peroxidázu (GPx), glutathion S-transferázu (GSTs) a askorbát peroxidázu (APOX) (Ahmad, 1995; obr. 3). SOD, CAT a GPx tvoří obranný komplex proti endogenně produkovaným RKM. SOD katalyzuje dismutaci superoxidového radikálu na H_2O_2 a molekulární kyslík, je také hlavní odpovědí na prooxidační působení allelochemikálií přijímaných s potravou. H_2O_2 je následně vychytáván CAT za vzniku vody a kyslíku (Ahmad & Pardini, 1990). APOX také vychytává H_2O_2, ale jen v nízkých koncentracích. GPx redukuje H_2O_2 a hydroperoxidy ve tkáních a buněčných membránách, u bezobratlých je zastoupena velmi málo. Oxidační stres vyvolávají například látky znečišťující životní prostředí nebo cíleně chemické pesticidy. Stanovení aktivity antioxidačních enzymů se tedy společně s přímým stanovením poškození organismu používá v ekotoxikologických studiích s využitím modelových zástupců kroužkovců (Panzarino et al., 2016; **příloha 4**) a hmyzu (Hyršl et al. 2007; **příloha 5**).



Obr. 3: Schéma působení hlavních antioxidačních enzymů.

Nodulace

Při vyšších dávkách antigenu dochází k tvorbě nodulí, což jsou útvary vznikající agregací cirkulujících hemocytů/coelomocytů kolem bakterií nebo jiného cizorodého materiálu podobné velikosti. Nodulace zabraňuje dalšímu šíření patogenů v organismu a zároveň je

při ní z hemocytů uvolňováno množství imunitních faktorů, které mají přímé cytotoxické účinky, účastní se jí složky fenoloxidázové kaskády, která je zároveň aktivována a dochází k melanizaci. Molekulární mechanismy nodulace nejsou zatím přesně známy; bylo však popsáno, že hlavní agregační reakce jsou zprostředkovány především lektiny (Marmaras & Lampropoulou, 2009). Někteří zástupci bezobratlých se vyznačují přirozeně nízkou nodulační aktivitou, do této skupiny patří i hojně používaná *D. melanogaster*. Mikroagregace nebo nodulace jako reakce na bakteriální infekce závisí na produktech kyseliny arachidonové – eikosanoidech, viz dále (Stanley-Samuelson et al., 1997; Miller et al., 1999).

Enkapsulace

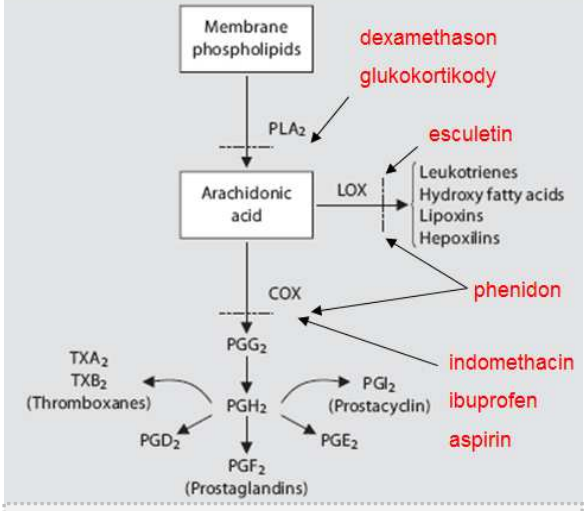
Podobně jako nodulace probíhá i enkapsulace. Ta se však uplatňuje zejména u invadujícího antigenního materiálu větších rozměrů, který nemůže být hemocyty/coelomocyty fagocytován - parazitičtí prvoci, hlísti a mnohobuněční nebo i nebiogenní substance (sklo, umělá hmota, latex). Bývá rozlišována buněčná a humorální enkapsulace (u druhů s malým počtem cirkulujících buněk). Není-li tvorba kapsule doprovázena následnou melanizací, je tento typ označován jako prostá enkapsulace, na rozdíl od častější melanotické enkapsulace (Gupta, 2002). Uvnitř vytvořené kapsule je parazit nejen imobilizován, ale také vystaven působení dalších složek imunity hostitele (např. RKM a RDM) a izolován od přístupu k živinám.

Regulace buněčné imunity pomocí eikosanoidů

Eikosanoidy jsou obecným termínem pro všechny biologicky aktivní, okysličené metabolity kyseliny arachidonové (AA) a dalších dvou C20 nenasycených mastných kyselin. Hlavní skupiny eikosanoidů zahrnují prostaglandiny (cyklooxygenázové produkty - COX), různé lipoxygenázové produkty (LOX) a epoxyeikosanoidové kyseliny. U savců eikosanoidy a hormony jako kortikosteron regulují imunitní odpověď, podobná funkce eikosanoidů je i u buněčné imunity hmyzu (např. Miller & Stanley, 2001; Dean et al., 2002). Stanley-Samuelson et al. (1991) jako první navrhl právě eikosanoidy jako prostředníky jedné nebo více buněčných reakcí zodpovědných za vychytávání bakterií z hemolymfy při infekci. Na základě těchto zjištění přišel Stanley (2000) s tzv. eikosanoidovou hypotézou, tj. že eikosanoidy zprostředkovávají nodulaci bakterií u většiny hmyzu, možná dokonce u všech druhů. Dále byla popsána jejich funkce při migraci hemocytů, fagocytóze a nodulaci (Stanley & Miller, 2006), účast na aktivaci

fenoloxidázové kaskády (Mandato et al., 1997) a jejich úloha v infekcích způsobených houbami, prvoky, parazitoidy a dokonce i viry (Beckage, 2008). Zapojení regulační kaskády eikosanoidů (konkrétně sekretované fosfolipázy A2) v odpovědi na nákazu hlísticemi je uvedeno v **příloze 6** (Hyršl et al., 2010).

Praktický přístup pro studium funkce eikosanoidů je založen na použití **inhibitorů biosyntézy eikosanoidů** (IBE). Použitím IBE dochází k inhibici jednotlivých imunitních reakcí. Látky patřící do této skupiny se mohou vzájemně lišit svou strukturou a především mechanismem působení. Mezi nejčastěji používané IBE patří např. dexamethason (9-α-fluor-16α-methyl-prednisolon), phenidon (1-phenyl-3-pryazolidinon), esculetin (6,7-dihydroxykumarin), indomethacin [1-(p-chlorophenycyl)-5-methoxy-2-methlyindol-3- octová kyselina] a ibuprofen [2-(4-isobutylphenyl) propionová kyselina], obr. 4. Dexamethason inhibuje enzym fosfolipázu A2, čímž blokuje uvolňování AA z buněčných fosfolipidů a znemožňuje tak její další zpracování na eikosanoidy. Účinek dexamethasonu může být obvykle vyrušen přídáním AA (Mandato et al., 1997; Tunaz, 2006). Phenidon, esculetin a indomethacin jsou inhibitory enzymů zprostředkujících přeměnu uvolněné AA (Mandato et al., 1997; Phelps et al., 2003; Tunaz, 2006). Phenidon inhibuje jak cyklooxygenázy, tak lipoxygenázy a brání tedy vzniku produktů obou těchto drah. Naproti tomu esculetin inhibuje pouze činnost lipoxygenáz (5- a 12-LOX) a indomethacin cyklooxygenáz (COX-1 a -2). Pomocí esculetinu a indomethacinu můžeme tedy určit závislost imunitní odpovědi na lipoxygenázové nebo cyklooxygenázové dráze metabolismu AA (Lord et al., 2002; Phelps et al., 2003).



Obr. 4: Schéma biosyntézy eikosanoidů, inhibitory jsou označeny červeně, upraveno podle Stanley, 2000.

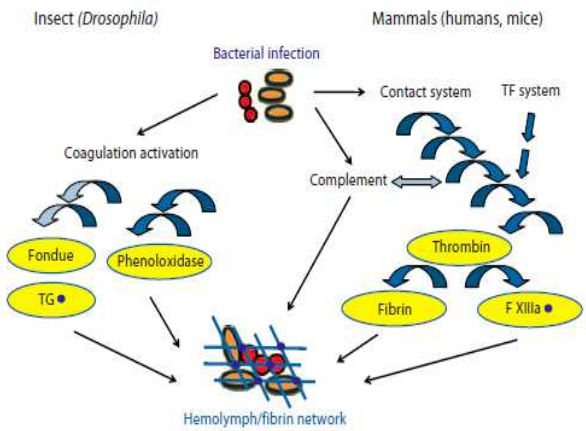
Další důležitou rolí eikosanoidů je jejich zapojení v antioxidačních mechanismech při zvýšené tvorbě RKM (oxidační stres), způsobené například podáváním prooxidantů larvám *G. mellonella* (Büyükgüzel et al. 2010; **příloha 7**). Kromě změny aktivity antioxidačních enzymů může být výsledným efektem také syntéza stresových proteinů (Hyršl et al., 2011; **příloha 8**).

Humorální imunita:

Humorální imunitu bezobratlých představují zejména dvě proteolytické kaskády (koagulační a fenoloxidázová), lysozym, lektiny, hemolin, aglutininy a řada antigenem stimulovaných antibakteriálních (s mikrobicidní nebo mikrobistatickou aktivitou) a regulačních peptidů (Gupta, 2001). Jednotlivé složky se nacházejí v cirkulující coelomové tekutině nebo hemolymfě, mohou být také složkou sekretů (typicky ve slizu) nebo vylučovány na povrch kutikuly jako součást bariérních antimikrobiálních mechanismů. Část z nich se nachází v organismu konstitutivně (fenoloxidázy ve formě profenoloxidáz, lysozym, lektiny, hemolin, aglutininy; stresová situace ale mnohonásobně zvyšuje jejich koncentraci), další část je exprimována pouze v případě infekce (typické pro baktericidní peptidy). Protože se jedná zejména o peptidy a proteiny, používá se pro analýzu proteinového spektra především elektroforéza. Hmyzí hemolymfa obsahuje velké množství transportních, zásobních, imunitních a jiných proteinů, jejich zastoupení se významně mění zejména během vývoje a také při stresových situacích, viz např. Hyršl & Šimek, 2005; **příloha 9**; Hyršl et al., 2008; **příloha 10**; Hyršl et al., 2011; **příloha 8**.

Koagulační kaskáda

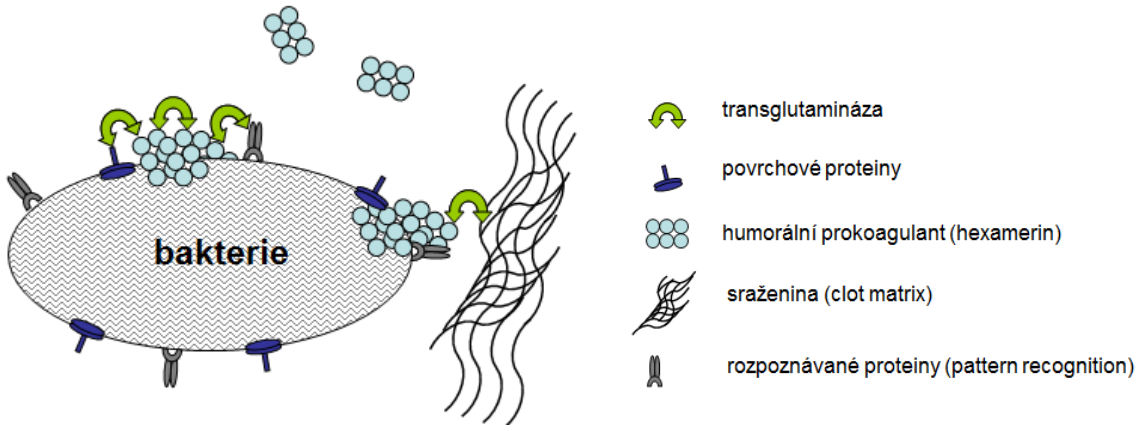
Po poranění dochází u téměř všech živočichů k aktivaci koagulační kaskády vedoucí ke srážení tělní tekutiny. U bezobratlých je koagulace zprostředkovaná různými typy hemocytů/coelomocytů, které se po stimulaci během poranění rozpadají, uvolňují faktory koagulační kaskády a aktivují ji. Jedná se tedy o ukázkové propojení buněčné a humorální imunity. Společně s fenoloxidázovou kaskádou se podílí na vytvoření „zátky“ v místě poranění (zastavení úniku hemolymfy/coelomové tekutiny a zabránění infekci, obr. 5). Bílkovinná síť v místě poranění je budována jednak z koagulogenu hemocytů/coelomocytů (koagulocytů) a jednak z koagulogenu plasmy, který je sekretován vnitřními orgány, např. tukovým tělesem u hmyzu. Polymerace obou proteinů je katalyzována transglutaninázou závislou na Ca^{2+}.



Obr. 5: Srovnání koagulace hmyzí hemolymfy a krve savců, Loof et al., 2011.

U relativně dobře probádaných členovců existují různé procesy koagulace a koagulační proteiny, obecně to je komplex enzymaticky řízených dějů vedoucích od srážecích proteinů (koagulogeny) k nerozpustnému gelu (koagulin). U korýšů jsou dvě základní složky – transglutamináza uvolňovaná z cirkulujících buněk po poranění a koagulační protein plasmy (homodimer 210 kDa), které polymerují do velkých agregátů (Cerenius et al., 2010). U ostrorepa se nachází proteolytická kaskáda, jejíž komponenty jsou v granulích hemocytů a jsou uvoňovány exocytózou v přítomnosti např. lipopolysacharidů. Koagulace u hmyzu vyžaduje transportní proteiny (lipophoriny), hexameriny (také označované jako larvální sérové proteiny, součást zásobních proteinů), jejich receptor FBP1 („fat body protein 1“), gp150 (glykoprotein 150) a další specializované srážecí faktory, jako jsou hemolektin (homolog savčího faktoru von Willebrand), Tiggrin a Fondue (Lesch et al., 2007; Dushay, 2009; Hyršl et al., 2010; **příloha 6**). Většina těchto látek slouží především jako substrát pro enzym tranglutaminázu, který vytváří vazby mezi glutaminem a lysinem, čímž dochází k provázání koagulační proteinů a vzniku sraženiny.

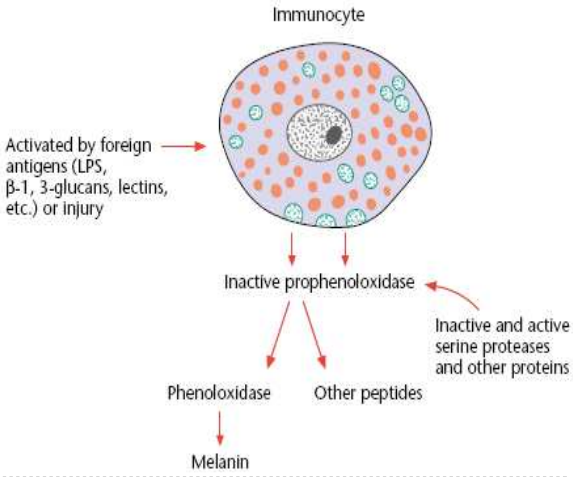
V místě poranění většinou probíhá aktivace fenoloxidázové kaskády a melanizace (Cerenius et al., 2010), poraněním může být také regulována syntéza antimikrobiálních peptidů (Zhu et al., 2016). Podobně jako u obratlovců je vytvrzení zátky závislé na transglutamináze (u obratlovců označované jako Faktor XIIIa, Lindgren et al., 2008, obr. 6). V síti polymerujících bílkovin jsou kromě vlastních buněk hemolymfy/coelomové tekutiny zachycované také pronikající patogeny, takže koagulace (a zejména evolučně konzervovaná tranglutamináza) hraje důležitou roli v imunitních reakcích jedince (Wang et al., 2010; obr. 6; **příloha 11**).



Obr. 6: Role transglutaminázy při formování sraženiny v hmyzí hemolymfě – zesíťování jednotlivých složek (podle Wang et al., 2010; **příloha 11**).

Fenoloxidázová kaskáda

Fenoloxidázová kaskáda je přítomná u měkkýšů, korýšů a hmyzu, u dalších skupin bezobratlých živočichů zcela chybí nebo nejsou známé informace. Zajímavá je její absence u některých klíšťat (Smith & Pal, 2014), i když blízké taxonomické skupiny ji mají. Fenoloxidázová kaskáda se podílí na tvorbě kutikulárních barviv, agregaci proniklých bakterií, opsonizaci, agregaci antigenu, enkapsulaci a tvorbě nodulí. Reakce probíhá přes několik mediátorů a celá je katalyzována pouze jedním enzymem, fenoloxidázou (80 kDa). Ta se vyskytuje v podobě profenoloxidázy (70 kDa), která je aktivovanými proteolytickými enzymy přeměněna na vlastní fenoloxidázu, celá reakce je aktivována složkami bakteriálních stěn, poraněním a enkapsulací (Gupta, 2001; obr. 7). Výsledkem kaskády je přeměna zbytků tyrosinu na polymer melanin (melanizace). Změny v aktivitě kaskády lze detekovat i během vývoje, např. u *G. mellonella* (Benešová & Hyršl, 2009; **příloha 12**).



Obr. 7: Aktivační systém profenoloxidázy vedoucí k melanizaci, Gupta, 2001.

Lysozym

Základní humorální složku přirozené imunity tvoří lysozym (14,5 kDa), který náleží ke skupině asi 20 příbuzných enzymů, označovaných jako N-acetylmuramylhydrolázy – E.C.3.2.1.17. Katalyzuje hydrolýzu polysacharidových řetězců N-acetylglukosaminových jednotek a zbytku N-acetylmuramové kyseliny v buněčných stěnách bakterií. Působí pouze na G^+ bakterie, protože mají odlišnou stavbu buněčné stěny než G^- bakterie (lytický, bakteriocidní faktor pro G^+ bakterie, bakteriostatický faktor pro G^- bakterie). Nachází se konstitutivně v tělních tekutinách a sekretech bezobratlých i obratlovců, po stimulaci jeho množství/aktivita stoupá.

Antimikrobiální peptidy

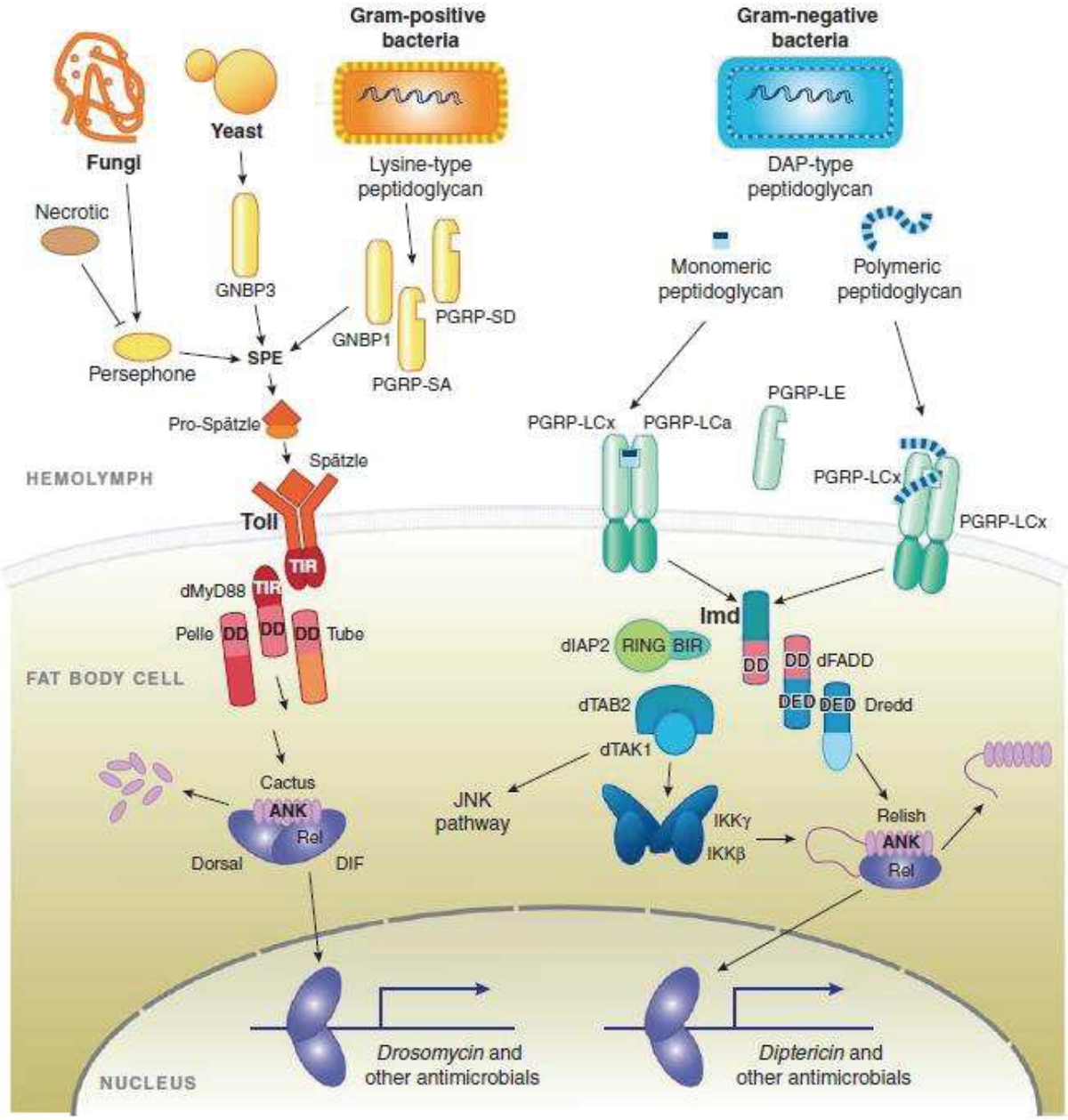
Invadující mikroorganismy jsou u bezobratlých po průniku mechanickými bariérami (kde mohou být zachyceny transglutaminázou během koagulace, viz výše) okamžitě likvidovány buněčnými složkami imunity. Uvádí se, že řádově v sekundách je 99,5%

mikroorganismů odstraněno fagocytózou a teprve proti zbývajícím jsou zapojeny složky humorální imunity (Schneider & Chambers, 2008). Kromě lysozymu, který se nachází u většiny živočichů, jsou to většinou inducibilní antimikrobiální peptidy (AMP), kterých je obrovské množství a jejich zastoupení se liší podle jednotlivých taxonů, vývojových stádií a zdroje nákazy. U většiny bezobratlých se předpokládá, že je současně přítomno několik AMP s odlišným působením, díky obrovskému množství druhů byly ale pouze u malé části z nich popsány a identifikovány. Výzkum AMP má velký potenciál i pro člověka ve farmacii a medicíně, mnoho přírodních látek z této skupiny může sloužit jako antibiotika a řada z nich vykazuje i protinádorové vlastnosti (Šíma & Trebichavský, 2001; Zasloff, 2002). Nejpodrobněji je prozkoumán aktivační systém syntézy AMP u hmyzu, který zahrnuje dvě hlavní dráhy Toll a Imd (obr. 8), které kontrolují syntézu malých kladně nabitých AMP aktivních proti bakteriím, houbám, ale i virům. Analogické signální dráhy najdeme i u obratlovců (signální dráhy přes Toll-like a TNF-α receptor, Hoffmann & Reichhart, 2002). V případě peptidů působících na bakterie rozhoduje také složení bakteriální buněčné stěny. Některé z AMP působí specificky pouze proti Gram negativním nebo pozitivním kmenům a na ostatní druhy mají jen bakteriostatický vliv. AMP lze dělit podle jejich chemické stavby a struktury, případně se označují podle taxonu, odkud byly izolovány (diptericiny z Diptera, cecropiny z *Hyalophora cecropia*, drosomycin z *Drosophila melanogaster*, atd.). Syntéza AMP je také tkáňově specifická (Ferrandon et al., 2007).

Pro studium aktivace signálních drah vedoucím k syntéze AMP jsme v našich studiích použili RNAi linie *D. melanogaster* a následně funkční test pomocí nematobakteriální infekce, viz dále. Antibakteriální aktivitu ve vzorcích hemolymfy je možné měřit různými metodami, v našich studiích jsme zavedli a optimalizovali bioluminiscenční metodu, která umožňuje sledovat přímé baktericidní působení v reálném čase na bioluminiscenční *E. coli* K12 (Vojtek et al. 2014; **příloha 13**) a zónovou difúzi pro stanovení aktivity lysozymu (Hyršl & Šimek, 2005; **příloha 9**).

Aglutininy

Aglutininy cirkulující v tělní tekutině jsou celkově neprobádanou skupinou proteinů, u nichž byla detekována aglutinační a opsonizační aktivita s rozdílnou intenzitou proti různým patogenům. Některé aglutininy patří zároveň mezi lektiny. Nacházejí se kromě tělní tekutiny i v hlenu na povrchu těla vodních i suchozemských bezobratlých



Obr. 8: Schéma aktivace Toll (vlevo) a Imd (vpravo) signální dráhy u hmyzu. Toll je aktivována složkami buněčných stěn hub a Gram pozitivních bakterií, kdežto Imd převážně peptidoglykany z Gram negativních bakterií. V obou případech dochází k postupné aktivaci několika intracelulárních mediátorů a nakonec k aktivaci exprese genů kódujících AMP. V případě Toll dráhy dochází po rozpoznání patogenu PRR k proteolytické aktivaci Spätzle – ligandu transmembránového Toll receptoru. Po dimerizaci se na Toll receptor vážou proteiny MyD88, Pelle a Tube obsahující death domény (DD) a následně je zatím neznámým mechanismem fosforylován a degradován protein Cactus. Jeho odstranění uvolňuje transkripční faktory Dif a Dorsal, které přecházejí z cytoplazmy do jádra, kde spouští transkripci kontrolovaných genů. Imd dráha je spouštěna po navázání DAP-type PG na PRR. Komplex elicitoru s PGRP-LC poté interaguje s adaptorovým proteinem Imd, na který se postupně váže protein FADD a apikální kaspáza Dredd. Výsledkem je štěpení proteinu Relish a přestup Rel domény do jádra, kde působí jako transkripční faktor. Aby mohl být protein Relish štěpen, musí být fosforylován. To zajišťuje komplex IKK, který je sám aktivován prostřednictvím proteinů TAK1 a Imd (převzato z Lemaitre & Hoffmann, 2007).

Lektiny a další PRR

Lektiny tvoří skupinu proteinů, které hydrofobně váží specifické glycidy, jedná se o bílkoviny s velkými molekulami o velikosti 70 - 150 kDa složené z podjednotek 30 - 40 kDa (Gupta, 2001). Lektiny jsou schopny rozeznávat patogeny a parazity a podílet se na obranné reakci. Syntetizují se v tukovém tělese po zranění, bakteriální infekci nebo během metamorfózy, což naznačuje, že jsou schopny podílet se na likvidaci patogenů nebo poškozených a rozpadávajících se tkání. Významný je např. limulin z ostrorepa – specificky rozpoznává strukturní složky bakteriálních stěn a je součástí testů zdravotnického materiálu na bakteriální kontaminace.

Inhibitory proteáz

U hmyzu bylo popsáno několik inhibitorů serinových proteáz, které se podílejí především na regulaci proteázových kaskád včetně fenoloxidázového systému a také je dobře znám jejich inhibiční vliv na proteázy houbových patogenů (Fröbius et al., 2000). Zatím poměrně málo prozkoumanou složkou vrozené imunity hmyzu jsou pak inhibitory metalloproteáz. Enzymy ze skupiny metalloproteáz jsou produkovány infekčními organismy včetně entomopatogenů, kterým napomáhají během invaze. Působením mikrobiálních proteáz vznikají v organismu fragmenty proteinů jako např. kolagenu IV, které jsou účinnými aktivátory fenoloxidázové kaskády a exprese genů kódujících lysozym, AMP i „vlastní“ inhibitory proteáz (Altincicek et al., 2007; Held et al., 2007).

Ig-like, complement-like molekuly

Molekuly po funkční stránce podobné působení protilátek (specifické „antibody-like“) a komplementového systému („complement-like“) byly popsány u některých bezobratlých. Tyto molekuly jsou důležité zejména pro studium evoluce imunitního systému jako možný přechod k adaptivní imunitě, více viz str. 26.

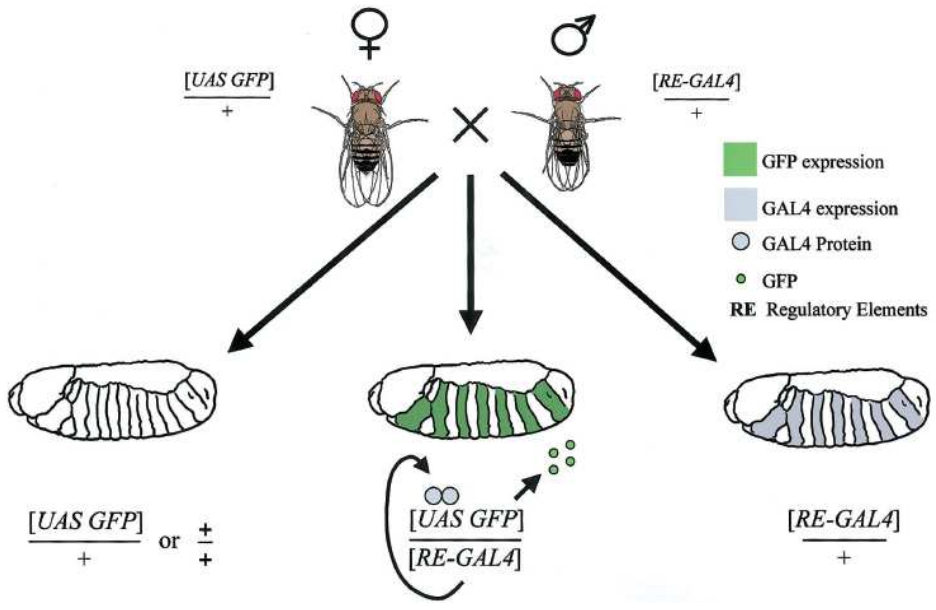
Další signální dráhy regulující humorální imunitu

Kromě již zmíněných Toll a Imd signální dráhy jsou také popsány další regulační mechanismy imunity. Pro opravu vlastních tkání slouží signální dráha JAK/STAT, uplatňuje se při opravě poškozených střevních buněk a v epiteliárních nádorech, její aktivace vede ke zvýšené tvorbě hemocytů a jejich aktivaci (Morin-Poulard, 2013). Během stresu v embryonálním vývoji některých zástupců hmyzu se uplatňuje ještě signální dráha

JNK (Wu et al., 2015) a homeostázu pomáhá udržovat signální dráha přes insulinový receptor („insulin/insulin-like growth factor signaling“; Shin et al., 2011).

Studium imunity hmyzu pomocí entomopatogenních hlístic

V našich studiích věnovaných imunitě hmyzu jsme používali hlavně dva modelové organismy. Tradičním modelem je motýl zavíječ voskový ***Galleria mellonella***, jehož výhodou je snadný laboratorní chov, stejně tak jako octomilka ***Drosophila melanogaster.*** Ta je jedním z nejstudovanějších druhů hmyzu včetně známého genomu, čehož se využívá pro genetické modifikace. Pro studium imunity a interakce hostitel-patogen je k dispozici mnoho metod molekulární biologie a genetiky využívajících přirozené patogeny (Keebaugh & Schlenke, 2014; Neyen et al., 2014). Možnost inaktivace („knockdown”) genů umožnila široké proteomické studie hemolymfy po stimulaci imunity (Lemaitre & Hoffmann, 2007); inaktivace genu může být i tkáňově specifická, což umožňuje tzv. UAS-Gal4 systém. Tento systém využívá kvasinkový promotor, který je spojený se specifickým genem v jedné linii *Drosophil* („responder line“) a kvasinkový transkripční aktivátor (Gal4) pod kontrolou tkáňově specifického promotoru v druhé linii („driver line“, Duffy, 2002, obr. 9). Zkřížení obou linií vede ke tkáňově specifické inaktivaci daného genu u potomstva, často se využívá spřažení s nějakým fenotypovým markerem nebo zeleným fluorescenčním proteinem (GFP).



Obr. 9: UAS-Gal4 systém pro knockdown genů u *Drosophily*, zde je ještě použito značení GFP (Duffy, 2002).

Dvě velké kolekce linií, které společně pokrývají většinu genomu *Drosophily*, jsou dostupné vědecké komunitě (jedna ve Vídni a jedna v Japonsku). V našich studiích jsme používali hlavně „driver lines“ specifické pro tukové těleso a hemolymfu jako hlavní imunitní tkáně.

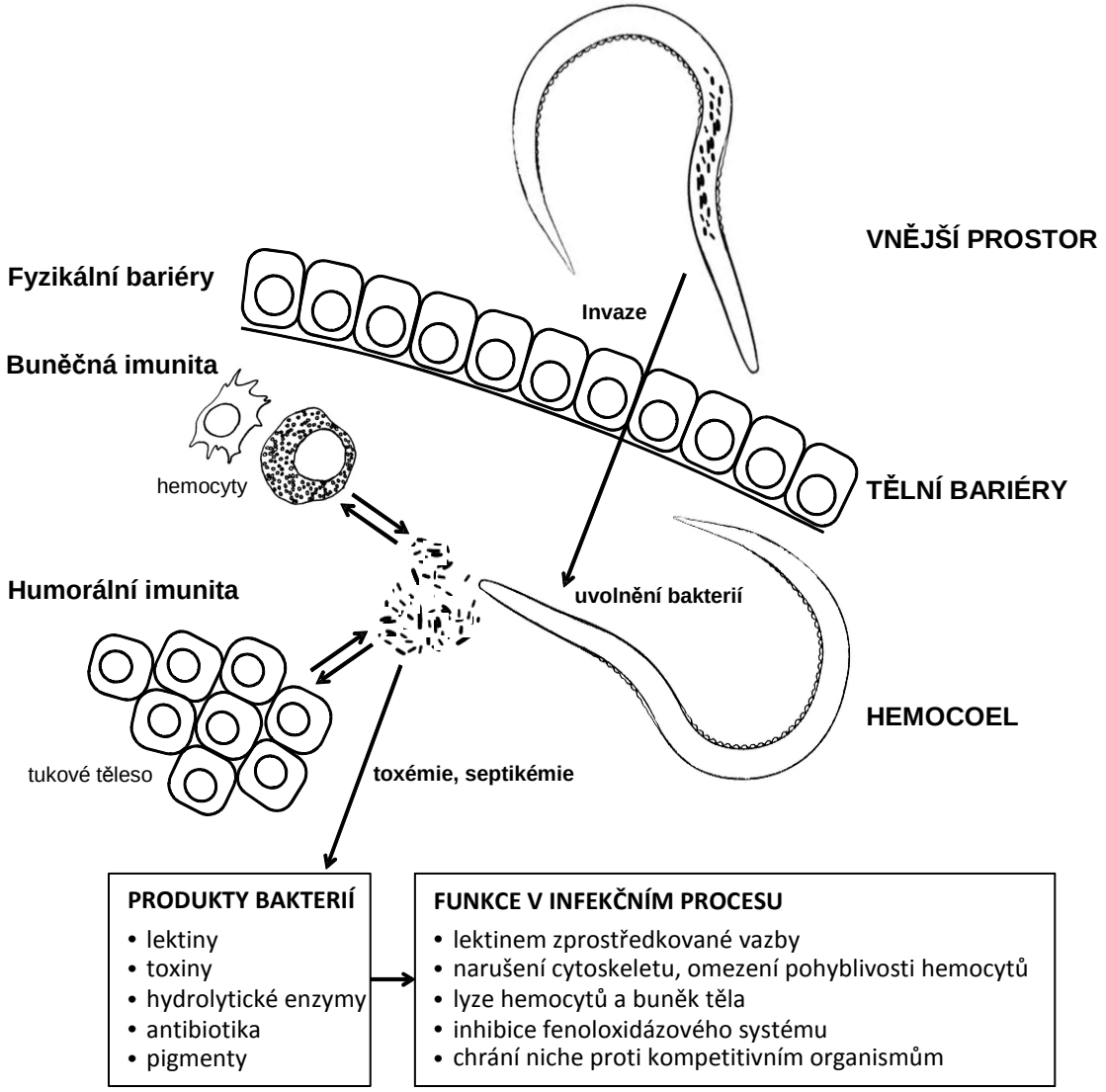
Jako přirozený infekční model jsme využívali v našich experimentech nákazu **entomopatogenními hlísticemi/hlístovkami** („entomopathogenic nematodes“, EPN). Tyto EPN rodu ***Heterorhabditis*** a ***Steinernema*** jsou obligátními hmyzími parazity, kteří usmrtí hostitele obvykle během 24-48 hodin. V posledních desetiletích jsou průmyslově namnožené hlístovky stále častěji využívány v biologickém boji proti škůdcům zemědělských plodin (např. Ehlers 2001). Třetí vývojové stádium hlístovek označované také jako IJ („infective juveniles“), se vyskytuje volně v půdě. IJ zde vyhledávají vhodného hostitele, kterého následně osidlují skrz přirozené tělní otvory a dokončují v něm svůj vývoj. Vzácně se podaří průnik spirakulami a systémem trachejí, zástupci rodu *Heterorhabditis* také mohou přímo penetrovat kutikulu hostitele. Hlavní rozdíl mezi rody *Heterorhabditis* a *Steinernema* je v tom, že *Heterorhabditis* má první generaci dospělců hermafroditickou a v druhé má amfimiktické samce a samice. Rod *Steinernema* má v obou generacích amfimiktické samce i samice. Podrobněji je životní cyklus popsaný např. v Burnell & Stock, 2000.

Po penetraci do tělní dutiny hostitele prolomí EPN obranné reakce hostitele a uvolní ze střeva svoje symbiotické bakterie, které způsobí toxémii a septicémii (hovoříme o tzv. nematobakteriálním komplexu a jeho vlivu na imunity hmyzu, viz dále). V napadeném organismu se vyvíjí několik dalších generací EPN, které se po spotřebování živin dostávají zpět do půdy a celý cyklus se tak opakuje. Jednotlivé druhy hlístic a jejich symbiotických bakterií vykazují odlišnou patogenitu vůči hostiteli, např. pro *G. mellonella* (Hyršl, 2011; **příloha 14**). I když se může zdát, že hlístovky jsou pouze biologickou schránkou pro svého bakteriálního symbionta, je vztah mezi těmito organismy klasickým mutualismem: růst hlístovek a jejich reprodukce závisí na podmínkách, které bakterie v těle hostitele vytvoří; bakterie dále přispívá antiimunitními proteiny, aby pomohla EPN překonat obranu hostitele, zvláště antimikrobiálními proteiny, které potlačují kolonizaci těla hostitelského hmyzu. Bakterie naopak postrádá invazivní vlastnosti, a využívá schopnosti EPN vyhledat hostitele a penetrovat do jeho tělní dutiny. U čeledi *Steinernematidae* se vyskytují jako symbionti bakterie ***Xenorhabdus*** sp. U čeledi *Heterorhabditidae* se vyskytují zejména bakterie ***Photorhabdus*** sp.

Během infekce jsou tyto bakterie uvolňovány do hostitelova organismu, což vede k jeho rychlému usmrcení a navíc bakterie napomáhají natrávení tkání. *Photorhabdus* je jediným dosud známým suchozemským druhem bakterie schopným bioluminiscence, čehož lze využít při zjišťování úspěšnosti nákazy. Jedinci, kteří byli zabiti hlísticemi *H. bacteriophora* a jejich symbiotickými bakteriemi, jsou prokazatelně bioluminiscenční a kadaver je červeně pigmentován, srovnání bioluminiscence jednotlivých druhů a poddruhů uvádí studie Hyršl et al. (2004; **příloha 15**). Navíc lze použít geneticky modifikovaný kmen *P. luminescens* TT01 (obr. 10), který obsahuje GFP, a takto snadno sledovat průběh nákazy a lokalizaci/množení bakterií. Sekvenovaný genom *Photorhabdus luminescens* je velmi blízký lidskému patogenu *Photorhabdus asymbiotica*, takže výzkum interakce hostitel-patogen je důležitý i z hlediska humánní medicíny.

Obr. 10: Infekční jedinci *H. bacteriophora* s GFP značenými bakteriemi *P. luminescens*.

Pro laboratorní chov EPN jsou využívány larvy zavíječe voskového *G. mellonella*, které jsou však na působení nematobakteriálního komplexu příliš citlivé a pro sledování imunitních procesů zapojených během invaze se tedy nehodí. Pro tyto účely je ideální *Drosophila melanogaster*, která je vůči nákaze EPN odolnější a lze na ní tedy testovat i vliv látek, příp. mutací zvyšujících úspěšnost nákazy. Kombinace všech tří výše zmíněných organismů (EPN, jejich symbiotických bakterií a *D. melanogaster*) je jedinečným modelem pro sledování interakce parazit-hostitel, přirozená infekce poskytuje ucelenější pohled na obranné mechanismy než umělé zásahy (např. injikace). Tento model byl zaveden Hallem a kol. (2007) a následně bylo prokázáno, že je při invazi EPN prostřednictvím působení symbiotických bakterií indukována u *D. melanogaster* syntéza některých AMP (ffrench-Constant et al., 2003) a předpokládá se také účast fenoloxidázové kaskády. Proti jejímu působení se EPN i symbiotické bakterie brání syntézou inhibitorů (Brivio et al., 2002; Eleftherianos et al., 2007), proteáz (Simoes et al., 2000) a toxinů (Eleftherianos et al., 2010). Souhrnné interakce hostitel-patogen zahrnuje obr. 11.



Obr. 11: Schéma obrany hmyzu proti nákaze nematobakteriálním komplexem.

V našich studiích jsme tuto nematobakteriální nákazu používali jako funkční test bioinformaticky vybraných kandidátních genů. Metodu jsme optimalizovali pro *D. melanogaster* (Dobeš et al. 2012; **příloha 16**), kdy byly porovnávány RNAi a mutantní linie s kontrolní skupinou. Larvy octomilky s defekty v indukci AMP, defekty ve fenoloxidázovém sytému a mutanti v receptorech fagocytózy nevykazovali žádné změny oproti kontrole, naopak mutanti v transglutamináze zprostředkovávající koagulaci hemolymfy a v substrátech pro tranglutaminázu vykazovali zvýšenou mortalitu po nematobakteriální infekci (Hyršl et al., 2010; **příloha 6**; Wang et al., 2010; **příloha 11**).

V navazující souhrnné studii využívající analýzu RNA před a po infekcí nematobakteriálním koplexem jsme identifikovali skupiny genů důležitých pro tuto infekci a v porovnání s jinými studiemi také společné nebo naopak specifické geny zapojené v

imunitní odpovědi hostitele (Arefin et al.; **příloha 17).** Vzhledem k tomu, že rozpoznání parazita je zásadní pro spuštění imunitních reakcí, byly testovány rozpoznávací molekuly ze skupiny PGRP a GNBP a také komplement-like proteiny („thioester-containing proteins“, TEP). V těchto experimentech bylo popsáno zapojení PGRP-LF, GNBP-like a TEP3 v nematobakteriální infekci. Dále bylo popsáno zapojení složek bazální membrány jako je např. glutaktin.

Sociální imunita

Kromě dříve popsaných mechanismů přirozené imunity bezobratlých se u některých setkáváme ještě s dalšími možnostmi, jak zvýšit úspěšnost organismu v kontaktu s patogeny v okolí. Tento typ imunitního systému vznikl u sociálně žijících druhů, jako jsou mravenci, včely, čmeláci a termiti (Schmid-Hempel, 1998). Sociální imunita je popsána jako série projevů chování ve skupině, které umožňují potlačit pravděpodobnou nákazu a přenos infekčních chorob v populaci (Cremer et al., 2007). Systém vzájemné spolupráce se soustřeďuje na čištění hnízda a také na vzájemné čistění jedinců, které zabraňuje šíření nákazy a v některých případech slouží i k sociální imunizaci, nezasahuje tedy pouze při onemocnění, ale vytváří také účinnou prevenci. Dalším příkladem mohou být antimikrobiální látky, které si sociální hmyz sám produkuje, nebo je sbírá ve svém okolí.

Mezi studované sociální druhy patří včely, které jsou zatížené řadou onemocnění, působením pesticidů a dalšími vnějšími faktory. Genom včel obsahuje pouze třetinu imunitních genů v porovnání s *Drosophilou* nebo *Anopheles* (Evans et al., 2006; Seaver, 2011); ztráta těchto genů musela být v průběhu evoluce kompenzována systémem sociální imunity, nebo nějakým jiným faktorem. V naší studii (Hyršl et al. 2016; **příloha 18**) jsme se zabývali možností posílení imunity včel pomocí rostlinného alkaloidu sanquinarinu a podáváním probiotických bakterií. Testování odolnosti včelího plodu proti nematobakteriální nákaze prokázalo stimulační efekt obou přípravků, což může být využito ve včelařské praxi.

Adaptivní imunita bezobratlých?

Adaptivní imunita založená na přítomnosti paměťových buněk, produkci protilátek a variabilitě hlavního histokompatibilního systému (MHC), jakou známe u obratlovců, u bezobratlých neexistuje. Absenci komplikovaných imunitních reakcí adaptivní imunity bezobratlí nahrazují jednak specifickými adaptacemi (pro patogeny neprostupná vnější

chitinová kostra, rychlé rozmnožování – krátký životní cyklus, velký počet potomků, krátká doba života, aj.) a potom také dobře fungujícím imunitním systémem, který se funkčně v některých případech adaptivním reakcím přibližuje. Experimenty prokázaly imunologickou specifitu a přinejmenším krátkodobou pamět u houbovců (Porifera), žahavců (Cnidaria), kroužkovců (Annelida), hmyzu (Insecta) a ostnokožců (Echinodermata), ale ne u hlístic (Nematoda) nebo měkkýšů (Mollusca; Turner, 1994). **Adaptivní projevy přirozené imunity** (alternativní adaptivní imunity) jsou v současnosti intenzivně studovány. Genetická variabilita bezobratlých může být analogická obratlovčím protilátkám, např. alternativním sestřihem molekuly Dscam („Down syndrome cell adhesion molecule“) u *Drosophily* (Kurtz & Armitage, 2006). Dalšími intenzívně studovanými molekulami jsou TEP, které pravděpodobně mají funkci podobnou komplementovému systému obratlovců (Bou et al., 2011). Přítomnost nových forem adaptivní imunity u bezobratlých není až tak překvapivá, protože již byla popsána adaptivní imunita i u bakterií a Archea, zde je založená na CRISPR/Cas systému proti virům a plasmidům (Horvath & Barrangou, 2010).

Základní schéma bezobratlých “rozpoznání – signál do buňky – reakce“ může být ve výsledku ovlivněno několika procesy. Ochranná **imunizace** se vyskytuje u řady druhů bezobratlých proti širokému spektru parazitů přesto, že postrádají T a B lymfocyty. Imunizace bezobratlých zahrnuje příklady obecné nespecifické imunitní ochrany, ale také specifickou **imunitní pamět** označovanou „**imunitní priming**“.

U **imunizace** hovoříme o snížené citlivosti na nákazu (parazita) při sekundárním setkání (Konrad et al., 2012). K aktivní imunizaci dochází, jestliže jedinec sám přijde do kontaktu s patogenem a jeho tělo je díky tomu lépe připraveno na sekundární setkání, u sociálně žijících druhů může dojít také k pasivní imunizaci výměnou antimikrobiálních prostředků mezi zdravým a nakaženým jedincem (Capinera, 2008).

Profylaktický efekt zvaný **„imunitní priming“** je soubor činností a opatření, který znamená určitou ochranu před chorobami (Capinera, 2008). Jedná se např. o odlišnou reakci na bakteriální infekci u naivních jedinců a těch, kterým byly předtím podány inaktivní bakterie (Wu et al., 2014). Konkrétních studií popisujících priming je již mnoho desítek, dokládá to, že bezobratlí si vytvořili všemožné varianty této obrany. Zajímavým případem je trofolaxe sociálního hmyzu - oboustranná či jednostranná výměna výživné tekutiny. Může probíhat buď přes ústa, to hovoříme o stomodeální, nebo konečníkem, tedy proktodeální trofolaxi. Např. u mravenců je trofolaxe jedním z důležitých faktorů, který jim dopomáhá k přežití a nárůstu kolonie. Priming prokazuje, že i u bezobratlých nalézáme

imunologickou specificitu a paměť (Little et al., 2005). **Termín specifita zde určuje konkrétní vztah hostitele a parazita, není tedy ve významu získané (specifické) imunity** obratlovců založené na produkci protilátek. Mnoho studií ukazuje, že díky prvotnímu vystavení patogenu může být hostitel při druhotném setkání více chráněn. Problémem však zůstává, že specificita odpovědi závisí na konkrétním genotypu patogenu (Kurtz & Franz, 2003), např. experiment provedený na buchance *Macrocyclops albidus* ukázal, že infekce po druhém setkání s parazitem je nižší, když jsou si parazité prvního a druhého setkání podobní. Kromě toho víme, že tuto **imunitu může matka předávat svým potomkům** (Moret & Schmid-Hempel, 2001). Rodiče své potomky zásobují lepší obranou schopností díky tomu, že oni sami již do kontaktu s patogenem přišli. Priming nalézáme tedy jak u obratlovců, tak i u bezobratlých. U obratlovců rozumíme tomuto mechanismu jak po funkční, tak i po mechanické stránce (Little & Kraaijeveld, 2004), u bezobratlých víme pouze o konkrétních příkladech, ale neznáme ještě mechanismus tohoto procesu.

2. Přirozená imunita obratlovců

Přirozená imunita je základem imunitních reakcí všech obratlovců, teprve na jejím základě se u většiny obratlovců vyvinula i imunita získaná, která je přítomna od chrupavčitých ryb evolučně výše. U nižších skupin strunatců (Urochordata, Cephalochordata) jsou přítomné pouze primitivní formy získané imunity, popřípadě mají po imunitní stránce blíže k bezobratlým. Také první skupina obratlovců - kruhoústí (Cyclostomata) jsou velmi zajímavou skupinou, kdy jedna část (mihule) jsou na mnohem vyšší úrovni než druhá (sliznatky). U těchto z hlediska imunologie důležitých přechodových skupin je známo jen velmi málo o jejich imunitním systému, nepatří sem ekonomicky významné druhy, přitom právě mezi ně ale spadají základy adaptivní imunity. Z hlediska imunologie tedy neexistuje jasné rozhraní mezi tradičním dělením na bezobratlé a obratlovce (nevyhovují ani jiná dělení jako např. na prvoústé a druhoústé živočichy).

Obratlovčí přirozená imunita je opět založena na přítomnosti PAMPs a DAMPs (rozpoznává asi 1000 geneticky kódovaných rozpoznávacích molekul, které následně aktivují intracelulární nebo povrchové PRR). Aktivované buňky nespecifické imunity se nacházejí v krevním oběhu (profesionální fagocyty) nebo ve tkáních – makrofágy, žírné a dendritické buňky spolu s fibroblasty. Tyto buňky produkují prozánětlivé cytokiny a další mediátory, jako např. matrix metaloproteinázy, které zahajují rozvoj zánětu v daném místě. Spolu s aktivovanými složkami komplementu a faktory krevního srážení indukují aktivaci endoteliálních buněk přilehlých cév, zejména kapilár, prostupující danou tkáň. Tato aktivace endoteliálních buněk umožňuje průnik imunitních buněk ze systémové cirkulace do místa zánětu. Aktivita jednotlivých složek nespecifické imunity se liší mezi jednotlivými taxony obratlovců, například komplementový systém může být aktivován třemi cestami, ale ne všechny jsou vždy přítomné, podobně je tomu s průběhem zánětu a odvržením transplantátu, které mohou být chronické nebo akutní. Také záleží na přítomných efektorových buňkách a imunitních orgánech (plaky v okolí střeva jsou přítomné již od kruhoústých, brzlík a slezina od ryb), kostní dřeň je vyvinuta až od bezocasých obojživelníků (žáby) a lymfatické uzliny jsou plně funkční až od ptáků, kde je také navíc Fabriciova bursa.

Získaná imunita se objevuje u mihulí, které mají adaptivní imunitu založenou místo na imunoglobulinech na VLR („variable lymphocyte receptor“), plně se ale vyvinula až u čelistnatých obratlovců. U čelistnatých obratlovců dochází k integraci a vzájemnému propojení reakcí adaptivní imunity, nejpravděpodobněji to je dáno přechodem prvotních čelistnatců na aktivnější způsob života a lov potravy. Postupně došlo ke strukturně-

anatomické přestavbě těla, vzniká výkonnější nervová soustava, smysly, pohybová ústrojí a podobně vznikaly i imunologické orgány (polykáním potravy bylo zvýšené riziko infekcí střevního traktu). Teprve čelistnatí obratlovci mají lymfocyty s receptory, které nejsou geneticky kódované, ale tvoří se přeskupováním řízeným produkty genů RAG1 a RAG2 („recombination activation genes“). Tyto geny kódují enzymy rekombinázy, které řídí přeskupování genových segmentů pro vazebné místo antigenu na molekule imunoglobulinu a tím generují diverzitu. Receptory vznikají během života na specifické podněty (typická je nadprodukce variant, až 98% lymfocytů „svůj“ antigen nikdy nepotká). Vrcholem získané imunity jsou protilátky, které mohou rozlišit prakticky každou antigenní strukturu, která se na Zemi vyskytuje nebo vyskytne. Obecně také platí, že variabilita jedince (založená ještě na MHC genech a Toll receptorech) určuje různou odolnost proti infekčním chorobám.

V našich studiích jsme se zaměřili na ekonomicky významné zástupce poikilotermních a homoiothermních obratlovců, konkrétně na ryby a ptáky. Část experimentů probíhalo také s izolovanými lidskými profesionálními fagocyty.

Přirozená imunita ryb

Přirozená imunita ryb je tvořena nespecifickými humorálními faktory, buněčnými mechanizmy a zánětem (Bols et al. 2001). Mezi **nespecifické humorální faktory** patří například inhibiční faktory proti bakteriím (transferrin, antiproteázy), lysiny (např. lysozym), C-reaktivní protein, bakteriální peptidy a **komplement** (Ellis 1999). S komplementem se setkáváme evolučně od chrupavčitých ryb výše, je to komplexní senzor z cca 30 proteinů, který nejen rozpozná, ale i zabíjí mikroorganismy. Dříve se vyskytující „komplement-like“ faktory měly pouze opsonizující roli u ostnokožců, případně rozpoznávací funkci u některých bezobratlých. Rozlišujeme tři možné cesty aktivace komplementu. Evolučně nejstarší alternativní cestu s využitím lektinů, klasickou cestu vyžadující protilátky a lektinovou cestu, která je u mnoha ryb diskutabilní. Antimikrobiální faktory (lysozym, komplement, lektiny a proteolytické enzymy) nejsou lokalizovány pouze v krvi, ale jsou také složkami kožního slizu a pravděpodobně i dalších povrchů rybího těla, které jsou v přímém kontaktu s okolním prostředím. **Nespecifická buněčná složka** je prezentována hlavně fagocytujícími leukocyty, dále pak retikuloendoteliálním systémem a nespecificky cytotoxickými buňkami (fylogenetičtí předchůdci NK buněk savců). Mezi fagocyty patří mononukleáry (tkáňové makrofágy a cirkulující monocyty) a polymorfonukleáry (hlavně neutrofily). Fagocyty obsahují mnoho

hydrolytických enzymů a po aktivaci produkují reaktivní kyslíkové metabolity během oxidačního vzplanutí, důležité jsou i reaktivní dusíkové metabolity. Podobně jako u bezobratlých je důležitá rovnováha pro- a antioxidačních mechanismů, aby nedocházelo k oxidačnímu stresu.

Faktory vnějšího prostředí jako jsou teplota vody a fotoperioda ovlivňují imunitní systém ryb a tím i jejich celkový zdravotní stav. V našich studiích jsme se zaměřili právě na studium **sezónní dynamiky** v aktivitě parametrů nespecifické imunity – oxidačního vzplanutí fagocytů, antibakteriální aktivity komplementového systému a antibakteriální aktivity lysozymu v kožním mukusu. Metodicky se jedná o podobný přístup jako v případě vzorků bezobratlých, postupně jsme metody optimalizovali pro kapra obecného (*Cyprinus carpio*; Buchtíková et al., 2011; **příloha 19**). Nejvyšší aktivita fagocytů byla naměřena v chladném období roku. Rozdíly v aktivitě celkové a alternativní cesty aktivace komplementu naznačují, že každá z těchto drah má odlišnou roli v jednotlivých sezónních obdobích. Potvrdili jsme také, že pohlavní hormony v období tření jsou významným inhibičním faktorem. Vliv sezóny a úrovně ploidie na hematologické parametry a parametry nespecifické imunity jsme sledovali také u lína obecného (*Tinca tinca*; Tolarová et al., 2014; **příloha 20**). Výsledky prokázaly silný vliv sezóny i rozdíly mezi diploidními a triploidními rybami. Podobná situace byla i při studiu hybridizace mezi kříženci kapra obecného a karase stříbřitého (*Carassius gibelio*; Šimková et al., 2015; **příloha 21**) a při studiu genetické diverzity karase zlatého (*Carassius auratus*; Šimková et al., 2015; **příloha 22**).

V navazujících studiích jsme se zabývali **imunoekologickým** vztahem reprodukce, imunity a parazitismu, kdy jedinec musí díky omezeným možnostem rozložit investice mezi tyto parametry (tzv. „trade-offs“). U kapra jsme opět zaznamenali výrazné sezónní rozdíly s oslabením imunity v období tření a odlišnou skladbou parazitů, přímý trade-off ale nebyl prokázán (Rohlenová et al., 2011; **příloha 23**). Naopak pozitivní vztah mezi parazitací a oxidačním vzplanutí fagocytů byl prokázán u jelce tlouště (*Leuciscus cephalus*, Poisot et al., 2009; **příloha 24**).

Stanovení faktorů přirozené imunity je velmi důležité i z hlediska jejich změn vyvolaných znečištěním životního prostředí (ekotoxikologie, imunotoxikologie). V komplexní studii Wenger et al. (2009; **příloha 25**) jsme tyto parametry sledovali u populace jelce tlouště (*L. cephalus*) na řece Bílině ve vztahu k chemické zátěži, parazitaci a celkovému zdraví ryb.

Přirozená imunita ptáků

Přirozený imunitní systém ptáků se velmi podobá lidskému s několika rozdíly. Během oxidačního vzplanutí ptačích heterofilů není přítomna myeloperoxidáza, produkce reaktivních kyslíkových metabolitů je tedy při fagocytóze výrazně nižší. Z hlediska ekoimunologie je zajímavá úloha antioxidantů, které hrají roli i v reprodukci - často pozorovaným jevem je upřednostňování samečků s více pestrými tóny barev či složitěji vyvinutými ornamenty. Za zbarvení ornamentů ptáků, které slouží nejen k zmiňované volbě partnera, ale i široké komunikaci, zodpovídají především karotenoidy – červené a žluté pigmenty přijímané spolu s rostlinnou potravou (Olson & Owens, 1998).

Důležitá je i imunita pohlavní soustavy (zajištění antibakteriální ochrany kloaky při oplození), stejně tak antibakteriální ochrana zárodku ve vejcích. Právě manipulací s hladinou lysozymu v bílku vajec křepelek japonských (*Coturnix japonica*) jsme se zabývali ve studii Javůrková et al. (2015; **příloha 26**). Zdá se, že kromě imunitní funkce je zde vliv i na regulaci růstu během embryonálního vývoje.

Přirozená imunita savců

Přirozená imunita savců obsahuje všechny dříve zmíněné komponenty obratlovčí imunity. V našich studiích jsme používali izolované lidské fagocyty jako standard pro porovnání produkce reaktivních kyslíkových metabolitů hmyzími hemocyty (Hyršl et al., 2004; **příloha 1**; Vašíček et al., 2011; **příloha 2**) nebo lidskou plasmu pro porovnání koagulace a prokázání imunitní funkce transglutaminázy (Wang et al., 2010; **příloha 11**). V další studii pomohlo měření celkové antioxidační kapacity plasmy norníků rudých (*Clethrionomys glareolus*) stanovit funkční rozdíly v mutaci hemoglobinu a tím následně zdůvodnit postup kolonizace Evropy na konci doby ledové (Kotlík et al., 2014; **příloha 27**). Antioxidační a antibakteriální vlastnosti vykazuje také řada rostlinných extraktů, které přijímáme s potravou. Testováním na izolovaných lidských fagocytech jsme přispěli k determinaci biologicky aktivních polyfenolických látek přítomných v různých plodech, tyto výsledky lze přímo aplikovat ve výživě člověka (Denev et al., 2014a; Denev et al., 2014b; **přílohy 28 a 28**).

Závěr

Přirozená imunita je vlastní všem živočichům a je jako první aktivována během poranění, infekce nebo parazitace. Její reakce jsou velmi rychlé a slouží k odstranění většiny patogenů, teprve následně jsou spouštěny složitější mechanismy, např. syntéza antimikrobiálních peptidů.

Po rozpoznání patogenu dochází zejména k fagocytóze, kde jsme studovali produkci reaktivních kyslíkových metabolitů hemocytů hmyzu ve srovnání s lidskými neutrofily. Jejich produkce je velmi nízká, což naznačuje jejich menší zapojení v mikrobicidních procesech u bezobratlých. Reaktivní metabolity kyslíku a dusíku souvisejí také s celkovou antioxidační kapacitou tělních tekutin a aktivitou jednotlivých antioxidačních enzymů. Prokázali jsme aktivaci antioxidačních mechanismů působením chemických podnětů a také jsme popsali reakce organismu na navozený oxidační stres včetně zapojení eikosanoidů. V dalších studiích jsme experimentálně prokázali imunitní funkci transglutaminázy při koagulaci tělních tekutin, což platí pro bezobratlé i obratlovce. Pomocí přirozeného infekčního modelu entomopatogenních hlístic byla popsána funkce řady genů vyselektovaných pomocí bioinformatických nástrojů. Jejich zapojení do imunitních reakcí proti nematobakteriálnímu komplexu umožnilo studium mutantních a RNAi linií *Drosophily*. Pomocí stejného modelu byly úspěšně testovány i přípravky pro zlepšení imunity včely medonosné. Zdánlivě jednodušší imunita bezobratlých má jasnou návaznost v přirozené imunitě obratlovců, proto jsme se zaměřili na problematiku poikilotermních i homoiotermních obratlovců, konkrétně ryb, ptáků a savců. Hlavní vliv na imunitu má sezóna, zejména okolní teplota a období rozmnožování, dále jsme studovali vliv ploidie, křížení, parazitismu a znečištění. Obecně ze všech experimentů vyplývá, že přirozená imunita bezobratlých i obratlovců má velmi podobné efektorové mechanismy a je základem udržení integrity organismu a jeho obranyschopnosti. Nově popsané detaily jednotlivých procesů a případové studie otevírají také nové otázky vývojové a srovnávací imunologie zejména mezi skupinami na rozmezí obratlovců a bezobratlých živočichů, kde vzniká imunita získaná. Stimulace mechanismů přirozené imunity může být klíčová pro udržitelnou produkci hospodářsky významných organismů, ale také pro léčbu řady lidských onemocnění.

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Přílohy

PŘÍLOHA Č. 1

HYRŠL Pavel, ČÍŽ Milan, KUBALA Lukáš, LOJEK Antonín

Silkworm (*Bombyx mori*) hemocytes do not produce reactive oxygen metabolites as a part of defence mechanisms. Folia Microbiologica, 49 (3), 315-319, 2004.

Charakteristika:

Srovnání produkce reaktivních kyslíkových metabolitů u lidských fagocytů a hmyzích hemocytů. Studie neprokázala jejich tvorbu u bource morušového (*Bombyx mori*) během aktivované fagocytózy pravděpodobně díky vysoké antioxidační kapacitě hemolymfy.

IF=1,000; citováno 1 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

VAŠÍČEK Ondřej, PAPEŽÍKOVÁ Ivana, HYRŠL Pavel: Fluorimetric determination of hydrogen peroxide production by the haemocytes of the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae). European Journal of Entomology, 108, 481-485, 2011.

PŘÍLOHA Č. 2

VAŠÍČEK Ondřej, PAPEŽÍKOVÁ Ivana, HYRŠL Pavel

Fluorimetric determination of hydrogen peroxide production by the haemocytes of the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae). European Journal of Entomology, 108, 481-485, 2011.

Charakteristika:

Hemocyty zavíječe voskového (*Galleria mellonella*) produkují peroxid vodíku během indukované fagocytózy jako součást mikrobicidních mechanismů podobně jako lidské fagocyty.

IF= 0,975; citováno 2 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Xi, Fengna; Zhao, Dongjiao; Wang, Xuewan; et al.: Non-enzymatic detection of hydrogen peroxide using a functionalized three-dimensional graphene electrode. Electrochemistry Communications, 26, 81-84, 2013.

PŘÍLOHA Č. 3

KRISHNAN Natraj, HYRŠL Pavel, ŠIMEK Vladimír

Nitric oxide production by hemocytes of larva and pharate prepupa of *Galleria mellonella* in response to bacterial lipopolysacharide: cytoprotective or cytotoxic? Comparative Biochemistry and Physiology, Part C, 142 (1-2), 103-110, 2006.

Charakteristika:

Souhrnné zpracování problematiky oxidu dusnatého u zavíječe voskového (*Galleria mellonella*).

IF=2,301; citováno 19 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Hillyer, Julian F.; Estevez-Lao, Tania Y.: Nitric oxide is an essential component of the hemocyte-mediated mosquito immune response against bacteria, Developmental and Comparative Immunology. 34, 2, 141-149, 2010.

PŘÍLOHA Č. 4

PANZARINO Onofrio, HYRŠL Pavel, DOBEŠ Pavel, VOJTEK Libor, VERNILE Pasqua, BARI Giuseppe, TERZANO Roberto, SPAGNUOLO Matteo, de LILLO Enrico

Rank-based biomarker index to assess cadmium ecotoxicity on the earthworm *Eisenia andrei*. Chemosphere, 145, 480-486, 2016.

Charakteristika:

Využití žížaly *Eisenia andrei* při studiu toxicity těžkých kovů v půdě, nalezení vhodných markerů toxicity.

IF=3,340; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 5

HYRŠL Pavel, BÜYÜKGÜZEL Ender, BÜYÜKGÜZEL Kemal

The effects of boric acid-induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella*. Archives of Insect Biochemistry and Physiology, 66, 23-31, 2007.

Charakteristika:

Kyselina boritá jako pomalu působící insekticid způsobuje oxidativní stres u zavíječe voskového *Galleria mellonella*.

IF=1,021; citováno 20 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Buyukguzel, Ender: Evidence of Oxidative and Antioxidative Responses by *Galleria mellonella* Larvae to Malathion, Journal of Economic Entomology. 102, 1, 152-159, 2009.

PŘÍLOHA Č. 6

HYRŠL Pavel, DOBEŠ Pavel, WANG Zhi, HAULING Thomas, WILHELMSSON Christine, THEOPOLD Ulrich

Clotting factors and eicosanoids protect against nematode infections. Journal of Innate Immunity, 2 (7), 1-6, 2010.

Charakteristika:

Během nákazy hmyzího hostitele entomopatogenními hlísticemi se uplatňují koagulační faktory a eikosanoidy. Jejich úloha byla studována na mutantních a RNAi liniích *Drosophila melanogaster*.

IF=4,352; citováno 15 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Loof, Torsten G.; Schmidt, Otto; Herwald, Heiko; et al.: Coagulation Systems of Invertebrates and Vertebrates and Their Roles in Innate Immunity: The Same Side of Two Coins? Journal of Innate Imunity, 3, 1, 34-40, 2011.

PŘÍLOHA Č. 7

BÜYÜKGÜZEL Ender, HYRŠL Pavel, BÜYÜKGÜZEL Kemal

Eicosanoids mediate hemolymph oxidative and antioxidative response in larvae of *Galleria mellonella* L. Comparative Biochemistry and Physiology A - Molecular & Integrative Physiology, 156 (2):176-83, 2010.

Charakteristika:

Popis funkce eikosanoidů během prooxidační a antioxidační odpovědi larev zavíječe voskového *Galleria mellonella* po navození oxidačního stresu xantotoxinem. Zapojení jednotlivých signálních drah bylo ověřeno použitím inhibitorů syntézy eikosanoidů.
IF=1,966; citováno 13 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Huang, Yufen; Xu, Zhibin; Lin, Xianyu; et al.: Structure and expression of glutathione S-transferase genes from the midgut of the Common cutworm, *Spodoptera litura* (Noctuidae) and their response to xenobiotic compounds and bacteria: Journal of Insect Physiology. 57, 7, 1033-1044, 2011.

PŘÍLOHA Č. 8

HYRŠL Pavel, BÜYÜKGÜZEL Ender, BÜYÜKGÜZEL Kemal

Effect of eicosanoid biosynthesis inhibitors on the haemolymph protein profile of *Galleria mellonella* larvae. Turkish Journal of Entomology. 35 (3), 397-405, 2011.

Charakteristika:

Popis proteinového spektra hemolymfy larev zavíječe voskového *Galleria mellonella* a jeho ovlivnění podáním inhibitorů syntézy eikosanoidů.

IF=0,272; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 9

HYRŠL Pavel, ŠIMEK Vladimír

An analysis of hemolymph protein profiles during the final instar, prepupa and pupa of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). Biologia, 60 (2), 207-213, 2005.

Charakteristika:

Analýza proteinového spektra hemolymfy během vývoje bource morušového *Bombyx mori*. Popsány byly hlavní skupiny proteinů během posledního larválního instaru a přeměny v kuklu.

IF=0,827; citováno 6 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Singh, Nitin Kumar; Pakkianathan, Britto Cathrin; Kumar, Manish; et al.: Vitellogenin from the Silkworm, *Bombyx mori*: An Effective Anti-Bacterial Agent, Plos One: 8, 9, 2013.

PŘÍLOHA Č. 10

HYRŠL Pavel, BÜYÜKGÜZEL Ender, BÜYÜKGÜZEL Kemal

Boric acid-induced effects on protein profiles of *Galleria mellonella* hemolymph and fat body. Acta Biologica Hungarica, Budapest: Akademiai Kiado, 59 (3), 281-288, 2008.

Charakteristika:

Pomalu působící insekticid kyselina boritá způsobuje změny v proteinovém spektru hemolymfy a tukového tělesa zavíječe voskového *Galleria mellonella*.

IF=0,589; citováno 4 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Buyukguzel, Ender; Buyukguzel, Kemal; Snela, Milena; et al.: Effect of boric acid on antioxidant enzyme activity, lipid peroxidation, and ultrastructure of midgut and fat body of *Galleria mellonella*: 29, 2, 117-129, 2013.

PŘÍLOHA Č. 11

WANG Zhi, WILHELMSSON Christine, HYRŠL Pavel, LOOF Torsten G., DOBEŠ Pavel, KLUPP Martina, LOSEVA Olga, MÖRGELIN Matthias, IKLÉ Jennifer, CRIPPS Richard M., HERWALD Heiko, THEOPOLD Ulrich

Pathogen entrapment by transglutaminase - a conserved early innate immune mechanism. PLoS Pathogens, San Francisco: Public Library Science, 6 (2), 1-9, 2010.

Charakteristika:

Na infekčním modelu zahrnujícímu tři organismy - hlístici *Heterorhabditis bacteriophora*, bakterie *Photorhabdus luminescens* a *Drosophila melanogaster* jako jejich hostitele, jsme identifikovali nové imunitní mechanismy podílející se na koagulaci hemolymfy. Klíčová je role transglutaminázy, které zprostředkovává vazbu patogenů na vznikající koagulační matrix. Stejná funkce byla popsána i u lidské plasmy, kdy pacienti deficientní na homolog transglutaminázy (faktor XIII) vykazují zvýšenou náchylnost k infekcím.

IF=7,562; citováno 62 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Loof, Torsten G.; Morgelin, Matthias; Johansson, Linda; et al.: Coagulation, an ancestral serine protease cascade, exerts a novel function in early immune defense, Blood: 118, 9, 2589-2598, 2011.

Pathogen Entrapment by Transglutaminase—A Conserved Early Innate Immune Mechanism

Zhi Wang[1☯], Christine Wilhelmsson[1☯], Pavel Hyrsl[1,2☯], Torsten G. Loof[3☯], Pavel Dobes[1,2], Martina Klupp[1], Olga Loseva[4], Matthias Mörgelin[3], Jennifer Iklé[5], Richard M. Cripps[5], Heiko Herwald[3], Ulrich Theopold[1]*

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Abstract

Clotting systems are required in almost all animals to prevent loss of body fluids after injury. Here, we show that despite the risks associated with its systemic activation, clotting is a hitherto little appreciated branch of the immune system. We compared clotting of human blood and insect hemolymph to study the best-conserved component of clotting systems, namely the *Drosophila* enzyme transglutaminase and its vertebrate homologue Factor XIIIa. Using labelled artificial substrates we observe that transglutaminase activity from both *Drosophila* hemolymph and human blood accumulates on microbial surfaces, leading to their sequestration into the clot. Using both a human and a natural insect pathogen we provide functional proof for an immune function for transglutaminase (TG). *Drosophila* larvae with reduced TG levels show increased mortality after septic injury. The same larvae are also more susceptible to a natural infection involving entomopathogenic nematodes and their symbiotic bacteria while neither phagocytosis, phenoloxidase or—as previously shown—the Toll or imd pathway contribute to immunity. These results firmly establish the hemolymph/blood clot as an important effector of early innate immunity, which helps to prevent septic infections. These findings will help to guide further strategies to reduce the damaging effects of clotting and enhance its beneficial contribution to immune reactions.

Citation: Wang Z, Wilhelmsson C, Hyrsl P, Loof TG, Dobes P, et al. (2010) Pathogen Entrapment by Transglutaminase—A Conserved Early Innate Immune Mechanism. PLoS Pathog 6(2): e1000763. doi:10.1371/journal.ppat.1000763

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Introduction

One of the major causes of organ failure during sepsis in humans is the systemic activation of coagulation which leads to the widespread deposition of fibrin deposits with the result of multiple organ failure due to reduced blood supply [1]. In contrast to these negative effects, it is less clear whether clotting also contributes to immunity in a positive way. The blood clot is ideally situated to prevent not only blood loss but also dissemination of infectious agents from the wound site [2] and has been proposed to have an immune-protective function during a very early stage of an infection [3–6]. Insects are injured frequently both by parasites such as nematodes [7] and parasitic wasps [8] as well as during copulation [9] and by predators increasing the risk of wound-borne systemic infections. Here we show that clotting has an important immune function by limiting the dissemination of infections. We focused on the enzyme transglutaminase/factor XIIIa, (TG and F XIII, respectively) which we studied both in the model insect *Drosophila melanogaster* and in humans. Chemically, TG and F XIII crosslink selected glutamines and lysines in proteins involved in clotting leading to ε-(γ-glutamyl)lysine bridges [10], which can be readily detected using artificial substrates. Phylogenetically, TG is the sole component of clotting cascades that has been conserved during evolution. Similarly in all species where coagulation has been studied, TG contributes to this process [11,12]. Finally to our knowledge TG is present in the genome of all animals studied so far. This includes *Drosophila* where TG-activity can be detected in the clot and the enzyme contributes to clot formation [13,14]. Like in other insects, coagulation of *Drosophila* hemolymph is based on an interaction between humoral and cellular procoagulants [15]. Humoral procoagulants in *Drosophila* comprise lipophorin, hexamerins, the hexamerin receptor (also called fat body protein 1, FPB1), the clotting factor fondue [5], and phenoloxidase, while hemolectin and tiggrin are derived from blood cells [16]. We hypothesized that *Drosophila* might be an ideal system to study the beneficial aspects of clotting since it has an open circulatory system in which obstruction of blood flow causes fewer problems than in vertebrates. We show that knockdown of *Drosophila* TG leads to increased mortality after injection of bacteria and in a natural infection model involving entomopathogenic nematodes and their associated bacteria. Both *Drosophila* hemolymph- and human blood clots sequester bacteria

Author Summary

One of the main functions of immune systems is to prevent the dissemination of microbes and the resulting sepsis. Blood clotting during sepsis has until now been primarily regarded as harmful, leading to the formation of widespread clots in blood vessels and as a result to organ failure. Here we show that clotting also has a protective function to limit and prevent infections. This is achieved by capturing bacteria in the clot. Our infection studies were performed in the insect model *Drosophila melanogaster* where, due to the presence of an open circulatory system, the negative effects of clotting are less pronounced. We show that clotting of hemolymph—the insect blood equivalent—is essential in *Drosophila* to prevent septic death arising from injection of bacteria or infection with a natural pathogen. We also show that both *Drosophila* transglutaminase and its human homologue clotting factor XIII are key enzymes for sequestration of bacteria in the clot matrix, indicating the conserved nature of the clot's function in immunity. Our data are expected to lead to a much stronger appreciation of the role of blood clotting in innate immunity, and will guide future therapies which target this process.

preventing their dissemination throughout the body. Our results firmly establish clotting as part of the innate immune system and relate it to other branches of immunity.

Results

Drosophila transglutaminase activity on microbial surfaces

To investigate whether TG actively participates in the host response to infection, we challenged *Drosophila* hemolymph with microbes or microbe-derived immune elicitors, and then tested whether each treatment triggered activation of TG. For this purpose, *Drosophila* hemolymph was mixed with yeast cell wall preparations (zymosan beads), and the resulting aggregates probed with an antibody that recognizes ε-(γ-glutamyl)lysine bridges. Fluorescence microscopy of the aggregates revealed a punctate pattern mostly located at the interface between the particles (Fig. 1A). Such aggregates were also observed when hemolymph and zymosan were mixed in the presence of biotin-cadaverine (B-cad), a small primary amine capable of replacing lysine during TG-mediated crosslinking and which can serve to mark host proteins involved in crosslinking (Fig. 1B: Zym). Using the biotin tag, TG activity was also detectable on the surface of both DAP peptidoglycan-containing Gram− (*Escherichia coli*) and Lys peptidoglycan-containing Gram+ (*Staphylococcus aureus*) bacteria (Fig. 1B: E.c and S.a) and on the surface of entomopathogenic nematodes (Fig. 1C), which had been incubated with hemolymph. In all cases, the pattern after B-cad incorporation appeared to localize to small deposits on the microbial surfaces.

Analysis of both *E.coli* and *S.aureus* lysates after incubation with B-cad and hemolymph showed one prominent protein that had bound to bacteria and was identified as the humoral procoagulant hexamerin (Fig. 2A, B, see also Figure S1 for additional controls and binding to *Photorhabdus luminescens*). Using affinity purification of bacterial lysates after incubation with the biotinylated cadaverine, we confirmed hexamerin subunits as the major constituent of the aggregates, while less abundant protein components included phenoloxidase and lipophorin (Fig. 2C). These results show that upon septic injury, TG mediates the local formation of small aggregates on microbial surfaces, which incorporate humoral procoagulants. This is in line with our and others' earlier results, showing that bacteria and zymosan beads are sequestered by the clot ([4,17] and Fig. S2).

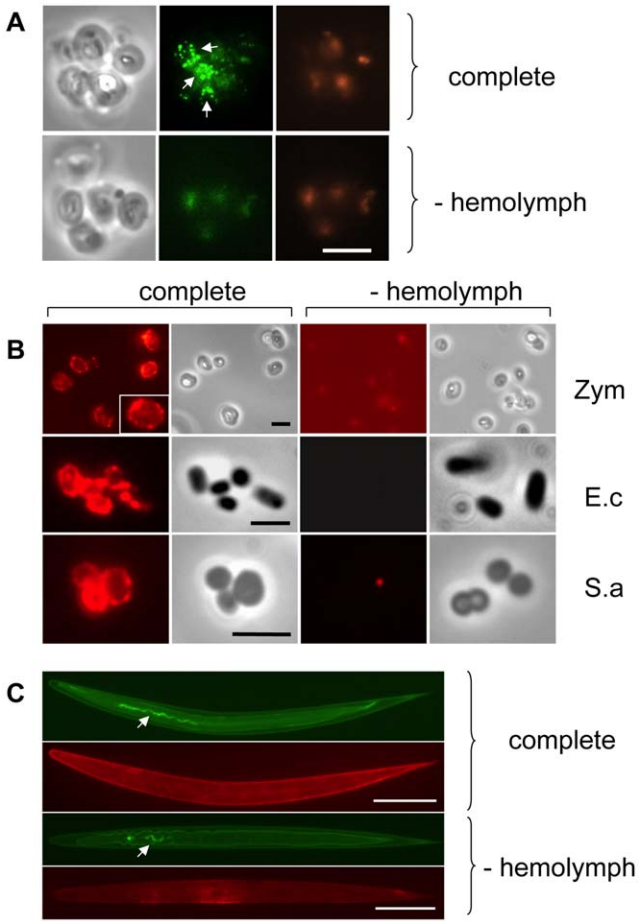

Figure 1. *Drosophila* Transglutaminase targets microbial surfaces. Microbes analyzed include: yeast zymosan particles (A,B: Zym), Gram− *E. coli* (E.c.) and Gram+ *S. aureus* (S.a.; B) and the entomopathogenic nematode *H. bacteriophora* (C); A: phase contrast exposure (left), immunocytochemistry with an antibody against ε-(γ-glutamyl)lysine bridges created by TG (middle) and autofluorescence in the red channel (right). Note the punctate deposits (arrows) on the zymosan particles which are absent in preparations that lack hemolymph. B-cad was used in B and C, showing TG-mediated incorporation of B-cad into Gln-containing protein substrates. The inset at the upper left in B is a twofold enlargement to show the punctate labelling. All exposures were analyzed using immunofluorescence detecting B-cad and the corresponding phase contrast exposures. Hemolymph was omitted as a control leading to a reduction of the signal for all microbes. The fluorescence exposure of the reaction with zymosan lacking hemolymph (Fig. 1B, right part) was 10× overexposed to underline the significant difference in labelling efficiency between the presence or absence of hemolymph. After omission of B-cad signals were undetectable in most cases (not shown). Scale bars in A and B correspond to 5μm. C: Nematodes (*Heterorhabditis bacteriophora*) were incubated with B-cad and hemolymph leading to the formation of aggregates on the cuticle (upper part). The lower part shows autofluorescence after omission of hemolymph. The presence of GFP-expressing *P. luminescens* is indicated by arrows. The scale bar corresponds to 100μm.
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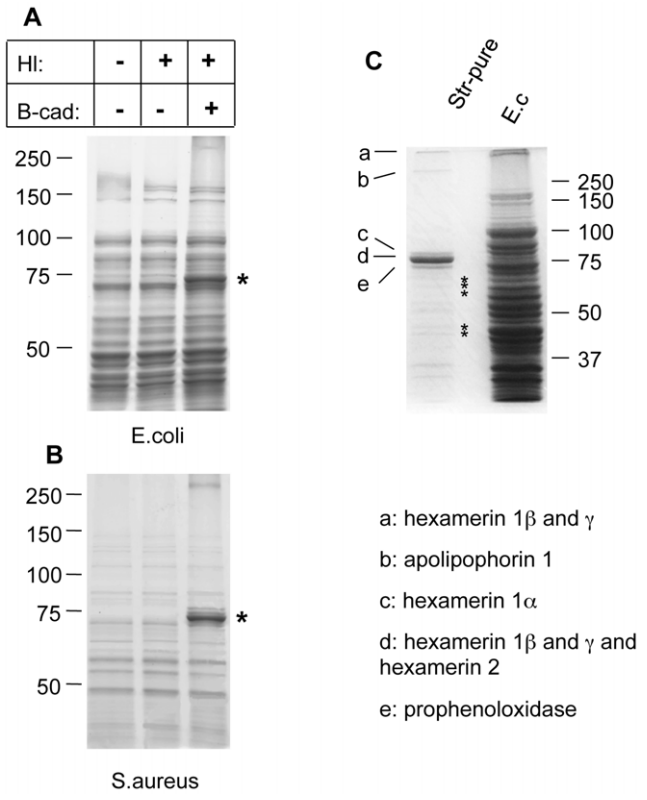

Figure 2. Humoral procoagulants bind to microbial surfaces. Lysates from *E. coli* (A) and *S. aureus* (B) were incubated in the presence of hemolymph (HI), B-cad or the combination of both and analyzed using polyacrylamide gel electrophoresis. The additional band in the samples with HI and B-cad (asterisk) represents hexamerin. Note that in the absence of B-cad hemolymph proteins form TG-crosslinked aggregates, thus preventing analysis with SDS-PAGE (see methods for further details). A similar pattern was obtained using *P. luminescens* (Figure S2). C: Proteins from an *E. coli* lysate treated like in Fig. 1 (right lane), were affinity-purified using streptavidin (Str-pure) and the identity of the purified proteins determined using mass spectrometry (E.c. shows a bacterial lysate, without hemolymph, the asterisks indicate breakdown products of hexamerin, see Table S1 for further details).
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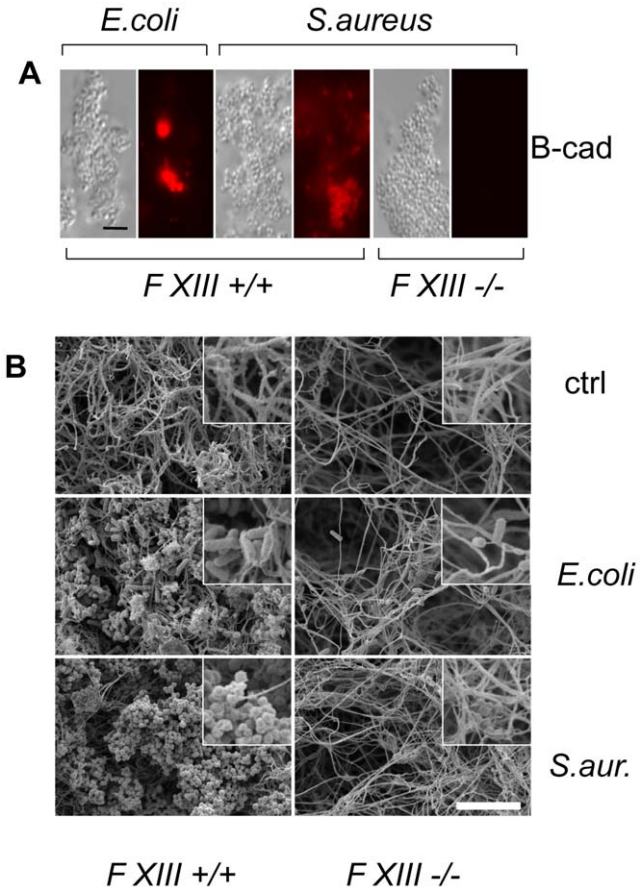

Figure 3. Human F XIII sequesters bacteria in the clot matrix. A: Plasma obtained from healthy donors (FXIII +/+) or donors with FXIII-deficiency (FXIII −/−) was activated with thrombin in the presence of *E. coli* or *S. aureus* (for details see Methods). B-cad was used to visualize FXIII-mediated incorporation at bacterial surfaces by immunofluorescence microscopy. Samples were also visualized by phase contrast to show the contour of the bacteria. B: SEM exposures of clots formed with normal plasma or FXIII-deficient plasma (the insets correspond to an 8-fold higher magnification). Clots were formed in the absence of bacteria (ctrl) or the presence of *E. coli* or *S. aureus* (scale bars correspond to 5 μm in A and 10 μm in B).
doi:10.1371/journal.ppat.1000763.g003

Factor XIII activity leads to sequestration of bacteria

To assess whether human F XIII has a role similar to *Drosophila* TG, we performed a parallel set of experiments by incubating B-cad with human plasma and either *E. coli* or *S. aureus*. In both samples, B-cad was deposited onto the bacterial surface albeit more efficiently with *S. aureus* as shown by fluorescence microscopy (Fig. 3A, left). No bacterial labelling was observed when F XIII-deficient plasma was used (Fig. 3A, right). This means that similar to *Drosophila* TG, human F XIII targets microbial surfaces. Subsequent scanning electron microscopy showed that the functional consequence of F XIII activity is the sequestration of bacteria by the clot matrix. Using normal plasma, both *S. aureus* and *E. coli* were efficiently immobilized (Fig. 3B left part). With both bacteria, sequestration was strongly reduced when F XIII-deficient plasma was used (Fig. 3B right part) or upon addition of monodansylcadaverine (MDC), a chemical inhibitor of TG with effects similar to B-cad ([14], Figure S3). Like in *Drosophila*, TG activity could also be detected on the surface of both *E. coli* and *S. aureus* using an antibody with specificity for TG crosslinks (Fig. 4). Altogether these data show that microbes are targeted by insect TG and human F XIII leading to their sequestration in the clot.

Transglutaminase plays a role in immunity

To test the functional requirement for TG activity in innate immunity, we used a previously described TG-RNAi line [14] with reduced expression of TG (inset in Fig. 5A) as well as a second independent TG knockdown line (see Methods). Aseptic injury of TG-RNAi larvae does not have a major effect upon survival, most likely due to the presence of redundant mechanisms [18]. In contrast, *black cells* (*Bc*) mutants lack phenoloxidase and show both poor clot formation [4] and strongly reduced viability upon wounding (Fig. 5A and [18,19]). Therefore, any increased mortality that arises upon introduction of pathogens into TG-RNAi larvae is not expected to result from increased loss of hemolymph. To test whether TG has a function in immunity we next injected normal and TG knockdown *Drosophila* larvae with *E. coli*, *S. aureus* and the entomopathogenic bacterium *Photorhabdus luminescens*, the symbiotic bacterium of the nematode *Heterorhabditis bacteriophora* (Fig. 1C, arrows). While only a marginal non-significant effect on survival was observed using the non-pathogenic *E. coli*, both the human and insect pathogen led to

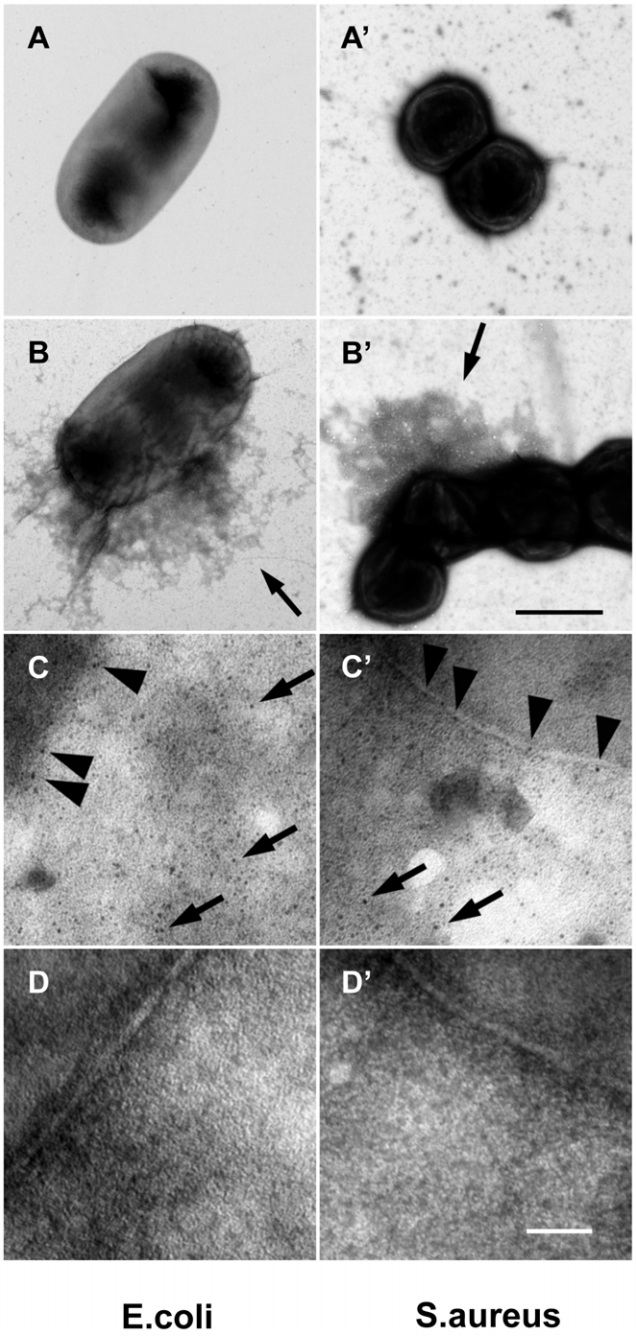

Figure 4. F XIII crosslinks are detectable on bacterial surfaces. *E. coli* (A–D) and *S. aureus* (A′–D′) bacteria were incubated with diluted and thrombin-activated normal (B–C′), F XIII-deficient plasma (D–D′) or left untreated (A–A′). Arrows in B and B′ point to plasma proteins crosslinked to the bacteria surface. Bacteria incubated with diluted and thrombin-activated normal (B–C′) or F XIII-deficient plasma (D–D′) were immunostained with a mouse anti-human gold-labeled ε-(γ-glutamyl) lysine-specific antibody. Arrowheads indicate crosslinking sites at the bacterial surface and arrows at crosslinking sites of crosslinked plasma proteins (scale bars correspond to 1 μm in B′ and 100 nm in D′). Please note that no colloidal gold staining was detected when bacteria were incubated with F XIII deficient plasma (D–D′).
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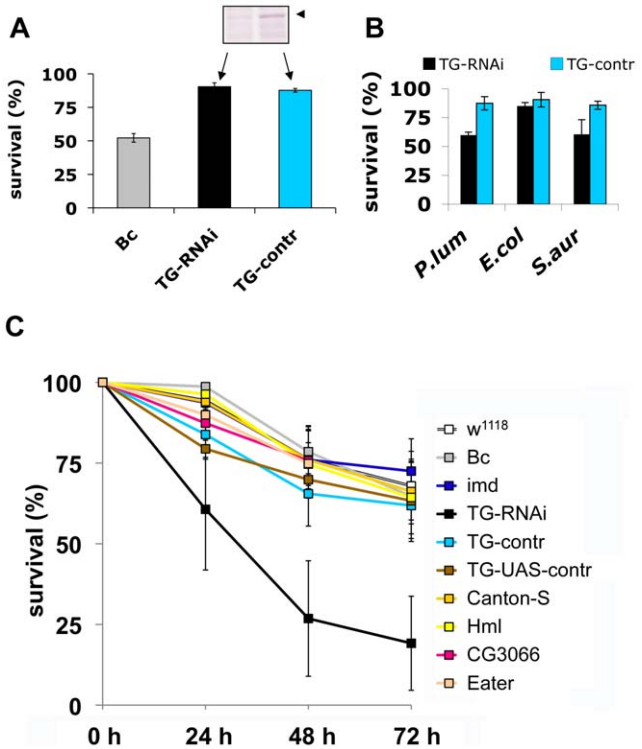

Figure 5. Larvae with reduced TG levels show immune defects. A: Lack of a wounding phenotype in TG knockdown lines. TG-knockdown larvae (*Act5C-Gal4>UAS-TG-RNAi*: labelled TG-RNAi); a control cross (*Act5C-Gal4>w*^{1118}: labelled TG-control); and *Bc* larvae were injured and survival determined after 24 hours. The insert shows the reduction in TG protein levels (arrowhead) detected using TG-specific antibodies. B: TG knockdown larvae are more susceptible to some bacteria than control larvae. TG-RNAi larvae and control larvae were injected with *P. luminescens*, *E. coli* and *S. aureus* and survival scored after 24 h. C: TG-RNAi larvae are more susceptible to nematode infections. Larvae from the same strains like in (A) as well as the strain used for construction of knockdown lines (*w*^{1118}); a homozygous *imd* mutant (*imd*^{y47}); a wildtype strain (Canton-S) Hml mutants (Hml); mutants lacking CG3066 and *eater* mutants were infected with *H. bacteriophora*. Mortality rates were determined at the indicated times post-infection. All data points in Figs. A–C represent at least triplicates (+/− s.d.), all experiments were performed at 22°C, see Methods for further details.
doi:10.1371/journal.ppat.1000763.g005

increased mortality in TG-RNAi larvae (Fig. 4B). Thus it appeared that loss of TG led to a specific immune defect in *Drosophila* larvae. To test the suspected immune function more stringently, we used the entomopathogenic nematode *H. bacteriophora*, which offers several advantages: i) this nematode is a natural invasive insect pathogen, thus larvae are infected in a much more reproducible way than by any artificial injection [7]; ii) the infection includes induction of septicaemia due to the massive release of the nematode's symbiotic bacteria (*P. luminescens*), which are essential for the nematode's success as an entomopathogen [20] and which we already had found to be more infectious in TG-RNAi larvae (Fig. 5B); and iii) although both *Toll* and *imd* pathway-dependent antimicrobial peptides are induced after infection with *H. bacteriophora*, survival of larvae after nematode infection was unaffected by mutations in either of the two pathways [20]. This suggests that previously uncharacterized immune mechanisms are involved in surviving nematode infections. To test the involvement of cellular procoagulants, we included mutants lacking hemolectin [18]. To cover other immune reactions we included mutants in additional effector pathways: *Bc*, which lack active crystal cells; CG3066 mutants, which lack a

protease required for prophenoloxidase activation [21] and mutants in the phagocytic receptor Eater [22].

Our experiments firstly confirm that *imd* mutant larvae have similar viability after infection with nematodes compared to control animals. In contrast, the TG-knockdown line used in Fig. 5A as well as the second TG RNAi line (see Methods) showed increased mortality, in line with a requirement for TG in immune function and survival after infection (Fig. 5C). Despite the wounding defects in *Bc* larvae, we found that *Bc* and CG3066 mutants showed normal viability after infection. We propose that, while phenoloxidase is critical to wound healing, it is less essential in the infection model we used here, most likely due to the production of a *P. luminescens* phenoloxidase inhibitor [23]. The cellular procoagulant hemolectin also appears dispensable for the response towards nematodes and their bacteria. Similarly, although we could confirm that lack of the phagocytic receptor eater reduces uptake of *P. luminescens* (Figure S4), this does not increase mortality indicating that phagoytosis too may be less critical for the defense towards *Heterorhabditis/Photorhabdus*. In contrast to *eater* mutants hemocytes from TG-RNAi larvae retained full phagocytic capacity (Figure S4). Further supporting the immune function of the clot, we found instead that *P. luminescens* is sequestered by the clot matrix (Fig. 6A). The clot's capacity to sequester bacteria is reduced in TG-RNAi larvae and the clot has a more brittle appearance in line with our previous results (Fig. 6B and C and [14]). Finally, the amount of hexamerin that binds to microbial surfaces is reduced in TG-RNAi larvae (Figure S5). Taken together these results firmly establish TG activity in the clot as an effective immune mechanism that plays a dominant role in infections such as with nematodes and their associated bacteria.

Discussion

We have identified a previously underappreciated mechanism in the arsenal of insect and human innate immunity. Upon contact with hemolymph or blood, microbes are almost instantaneously targeted by TG activity leading to formation of small aggregates and ultimately to sequestration by the clot matrix (Fig. 7). Glutamine and lysine residues required for TG-crosslinking may potentially be present on different classes of proteins including: i) hemolymph proteins, such as hexamerin, assembled at the bacterial surface (see Fig. 2, of note hexamerins have been implied in immunity before [24,25]); ii) bacterial proteins such as secretion systems or other virulence factors; or iii) host-derived recognition proteins with specificity for microbial patterns which have bound to the bacterial surface. Interestingly some hexamerins display lectin-like activity [26] and may act as recognition molecules in their own right. TG-substrates on microbes are subsequently linked to TG-substrates in the clot. We propose that in cooperation with phagocytosis, sequestration by the clot prevents dissemination of bacteria and systemic infections leading to a fast

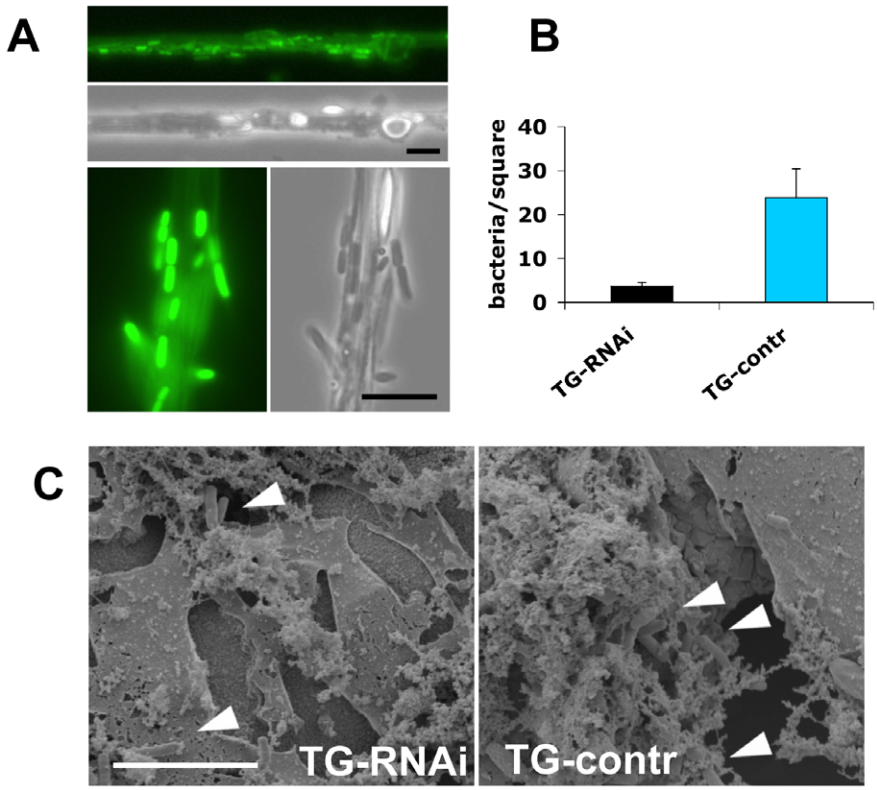

Figure 6. Clots from larvae with reduced TG levels sequester fewer bacteria. A: The clot from normal larvae captures *P. luminescens*. A clot bled from larvae expressing Fondue-FGP [14] was drawn out from hemolymph in the presence of GFP-expressing *P. luminescens* as described [16]. The clot is weakly labelled with Fondue-GFP [14]. The bacteria, which are immobilised in the clot [4] show a strong GFP signal (two sections are shown at different magnifications, the scale bars correspond to 10 μm). B: Hemolymph clots prepared as described [4] from larvae with less TG (TG-RNAi) and control larvae were captured and the number of sequestered bacteria determined under the microscope ($P<0.01$, performed in triplicates). C: Clots from both types of larvae were also analyzed using scanning electron microscopy (note the more brittle appearance of the clot from TG-RNAi larvae, which is also observed after addition of MDC: see [14]). The scale bar corresponds to 10 μm, the arrowheads indicate bacteria which have been incorporated into the clot.
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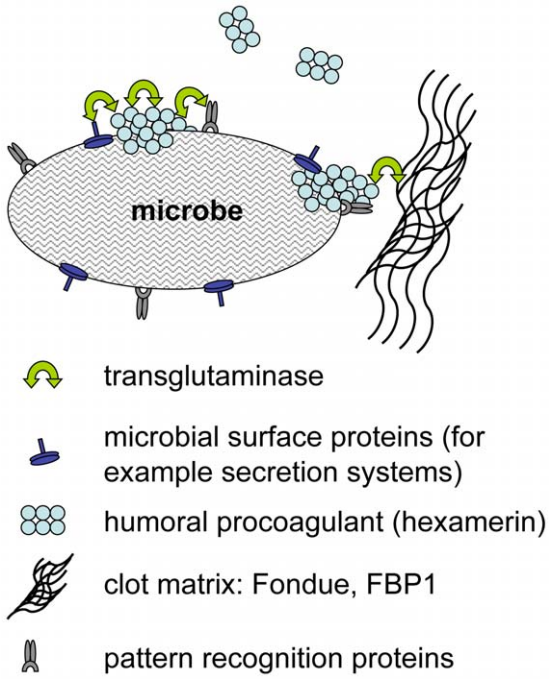

Figure 7. Hypothetical mechanisms for transglutaminase-mediated sequestration of microbes by the clot matrix. Transglutaminase crosslinks humoral procoagulants such as hexamerin and Fondue leading to their incorporation into the clot. Additional possible TG-substrates on microbes include microbial surface proteins such as secretion systems and recognition proteins with specificity for microbial patterns.
doi:10.1371/journal.ppat.1000763.g007

reduction in bacterial titres [6]. Alternatively the small aggregates we observe on microbial surfaces (Fig. 1) might play a role in immunity in their own right. Irrespective of the exact mechanism, TG- dependent activity appears to be the dominant immune mechanism during massive infiltration of bacteria such as after release from the nematode gut (Fig. 5C). In this case clot formation occurs in the absence of injury and is most likely identical to the formation of nodules to which it has been likened previously based on histological observations [27]. Future work will help to elucidate the exact route of *Photorhabdus* after their release into the hemolymph and whether TG contributes more to resistance or tolerance towards the bacteria [28,29].

We observe that although TG activity can be detected on all microbial surfaces tested and targets the same hemolymph proteins on *E. coli*, *S. aureus* (Fig. 2A, B) and *P. luminescens* (Figure S3), TG knockdown lines show increased susceptibility to only some microbes (Fig. 3B). Further work will be required to show whether there are any qualitative differences between the aggregates that bind to different microbes and whether these explain the different efficacy of TG-mediated crosslinking. Regardless of the evolutionary variability of TG substrates in blood/hemolymph, TG itself is widely conserved and has been shown to contribute to clot formation in almost every species where clotting has been studied in any detail [15,30]. For several animal models and for humans, evidence has been provided that the clot has a function in entrapping microbes [17,31,32]. Here we show for the first time its functional importance in a natural infection model. The mode in which TG contributes to immunity appears to be evolutionarily conserved providing yet another example for the successful use of insect models to decipher mechanisms that contribute to human immunity. TG activity has to be kept local in both *Drosophila* and humans but in contrast to insects with their open circulatory system systemic activation of TG in humans bears the additional risk of obstructing small blood vessels. Until now, focus on this negative aspect might have prevented full appreciation of the beneficial aspects of clotting. Our results fully agree with the observation that certain polymorphisms in clotting factors such as factor V Leiden which leads to a hypercoagulable state appear to be under balancing selection [33]. Epidemiological studies in humans and studies in animal models indicate that in addition to preventing bleeding more efficiently, Factor V Leiden might also protect from severe sepsis [34]. Future therapeutic strategies will thus have to aim at enhancing the helpful local effects of clotting while preventing its detrimental systemic effects. This appears even more vital in the light of the fact that we observe strong support for TG's immune function when using pathogenic bacteria such as *S. aureus* or *P. luminescens*.

Methods

Fly stocks

Flies were kept under standard conditions. The *Drosophila* strains included: a TG knockdown strain (Stock ID: 7356R-2, National Institute of Genetics Fly Stock Center, [14]). A second TG-RNAi strain produced independently with a different construct (Stock ID: 26101, Construct ID: 10774 from Vienna collection) showed similar reduced survival at all time points studied (24, 48 and 72 hours; $p<0.05$) although stronger effects were observed with 7356R-2 which was used for further studies. Crosses between TG-RNAi and *Act5C*-Gal4 show no morphological defects at 22°C and survive wounding equally well as control larvae (Fig. 4B); at higher temperatures, larvae appear normal although pupae displayed decreased eclosion rates. Additional strains include: *Canton-S*, *Black cells (Bc)*, and a P-element insertion mutant in CG3066 [28,35] and imd^{1547}. Driver lines were *Act5C*-Gal4 and *ppl*-GAL4 (kindly provided by B. Lemaitre, Lausanne).

Histochemistry with anti-crosslink antibody

Hemolymph from ten w^{1118} larvae was incubated at a 50 fold dilution for five minutes at room temperature with *Drosophila* Ringer's solution containing the phenoloxidase inhibitor phenylthiourea (PTU) and Zymosan A (at a final concentration of 3×10^5 beads/ml). The preparation was analyzed with the ε-(γ-glutamyl) lysine-specific antibody (at a dilution of 1:100, Covalab mab0012). No signal was detected with secondary antibodies alone (not shown) or when hemolymph was omitted (Fig. 1A).

Sequestration of bacteria by *Drosophila* clot

To analyze the sequestration of bacteria to fly clot, 10 larvae were bled into 2µl of bacterial suspension (*P.luminescens* expressing GFP) as described (hanging drop method, [4]). The clot was captured on an electron microscopy grid, washed 5 times with PBS and subsequently mounted on a new slide and the number of bacteria/square counted using fluorescence microscopy.

Incorporation of B-cad into microbial surfaces

Ten w^{1118} larvae were bled as described [14] followed by addition of either washed Zymosan A beads (SIGMA), or bacterial suspensions (*S. aureus* SH1000 or *E. coli* MG 1655, kind gifts from Håkan Steiner, Stockholm). After addition of biotincadaverine (Zedira) to 5 mM the preparation was incubated for 80 minutes at room temperature, centrifuged at 4000 g for 5 minutes, washed 3 times with *Drosophila* Ringer's solution and visualised using

Streptavidine-Cy3 (SIGMA, note that due to competition with TG-mediated crosslinking, B-cad reduces aggregation of zymosan beads). Control preparations without biotincadaverine, which were prepared the same way as above showed labelling of just a few dead bacteria (see Fig. 1 for additional controls).

Infection of *D. melanogaster* larvae with *H. bacteriophora*

Infection of *D. melanogaster* larvae with infective juveniles was modified according to Hallem et al. [20]. Infective juveniles from wildtype *H. bacteriophora* (H222, isolated from Pouzdřany, Czech Republic, kindly provided by Dr. Z. Mráček, Institute of Entomology, České Budějovice, Czech Republic) were collected after multiplying on *G. mellonella* larvae and used for infection according to [20] with the exception that the nematodes were applied using tissue paper at a multiplicity of 100 nematodes/larva. All experiments were performed at 22°C.

TG antibody synthesis

Anti-TG guinea pig polyclonal antibody was produced by Invitrogen Corp. (Carlsbad, CA). Amino acid residues 757–773 were selected as the antigen (NH_2-CQPNGSHRSSNIIRRRTD). The cysteine at the N-terminus is inserted to allow for conjugation with keyhole limpet hemocyanin. 50 µg of conjugate in Incomplete Freund's Adjuvant was injected into each of two guinea pigs at weeks 3, 5, and 8. Each guinea pig was "boosted" with 200 µg in Complete Freund's Adjuvant at week 10, and 100 µg in Incomplete Freund's Adjuvant at week 11. Exsanguination was carried out at week 21.

Interactions of human F XIII with microbial surfaces in plasma

S. aureus SH1000 or *E. coli* MG 1655 bacteria were grown overnight in Todd-Hewitt-Broth or LB-Medium and washed 3× with sterile PBS. Human plasma obtained from healthy donors (purchased from the blood bank at Lund University Hospital, Lund, Sweden) or from donors with F XIII-deficiency (F XIII−/− plasma, purchased from George King BioMed Inc., Overland Park, KS, USA) was incubated with thrombin (Sigma, St. Louis, MO, USA) and bacteria. The peptide H-1998 (H-Gly-Pro-Arg-Pro-NH_2) (Bachem, Bubendorf, Switzerland) was added to avoid clotting. Finally, biotincadaverine (Zedira, Darmstadt, Germany) was added to 5 mM and preparations were incubated for 1.5 h rotating at 37°C. After centrifugation at 8000 rpm and washing 3× with PBS streptavidin-Cy3 (Sigma, St. Louis, MO, USA) was added and the samples were incubated for 1 h rotating at room temperature. After 3× washing with PBS the preparations were mounted in glycerol and analyzed with a fluorescence microscope (Nikon, Tokyo, Japan) using a 100× objective. Control samples without biotincadaverine were prepared the same way as described above.

Preparation of clots

Overnight cultures of *S. aureus* SH1000 or *E. coli* MG 1655 were grown in Todd-Hewitt-Broth or LB-Medium and washed 3× with sterile PBS. 50 µl of human plasma obtained from healthy donors or donors with F XIII-deficiency were incubated for 60 sec. at 37°C in a coagulometer (Amelung, Lemgo, Germany). 50 µl of bacterial solution were added followed by 60 sec. incubation at 37°C. Clotting was initiated by adding 100 µl of Hemoclot-Thrombin (Hyphen Bio-Med, Neuville-sur-Oise, France). Control clots without bacteria were generated by adding 100 µl of Hemoclot-Thrombin to 100 µl of human normal or F XIII−/− plasma. All clots were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2) and analyzed by scanning electron microscopy. Samples were dehydrated with a graded series of ethanol, critical-point dried with CO_2, and sputter coated with gold before examination in a JEOL JSM-350 scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at 5 kV accelerating voltage and a magnification of 2000. (as described elsewhere [36]). In some experiments the transglutaminase inhibitor monodansylcadaverine (MDC) (Sigma, St. Louis, MO, USA) was added to a final concentration of 5 mM to the plasma prior to the incubation with bacteria and the initiation of clotting.

Negative staining

E. coli or *S. aureus* were grown overnight and 2×10^9 bacteria per ml were incubated with human plasma obtained from healthy donors or patients with FXIII-deficiency. Plasma (diluted 1:100 in 13 mM sodium citrate to avoid clotting) and bacteria were incubated for 30 Min at 37°C in the presence of thrombin and a mouse anti-human gold-labeled N ε gamma glutamyl Lysine [153-81D4] antibody (GeneTex Inc., Irvine, CA, USA), recognizing the crosslinking site of FXIII. Subsequently samples were adsorbed to 400 mesh carbon-coated copper grids for 1 minute, washed briefly with two drops of water, and stained with two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Samples were observed in a Jeol 1200 EX transmission electron microscope operated at 60kV accelerating voltage as described earlier [37]. Control experiments were performed in the absence of bacteria and the antibody.

Purification of proteins with a B-cad tag

Proteins containing a biotin tag were purified from sonicated bacterial lysates which had been treated as described above (see: Incorporation of B-cad into microbial surfaces) using streptavidin-containing magnetic beads (Dynal) according to the manufacturer's instruction except that *Drosophila* Ringer's solution was used for washes. Proteins were eluted using SDS-PAGE loading buffer and separated using PAGE.

MALDI-TOF mass spectrometry analysis and protein identification

After affinity purification on streptavidin proteins (see Fig. 2C) were identified as described [16]. The hexamerin bands in Figs. 2A and B were in sufficient amounts to identify them without further purification. The results of the complete identification are summarised in Table S1.

Statistical analysis

Samples from 5 infection experiments using w^{1118} were initially tested positive for normality (Lilliefors test). Strain mortality was subsequently compared using ANOVA followed by Tukey's test for significance. The results were confirmed using a log-Rank test on survival curves.

Supporting Information

Figure S1 Humoral procoagulants bind to *E. coli* (A) and *P. luminescens* (B) surfaces. Bacterial lysates were incubated in the presence of hemolymph (Hl), B-cad or the combination of both or with B-cad alone (in the case of *E. coli*) and analyzed using polyacrylamide gel electrophoresis. The additional band in the samples with Hl and B-cad (asterisks) represents hexamerin. Note that in the absence of B-cad hemolymph proteins form TG-crosslinked aggregates, thus preventing analysis with SDS-PAGE (see methods for further details).

Found at: doi:10.1371/journal.ppat.1000763.s001 (0.53 MB TIF)

Figure S2 Zymosan particles are sequestered by the clot matrix. A drawout (A and [16] was performed in the presence of zymosan and the resulting fibers analyzed under fluorescence microscopy (B) and phase contrast (C). Zymosan beads visible due to autofluorescence are indicated by arrowheads, fat body debris released during wounding is also incorporated (*).
Found at: doi:10.1371/journal.ppat.1000763.s002 (1.27 MB TIF)

Figure S3 Sequestration of bacteria is inhibited by the TG inhibitor monodansylcadaverine (MDC). Clots were prepared as described (see Fig. 3B) in the presence and absence of MDC alone or in the presence of either *E. coli* or *S. aureus* SH1000. The scale bar corresponds to 10 μm.
Found at: doi:10.1371/journal.ppat.1000763.s003 (3.75 MB TIF)

Figure S4 Hemocytes from eater mutants but not from TG-RNAi larvae show reduced phagocytosis of *P. luminescens*. The percentage of hemocytes that had taken up bacteria was counted essentially as described [22] after mixing with GFP-expressing *P. luminescens* and incubation for 30 minutes. Note that in contrast to eater mutants ($p = 8.1 \times 10^{-8}$ compared to controls: TG-ctrl), hemocytes from TG-RNAi lines show normal phagocytic capacity.
Found at: doi:10.1371/journal.ppat.1000763.s004 (0.11 MB TIF)

Figure S5 Hexamerin binding to microbes is reduced in TG-RNAi larvae. Proteins binding to zymosan in the presence of biotincadaverine were analyzed using polyacrylamide-gelelectrophoresis. Both complete zymosan beads (−) as well as beads after pullout [5] on peanut agglutinin (PNA, +) are shown for a control cross (TG-contr) and a TG-knockdown (TG-RNAi). Note that the amount of hexamerin is reduced after TG-RNAi for both treatments.
Found at: doi:10.1371/journal.ppat.1000763.s005 (0.31 MB TIF)

Table S1 Identification of microaggregate proteins.
Found at: doi:10.1371/journal.ppat.1000763.s006 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: ZW CW PH TGL PD MK HH UT. Performed the experiments: ZW CW PH TGL PD MK OL MM UT. Analyzed the data: ZW CW PH TGL PD MK OL MM RMC HH UT. Contributed reagents/materials/analysis tools: JI RMC. Wrote the paper: ZW CW PH TGL OL MM RMC HH UT.

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PŘÍLOHA Č. 12

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Developmental changes in phenol-oxidizing activity in the greater wax moth *Galleria mellonella* L. Bulletin of Insectology, Bologna, 62 (2), 237-243, 2009.

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Developmental changes in phenol-oxidizing activity in the greater wax moth *Galleria mellonella*

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Abstract

Activity of enzymes oxidizing phenolic substrates commonly termed phenoloxidases (POs) was investigated during normal development of *Galleria mellonella* L. in haemolymph, intact cuticle and homogenized cuticle. PO activities were determined colorimetrically following chemical activation of prophenoloxidase with acetone or methanol using hydroquinone and pyrogallol as substrates. Hydroquinone with *para*-positioned hydroxyl group seems to be most suitable for spectrophotometrical detection of phenol-oxidizing activity in various insect tissues because its oxidation products interact only minimally with tissue material. Enzymatic activity changed markedly during development in larvae, pupae and adults. The highest levels of PO in haemolymph were detected using hydroquinone on the fifth day of the last larval instar and at the beginning and at the end of pupal stage. PO activity changed gradually during development except during larval-pupal and pupal-adult metamorphosis, where marked increases in activity occurred. Comparable changes with slight time shifts were determined in cuticle after its homogenization. Intact cuticles showed only minimal PO activity without distinct developmental changes. Experiments using pyrogallol as substrate confirmed the temporary decrease of overall PO activity during pupal stage of development and the subsequent return to high PO levels in adults. Developmental changes in phenol-oxidizing activity are independent of sex in both pupal and adult stage of *G. mellonella*.

Key words: development, *Galleria mellonella*, hydroquinone, laccase, phenol-oxidazing activity, phenoloxidase, pyrogallol.

Introduction

Insect immune responses against invading pathogens can be broadly categorized into two processes: cellular also called haemocytic and humoral. Both of these branches cooperate to protect animals against invaders that have breached their physical barriers such as integument, midgut epithelium and peritrophic membrane (Elrod-Erickson *et al.*, 2000). Cellular responses involving direct interaction of haemocytes with antigens include phagocytosis, microaggregation, nodulation and encapsulation. Bacteria that are entrapped in nodules (Stanley and Miller, 2006) as well as encapsulated larger objects such as parasites or parasitoid eggs (Gupta, 2002) can subsequently be melanized by action of phenoloxidases (POs). Humoral defense reactions involve the action of molecules constitutively present in tissues and haemolymph such as lectins or lysozyme, induced synthesis of antimicrobial peptides and the phenoloxidase cascade (Gupta, 2001). At the boundary of cellular and humoral immunity lies the coagulation cascade which includes action of both whole haemocytes and many coagulation factors released by them. The coagulation cascade closely cooperates with the PO cascade, which contributes to protein cross-linking, elimination of the pathogens and melanization in forming clot (Li *et al.*, 2002; Theopold *et al.*, 2004). In addition to its role in immune reactions POs in insects are essential to the fundamental physiological processes of pigmetation and sclerotization of new cuticle after each moult (Arakane *et al*., 2005).

The PO cascade or prophenoloxidase-activating system comprises several serine proteinase zymogens and pattern-recognition proteins that are able to detect minute amounts of antigens. Its function is to activate the enzyme PO which generally occurs as an inactive prophenoloxidase (proPO) (Ashida and Yamazaki, 1990; Jiravanichpaisal *et al.*, 2006; Cerenius *et al.*, 2008). POs sensu lato include enzymes with monophenoloxygenase (tyrosinase-type, EC 1.14.18.1), *o*-diphenoloxidase (tyrosinase-type, EC 1.10.3.1) and *p*-diphenoloxidase (laccase-type, EC 1.10.3.2) activity. In *Galleria mellonella* L. (Lepidoptera Pyralidae) as in other insects the presence of multiple proPO genes is assumed (Li *et al.*, 2002; Cerenius and Söderhäll, 2004) and some of the corresponding proteins were already purified (Kopáček *et al.*, 1995). Although laccase-type and tyrosinase-type POs prefer various substrates and differ in their primary function, both of them belong to the phenoloxidase group of copper-binding proteins (Hughes, 1999; Arakane *et al*., 2005).

During normal development laccase-type POs commonly termed laccases participate especially in sclerotization of cuticle (Sugumaran *et al*., 2000; Sugumaran, 2002). In this non-immune process laccases in conjunction with other enzymes such as quinone isomerase catalyze the production of highly reactive quinones and quinone methides. Products of these cuticular reactions are responsible for cross-linking of cuticular proteins and their incorporation into the cuticle leads to its hardening and coloration. In contrast to tyrosinase-type POs the role of laccases during immune responses is still unclear.

Tyrosinase-type POs sometimes referred to simply as phenoloxidases or tyrosinases mediate melanin synthesis, protection against potential pathogens and are also involved in immune reaction such as nodulation and encapsulation (Mandato *et al.*, 1997; Zhao *et al.*, 2007).

Reminiscent of laccases the tyrosinases act in conjunction with other enzymes especially with dopa decarboxylase (Sideri *et al.*, 2007). During immune response tyrosinases respond on the presence of antigen, proPO is activated and the production of melanin leads to the sequestration of invaders. Furthermore, intermediates with potential cytotoxic activity are produced during proPO activation which contribute to the elimination of pathogens (Nappi and Ottaviani, 2000). Similarly, tyrosinases are important in wound healing and clot formation, where they contribute to protein cross-linking and microbe killing (Theopold *et al.*, 2002).

The greater wax moth *G. mellonella* is frequently used as a model for investigating insect immunity and physiology, but little is known about the development-dependence of immune reactions, including PO activity, both in this and other insect species. The aim of the present work was to describe developmental changes in oxidation activity for phenolic substances in *G. mellonella* with accent on cuticular laccases.

The PO activity was determined in several tissues of *G. mellonella* including haemolymph, intact cuticle (IC) and homogenized cuticle (HC). Inactive proPO is generally produced by subsets of insect haemocytes and released into haemolymph. It was proven in *Bombyx mori* L. that some haemocyte-derived proPO can be post-translationally modified and then transported through the cuticle (Asano and Ashida, 2001). Moreover, laccase genes are highly expressed in epidermal cells participating in cuticle formation (Dittmer *et al*., 2004) and the presence of inactive form of laccase-type PO in cuticle of *B. mori* was proven in recent study (Yatsu and Asano, 2009). Here POs are involved, apart from other functions, in sclerotization and pigmentation thus developmental changes in PO activities linked to growth and molting are likely.

Materials and methods

Insects

Larvae of greater wax moth were reared on an artificial diet (Haydak, 1936) at 30 ± 1 °C in constant darkness. For the experiments larvae from the same day of development were used. We established several experimental groups covering each day of the 7th instar of larval stage, including the last two days also called prepupa. In some experiments selected days of pupae or adults were used. To discover possible differences between sexes we divided pupae into two subgroups. There were at least ten larvae in each experimental group.

Reagents

In all experiments sodium phosphate buffer (pH 5.8) consisting of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 was used. DOPA [3-(3,4-Dihydroxyphenyl)-DL-alanine]; hydroquinone (1,4-Dihydroxybenzene); pyrocatechol (1,2-Dihydroxybenzene); pyrogallol (1,2,3-Trihydroxybenzene) and tyrosine [3-(4-Hydroxyphenyl)- DL-alanine] were purchased from Sigma Aldrich Chemical Co.

Determination of phenol-oxidizing activities in haemolymph

Haemolymph was collected by amputation of a proleg and pooled into cold tubes without using any anticoagulant. 50 µl of haemolymph was mixed with 3 ml of ice-cold acetone and incubated 30 min at -4 °C. After centrifugation (5 min, 5000 g, -4 °C) the supernatant was removed and the sediment dried at room temperature. The acetone-treated dry material was then mixed with 3 ml of phosphate buffer (pH 5.8) and preincubated 10 min at 30 °C before 0.6 ml of 0.1 M hydroquinone (or 0.1 M pyrogallol) were added. The reaction was allowed to proceed for 25 min at 30 °C and then stopped by adding a few drops of inhibitor (2.5% phenylthiourea in distilled water). Controls with no PO activities used for determination of background absorbance values were prepared in the same way apart from phenylthiourea solution which was added before the substrate. After centrifugation (5 min, 5000 g, RT) the absorbance was measured spectrophotometrically at 470 nm (410 nm for pyrogallol) using a cuvette spectrophotometer (Spekol 210, Germany). The PO activities determined as the difference between absorbance values of controls and experimental samples was expressed in absorbance units per µl of haemolymph.

Determination of phenol-oxidizing activities in intact cuticle (IC)

Freshly prepared cuticles from the abdomen were divided lengthwise into halves, which facilitated processing of samples and controls simultaneously. To adjust for weight differences between six and eight halves of cuticle were mixed with 3 ml of ice-cold acetone and incubated 30 min at -4 °C. Concentration of cuticular material in all samples was adjusted to 5 mg/ml in order to ensure identical conditions for all groups. After centrifugation (5 min, 5000 g, -4 °C) the supernatant was removed and cuticles were dried at room temperature. Cuticles were then mixed with 3 ml of phosphate buffer (pH 5.8); preincubated 10 min at 30 °C and 0.6 ml of 0.1 M hydroquinone (or 0.1 M pyrogallol) was added as substrate. After 25 min at 30 °C the reaction was stopped by adding 2.5% phenylthiourea solution. Controls were prepared simultaneously from the same cuticles as the samples and treated with phenylthiourea to block PO activities before substrate addition. Finally, cuticles were removed carefully from both samples and controls and the absorbance of supernatants was measured at 470 nm or 410 nm for hydroquinone and pyrogallol, respectively. The PO activity was expressed as absorbance per mg of cuticle.

Determination of phenol-oxidizing activities in homogenized cuticle (HC)

Homogenized cuticle was prepared by mixing ten to fifteen cuticles from the abdomen (fixed concentration as above) with 6 ml of phosphate buffer (pH 5.8) and subsequent homogenization on ice. For each HC assay 30 mg of cuticular material was used. Compared to IC assay double amount of cuticle was used due to ensure optimal absorbance values. The homogenized mixture was centrifuged (5 min, 5000 g, -4 °C) and the super-

natant was used for determination of PO activity. 2 ml of supernatant was mixed with 0.2 ml of methanol and 0.5 ml of 0.1 M hydroquinone. The reaction was allowed to proceed for 25 min at 30 °C and then stopped by adding 2.5% phenylthiourea. In controls the phenylthiourea solution was added at the same time as methanol. Debris was removed by centrifugation (5 min, 5000 g, RT) and absorbance was measured spectrophotometrically at 470 nm. The enzymatic activities were determined as the difference between absorbance of samples and controls and expressed in absorbance units per mg of homogenized cuticle.

Statistical analysis

To determine significant differences between means Kruskal-Wallis analysis of variance (Statistica 8.0 software) followed by Tukey's test was used. The results were expressed as mean ± standard deviation and considered significantly different at $p < 0.05$.

Results

In preliminary tests the dependence of PO activity on time was determined. Samples from both haemolymph and cuticle were prepared as described above and absorbance was measured spectrophotometrically in 5min intervals during 30 min after addition of substrate. Both 0.1 M hydroquinone and 0.1 M pyrogallol were used as substrates measured at 470 nm and 410 nm, respectively. In all samples the increase in absorbance was nearly linear during this time period both with hydroquinone (figure 1) and pyrogallol (data not shown) which confirms earlier observations (Goldsworthy *et al.*, 2002; Adamo, 2004). Because PO activity can be considered constant under the used conditions during the first 30 min of the reaction and for simplicity, we decided to express PO activity as the absorbance units (au) reached after 25 min of incubation with substrate per µl of haemolymph or mg of cuticle.

Significant differences in laccase-type PO activity during development were detected using hydroquinone as substrate in haemolymph samples (figure 2). Enzymatic activity increased gradually from the first to the fifth day of 7th instar larvae where it reaches the maximum (0.00634 ± 0.00058 au_{470}/µl). During subsequent days of the larval stage the laccase activity decreased until the end of this stage. Rapid growth in activity appeared during the first day of the pupal stage and then in the last day of the same stage. Enzymatic activity on these days was as high as in five day old 7th instar larvae. There was no significant difference in laccase activity between male and female pupae.

When pyrogallol was used as substrate we weren't able to detect any developmental changes of PO activity in haemolymph except a small non-significant decrease connected with change of prepupae to pupae (figure 3). This decrease appeared in both males and females. During other days of the 7th larval instar overall PO activity changed only minimally with no significant differences.

Levels of laccase-type PO activity determined in IC were very low if hydroquinone was used as substrate

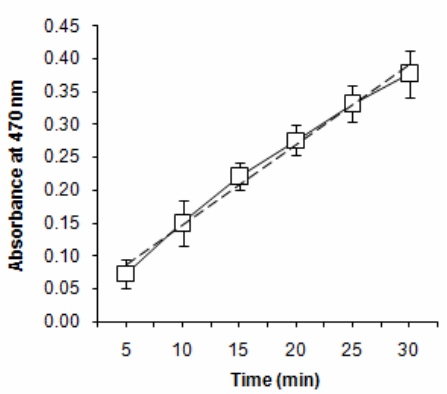

Figure 1. Time course of hydroquinone conversion mediated by laccase-type PO from haemolymph (means ± SD, $n = 4$). Dashed line demonstrates linearity for comparison with observed increase in absorbance (solid line).

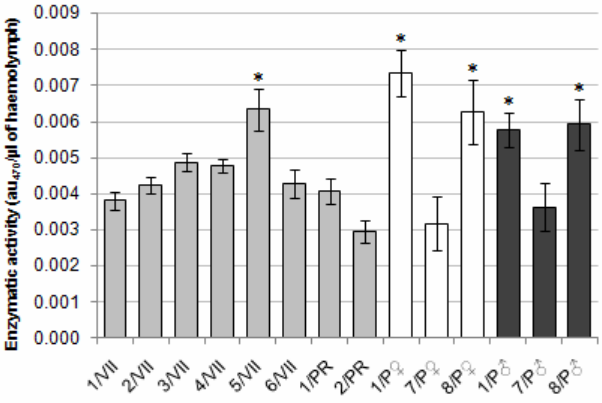

Figure 2. Enzymatic activity in *G. mellonella* haemolymph using hydroquinone as substrate [means ± SD, $n = 3$, significance level is <0.05 (∗)] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

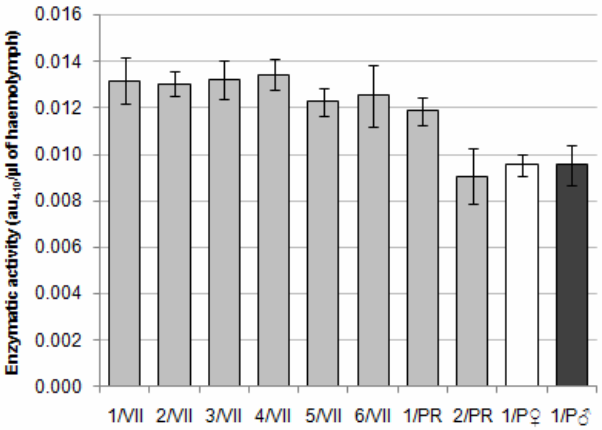

Figure 3. Enzymatic activity in *G. mellonella* haemolymph using pyrogallol as substrate (means ± SD, $n = 3$) and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

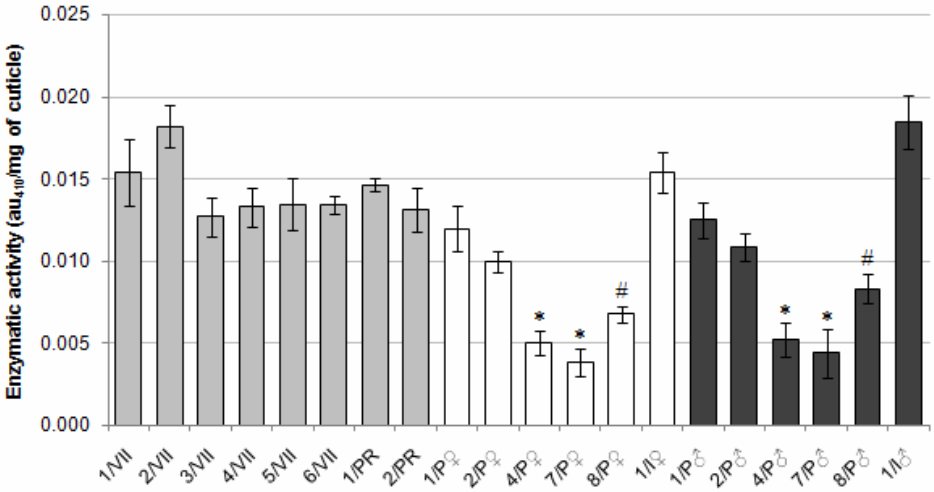

Figure 4. Enzymatic activity in *G. mellonella* intact cuticle using pyrogallol as substrate [means ± SD, $n = 3$, significance level is <0.05 (∗), marked values (#) indicate no significant difference to all other groups] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR); pupae (P) and imagoes (I). Sex not determined (grey); females (white); males (black).

(data not shown) and almost all led to OD values of 0.00100 au_{470}/mg independently on age of larvae or pupae. Distinctively different laccase activity was determined only on the first day of 7th instar larvae where the activity was nearly three times lower (0.00038 ± 0.00002 au_{470}/mg). In IC as in haemolymph there was no difference between sexes.

Much more data from IC were gained using pyrogallol as substrate (figure 4). Also here PO activity changed minimally during larval stages, but there was a substantial decrease in enzymatic activity during the pupal stage. In cuticles from one day old female imagoes the PO activity was again as high as in larvae; in males it was even slightly higher. Except for this difference the changes in PO activity during pupal stage were the same both in males and females.

In HC using hydroquinone as substrate the changes of phenol-oxidizing activity were much more pronounced (figure 5). During the first two days of the 7th instar the laccase-type PO activity was only minimal, but then gradually increased with the age of the larvae similar to haemolymph. This time the maximum of enzymatic activity was reached on about the sixth day of larval stage (0.00779 ± 0.00052 au_{470}/mg) and first day of prepupae (0.00799 ± 0.00053 au_{470}/mg). Then the activity started to decrease again and at the beginning of the pupal stage it was more than three times lower both in males and females.

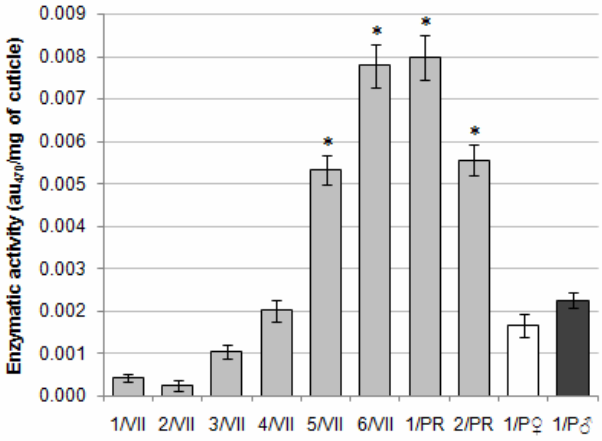

Figure 5. Enzymatic activity in *G. mellonella* homogenized cuticle using hydroquinone as substrate [means ± SD, $n = 3$, significance level is <0.05 (∗)] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

Discussion and conclusions

In this work we show that the activity of phenol-oxidizing enzymes is strongly influenced by development in *G. mellonella* and that the developmental changes appear both in haemolymph and in the cuticle. However, we detect a difference of about two days in the maxima of activities between haemolymph and cuticle in larval stage. This may confirm the transport of proPO from haemolymph, where it is synthesized by haemocytes, to the cuticle as suggested before (Ashida and Brey, 1995; Asano and Ashida, 2001). On the other hand, laccase genes are expressed mainly in epidermal cells, fat body and other tissues, but their expression in circulating haemocytes is low as observed in *Manduca sexta* L. (Dittmer *et al.*, 2004). Therefore, regarding laccases it is conceivable that there exist two different enzymes with similar functions, one responsible for *p*-diphenoloxidase activity in cuticle and a second functioning in haemolymph.

It is known that POs are able to process various substrates (Hall *et al.*, 1995; Ashida and Yamazaki, 1990; Dittmer *et al.*, 2004; Munoz *et al.*, 2006). Therefore the PO assays used in this article were also tested with DOPA, hydroquinone, pyrogallol, pyrocatechol and tyrosine as

substrates and their suitability for our experiments was compared. While DOPA is traditionally used as substrate for determining of insect POs (Li *et al.*, 1994; Kopáček *et al.*, 1995; Mandato *et al.*, 1997; Goldsworthy *et al.*, 2002; Hattori *et al.*, 2005), we found it unsuitable for spectrophotometrical detection of PO activity in samples containing whole tissues or tissue debris. Reaction products generated by enzymatic conversion of DOPA and pyrocatechol were kept on tissue surfaces causing its darkening and only a small fraction was released into solution where it could be measured spectrophotometrically. This was a problem mainly in HC assay during which substrates and cuticular material are both present in the reaction mixture. Tyrosine-based reactions were very slow and produced weak coloration. Therefore, we used diphenol hydroquinone and triphenol pyrogallol as substrates, which both appear to be efficient in detecting PO activity.

Hydroquinone was chosen as the most suitable substrate for our experiments, because only minimal interaction of its oxidation products with tissue debris and cuticular surfaces was observed. This enables the use of hydroquinone for colorimetric assays of PO activity both in haemolymph and cuticle. The disadvantage of hydroquinone is its *para*-positioned hydroxyl groups which makes it suitable substrate for laccase-type PO but not for tyrosinases with *o*-diphenoloxidase activity (Chase *et al*., 2000). That is why we used also pyrogallol as substrate wherever possible. This triphenolic substance can be processed by both laccase-type and tyrosinase-type PO, but causes the darkening of tissues containing POs, which complicate its use in HC assay. Discrepancies between chemical structures of used substrates can also lead to differences in PO levels; using hydroquinone we found greater fluctuations in PO activity than with pyrogallol as substrate. The fact that different activity profiles were observed when pyrogallol and hydroquinone were used as substrates suggests that hydroquinone was detecting a different enzyme(s) than pyrogallol.

Because the level of PO activity is very low in haemolymph and tissues of *G. mellonella* that are not immune challenged with pathogens or their components (unpublished observation), we decided to measure and compare PO activity obtained after in vitro conversion of proPO to active enzyme by organic solvents (Goldsworthy *et al.*, 2002; Adamo, 2004). Samples were treated with acetone or methanol that interact with proPO and are able to change it into active PO. Although methanol is a more effective activator of proPO in *G. mellonella* than acetone we observed the developmental changes in total PO activity are not affected by the type of used organic solvent (unpublished observation). The exact molecular mechanism of this chemical activation is still not fully understood and remains to be elucidated.

During the larval stage the laccase activity in haemolymph determined using hydroquinone gradually increases, followed by a partial decrease prior the pupal stage. The same trend was observed before in larval haemolymph proteins of *G. mellonella* using SDS-PAGE (Godlewski *et al.*, 2001). The largest increase in laccase activity was detected in haemolymph at the beginning and at the end of the pupal stage. This makes sense since the animal undergoes large developmental changes in this period connected with pupating and adult eclosure (Krämer *et al.*, 2002; Hyršl and Šimek, 2005). At these two important milestones of development laccases participate to a large extent in sclerotization and pigmentation of the new cuticle which forces the organism into the larger synthesis of PO precursors that is subsequently demonstrated in observed increase of enzymatic activity. These results agree with former observations in other insect species, which showed increased expression of laccase-like gene in epidermal cells of the pharate pupae of *M. sexta* (Dittmer *et al.*, 2004) and prepupae of *B. mori* (Yatsu and Asano, 2009). Insect development is controlled hormonally and the direct correlation was shown before between hormones and proPO gene expression. In *Anopheles gambiae* Giles 20-hydroxyecdysone (Ahmed *et al.*, 1999; Müller *et al.*, 1999) and in *M. sexta* the juvenile hormone (Hiruma and Riddiford, 1988) were shown to modulate proPO gene expression and thus are able to cause the observed changes in phenol-oxidizing activity during moulting.

In IC laccase activity changed only slightly during development of the larvae. This is probably caused by the small amount of enzyme bound to the inner and outer surface of the cuticle, which is able to interact directly with substrate present in reaction mixture. On the other hand, considerable age-dependent fluctuations in PO levels were determined in HC. Homogenization combined with methanol treatment supposedly led to the release of proPO from cells and its conversion to the active enzyme.

Unlike laccase activity, the phenol-oxidizing activity determined using pyrogallol in haemolymph and intact cuticles was more uniform during development. As mentioned above this is caused by different chemical structure of pyrogallol, which makes it suitable for enzymes with both *o*-diphenoloxidase and *p*-diphenoloxidase activity. With this in mind we propose that not only the activity of one enzyme, but common phenol-oxidizing ability including activity from laccases, tyrosinases and other enzymes with potential phenoloxidase activity was measured using pyrogallol. Our results suggest that even if the activity of individual enzymes such as laccases changes markedly during development, the overall phenol-oxidizing activity shows less fluctuation during development.

It seems that developmental changes of PO activities are not affected by sex in *G. mellonella*. Similarly, no gender-specific differences in cecropin-like antibacterial activity were detected in *G. mellonella* haemolymph (Meylaers *et al.*, 2007).

To conclude, our study provides new data on phenol-oxidizing activities in the haemolymph and cuticle of *G. mellonella*. We show strong effect of development especially on laccase-type POs in haemolymph and homogenized cuticle.

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PŘÍLOHA Č. 13

VOJTEK Libor, DOBEŠ Pavel, BÜYÜKGÜZEL Ender, ATOSUO Janne, HYRŠL Pavel Bioluminescent assay for evaluating antimicrobial activity in insect haemolymph. European Journal of Entomology, 3, 1-6, 2014.

Charakteristika:

Popis nové metody studia antibakteriální aktivity hemolymfy hmyzu. Jedná se o detekci přímé mikrobicidní aktivity v reálném čase pomocí bioluminiscenčních bakterií.

IF=0,975; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 14

HYRŠL Pavel

Pathogenicity of four entomopathogenic nematodes species to *G. mellonella* larvae. Karaelmas Science and Engineering Journal, 1 (1), 1-6. 2011.

Charakteristika:

Studium patogenity čtyř druhů entomopatogenních hlístic vůči hostiteli – zavíječi voskovému *Galleria mellonella*.

IF= 0,765; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 15

HYRŠL Pavel, ČÍŽ Milan, LOJEK Antonín

Comparison of the bioluminescence of *Photorhabdus* species and subspecies type strains. Folia Microbiologica, 49 (5), 539-542, 2004.

Charakteristika:

Bakterie rodu *Photorhabdus* mají vlastní systém bioluminiscence, která je druhově specifická. Porovnány byly všechny dostupné druhy a poddruhy tohoto rodu.

IF=1,000; citováno 4 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Peat, Scott M.; Ffrench-Constant, Richard H.; Waterfield, Nick R.; et al.: A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: A case for 16S, gyrB, and glnA, Molecular Phylogenetics and Evolution: 57, 2, 728-740, 2010.

PŘÍLOHA Č. 16

DOBEŠ Pavel, WANG Zhi, MARKUS Robert, THEOPOLD Ulrich, HYRŠL Pavel

An improved method for nematode infection assays in *Drosophila* larvae. Fly, Austin (USA): Landes Bioscience, 6 (2), 2012.

Charakteristika:

Popis optimalizace metody nákazy larev octomilky *Drosophila melanogaster* entomopatogenními hlísticemi. Srovnány byly různé druhy hlístic a jejich množství na jednoho hostitele.

IF=3,325; citováno 9 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Krautz, Robert; Arefin, Badrul; Theopold, Ulrich: Damage signals in the insect immune response, Frontiers in Plant Science: 5, 342, 2014.

PŘÍLOHA Č. 17

AREFIN Badrul, KUČEROVÁ Lucie, DOBEŠ Pavel, MARKUS Robert, STRNAD Hynek, WANG Zhi, HYRŠL Pavel, ŽUROVEC Michal, THEOPOLD Ulrich

Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. Journal of Innate Immunity, 6 (2):192-204, 2014.

Charakteristika:

Pomocí microarray analýzy jsme porovnali genovou expresi larev *Drosophila melanogaster* infikovaných entomopatogenními hlísticemi a jejich symbiotickými bakteriemi s larvami neinfikovanými. Exprese vybraných infekcí ovlivněných genů byla následně u larev utlumena pomocí UAS-Gal4 systému nebo mutována a byla testována obranyschopnost daných larev proti přirozené nákaze hlísticemi. Mezi geny významně ovlivněnými infekcí byly zejména ty, které jsou zapojeny v imunitních reakcích, buněčných a vývojových procesech. Pomocí experimentálních nákaz jsme identifikovali několik imunitních genů, kódujících např. složky koagulační kaskády nebo rozpoznávací molekuly, jako klíčové pro zdolání entomopatogenní nákazy.

IF=4,352; citováno 6 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Krautz, Robert; Arefin, Badrul; Theopold, Ulrich: Damage signals in the insect immune response, Frontiers in Plant Science: 5, 342, 2014.

PŘÍLOHA Č. 18

HYRSL Pavel, DOBES Pavel, VOJTEK Libor, HRONCOVA Zuzana, TYL Jan, KILLER Jiri

Plant alcaloid and probiotics promotes resistance of honey bees to nematobacterial infection (submitted to Bulletin of Insectology)

Charakteristika:

Úspěšná infekce včelího plodu nematobakteriálním komplexem umožnila otestovat účinek přípravků pro podporu včelí imunity. Aplikace směsi probiotických bakterií nebo rostlinného alkaloidu sanquinarinu zlepšila přežívání včelího plodu po experimentální infekci.

IF=1,494; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 19

BUCHTÍKOVÁ Soňa, VETEŠNÍKOVÁ ŠIMKOVÁ Andrea, ROHLENOVÁ Karolína, FLAJŠHANS Martin, LOJEK Antonín, LILIUS Esa-Matti, HYRŠL Pavel

The seasonal changes in innate immunity of the common carp (*Cyprinus carpio*), Aquaculture, 318, 169-175, 2011.

Charakteristika:

Tato studie popisuje změny parametrů přirozené imunity kapra obecného (*Cyprinus carpio*) během ročních období, vliv sezóny souvisí zejména s teplotou vody, ale také s inhibičním účinkem pohlavních hormonů během období rozmnožování.

IF=1,878; citováno 15 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Kokou, Fotini; Rigos, George; Henry, Morgane; et al.: Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal, Aquaculture, 364, 74-81, 2012.

PŘÍLOHA Č. 20

TOLAROVÁ Soňa, DÁVIDOVÁ Martina, VETEŠNÍKOVÁ ŠIMKOVÁ Andrea, FLAJŠHANS Martin, HYRŠL Pavel

The seasonal changes of innate immunity of tench, *Tinca tinca* (L.) with different ploidy level. Aquaculture, 432, 46-52, 2014.

Charakteristika:

Tato studie popisuje změny parametrů přirozené imunity lína obecného (*Tinca tinca*) během ročních období, vliv sezóny souvisí zejména s teplotou vody, ale také s inhibičním účinkem pohlavních hormonů během období rozmnožování. Výsledky prokázaly silný vliv sezóny i rozdíly mezi diploidními a triploidními rybami.

IF=1,878; citováno 1 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Sharma, Neeraj Kumar; Akhtar, M. S.; Pandey, Nityanand; et al.: Seasonal variation in thermal tolerance, oxygen consumption, antioxidative enzymes and non-specific immune indices of Indian hill trout, *Barilius bendelisis* (Hamilton, 1807) from central Himalaya, India, Journal of Thermal Biology: 52, 166-176, 2015.

PŘÍLOHA Č. 21

VETEŠNÍKOVÁ ŠIMKOVÁ Andrea, VOJTEK Libor, HALAČKA Karel, HYRŠL Pavel, VETEŠNÍK Lukáš

The effect of hybridization on fish physiology, immunity and blood biochemistry: A case study in hybridizing *Cyprinus carpio* and *Carassius gibelio* (Cyprinidae). Aquaculture, 435, 381-389, 2015.

Charakteristika:

Studie se zabývá přirozenou hybdridizací kapra obecného (*Cyprinus carpio*) a invazního karase stříbřitého (*Carassius gibelio*). Původní druhy i kříženci byli charakterizování z hlediska krevních rozborů, fyziologie a imunologie.

IF=1,878; citováno 1 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Simkova, Andrea; Hyrsl, Pavel; Halacka, Karel; et al.: Physiological and condition-related traits in the gynogenetic-sexual *Carassius auratus* complex: different investments promoting the coexistence of two reproductive forms? BMC Evolutionary Biology: 15, 154, 2015.

PŘÍLOHA Č. 22

ŠIMKOVÁ Andrea, HYRŠL Pavel, HALAČKA Karel, VETEŠNÍK Lukáš

Physiological and condition-related traits in the gynogenetic-sexual *Carassius auratus* complex: different investments promoting the coexistence of two reproductive forms? BMC Evolutionary Biology, BioMed Central, 2015, 15: 154-167.

Charakteristika:

V této studii je popsána genetická diverzita karase zlatého (*Carassius auratus*) a rozdíly ve fyziologii a imunitě jednotlivých reprodukčních forem.

IF=3,368; citováno 0 x (údaje k 31.1.2016).

Šimková et al. BMC Evolutionary Biology (2015) 15:154
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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Physiological and condition-related traits in the gynogenetic-sexual *Carassius auratus* complex: different investments promoting the coexistence of two reproductive forms?

Andrea Šimková[1*], Pavel Hyršl[2], Karel Halačka[3] and Lukáš Vetešník[3]

Abstract

Background: *Carassius auratus complex* is an extraordinary species complex including the diploid and polyploid forms exhibiting asexual and sexual reproduction modes. The coexistence of both forms in the same habitats is currently reported. The stable coexistence of asexual and sexual forms assumes some disadvantages for asexuals that balance the costs of sex. In our study, we hypothesized and tested the differences in physiological (including heamatological and immunological), growth-related, condition-related, and fitness-related traits between gynogenetic females and sexuals.

Results: Our results revealed similar growth performance in gynogenetic females and sexuals measured by body size and weight, or expressed by condition factor. The energy allocation in reproduction measured by the relative size of gonads revealed no difference between gynogenetic and sexual females; in addition, both females in spawning expressed the same estradiol levels in blood plasma. We found a gender specific trade-off between investment in reproduction and immunocompetence (measured by the spleen-somatic index). Higher aerobic performance expressed by the heart index and higher oxygen-carrying capacity were found in sexual males, with increasing values before and during spawning. Our study evidenced significantly lower aerobic performance but higher oxygen-carrying capacity per erythrocyte in gynogenetic females when compared to sexuals. IgM production differed between gynogens and sexuals of *C. auratus* complex.

Conclusions: Our study indicates that a similar amount of energy is invested by both gynogenetic and sexual females of *C. auratus* complex in reproductive behaviour. We suggest that lower aerobic performance in gynogens may represent their physiological disadvantage balancing the cost of sexual reproduction. A trade-off between the number of erythrocytes and the oxygen-carrying capacity per erythrocyte in sexual males and gynogenetic females may contribute to the coexistence of gynogenetic and sexual forms. In addition, the differences in specific immunity between gynogens and sexuals may also reduce the evolutionary disadvantage of sexual reproduction. In conclusion, we propose that several mechanisms contribute to the coexistence of the gynogenetic-sexual *C. auratus* complex.

Background

The coexistence of sperm-dependent asexuals and their sexual "hosts" is a phenomenon rarely reported in vertebrates [1]. In fish, such sperm-dependent asexuals often reproduce by gynogenesis (i.e. egg development is induced by sperm of conspecific or closely-related sexual species, but there is no syngamy resulting in fully clonal progeny). To compensate the evolutionary two-fold cost of sex, the sexuals need a short-term advantage [2]. Such an advantage (i.e. some advantageous fitness components) must be frequency-dependent to stabilize the coexistence of asexual and sexual forms [3, 4].

Most models explaining the stable coexistence of asexual and sexual forms assume some disadvantages for asexuals that balance the costs of sex. According to the Red Queen hypothesis [5–7], asexual genotypes are the

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target of parasite adaptation, and as a result, parasites causing low fecundity and high mortality in asexual hosts may promote the coexistence of asexual and sexual forms. In relation to this hypothesis, differences in immune effectiveness between asexual and sexual forms have been predicted [8]. Potential differences in life-history traits between asexual and sexual females have been proposed on the basis of life-history regulation hypothesis [9]. Other mechanisms such as male mate choice (the behaviour regulation hypothesis [9, 10]), different feeding efficiency and competitive abilities [11, 12], and apostatic selection, i.e. the negative frequency-dependence in reproductive success or other fitness components [13], were postulated to play an important role in the coexistence of asexual and sexual forms. Finally, ecological differentiation or specialization may also mediate the coexistence of asexual and sexual forms (Frozen Niche Variation Model, [14]).

Carassius auratus complex [15, 16] is represented by the diploid and the polyploid forms exhibiting asexual and sexual reproduction modes. In the areas of Czech Republic, this complex recently includes the forms belonging to four mitochondrial lineages – *C. gibelio*, *C. auratus*, *C. langsdorfii* and so-called M-lineage [17]. *Carassius gibelio*, Prussian carp, is the most abundant and widespread species of *C. auratus* complex in Europe [16]. The most recent analyses of *C. auratus* complex sampled in the areas of South Moravia (Southeast part of the Czech Republic), where the presented study was conducted, indicated that 96 % of specimens morphologically identified as the representatives of *C. auratus* complex belong to the *C. gibelio* mt DNA lineage (our unpublished data). The origin of *C. gibelio* is not yet fully resolved [16]. This species is usually considered as native from central Europe to Siberia or introduced to European waters from eastern Asia [16, 18, 19]. Nevertheless, it is documented that *C. gibelio* permeated into the Czech hydrologic system by migration from the Danube River in 1975 [20]. The successful invasion of Prussian carp into novel habitats was linked with rapid reproduction due to gynogenesis (females are able to use the sperm of conspecific males or males of other cyprinid species for the activation of embryogenesis e.g. Paschos et al. [21]). Interestingly, Zou et al. [22] showed that the eggs of Prussian carp activated by sperm from common carp grew faster than those activated by sperm from conspecifics. In the experimental studies, it was showed that even triploid females are able of sexual reproduction (i.e. the eggs of triploid females may also incorporate the sperm nuclei when coexisting with diploid bisexual specimens as demonstrated by Zhou et al. [23] and the mature eggs of polyploid *C. gibelio* have three various development modes in response to sperm as demonstrated by Zhang et al. [24]). However, the low survival rates at the hatching and first-feeding larval stages of sexual triploid offspring of *C. gibelio* [23] may indicate that sexual reproduction is not a commonly adapted and successful strategy for the reproduction of triploid *C. gibelio* when coexisting with sexual diploid in the mixed populations.

In the early invasive populations of Prussian carp in the Czech Republic triploid females with gynogenetic reproduction were detected. Such a unisexual triploid character of populations had been recorded up to 1992, when the first males were recorded in the populations of Prussian carp. A few years later, *C. gibelio* had formed mixed populations composed of the sexual diploid form (with a similar proportion of females and males) and gynogenetic triploid females [19, 20, 25]. In the few last years, an increase in the numbers of sexual diploids in mixed populations of *C. gibelio* has been reported and the occurrence of solely triploid female populations seems to be very rare (Vetešník, personal observation).

A historical shift from the female gynogenetic form toward the sexual form of Prussian carp resulting in mixed populations in which both forms coexist in the same habitats may indicate that sexual diploids are currently favored over gynogens. Several studies have recently been published investigating the factors potentially contributing to the coexistence of gynogenetic and sexual forms of Prussian carp (or other representatives of the *C. auratus*-complex with dual reproduction strategies). Vetešník et al. [26], investigating the biochemical profile of blood, suggested some potential advantage for the triploid gynogenetic form (i.e. a high total protein concentration reflecting better body condition) when compared to the diploid sexual form of Prussian carp. However, they proposed that such advantages may be offset by some disadvantages such as higher concentrations of triacylglycerols and cholesterol in gynogenetic females, which may indicate a higher metabolic rate and higher energy intake when compared to sexual diploids. A parasitological survey of a mixed population of Prussian carp showed the weakened immunocompetence of triploid gynogenetic females (especially of the most common triploid major histocompatibility complex (MHC) genotype) measured by parasite load, i.e. the level of infection by gill monogenean parasites, when compared to that in diploid sexuals [27]. In addition, differential gene expression in fully-grown oocytes between gynogenetic and sexual forms of Prussian carp was recorded, suggesting different responses and behaviours of the oocytes towards sperm [28].

In the present study, we tested whether the two forms of *C. auratus* complex with different reproduction modes (i.e. gynogenetic versus sexual) should exhibit some differences in physiological, condition-related, growth-related and fitness-related traits in order to facilitate their coexistence.

Such differences may reflect the advantages of one form relative to the other and may also potentially explain the historical shift from gynogenetic unisexual populations to the mixed diploid-polyploid populations.

Methods

Fish samples

A total of 157 individuals of *C. auratus* complex was collected by electrofishing from the River Dyje near the city of Břeclav (48° 38′ N; 16° 56 E; the Morava River basin) on 2nd November (7 diploid males, 14 diploid females and 19 triploid females; water temperature 5.4 °C), on 22nd March (5 diploid males, 14 diploid females and 20 triploid females; water temperature 7.7 °C), on 31st May (10 diploid males, 13 diploid females and 16 triploid females; water temperature 16.9 °C), and on 24th August (15 diploid males, 12 diploid females and 12 triploid females; water temperature 19.8 °C). The samples represented autumn, early spring, late spring, and summer, respectively. For our study, fish of the same age (5+ year old) were selected. Age was determined using scales following Holčík & Hensel [29].

A sample of blood sampling was collected from each individual by puncturing the caudal blood vessel using a heparinized syringe. Heparin was used as an anticoagulant (Zentiva a.s., Prague, Czech Republic) at a concentration of 50 U/ml. After sampling, the blood was centrifuged and plasma samples were stored in a freezer.

From each fish, finclip about 1 cm^2 was taken for ploidy detection and fixed in 70 % ethanol. Before analysis this tissue was homogenized with scissors on Petri dish in 2 ml solution of CyStain DNA 1 step PARTEC and relative DNA content was estimated using Partec CCA I flow cytometer (Partec GmbH; www.sysmex-partec.com). Fresh blood of diploid *Carassius auratus* was used as reference standard.

At the end of the experiment, all individuals were euthanized by an overdose of anaesthetic (2-phenoxyethanol). For each individual, the standard length and total weight were measured, and the sex was determined. In addition, the somatic body weight, spleen weight, hepatopancreas weight, gonad weight, heart weight, intestine weight and length (in mm) were measured. Intestine weight was measured after removing all intestinal content. In all statistical analyses (see below) the intestine length was corrected for standard length using ratio of intestine length and standard length and intestine weight was corrected for total weight using the ratio of intestine weight and total weight. The following body indexes reflecting investments in body condition, vitality, and reproduction were calculated: condition factor, spleen-somatic index, hepato-somatic index, gonado-somatic index, and heart index. The condition factor (K) representing the relative body weight was calculated using the equation: K = constant × body weight (g)/(standard length [cm])^3 according to Bolger and Connolly [30]. To evaluate the potential difference in growth performance between gynogens and sexuals, this index was calculated (1) using somatic weight and (2) using total body weight. The spleen–somatic index (SSI)—a measure of immunocompetence—was calculated as spleen weight (g)/total weight (g) × 100. Similarly, the relative size of the gonads (i.e. the gonado-somatic index, GSI) was calculated as GSI = gonad weight (g)/total weight (g) × 100, and the relative size of the liver (i.e. the hepato-somatic index, HSI) as HSI = hepatopancreas weight (g)/total weight (g) × 100. Finally, the heart index (HI), reflecting heart functional capacity, was calculated as heart weight (g)/total weight (g) × 100.

Animal care was in accordance with Law No. 207/2004 of the Collections of Laws of the Czech Republic on the Protection, Breeding and Use of Experimental Animals. This study was performed following the project of experiments n. 031/2011 approved by the Animal Care and Use Committee of the Faculty of Science, Masaryk University (Czech Republic).

Haematological analyses

Immediately after sampling, erythrocyte count, haematocrit value, haemoglobin content, and leukocyte count were determined according to Svobodová et al. [31]. Erythrocyte and leukocyte counts were performed in Bürker's haemocytometer after staining with Natt–Herrick solution. Heparinized microcapillaries (75 mm) were used to measure haematocrit and leukocrit. Blood samples were centrifuged in microcapillaries using a haematocrit centrifuge at 12.000 g for 3 min. Haemoglobin content (Hb) was analyzed photometrically (540 nm; Helios Unicam, USA) in Kampen–Zijlster transformation.

Immunological analyses

Lysozyme concentration, complement activity, and respiratory activity were analyzed as the measures of non-specific immunity. The lysozyme concentration in skin mucus (in $mg.ml^{-1}$) was determined by radial diffusion in agarose containing *Micrococcus luteus* (CCM 169) according to Poisot et al. [32].

Complement activity was measured according to Buchtíková et al. [33]. The total complement activity (including all activation pathways) of plasma was determined using a bioluminescent strain of *Escherichia coli* K12 (luxAmp, kindly provided by the University of Turku, Finland). The light emission measured by an LM01-T luminometer was positively correlated with the viability of *E. coli*. The relative measure of complement activity was estimated by computing the difference between the maximum time of measurement (equal to

4 h) and the time necessary to kill 50 % of *E. coli* by complement (in h).

Respiratory burst activity was measured as luminol-enhanced chemiluminescence using an LM01-T luminometer (Immunotech, Czech Republic) and opsonized Zymosan A as activator. The reaction mixture contained 50x diluted blood in Hank's balanced salt solution, luminol (Molecular Probes, Eugene, Oregon USA, dissolved in borate buffer, pH = 9, final concentration 10^{-3} mol.l^{-1}) and Zymosan A (from *Saccharomyces cerevisiae*; Sigma, USA, opsonized by incubation with serum). The final concentration of Zymosan A in the reaction mixture was 0.25 mg.ml^{-1}. The maximum intensity of respiratory burst (peak in relative light units–RLU) and total intensity of respiratory burst defined as the integral of the reaction curve area (RLU*s) were included as the measures of respiratory burst. For other details, see Buchtíková et al. [33].

IgM was analyzed as a measure of specific immunity. The total IgM level was determined using precipitation with zinc sulphate (0.7 mM $ZnSO_4.7H_2O$, pH = 5.8) [34]. IgM quantification was based on the total level of proteins in the sample, determined using commercially available kit (Bio-Rad, USA) before and after precipitation. The concentration of IgM in the sample (in g/l) was calculated as the difference between total plasma proteins and proteins in the supernatant after precipitation and centrifugation.

Steroid hormones analyses

The level of 11-ketotestosterone (11-KT) in blood plasma was analysed using the commercial competitive enzyme immunoassay (EIA) kit (Cat. No. 582751, Cayman Chemical, Estonia). Duplicates of each sample were run in two dilutions (50× and 200×) on the plate containing wells for a blank, standards and interassay variance. All plates were then analyzed using a plate reader at 412 nm (Tecan Sunrise, USA) and the concentration of 11-KT (in pg ml^{-1}) was calculated according to the manufacturer's instructions.

Plasma estradiol levels were analysed by an EIA method (Cat. No. 582251, Cayman, Estonia). Samples diluted ten times were run in triplicate and each plate contained the wells for interassay variance, standards, and a blank. The plates with samples were analysed with a plate reader at 422 nm (Tecan Sunrise, USA) and the concentration of estradiol (in pg ml^{-1}) was calculated according to the manufacturer's instructions.

The stress hormone cortisol was determined in the plasma by a solid phase enzyme-linked immunosorbent assay (Cat. No. 1887, DRG® Cortisol ELISA, Germany), based on the principle of competitive binding. Non-diluted samples were run in duplicate and each plate contained the wells for standards and a blank. The plates with samples were analysed with a plate reader at 450 nm (Tecan Sunrise, USA) and the concentration of cortisol (in ng ml^{-1}) was calculated according to the manufacturer's instructions.

Statistical analyses

General linear models (GLM) were used to analyze the effects of fish group (i.e. this factor takes into account the effects of ploidy and sex—triploid gynogenetic females, diploid sexual females, and diploid sexual males) and season (i.e. four periods of collection spanning one year); fish body size was included in the models as a covariate. The Tukey post-hoc test was applied for multiple comparisons. Factor ANOVA was applied to analyze the effect of fish group on standard length, total weight and somatic weight. All variables were checked for normal distribution and homogeneity of variance prior to performing GLM and ANOVA analyses. Alternatively, log-transformation, root-square transformation, or arcsin-transformation were applied to fulfill the above mentioned criteria. Statistical analyses were performed in Statistica 12 for Windows, StatSoft Inc.

Results

No significant effect of fish group on body size parameters was found ($p > 0.05$), i.e. standard length, total body weight, and somatic body weight did not differ between fish groups. However, body size parameters differed between seasonal samples (factor ANOVA, $p < 0.001$), with fish exhibiting the smallest body parameter (i.e. concerning all above mentioned body parameters) in spring and the largest body parameters in summer when compared to early spring and autumn samples (Tukey post hoc test, $p < 0.001$). No significant effect of fish group on intestine length corrected for body length was found ($p > 0.05$), but a significant effect of season on the same parameter was determined ($p < 0.001$), with a higher value in autumn compared to other seasons ($p < 0.001$) and a higher value in summer compared to spring ($p < 0.001$). Intestine weight corrected for body weight was affected by both fish group and season (factor ANOVA, $F = 6.196$, $p < 0.001$; for fish group, $F = 10.528$, $p < 0.001$; and for season, $F = 8.250$, $p < 0.001$). The highest intestine weight was found in late spring when compared to summer and early spring (Tukey post hoc test, $p < 0.001$) and autumn (but with $p = 0.084$). A significantly lower intestine weight was found in triploid gynogenetic females when compared to both sexual females and males ($p < 0.01$). This pattern was evidenced in all samples except for summer, where both females reached a similar intestine weight (Fig. 1a). A higher condition index was recorded in autumn when compared to other seasons ($p < 0.01$) and it was also higher in early spring when compared to summer

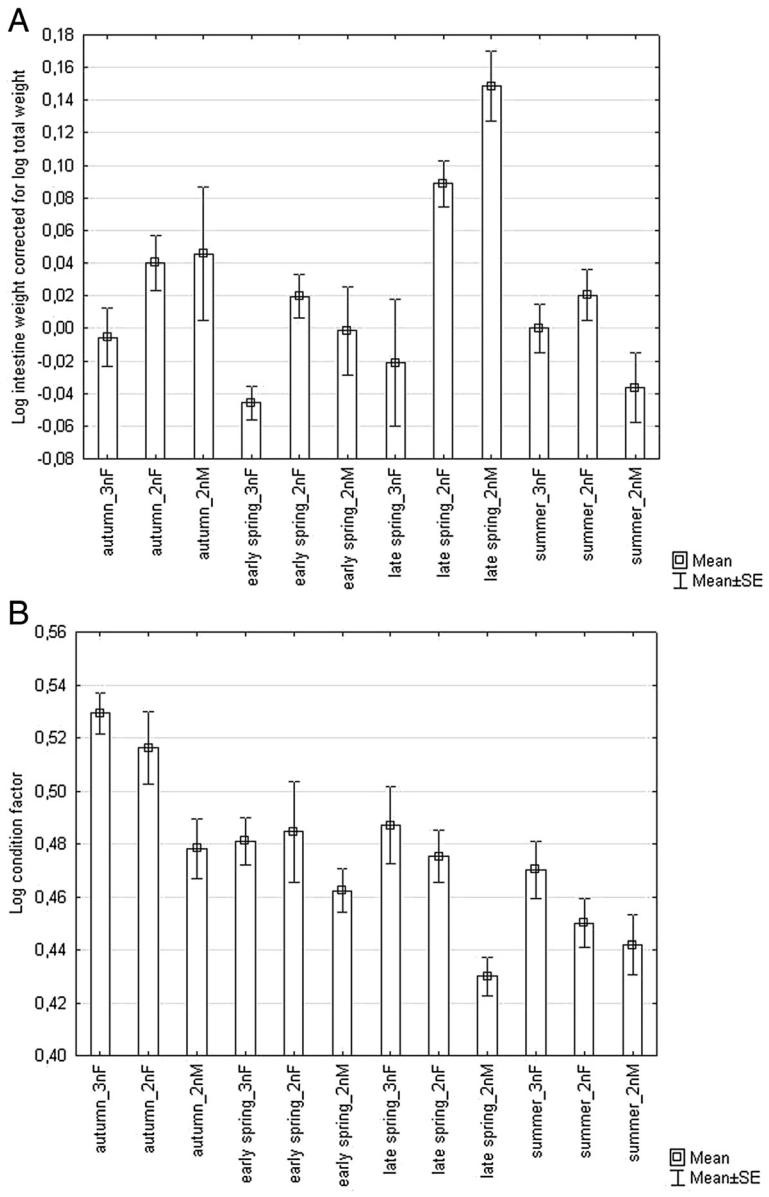

Fig. 1 The effects of season and fish group (including 3 nF – triploid gynogenetic females, 2 nF – diploid sexual females and 2 nM – diploid sexual males) on relative weight of intestine (**a**) and condition factor (**b**)

($p = 0.034$). Significant effects of sampling period and fish group on condition factor were found (Table 1). Condition factor in males was lower when compared to both gynogenetic and sexual females ($p < 0.001$) (Fig. 1b). However, the effect of fish group disappeared when somatic body weight was used in the calculation of condition factor.

Concerning organo-somatic indexes (Table 1), all of them except for HI were affected by season. A significant effect of fish group on all indexes was found. The index of reproductive investment, GSI, was also significantly affected by the interaction of season and fish group. SSI reached its significantly lowest values in autumn and early spring; it increased in late spring ($p < 0.01$) and reached its highest values in summer ($p < 0.001$). Higher values of SSI in diploid sexual males when compared to both diploid sexual and triploid gynogenetic females ($p < 0.001$) were found. This pattern was recognized within each season (Fig. 2a). The significantly highest value of GSI was recorded in late spring ($p < 0.001$); this index then decreased in summer, but increased again from summer to autumn and from autumn to early spring ($p < 0.001$). The GSI of both female forms was significantly higher than GSI in males ($p < 0.001$), whilst no significant difference in GSI between sexual and gynogenetic females was found ($p > 0.05$). This pattern was well evidenced within each season (Fig. 2b). A significant effect of fish group on relative heart

Table 1 The effects of fish group (including the effects of sex and ploidy associated with reproduction mode) and season on body condition and reproduction indexes

Dependent variable	Predicted variables	SS	Df	F	p	Total F	p
SSI	season	1.441	3	19.791	**<0.001**	10.458	**<0.001**
(*N* = 155)	fish group	0.666	2	13.709	**<0.001**		
	season*fish group	0.097	6	0.666	0.677		
HSI	season	9.460	3	234.060	**<0.001**	75.553	**<0.001**
(*N* = 155)	fish group	0.257	2	9.534	**<0.001**		
	season*fish group	0.110	6	1.358	0.235		
GSI	season	2472.748	3	186.352	**<0.001**	98.951	**<0.001**
(*N* = 156)	fish group	948.170	2	107.184	**<0.001**		
	season*fish group	673.676	6	25.385	**<0.001**		
HI	season	0.010	3	0.567	0.638	7.671	**<0.001**
(*N* = 155)	fish group	0.463	2	38.633	**<0.001**		
	season*fish group	0.029	6	0.807	0.566		
Condition factor	season	0.058	3	10.012	**<0.001**	5.958	**<0.001**
(*N* = 157)	fish group	0.032	2	8.327	**<0.001**		
	season*fish group	0.008	6	0.715	0.638		

The statistically significant p-values are shown in bold

size (HI) was found; this index reached its highest value in 2n sexual males followed by sexual 2n females. The lowest HI values were found in triploid gynogenetic females (Fig. 2c). On the other hand, HSI (Fig. 2d) reached its highest values in autumn ($p < 0.001$) and decreased significantly in the following order: early spring, summer, and late spring ($p < 0.001$). HSI was significantly higher in triploid gynogenetic females when compared to both sexual males and females ($p < 0.001$).

Haematological parameters were variable throughout the different seasons. In addition, erythrocyte count, haematocrit, and Hb were significantly affected by fish group (Table 3). More specifically, all these variables had significantly higher values in sexual males when compared to gynogenetic and sexual females ($p < 0.01$). In addition, erythrocyte count in gynogenetic females was lower when compared to sexual females ($p < 0.001$) (Fig. 3a). All three variables followed the same seasonal changes, i.e. the highest values of Hb were found in late spring and the highest values of erythrocyte count and haematocrit were found in late spring and summer ($p < 0.01$). The seasonal variation in these parameters was the most obvious in sexual males (Fig. 3a, b). When Hb was adjusted for erythrocyte count (i.e. the oxygen-carrying capacity of individual erythrocytes) GLM revealed the significant effects of sampling period and fish group (Table 2). No significant difference was found between sexual females and males ($p > 0.05$), whilst gynogenetic females reached higher Hb adjusted for the number of erythrocytes (Fig. 3c). The same result was found after adjusting Hb for haematocrit values. Leukocyte count was higher in autumn when compared to other seasons ($p < 0.001$), whilst leukocrit reached its highest values in summer ($p < 0.01$).

Concerning immune parameters, a significant effect of season on all immune measures was found (Table 3), with an increase in their values in spring. However, only IgM level, the single analyzed parameter of specific immunity, differed between fish groups. The IgM was significantly higher in late spring when compared to other seasons ($p < 0.01$), and significantly higher in gynogenetic females when compared to sexual males and females ($p < 0.001$). This pattern was evidenced within each period (Fig. 4). In addition, the IgM level of sexual males was also lower than that of sexual females ($p = 0.034$). Concerning measures of non-specific immunity, the highest values were found in late spring (lysozym concentration and complement activity) or in both late spring and summer (oxidative burst) ($p < 0.001$).

The levels of three steroid hormones—estradiol, cortisol and 11-ketotestosterone—were analyzed. Cortisol level was significantly affected by sampling period whilst the effect of fish group was not significant in the GLM model (Table 3). Estradiol level was significantly affected by both sampling period and fish group using the GLM model (Table 3). A significantly higher estradiol level was found in early spring ($p < 0.001$) when compared to other sampling periods. Males expressed a lower estradiol level when compared to both gynogenetic and sexual females ($p < 0.001$). No significant difference was found between gynogenetic and sexual females ($p > 0.05$). Concerning 11-ketotestosterone, the GLM model revealed the significant effects of fish group and sampling period (Table 3). Males

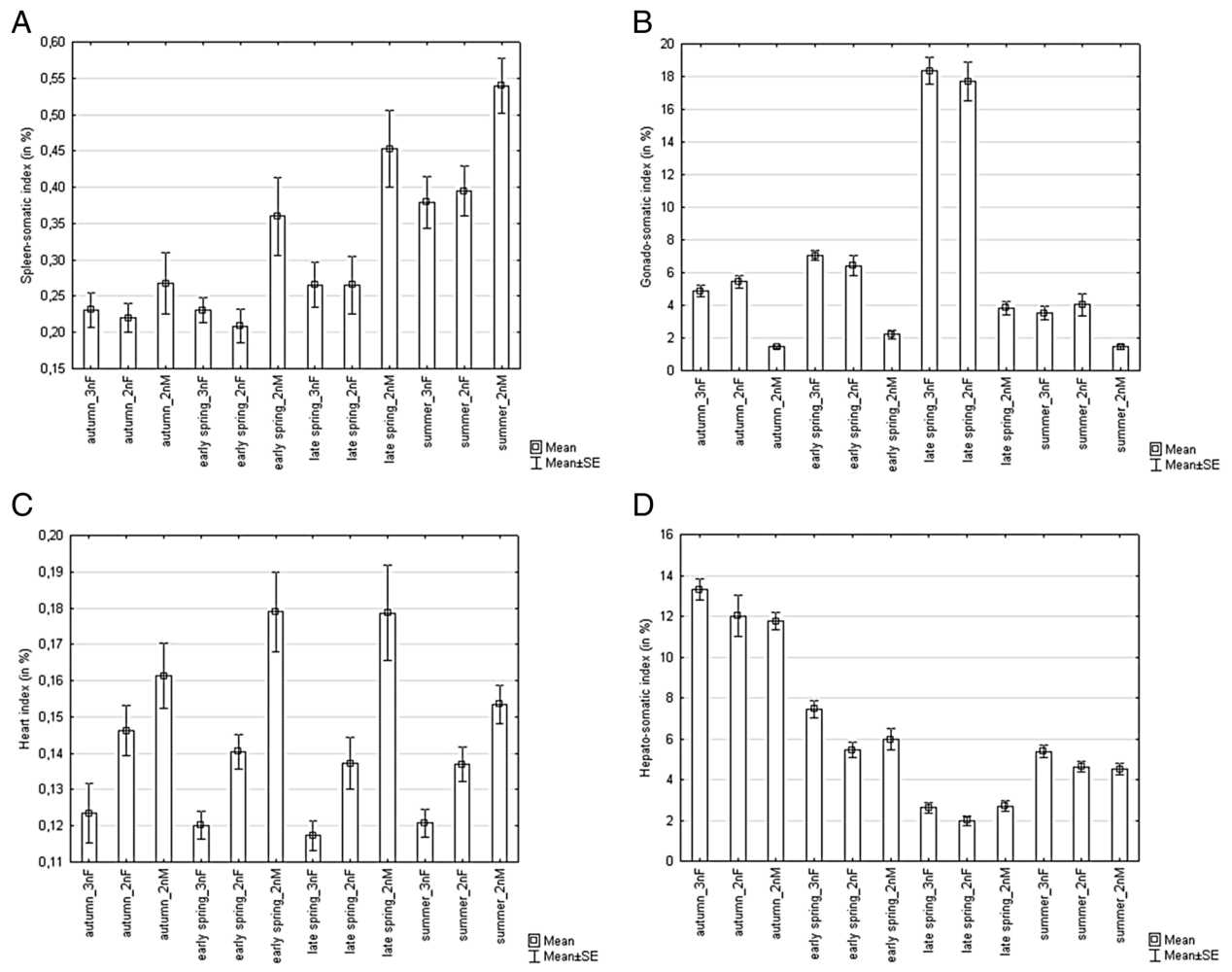

Fig. 2 The effects of season and fish group (including 3 nF – triploid gynogenetic females, 2 nF – diploid sexual females and 2 nM – diploid sexual males) on condition-related and fitness - related traits **a** spleen-somatic index, **b** gonado-somatic index, **c** heart index, **d** hepato-somatic index

expressed a higher 11-ketotestosterone when compared to both gynogenetic and sexual females ($p < 0.001$), but no significant difference was found between two groups of females. The highest values of 11-KT were found in early spring and late spring samples for males.

Discussion

In the present study, we focussed on selected physiological, condition-related, growth-related, and fitness-related traits in the gynogenetic-sexual complex of *C. auratus*, hypothesizing some disadvantage for asexuals which should compensate the cost of sexual reproduction and facilitate the coexistence of the gynogenetic and sexual forms of *C. auratus* complex in the same habitats. In our study, we investigated specimens of the same age for both forms collected in nature.

First, we focussed on parameters of the intestine which may reflect some feeding differences between the two forms. Our analyses revealed that intestines of similar total length in gynogens and both male and female sexual forms differed in total intestine weight, i.e. significantly lower intestine weight was found in gynogenetic females when compared to the sexual forms. This finding may suggest some differences in feeding efficiency between the two reproductive forms and may indicate a disadvantage for gynogens when competing for food with sexual counterparts. However, this should be tested in the future under experimental conditions. Scharnweber et al. [12] hypothesized that the costs of sexual reproduction could be balanced if asexuals were inferior in acquiring resources. They investigated whether feeding efficiency and food competition might promote the coexistence of sexual and asexual livebearing fishes of the genus *Poecilia*. However, their study did not reveal that gynogens were less efficient foragers when compared to sexuals, and the two reproductive forms did not differ in feeding efficiency measured by gut fullness.

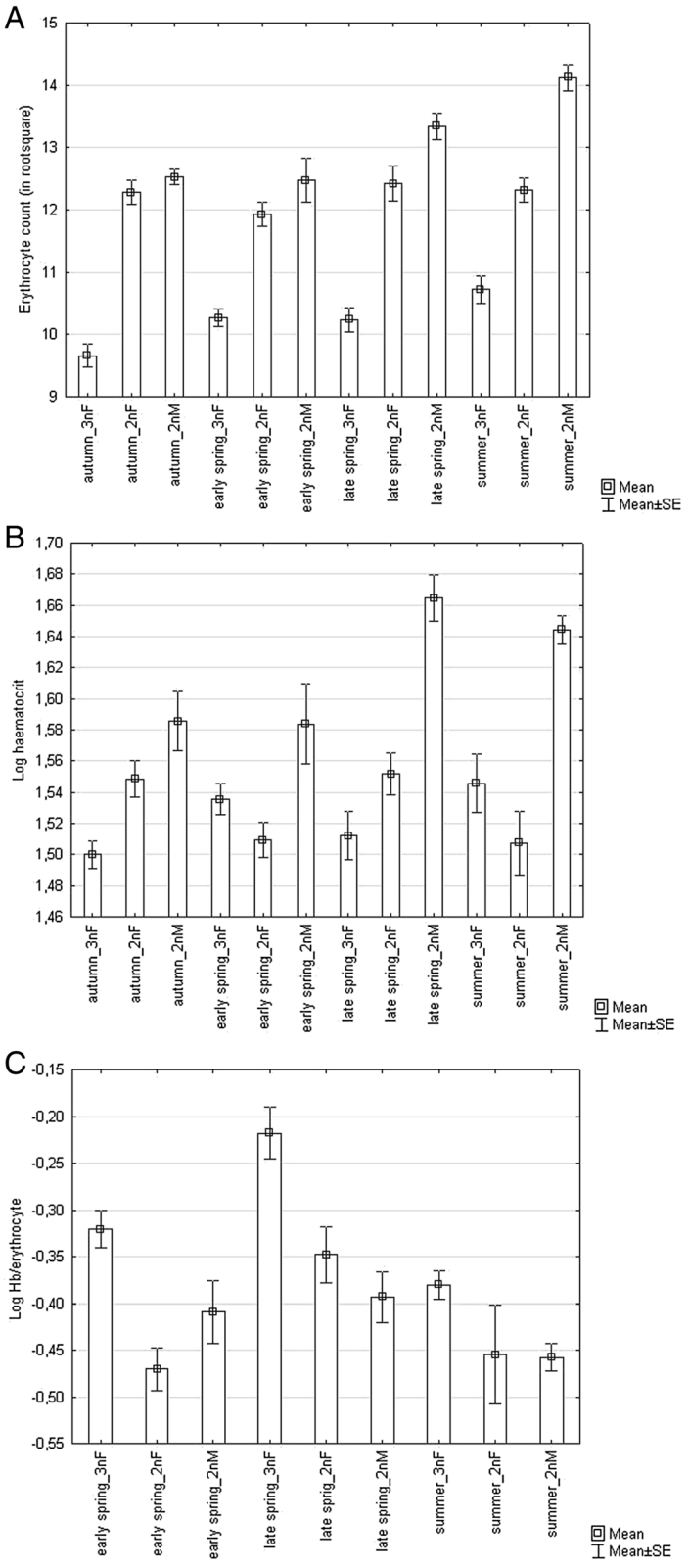

Fig. 3 The effects of season and fish groups (including 3 nF – triploid gynogenetic females, 2 nF – diploid sexual females and 2 nM – diploid sexual males) on red blood cell parameters **a** erythrocyte count, **b** heamatocrit and **c** oxygen-carrying capacity per erythrocyte

Table 2 The effects of fish group (including the effects of sex and ploidy associated with reproduction mode) and season on haematological parameters

Dependent variable	Predicted variables	SS	Df	F	p	Total F	p
Erytrocyte count	body size	0.214	1	0.375	0.541	44.464	**<0.001**
(*N* = 155)	season	16.551	3	9.675	**<0.001**		
	fish group	214.126	2	187.742	**<0.001**		
	fish group*season	10.868	6	3.176	**0.006**		
Leukocyte count	body size	0.025	1	0.019	0.891	3.809	**<0.001**
(*N* = 156)	season	47.142	3	11.749	**<0.001**		
	fish group	2.774	2	1.037	0.357		
	fish group*season	5.910	6	0.736	0.621		
Haematocrit	body size	0.004	1	1.437	0.233	13.613	**<0.001**
(*N* = 155)	season	0.027	3	3.653	**0.014**		
	fish group	0.220	2	44.804	**<0.001**		
	fish group*season	0.063	6	4.259	**<0.001**		
Haemoglobin (Hb)	body size	0.001	1	0.107	0.744	5.459	**<0.001**
(*N* = 117)	season	0.152	2	6.974	**0.001**		
	fish group	0.162	2	7.424	**0.001**		
	fish group*season	0.057	4	1.297	0.276		
Hb/erythrocyte	body size	0.028	1	1.775	0.186	6.295	**<0.001**
(*N* = 116)	season	0.207	2	6.477	**0.002**		
	fish group	0.365	2	11.421	**0.001**		
	fish group*season	0.088	4	1.377	0.247		
Leukocrit	body size	0.044	1	1.646	0.202	3.762	**<0.001**
(*N* = 147)	season	0.813	3	10.217	**<0.001**		
	fish group	0.003	2	0.058	0.943		
	fish group*season	0.088	6	0.551	0.768		

The statistically significant p-values are shown in bold

Next, we focussed on fitness-and condition-related traits. Mee et al. [35], predicting low asexual fitness in order to facilitate coexistence with sexual hosts, analyzed fitness-correlated traits (i.e. fecundity, egg viability, and hatching growth rate) in sperm-dependent asexual *Phoxinus eos-neogaeus* and their sexually-reproducing parental species *P. eos* and *P. neogaeus*. However, contrary to their expectation, they found a weak fitness advantage for the asexuals, which indicates that other factors should contribute to the maintenance of the coexistence of sexuals and asexuals in *Phoxinus*. In our study, we analyzed several simple measurable fitness- and condition-correlated traits. Energy allocation in the development of gonads, considered as such a fitness-related trait, was measured according to the relative size of gonads. Our study revealed that GSI did not differ between gynogenetic and sexual females of *C. gibelio*, suggesting similar allocations in gonad formation in the females of both reproductive forms. This is in line with the study by Vetešník et al. [26], who showed a high blood plasma calcium concentration (important for the development of eggs) in a spring sample for both gynogenetic and sexual females of *C. gibelio* (even the calcium in gynogenetic females was slightly higher than that in sexual females). Asexuality in fish is linked with genome polyploidization and/or hybridization, which was also the case with the *C. auratus* complex investigated here. However, the ovaries of artificially induced triploid females (in cases where polyploidization is produced for commercial purpose) are reduced in size, which results in a lower GSI and may indicate the diversion of energy from vitellogenesis to body growth [36]. Piferrer et al. [37] summarized the effects of induced triploidy on gonadal development and showed that triploids often exhibit reduced gonadal development; the presence of vitellogenic oocytes is rare in triploid females and functional gonadal sterility in females and males is observed. However, this is not the case of *C. auratus* complex when gynogenetic polyploids (triploids and rarely also tetraploids) are generated naturally. The similar values of GSI and similar body size (suggesting almost equal

Table 3 The effects of fish group (including the effects of sex and ploidy associated with reproduction mode) and season on immunity parameters and steroid hormone levels

Dependent variable	Predicted variables	SS	Df	F	p	Total F	p
IgM	body size	0.032	1	0.002	0.962	8.981	**<0.001**
(*N* = 155)	season	261.658	3	6.224	**<0.001**		
	fish group	615.606	2	21.965	**<0.001**		
	fish group*season	415.222	6	4.938	**<0.001**		
Oxidative burst (integral)	body size	0.038	1	2.343	0.128	13.611	**<0.001**
(*N* = 148)	season	1.996	3	40.764	**<0.001**		
	fish group	0.012	2	0.382	0.683		
	fish group*season	0.110	6	1.120	0.354		
Oxidative burst (peak)	body size	0.272	1	4.703	**0.032**	10.272	**<0.001**
(*N* = 152)	season	4.995	3	28.742	**<0.001**		
	fish group	0.060	2	0.522	0.595		
	fish group*season	0.717	6	2.062	0.062		
Lysozyme concentration	body size	0.135	1	0.572	0.451	6.322	**<0.001**
(*N* = 132)	season	8.558	3	12.086	**<0.001**		
	fish group	0.704	2	1.492	0.229		
	fish group*season	1.955	6	1.380	0.228		
Complement activity	body size	0.010	1	1.139	0.288	14.384	**<0.001**
(*N* = 135)	season	1.287	3	48.703	**<0.001**		
	fish group	0.021	2	1.183	0.310		
	fish group*season	0.197	6	3.730	**0.002**		
Cortisol level	body size	0.189	1	2.955	0.089	5.363	**<0.001**
(*N* = 108)	season	2.801	3	14.630	**<0.001**		
	fish group	0.312	2	2.446	0.092		
	fish group*season	0.332	6	0.866	0.523		
Estradiol level	body size	0.000	1	0.000	0.984	40.453	**<0.001**
(*N* = 88)	season	10.453	3	51.065	**<0.001**		
	fish group	2.040	2	14.950	**<0.001**		
	fish group*season	6.691	6	16.342	**<0.001**		
11-ketotestosterone level	body size	0.125	1	1.131	0.295	27.994	**<0.001**
(*N* = 43)	season	5.144	3	15.477	**<0.001**		
	fish group	17.156	2	77.423	**<0.001**		

The statistically significant p-values are shown in bold

growth rates) most likely linked to the fertility of both sexual and gynogenetic forms were previously also documented in *Carassius auratus* by Takada and Tachihara [38]. However, other aspects of *C. gibelio* reproduction, including gonadal histology, egg viability, hatching success, and potential male mating discrimination (see below) should be examined in detail in future studies to clarify whether or not the gynogenetic form of *C. gibelio* exhibits some reproductive disadvantage promoting its coexistence with its sexual hosts.

Concerning condition-related traits, three measures of fish vigour were applied in our study—condition factor, spleen-somatic index, and hepato-somatic index. Condition factor is also considered as a measure of growth performance in fish. When comparing the growth rates of triploid and diploid fish, increased cell size does not appear to confer a growth advantage to triploids, due to the concomitant decrease in cell numbers [36]. Vetešník et al. [39], analyzing the growth of gynogenetic and sexual Prussian carp on the basis of standard length and using temporal and spatial data series collected both ten and twenty years after the introduction of Prussian carp to the Czech Republic, showed that gynogenetic triploid females had significantly higher growth rates than sexual diploids. However, condition factor calculated using somatic weight in

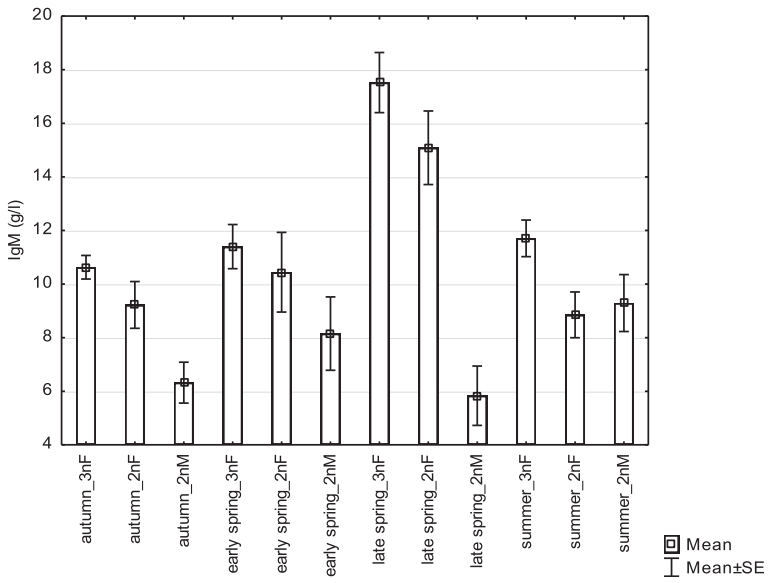

Fig. 4 IgM concentration affected by reproductive forms (including the effect of sex) and season (3 nF – triploid gynogenetic females, 2 nF – diploid sexual females and 2 nM – diploid sexual males)

the representatives of *C. auratus* analyzed in our study does not indicate the difference in growth performance between gynogenetic triploid females and sexual diploids. Using condition factor based on the total weight showed the difference between gynogenes and sexuals, which likely reflects the differences in gonad sizes between females and males rather than the difference in growth performance.

McLean et al. [40], in a study of coho salmon (*Oncorhynchus kisutch*), showed a reduction in condition factor caused by growth hormone in diploids but was not evident in triploids, and this hormone also caused triploids to deplete lipid energy stores more rapidly than diploids. This is in line with Vetešník et al. [26], who identified a higher metabolic rate and higher energy intake in gynogenetic females of Prussian carp when compared to sexuals. Concerning the representatives of *C. auratus* complex in our study, a higher condition was found in both female forms when compared to sexual males; the trend of a slightly higher condition was even reported for gynogenetic females when total body weight was applied in the calculation of condition factor. However, no significant difference was found when somatic weight was applied to this calculation. This finding indicates similar growth performance in both reproductive forms, but suggests the effect of ploidy and reproduction on the weight of internal organs. This is in accordance with the observation that the hepato-somatic index (considered as an indicator of fish vigour) of the representatives of *C. auratus* complex was clearly affected by the reproduction mode, with high values of the index for the gynogenetic form, supporting the hypothesis of a better condition in gynogenetic triploid females when compared to diploid sexuals, as suggested by Vetešník et al. [26].

The spleen-somatic index is often considered a simple measure of immunocompetence. Our study indicates no obvious difference in immunocompetence when comparing the two reproductive modes of *C. auratus* complex, but it does suggest gender differences in energy allocation. Thus, it seems that the high investment in gonad development in both gynogenetic and sexual females is compensated by low investment in immunocompetence (measured by spleen size), whilst the opposite trend in investments in reproduction and immunity was recorded for sexual males of *C. auratus* complex.

The heart index reflects heart functional capacity, i.e. aerobic performance, organ aerobic activity, and the animal's ability to engage in physical activity. Generally, it has been documented that the hearts of males are larger, thinner, and have indexes of diminished performance (including lower resting heart rates) when compared to females. The differences in cardiovascular performance between genders are related to the difference in endocrine systems, i.e. the production of steroid hormones. Concerning fish, a larger heart was evidenced for mature males of migratory salmonid species investigated in the spawning period (e.g. Franklin and Davie [41]; Armstrong and West [42]; Altimiras et al. [43]; Clark et al. [44]) indicated the increased functional demands placed on the hearts of males during spawning [45]. During this period, the elevated levels of steroid hormones in salmonid males (testosterone, 11-ketotestosterone) induce hearth growth [46, 47]. On the other hand, salmonid females approaching their spawning grounds had higher heart rates

and higher levels of cortisol and estradiol when compared to males [48]. Clark et al. [44] hypothesized that the increase in heart volume in salmonid males may be beneficial for increasing oxygen transport capacity during reproduction. However, they found that even if the males of sockeye salmon (*Oncorhynchus nerka*) consume significantly more oxygen than females, the females exhibited higher blood oxygen-carrying capacity measured by Hb concentration. Our data support the fact of larger heart mass in fish males when compared to females, but also show high Hb concentration in males of *C. auratus* complex, which may suggest their higher oxygen-carrying capacity. In addition, the highest heart mass and the highest Hb in males collected in spring were linked to the highest levels of 11-ketotestosterone, supporting the idea of androgen-induced heart growth with increasing aerobic performance.

To our knowledge, cardiovascular systems in diploid-polyploid complexes of fish species have not yet been analyzed. The polyploidization of the myocardium in birds and mammals is correlated with a reduction in cardiac aerobic performance expressed by heart index (for birds, see Anatskaya and Vinogradov, [49]; for mammals, see Anatskaya and Vinogradov, [50]). Genome polyploidization seems to be linked to a severe decline in aerobic respiration and to the stimulation of sugar and fatty acid metabolism, and promotes cell survival and tissue regeneration under stressful conditions in mammals, as demonstrated by analyses of the ploidy-associated changes in the expressions of non-tissue specific genes in heart and liver of human and mouse [51]. The low heart index in the triploid gynogenetic females of *C. auratus* complex may indicate low functional capacity (probably low aerobic performance). Even if the high Hb concentration in sexual males suggests their higher total oxygen-carrying capacity when compared to females (including both forms—sexual and gynogenetic), our results also demonstrate the higher oxygen-carrying capacity of individual erythrocytes of gynogenetic females when compared to sexuals. Thus, we propose that a trade-off between the high number of erythrocytes with lower oxygen-carrying capacity per erythrocyte in sexual males and the low number of erythrocytes with high oxygen-carrying capacity per erythrocyte in gynogenetic females may significantly contribute to the coexistence of both forms. Concurrently, both the increase in erythrocyte count and total oxygen-carrying capacity in sexual males and the increase in oxygen-carrying capacity per individual erythorocyte in gynogenetic females were reported in spring, i.e. during the spawning period. This phenomenon of different oxygen-carrying capacities in gynogenetic and sexual forms of *C. auratus* complex did not vary seasonally. However, morphological and functional analyses (i.e. of the cardiac energy metabolism) of cardiovascular systems as well as analyses of oxygen consumption rate may represent helpful tools for understanding the cardiovascular aspects of the evolution of physiology in the two coexisting reproductive forms of this unique fish species.

The role of immunity in the dynamics of the asexual-sexual complex was highlighted by Hakoyama et al. [52], as differences in immunity between gynogens and sexuals may reduce the evolutionary two-fold cost of sex. Whilst non-specific immunity is independent of the relative frequency of the two forms in the gynogenetic-sexual complex, differences in specific immunity may cause negative density-dependent regulation in this complex. Using parasite infection level as a measure of non-specific immunity, Hakoyama et al. [52] found the gynogenetic form to be more susceptible to parasite disease. However, Šimková et al. [27] focused on the MHC genotyping of gynogenetic and sexual specimens from the selected mixed population. They showed that infection by highly specific gill ectoparasites (*Dactylogyrus*) is higher in the most common MHC genotypes of gynogens when compared to rare MHC genotypes of gynogens or highly variable MHC genotypes of sexuals, suggesting a co-evolutionary arms-race between host immunity and parasite virulence. Our study did not reveal differences in non-specific immunity between gynogenetic and sexual forms of *C. auratus* complex measured either by respiratory burst (reflecting phagocyte activity), complement activity, or lysozym activity. We showed for the first time the clear difference in IgM production between gynogenetic and sexual forms, and this pattern was evidenced in different sampling periods throughout the year. In addition, we identified a difference in IgM production between females (both sexual and gynogenetic) and males, probably indicating the immunosuppressive roles of 11-ketotestosterone in males of *C. auratus* complex, which was especially evidenced in the spawning period. Such gender differences in IgM level were recently demonstrated in a hybridizing system of sexual Prussian carp and common carp (*Cyprinus carpio*), probably reflecting the different costs of reproductive investment in males and females [53].

The sex steroid hormones were previously suggested to play an important role in species recognition in the complex of gynogenetic and sexual fish (e.g. Gabor et al. [54]). Gynogenetic Amazon molly (*Poecilia formosa*) arose via the hybridization of female Atlantic molly (*P. mexicana*) and male sailfin molly (*P. latipinna*), this gynogenetic species requiring the sperm of their parental species. When living in sympatry with gynogenetic Amazon molly, the males of sailfin and Atlantic mollies showed a mating preference for conspecific females [55–57]. However, Gabor et al. [54] showed that males of Atlantic molly did not discriminate against gynogenetic Amazon molly as

strongly as males of sailfin molly. They also showed that the levels of premating and postmating estradiol and 11-ketotestosterone are important for mating behaviour. Thus, we focused on the levels of three steroid hormones-11-ketotestosterone, estradiol, and cortisol. Whilst 11-ketotestosterone and estradiol regulate reproductive behaviour in fish, cortisol is considered as an indicator of stress (e.g. Eslamloo et al. [58]) affecting disease resistance (e.g. Fast et al. [59]; Tort [60]). Our study showed a high level of 11-ketotestosterone in sexual males of the representatives belonging to *C. auratus* complex in early and late spring, and a high level of estradiol in both gynogenetic and sexual females of *C. auratus* complex in early spring. However, cortisol level was not different in gynogenetic and sexual reproductive forms. This hormone increased during spawning (in late spring sample) for gynogens and both sexual females and males. The females of both gynogenetic and sexual forms collected during the spawning period expressed the same estradiol levels in blood plasma, probably indicating that the same amount of energy is devoted to reproductive behaviour in the females of both forms. However, only further experimental studies can clarify whether males of *C. auratus* complex discriminate between gynogenetic and sexual females in order to conserve the energy associated with sperm production [61] and, thus, whether mating choice is a factor facilitating the coexistence of gynogenetic and sexual forms belonging to *C. auratus* complex.

Conclusions

Our study provides the first investigation of physiological and immune parameters in gynogenetic-sexual *C. auratus* complex. In addition to the limited ability of the gynogenetic form to escape parasitism as a potential mechanism promoting the coexistence of gynogenetic-sexual *C. auratus* complex [27], we hypothesized that the different investments in condition-, growth- and fitness-related traits may represent another mechanism contributing to the coexistence of gynogenetic and sexual forms of this complex. However, both gynogenetic and sexual forms of *C. auratus* complex showed comparable growth performance. Even if our study seems to indicate that similar amount of energy is devoted to the reproductive investment in gynogenetic and sexual females suggesting no reproductive disadvantage for gynogenetic form, we suggest that lower aerobic performance in gynogens (this may be amplified in eutrophic habitats with low ogygen level) may represent their physiological disadvantage balancing the cost of sexual reproduction. The different investments on the basis of a trade-off between the number of erythrocytes and the oxygen-carrying capacity per erythrocyte in sexual males and gynogenetic females may also facilitate the coexistence of gynogenetic and sexual forms. In addition, the differences in specific immunity between gynogens and sexuals may also compensate the evolutionary disadvantage of sexual reproduction. However, other mechanisms such are feeding competition, mating choice or different metabolic costs should be investigated in the future as other potential mechanisms contributing to the coexistence of this unique system. We highlight that this study may further help for the understanding invasion success of the species belonging to *C. auratus* complex in the recently invaded areas.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS designed this study and drafted the manuscript. LV and KH acquired fish material and measured the data. PH carried out the immunological analyses. AS analyzed the data. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final manuscript.

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PŘÍLOHA Č. 23

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Are fish immune systems really affected by parasites? An immunoecological study of common carp (*Cyprinus carpio*). Parasites and Vectors, 4 (1), 120-137, 2011.

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Parasites & Vectors

RESEARCH Open Access

Are fish immune systems really affected by parasites? an immunoecological study of common carp (*Cyprinus carpio*)

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Abstract

Background: The basic function of the immune system is to protect an organism against infection in order to minimize the fitness costs of being infected. According to life-history theory, energy resources are in a trade-off between the costly demands of immunity and other physiological demands. Concerning fish, both physiology and immunity are influenced by seasonal changes (i.e. temporal variation) associated to the changes of abiotic factors (such as primarily water temperature) and interactions with pathogens and parasites. In this study, we investigated the potential associations between the physiology and immunocompetence of common carp (*Cyprinus carpio*) collected during five different periods of a given year. Our sampling included the periods with temporal variability and thus, it presented a different level in exposure to parasites. We analyzed which of two factors, seasonality or parasitism, had the strongest impact on changes in fish physiology and immunity.

Results: We found that seasonal changes play a key role in affecting the analyzed measurements of physiology, immunity and parasitism. The correlation analysis revealed the relationships between the measures of overall host physiology, immunity and parasite load when temporal variability effect was removed. When analyzing separately parasite groups with different life-strategies, we found that fish with a worse condition status were infected more by monogeneans, representing the most abundant parasite group. The high infection by cestodes seems to activate the phagocytes. A weak relationship was found between spleen size and abundance of trematodes when taking into account seasonal changes.

Conclusions: Even if no direct trade-off between the measures of host immunity and physiology was confirmed when taking into account the seasonality, it seems that seasonal variability affects host immunity and physiology through energy allocation in a trade-off between life important functions, especially reproduction and fish condition. Host immunity measures were not found to be in a trade-off with the investigated physiological traits or functions, but we confirmed the immunosuppressive role of 11-ketotestosterone on fish immunity measured by complement activity. We suggest that the different parasite life-strategies influence different aspects of host physiology and activate the different immunity pathways.

Background

Physiology and immunity in fish, a group of poikilothermic vertebrates, are strongly influenced by both abiotic and biotic factors. Water temperature is generally considered as the strongest abiotic factor which affects fish physiology including immune functions. However, the infection dynamics of fish parasites and pathogens is also strongly influenced by water temperature changes [1,2].

To determine whether the observed status of fish physiology results from abiotic changes or reflects the level of parasite infestation is very difficult in natural conditions because of the confounding effects of several abiotic and biotic factors including parasitism, often varying in space and time. Recently, many studies have focused on the abiotic effects, especially of water temperature, on physiological and immunological mechanisms in poikilothermic organisms, like fish. The majority of

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immunological studies have suggested an immune-suppression effect associated with a decrease in water temperature [3-7]. Moreover, the immunosuppressive effects of polychlorinated biphenyls are known in fish species [e.g. [8,9]]. Teleost fish possess similar immune system mechanisms to mammals - both non-specific (innate or natural) and specific (acquired or adaptive) [10]. However, substantial differences exist between the immune systems of poikilo- and homoiothermic organisms. According to Ainsworth et al. [11], the specific branch of immunity is more sensitive than the non-specific defence at lower water temperature, and was assumed to be more important for poikilothermic than for homoiothermic vertebrates [12]. Moreover, Le Morvan et al. [5] suggested that, at low water temperature, the non-specific defence of fish immune system tends to offset specific immune suppression until the specific immune system adapts.

Several studies have reported that the decrease in water temperature may cause the suppression of acquired immunity, with the components of innate immunity being relatively independent of water temperature [13]. Other seasonally-dependent events like spawning in fish could more strongly influence immunity than water temperature [14]. However, many studies have also shown how water temperature drives the seasonal changes in parasite infection, mainly because parasite reproduction and survival of free-living infective stages of parasites are dependent on a specific range of temperature [15].

Close interactions occur likewise between fish host and parasites. The interactions between fish physiology (associated with host size, age, sex etc.) and the level of parasite infection have been relatively well documented [16,17]. However, there have been few studies investigating the effects of seasonal changes on selected measures of host physiology in relation to parasite load [e.g. [18]], and fish immune response has mostly been studied solely in relation to parasite infection [19,20].

The contribution of immunoecological studies has gained a place of central importance [21,22]. Life-history theory is at the core of immunoecological studies. The principle assumption is that each organism has a limited amount of energy which is allocated to different fundamental functions (i.e. maintenance including immune defence, reproduction and growth) in accordance with current needs [23]. The activation of the immune system is energetically costly [24,25]. Therefore, trade-offs are expected to occur as hosts infected by parasites should invest energy into immune responses, at the expense of other physiological tasks [26]. However, optimum host defence is governed by a parasite-mediated allocation trade-off between growth and immune function (see Tschirren and Richner [27]).

Furthermore Folstad and Karter [28] introduced the immunocompetence handicap hypothesis, which predicts that the steroid hormones responsible for the production of sexual signals in males may cause immunosuppression especially during the reproductive period. Many recent studies have been conducted to test whether the expression of sexual ornamentation is associated to immunosuppression in fish [e.g. [29,30]].

The aims of the present study were to analyze the effects of seasonal changes (i.e. temporal variability) on (a) selected physiological and immune parameters and on (b) the infection by metazoan parasites. We examined the potential associations between immunity, physiology and parasite infection following the assumption of trade-offs in energy allocation using the total investment in immunity, physiology and total measure of parasitism as well as analyzing each measure of immunity and physiology separately. We tried to estimate whether the seasonal changes of abiotic environment are the main driver of immune activation in order to face risks of being parasitized or whether the observed associations between immunity and physiology are the results of trade-off without being affected by seasonal changes. Finally, we tested the hypothesis of immunosuppression by 11-ketotestosteron in fish males.

Methods

Fish sampling

A total number of 160 three-to-four-year-old individuals of common carp (*Cyprinus carpio* Linnaeus 1758, Cyprinidae), including 87 males and 73 females were collected from a pond-farmed population (Vodňany, South Bohemia, Czech Republic) in five selected months in 2007 and 2008. Each sample represented a different season and diverse water temperature, i.e. June 2007 - early summer (16°C), August 2007 - late summer (18.4°C), November 2007 - autumn (4.9°C), February 2008 - winter (2.8°C) and April 2008 - spring (7.5°C). The temperature on the day of collection was measured. The fish were sampled using seine netting and then separated according to their sex. Samples of blood were taken immediately from each specimen from the caudal vein using heparinized syringes following Pravda and Svobodová [31]. Blood was preserved in microtubes with heparin (50 U/ml, Zentiva). Blood samples used for measuring oxidative burst activity and haematology (differential leukocyte counts and total leukocyte counts) were processed immediately after blood collection. Blood samples used for other immunological analyses (complement activity, IgM) and 11-ketotestosterone concentration were deep-frozen at -80°C.

Each fish individual was intramuscularly tagged for later identification using a P.I.T. tag (134.2 kHz, AEG ID-162, AEG Co., Ulm, Germany) in the left side of the

dorsal part close to the first hard dorsal fin ray. Fish were transported to the laboratory in tanks containing original water from the pond. Fish were killed by severing the spine. Each individual was measured (total length in centimetres) and weighed (in grams). A complete parasitological dissection for all metazoan parasites was immediately performed for 145 individuals (including 80 males and 65 females) according to Ergens and Lom [32].

Respiratory burst activity

Phagocytes (granulocytes and monocytes/macrophages) are considered to be the first line of immune defence against pathogens overcoming the natural barriers. These cells have the ability to engulf and kill pathogens during the so-called "oxidative burst" leading to production of reactive oxygen species [33]. Seasonal changes in phagocytic activity have been studied in different fish species [34,11].

Respiratory burst activity was measured as luminol-enhanced chemiluminescence using a luminometer (LM01-T, Immunotech, Czech Republic) and opsonised Zymosan A as activator [35,36]. The maximal intensity of respiratory burst (peak in relative light units - RLU) was evaluated in this study.

The measurement of complement activity in plasma

Among other non-specific humoral factors (i.e. non-cellular defence mechanisms) complement system plays an important role in natural defence against pathogens. Complement contains a series of serum proteins that are activated using either a classical (antibody dependent) or alternative pathway. Complement participates in lytic, pro-inflammatory, chemotactic and opsonic activities, thus it forms the connection between non-specific humoral and cellular mechanisms (i.e. phagocyte responses) [37]. One of the most important and well known complement functions is the capacity to create pores in the membrane of the pathogens' surface and thereby kill them. Hernández et al. [7] reported a close relationship between water temperature and the level of complement activity. The role of complement in monogenean infection was demonstrated in salmonid fish [38,39].

Complement activity was measured according to Virta et al. [40] and Nikoskelainen et al. [41] with modifications. The total complement activity (including all activation pathways) of plasma was determined using a bioluminescent strain of *Escherichia coli* (K12luxAmp, kindly provided by University of Turku, Finland). The light emission measured by LM01-T luminometer was positively correlated with the viability of *E. coli*. The relative measure of complement activity was estimated by computing the difference between the maximal time of measurement (equal to 4 h) and the time necessary for killing 50% *E. coli* by complement (in h). For the details see Buchtíková et al. [42].

The determination of total IgM level

The major component of fish specific humoral defence is immunoglobulin M (IgM), although IgD [43] and even IgZ and IgT [44,45] have also been recently described. Clear seasonal changes of plasma IgM levels were found to be related to water temperature and/or gonad maturation [46]. The production of specific immunoglobulin against gill monogeneans [16] or other helminths [47] was observed. Specific antibodies play an essential role in cytolytic or cytotoxic mechanisms, such as in the activation of the complement system (classical pathway) or helping leukocytes to adhere to the parasite surface, presumably through Fc-like receptors [48].

The total IgM level was determined using precipitation with zinc sulphate (0.7 mM $ZnSO_4.7H_2O$, pH = 5.8) [49]. The concentration of IgM in the sample (in g/l) was calculated as the difference between total proteins (commercial kit, Bio-Rad, USA) and proteins in the supernatant after precipitation and centrifugation.

11-ketotestosterone level

The immune system is affected by the level of steroid hormones. The 11-ketotesterone (11-KT) is a major androgen in the majority of teleost fish, responsible for sexual behaviour and spermatogenesis, found in higher levels in the blood plasma or serum in males than in females [50]. As already mentioned, these hormones have an immunosuppressive effect. The level of 11-ketotestosterone in male plasma was analyzed following the protocol provided in the commercial competitive enzyme immunoassay kits (Cayman Chemical, Estonia). For the details see Buchtíková et al. [42].

Haematological analyses

The determinants of white-blood cell count (including leukocyte and lymphocyte counts, and differential leukocyte counts) are considered to be an important parameter of fish health status. Like other haematological parameters, white-blood cell counts depend on various abiotic and biotic factors such as water temperature, environmental stress, fish sex and age [51,52]. According to Ruane et al. [53], fish infected by parasites significantly changed their haematological parameters. Leukocyte counts can be applied as a measure of general immune response. The increased leukocyte counts and shift values towards the myeloid line (especially a high number of myelocytes and metamyelocytes) reflect the current infection or inflammation [54].

Leukocyte counts (in g/l) were determined according to the methodology of Svobodová et al. [55] and

Lusková [56] using Natt-Herrick solution. A differential leukocyte profile was assessed following Lamková et al. [18]. We estimated the percentage distribution of all types of white blood cells. However, we used only the leukocyte count and the relative count of lymphocytes and phagocytes (in g/l) for statistical analyses (see Rohlenová et al. [30]). Finally, haemoglobin content (Hb) was analyzed photometrically (540 nm; Helios Unicam, USA) in Kampen-Zijlster transformation medium.

Spleen size

The spleen, the thymus, but also the head and trunk of the kidney belong to the principal lymphomyeloid tissues of teleosts. The spleen as a secondary lymphatic and scavenging organ plays an important role in haematopoiesis, antigen degradation and antibody production processing [57]. This organ is also known to act as an erythrocyte reservoir [58]. The spleen size of fish is widely used as a simple measurable immune parameter with a potential role in immune response against parasite infection [59-61]. A link between spleen size and fish condition was also previously documented [62]. In this study, we measured spleen weight (in grams at accuracy 0.001 g). The spleen-somatic index (SSI) was calculated as spleen weight (g)/body weight (g) × 100.

Other physiological parameters

We measured liver and gonad weight (in grams). We calculated the relative body weight (i.e. condition factor, K) using the equation: K = constant × somatic weight (g)/(standard length [cm])^3 according to Bolger and Connolly [63]. The relative size of gonad (i.e. gonado-somatic index, GSI) was calculated as follows: GSI = gonad weight (g)/body weight (g) × 100, and the relative size of liver (i.e. hepato-somatic index, HSI) was calculated as follows: HSI = liver weight (g)/body weight (g) × 100.

Parasite collection and determination

Fish hosts were investigated for all metazoan parasites (Table 1). Collected parasites were determined using recent keys [64-68]. Due to the very high parasite abundance on fish gills and because *Dactylogyrus* (see Table 1), in particular, die quickly after the killing of the fish, we collected these parasites only from the right side of the gills following Kadlec et al. [69]. For the details on parasite fixation and identification see Lamková et al. [18]. Epidemiological characteristics such as prevalence (percentage of infected host individuals in each sample), intensity of infection (number of parasites per infected host), and abundance (mean number of parasites per host individual in each seasonal sample) were calculated for each parasite species according to Bush et al. [70].

Statistical analyses

Most of the measured parameters did not fit a normal distribution and required transformation logarithm, hyperbolic arcsine, hyperbolic arcsine square root or square root transformations. First, preliminary analyses testing the effects of season and sex on parasitism, physiology and immunity were performed using one-way ANOVAs.

Following Poisot et al. [71], we used an indicator of parasite community structure based on principal component analysis (PCA) that takes into account all parasite species and the number of individuals for each parasite species within host individuals. In the same manner, we used an indicator of immunity based on PCA that takes into account all immune variables (spleen-somatic index, leukocyte, lymphocyte and phagocyte counts, IgM level, respiratory burst and complement activity). Finally, an indicator of physiology was also based on PCA that takes into account parameters linked to physiology (condition factor, gonado-somatic index, hepato-somatic index and haemoglobin).

We extracted values of the first three principal components (PCs) of each of these PCAs (i.e. principal components having eigenvalues over one were considered following Vainikka et al. [72]). These PCs represent a measure of parasitism (PCs of parasites), a measure of immunity (PCs of immune variables) or a measure of physiology (PCs of physiological variables) respectively. Then, we performed Pearson correlation between sampling period and all three PCs for each parasitism, immunity and physiology. Further, partial correlations controlling for sampling period were performed to analyze the potential relationships between parasitism, immunity and physiology. Next, GLM analyses were performed to investigate the potential associations between parasite abundance and all immune and physiological variables taking into account the sampling period. Last, GLM analyses were conducted to analyze the associations between immune and physiological variables taking into account the effect of sampling period or alternatively the effects of both sampling period and sex following the preliminary analyses. As the spleen, gonad and liver weights were correlated with total body weight; we used SSI, GSI or HSI. All statistical analyses were executed using Statistica 9.0 for Windows.

Results

Seasonal changes of parasite infection and gender differences

The basic characteristics of parasite infection were estimated for each parasite species within each sampling period (Table 1). The metazoan parasites belonging to six parasitic groups were found on common carp including ectoparasitic Monogenea, Crustacea, Mollusca

Table 1 Parasite abundance, intensity of infection and prevalence

		Abundance ± SD	Intensity of infection (min-max)	Prevalence (%)	Abundance ± SD	Intensity of infection (min-max)	Prevalence (%)	Abundance ± SD	Intensity of infection (min-max)	Prevalence (%)
Parasites	Parasite species	Early summer			Late summer			Autumn		
Monog	*D. molnari* Ergens & Dulma, 1969	79.04 ± 54.61	6-204	100	1065.63 ± 592.69	267-2450	100	1816.62 ± 1623.86	159-6879	100
	D. extensus Mueller & Van Cleave, 1932	16.33 ± 9.55	1-40	100	179.39 ± 132.67	4-611	100	99.95 ± 222.96	0-1212	97
	D. falciformis Achmerow, 1952	7.16 ± 8.77	0-32	84	63.9 ± 46.05	3-167	100	207.54 ± 156.32	30-528	100
	D. achmerowi Gussev, 1955	3.55 ± 5.08	0-20	72	36.21 ± 21.65	0-81	97	46.16 ± 35.16	0-133	97
	D. anchoratus (Dujardin, 1845)	0.08 ± 0.28	0-1	8	2.3 ± 3.82	0-12	30	1 ± 3.93	0-19	7
	Gyrodactylus spp.	0.88 ± 1.9	0-8	28	-	-	-	0.8 ± 1.32	0-4	33
	Eudiplozoon nipponicum (Goto, 1891)	8.44 ± 4.08	2-20	100	22.77 ± 17.45	0-53	97	1.1 ± 1.63	0-7	47
Crusta	*Argulus foliaceus* (Linnaeus, 1758)	13.4 ± 11.22	3-47	100	9.27 ± 7.17	0-27	87	8.57 ± 5.79	0-25	97
	Ergasilus sieboldi Nordmann, 1832	0.04 ± 0.2	0-1	4	-	-	-	0.07 ± 0.25	0-1	7
Cesto	*Antractolytocestus huronensis* Anthony 1958	6.24 ± 15.30	0-56	28	472.83 ± 964.82	0-5014	83	19.73 ± 36.26	0-119	43
	Khawia sinensis Hsü, 1935	0.16 ± 0.55	0-2	8	-	-	-	0.5 ± 1.43	0-6	17
	Valipora campylancristrota (Wedl, 1855)	-	-	-	0.17 ± 0.65	0-3	7	-	-	-
Dige	*Diplostomum* larv sp.	5.28 ± 6.94	0-31	68	1.97 ± 3.50	0-12	33	6.13 ± 7.74	0-31	73
Moll	Glochidium spp.	-	-	-	0.07 ± 0.37	0-2	3	-	-	-
Hirud	*Piscicola geometra* (Linnaeus, 1761)	0.04 ± 0.2	0-1	4	-	-	-	0.13 ± 0.43	0-2	10
		Winter			Spring					
Monog	*D. molnari* Ergens & Dulma, 1969	81.72 ± 144.99	0-825	97	229.56 ± 662.57	23-3653	100			
	D. extensus Mueller & Van Cleave, 1932	1.25 ± 2.55	0-9	33	2.59 ± 7.08	0-38	59			
	D. falciformis Achmerow, 1952	3.99 ± 12.15	0-67	67	30.58 ± 148.64	0-803	86			
	D. achmerowi Gussev, 1955	12.69 ± 30.36	0-161	90	12.92 ± 31.04	0-172	97			
	D. anchoratus (Dujardin, 1845)	-	-	-	-	-	-			
	Gyrodactylus spp.	303.83 ± 1094.16	0-5664	70	2.14 ± 2.29	0-8	66			
	Eudiplozoon nipponicum (Goto, 1891)	0.43 ± 0.86	0-3	27	0.3 ± 0.53	0-2	33			

Table 1 Parasite abundance, intensity of infection and prevalence *(Continued)*

Crusta	*Argulus foliaceus* (Linnaeus, 1758)	-	-	-	0.03 ± 0.19	0-1	3
	Ergasilus sieboldi Nordmann, 1832	0.03 ± 0.18	0-1	3	-	-	-
Cesto	*Antractolytocestus huronensis* Anthony 1958	-	-	-	-	-	-
	Khawia sinensis Hsü, 1935	1.37 ± 3.52	0-15	20	0.62 ± 1.40	0-6	24
	Valipora campylancristrota (Wedl, 1855)	0.03 ± 0.18	0-1	3	-	-	-
Dige	*Diplostomum* larv sp.	3.7 ± 4.02	0-16	80	4.05 ± 3.43	0-14	86
Moll	Glochidium spp.	-	-	-	-	-	-
Hirud	*Piscicola geometra* (Linnaeus, 1761)	0.03 ± 0.18	0-1	3	-	-	-

Parasite abundance (mean with standard deviation), intensity of infection (minimum and maximum values) and prevalence for each metazoan parasite species found on common carp collected within each sampled period. Monogenea (Monog), Crustacea (Crusta), Cestoda (Cesto), Digenea (Dige), Mollusca (Moll), and Hirudinea (Hirud).

and Hirudinea, and endoparasitic Cestoda and Digenea. No Nematoda or Acanthocephala were observed. Monogenea was the species' richest and most numerous group (almost 90% of total parasite abundance) and included *Eudiplozoon nipponicum*, five *Dactylogyrus* species (four of them present in all sampling periods) and viviparous *Gyrodactylus* species. In addition, three species of Cestoda, two species of Crustacea, one species of Hirudinea and one species of Digenea were found. The larval stages of Mollusca were undetermined. Following the variability in abundance among the various metazoan parasites (see Table 1), only the parasite groups with high abundance were included in statistical analyses i.e. Monogenea as the most abundant group, Crustacea and Cestoda characterized by the presence of one dominant species and one or two rare species, and Digenea represented only by larval stages of *Diplostomum* species. Due to low abundance Hirudinea and Mollusca were not included into statistical analyses.

Using one-way ANOVA, significant effects of sampling period were observed on the abundance of Monogenea ($F_{4,139}$ = 66.951, p < 0.0001), Cestoda ($F_{4, 139}$ = 44.108, p < 0.0001) and Crustacea ($F_{4, 139}$ = 25.707, p < 0.0001). A marginal but significant effect of sampling period on the abundance of Digenea was found ($F_{4, 132}$ = 2.488, p = 0.046). Clear patterns emerge for Monogenea with the highest values of abundance observed in late summer and autumn (Figure 1A) due to peak infection of *Dactylogyrus. D. molnari*, in particular, reached extremely high abundance. Viviparous *Gyrodactylus* species were present only in winter (in very high abundance) and spring (Table 1). Abundances in Crustacea decreased from early summer to spring (Figure 1B) resulting from the seasonal variation of *Argulus foliaceus* (see Table 1). Cestoda reached highest abundance in late summer (Figure 1C) resulting from the seasonal variation of *Antractolytocestus huronensis* (see Table 1). Only a slight seasonal variation in abundance of larval Digenea was found (Figure 1D). ANOVA showed a weak significant effect of host sex only on the abundance of Cestoda ($F_{1, 142}$ = 4.1041, p = 0.045) with higher abundance values recorded in females.

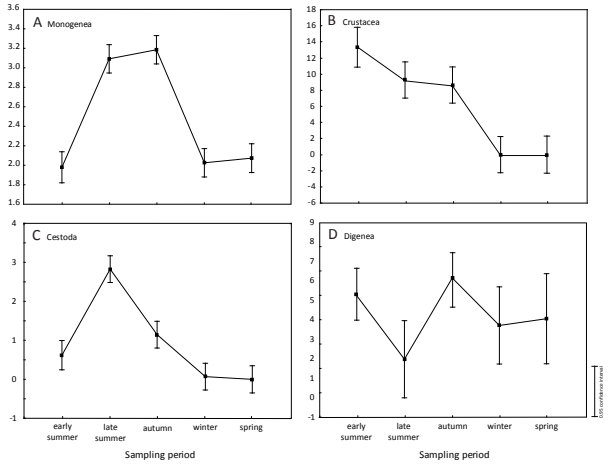

Figure 1 The changes in parasite abundance in relation to sampling period. Detailed legend: The changes in abundance of (A) Monogenea, (B) Crustacea, (C) Cestoda and (D) Digenea in relation to sampling period. Log transformation for abundance of Monogenea and hyperbolic arcsine square root transformation for Cestoda were applied.

Seasonal effect on host physiology and immunity and gender differences

All measured variables were influenced by the sampling period: condition factor ($F_{4, 139}$ = 11.619, p < 0.0001), GSI ($F_{4, 137}$ = 19.927, p < 0.0001), HSI ($F_{4, 139}$ = 42.483, p < 0.0001), haemoglobin concentration ($F_{4, 150}$ = 30.747, p < 0.0001), SSI ($F_{4, 139}$ = 8.630, p < 0.0001), leukocyte count ($F_{4, 150}$ = 26.741, p < 0.0001), lymphocyte count ($F_{4, 149}$ = 7.484, p < 0.0001), phagocyte count ($F_{4, 150}$ = 17.871, p < 0.0001), respiratory burst ($F_{4, 150}$ = 14.131, p < 0.0001), IgM concentration ($F_{4, 149}$ = 7.484, p < 0.0001), activity of complement ($F_{4, 133}$ = 29.293, p < 0.0001) and concentration of 11-ketotestosterone measured in males ($F_{4,84}$ = 4.541, p < 0.01).

The highest values of condition factor and HSI were detected in winter and spring (Figure 2A, C), whereas GSI reached the highest values in summer and autumn (Figure 2B). The highest values of haemoglobin concentration (Figure 2D) and SSI (Figure 3A) were found in early summer. Lymphocyte counts (Figure 3B) and total leukocyte counts (not shown) showed similar seasonal variations with high values in autumn and spring and low values in winter. On the other hand, the phagocyte count increased from summer to winter and reached maximum values in spring (Figure 3C). Values of respiratory burst were low in late summer and significantly increased in autumn and the following periods (Figure 3D). The highest IgM concentration level was recorded in early summer and autumn (Figure 3E). The concentration level of 11-ketotestosterone in males increased in spring (not shown), whereas complement activity (considered for both males and females) achieved its lowest values in spring (Figure 3F).

The effect of sex on each immune and physiological variable was tested using ANOVA. Significant differences were revealed only for IgM concentration ($F_{1,152}$ = 34.265, p < 0.0001) with significant higher values in females, and for haemoglobin concentration ($F_{1, 153}$ = 24.588, p < 0.0001) with significant higher values in males.

Parasitism versus immunity and physiology: the effect of sampling period

PCA performed on parasites showed that the first three axes accounted for most of the total variability in the

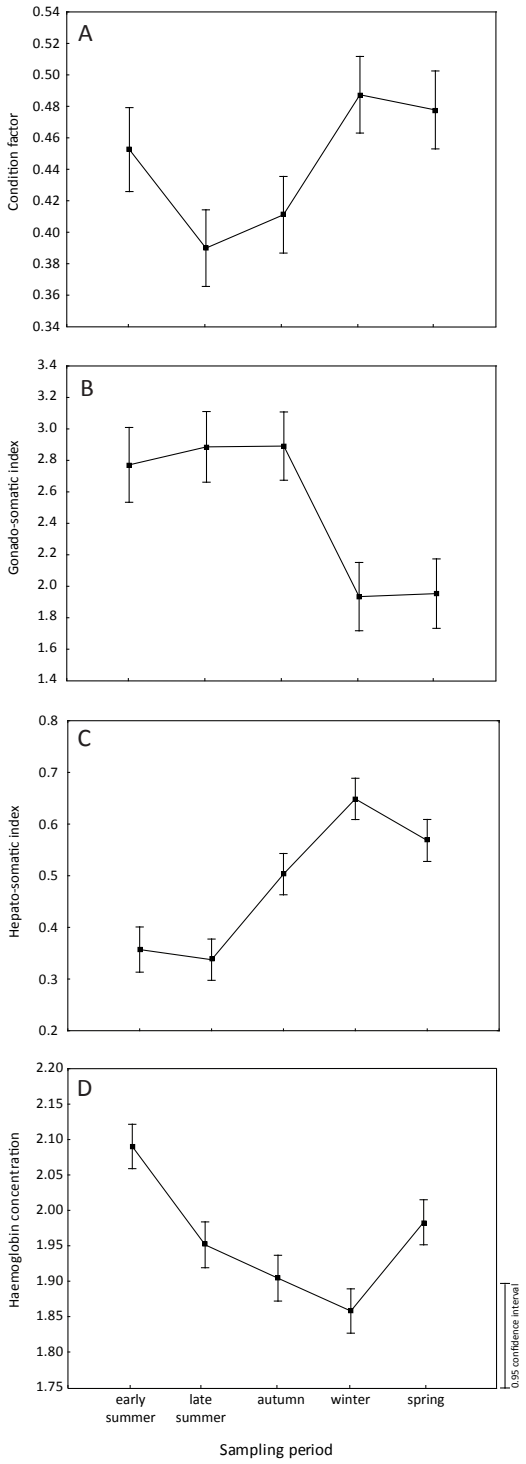

Figure 2 The changes in physiological variables. Detailed legend: The changes in the following physiological variables (A) condition factor, (B) gonado-somatic index, (C) hepato-somatic index and (D) haemoglobin concentration in relation to sampling period. Log transformation for condition factor, hepato-somatic index and haemoglobin concentration; and hyperbolic arcsine square root transformation for gonado-somatic index were applied.

data set. The first axis explained 42.0%, the second explained 26.3% and the third explained 21.1% of the total variability in the parasitological data set (see Figure 4A for the representation using first and second axes). The values of the first three PCs were extracted as a measure of parasitism (PCs 1, 2 and 3 for parasitism). PC1 for parasitism was negatively related to abundance of Monogenea, Cestoda and Crustacea. PC2 for parasitism was positively related to abundance of Digenea and finally, PC3 for parasitism was positively related to abundance of Crustacea and negatively to abundance of Monogenea (Table 2). The PCA on physiology showed that the first three axes accounted for most of the total variability in the physiological data set. The first axis explained 52.9%, the second explained 20.5% and the third axis explained 17.2% of the variability in the data set (see Figure 4B for the representation using first and second axes). The values of the first three PCs were extracted as a measure of physiology (PCs 1, 2 and 3 for physiology). PC1 for physiology was negatively related most clearly to LSI and condition and positively to GSI and haemoglobin concentration. PC2 for physiology was negatively related to haemoglobin concentration and condition. PC3 for physiology was positively related to GSI (Table 2). Finally, we performed similar analyses for the immune variables. PCA showed that the first three axes accounted for most of the total variability in the data set, with the first axis explaining 33.1%, the second explaining 22.0% and the third explaining 16.4% of the variability in the data set (see Figure 4C for the representation using first and second axes). The values of the first three PCs were extracted as a measure of immunity (PCs 1, 2 and 3 for immunity). PC1 for immunity was most clearly related to leukocyte, lymphocyte and phagocyte counts, and respiratory burst. PC2 for immunity was positively related to phagocyte count, respiratory burst and SSI, but negatively related to lymphocyte count. Finally, PC3 for immunity was indicative of the complement system activity and, IgM level (Table 2).

Sampling period was significantly correlated with PC1 and PC3 for parasitism, PC1 for physiology and PC1 and PC2 for immunity ($p < 0.001$). The seasonal variation of parasitism, immunity and physiology using PC1 is shown in Figure 4D-F). After correcting for sampling period (Table 3), the significant negative correlation between PC1 for parasitism and two PCs for physiology as well as between PC3 for parasitism and PC2 for physiology were found. Moreover, PC1 for parasitism with PC2 for immunity and PC2 for parasitism with PC1 for immunity were significantly positively correlated. Finally, PC2 for immunity was significantly negatively correlated with PC1 and PC2 for physiology, but PC3 for immunity was significantly positively correlated with PC2 and PC3 for physiology.

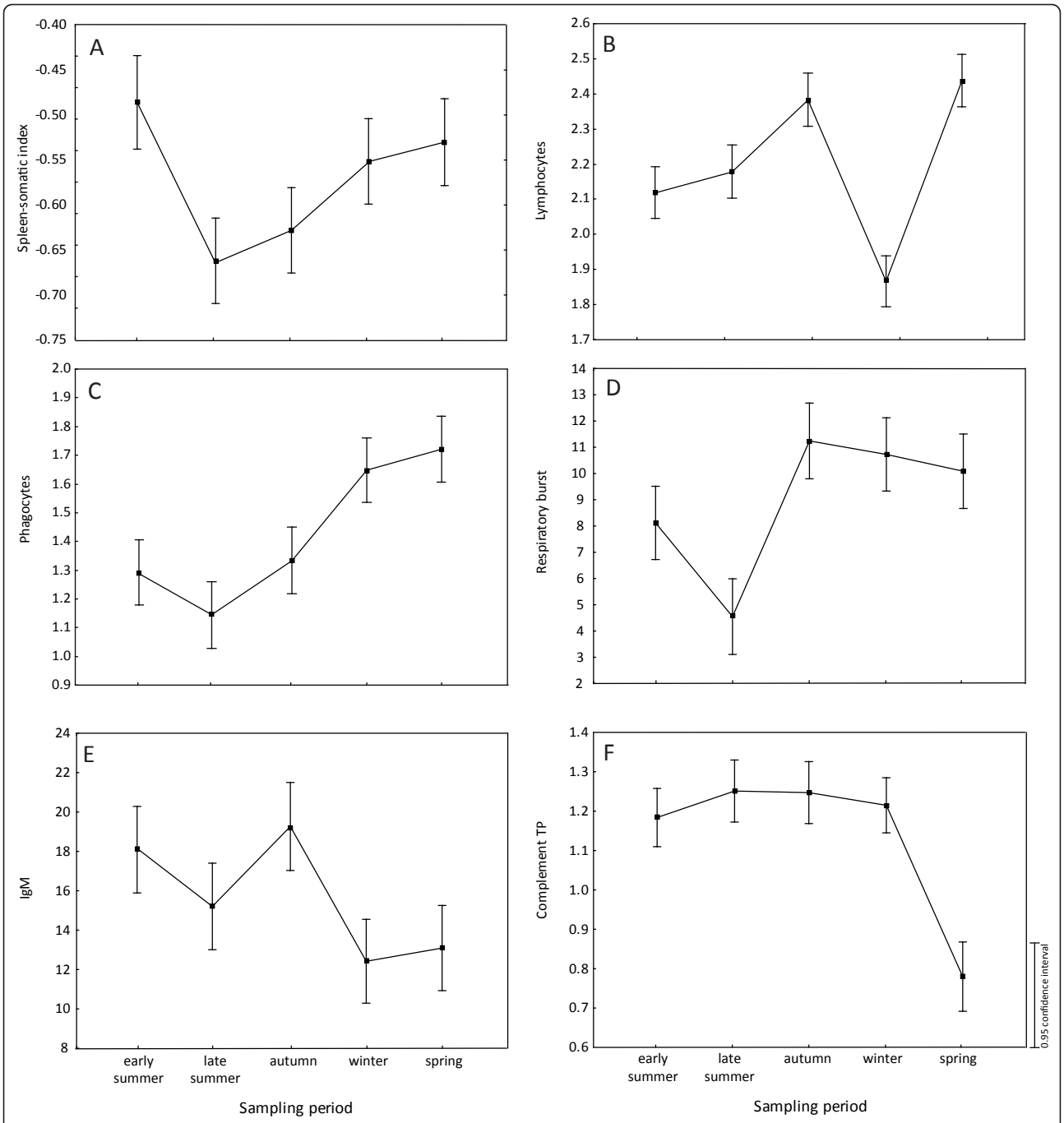

Figure 3 The changes in immune variables. Detailed legend: The changes in the following immune variables (A) spleen-somatic index, (B) lymphocyte count, (C) phagocyte count, (D) respiratory burst activity, (E) IgM level and (F) complement activity in relation to sampling period. Log transformation for spleen-somatic index; hyperbolic arcsine square root transformation for phagocyte count, lymphocyte count and complement activity; and square root transformation for respiratory burst were applied.

The different parasite life strategies: a link with host physiology and immunology

GLM analyses on the abundance of different parasite groups (representing parasites with different life strategies) as a function of immune and physiological variables and taking into account sampling periods were performed (Table 4). Except for the case of Digenea, the abundance of all other analyzed parasite groups (i.e. Monogenea, Cestoda and Crustacea) was found to be significantly dependent on sampling period. Moreover, significant relationships between the abundance of Monogenea and two physiological variables, i.e.

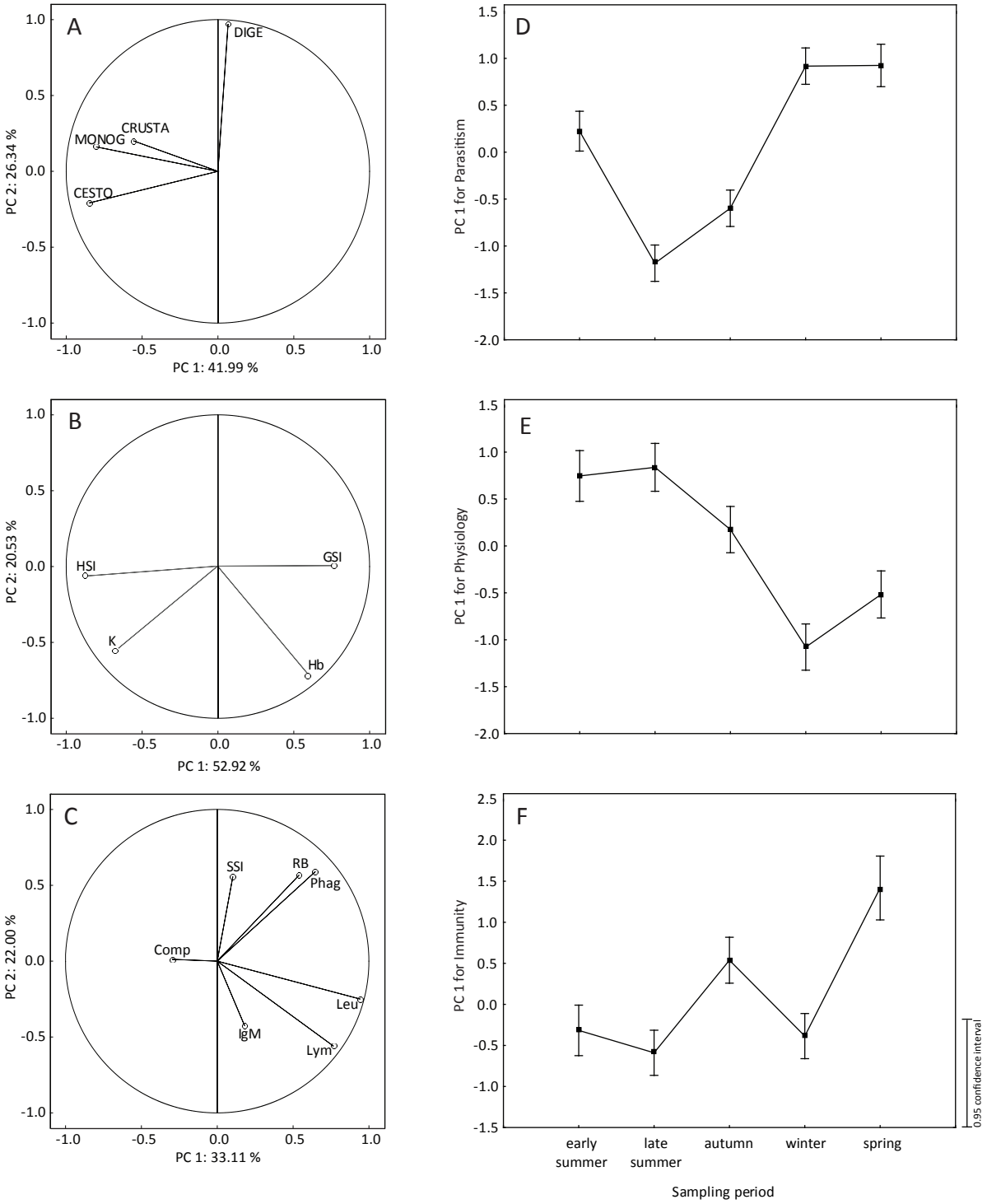

Figure 4 PCA on parasitism, physiology and immunity in common carp. Detailed legend: Principal component analyses on (A) parasitism including abundance of Monogenea (MONOG), Crustacea (CRUSTA), Cestoda (CESTO), and Digenea (DIGE), (B) physiological variables including condition factor (K), gonado-somatic index (GSI), hepato-somatic index (HSI), haemoglobin concentration (Hb), and (C) immune variables including spleen-somatic index (SSI), leukocyte (Leu), lymphocyte (Lym) and phagocyte count (Phag), respiratory burst (RB), IgM concentration (IgM) and complement activity (Comp). The changes of (D) index of parasitism (using PC1 of parasitism), (E) index of physiology (using PC1 of physiology) and (F) index of immunity (using PC1 of immunity) in relation to sampling periods are shown.

Table 2 Component scores of the three principal components of host physiology, host immunity and parasitism

Physiology	PC1	PC2	PC3
K	**-0.67**	**-0.55**	0.46
GSI	**0.75**	0.00	**0.60**
LSI	**-0.87**	-0.07	-0.07
Haemoglobin	**0.60**	**-0.72**	-0.34
Cumulative R^2	*52.92*	*73.45*	*90.62*
Immunity	PC1	PC2	PC3
SSI	0.10	**0.56**	-0.11
Leukocyte count	**0.94**	-0.25	-0.04
Respiratory burst	**0.54**	**0.57**	0.41
Complement	-0.30	0.01	**0.76**
IgM concentration	0.18	-0.43	**0.61**
Lymphocyte count	**0.77**	**-0.56**	-0.10
Phagocyte count	**0.65**	**0.59**	0.03
Cumulative R^2	*33.11*	*55.11*	*71.52*
Parasitism	PC1	PC2	PC3
Monogenea	**-0.80**	0.16	**-0.40**
Crustacea	**-0.56**	0.20	**0.80**
Cestoda	**-0.85**	-0.21	-0.16
Digenea	0.07	**0.97**	-0.13
Cumulative R^2	*41.99*	*68.33*	*89.45*

The most important parameters contributing to the principal components are shown in bold.

condition factor and haemoglobin concentration, were found. The abundance of Cestoda was significantly related to phagocyte count and respiratory burst when taking into account the effects of sampling period and sex (both effects were included in GLM following the results of one-way ANOVA). GLM analyses showed a significant partial relationship between the abundance of Digenea and SSI, although the model was not significant (see Table 4).

Associations between host immunity and physiology

Using GLM analyses, associations between host immunity (i.e. SSI, phagocyte count, respiratory burst, complement activity and IgM concentration) and physiology (condition factor, GSI, HSI and haemoglobin) taking into account the effect of sampling period or, alternatively, the effects of both sampling period and sex, were analyzed. All variables of immunity and physiology, except 11-ketotestosterone concentration, appeared statistically dependent on sampling period (Table 5). Significant relationships between condition factor, GSI and HSI were found when taking into account the sampling period. Haemoglobin concentration was related to GSI and affected by both sampling period and sex. In addition, 11-ketotestosterone concentration measured in males was significantly related to gonad weight (measured by GSI) and linked to host immunity measured by respiratory burst and complement activity, which suggests a potential trade-off between immunity and reproduction. The reversed patterns of seasonal changes for 11-KT (not shown) and complement activity (Figure 3F) levels were revealed. Phagocyte counts were related only to respiratory burst (a measure of phagocyte activity). However, no relationships among the different measures of immunity or between immunity and physiology were found when taking into account the sampling period ($p > 0.05$).

The last analysis was restricted to the spring season and to males as the level of 11-ketotestosterone was only significantly higher in this period (following the results of ANOVA). A significant negative relationship between 11-ketotestosterone and the level of total complement pathway was found ($N = 12$, $b = -0.78$, $p > 0.01$) suggesting the potential immunosuppression by steroid hormones (Figure 5). There were no significant relationships for any other immune variables in the spring period ($p > 0.05$).

Discussion

The relationship between abiotic environment and parasite infection

Changes in parasite abundance in relation to their life-cycle have been generally considered to be influenced by both host environment and host physiology [2,73]. Differences in the seasonal dynamic of abundance changes among different parasite groups are then predetermined by parasite life-strategies. Moreover, both the presence and efficiency of intermediate hosts play an important role in the transmission of endoparasites (e.g. [74]). In our study, we confirmed that seasonality influence the abundance of Monogenea, Crustacea and Cestoda. Change in water temperature (one of the principal cues of seasonality) is commonly regarded as one of the most important factors determining the presence and abundance of Monogenea [1]. We observed a different seasonal pattern of the abundance changes in oviparous gill parasites of *Dactylogyrus* and *Eudiplozoon* (with maximum abundance observed in summer) compared to viviparous *Gyrodactylus* species (with maximum abundance in winter). *Dactylogyrus* species were the most abundant parasites. The general trend associated with their life-cycle (direct-transmitted ectoparasites) is that any increase in temperature leads to an increase of their population densities [75]. Our results demonstrating the high abundance of all *Dactylogyrus* species of common carp in summer confirmed that water temperature is the main factor determining the high abundance of all *Dactylogyrus* species of common carp.

Adult stages of *Atractolytocestus huronensis* represented the dominant cestode species of common carp in

Table 3 Partial correlations controlling for sampling period

	PC1 for Parasitism	PC2 for Parasitism	PC3 for Parasitism	PC1 for Physiology	PC2 for Physiology	PC3 for Physiology	PC1 for Immunity	PC2 for Immunity
PC1 for Parasitism	1							
PC2 for Parasitism	0.039	1						
PC3 for Parasitism	**0.221**	-0.070	1					
PC1 for Physiology	**-0.300**	0.058	-0.138	1				
PC2 for Physiology	**-0.464**	0.145	**-0.333**	0.121	1			
PC3 for Physiology	-0.108	-0.043	-0.026	-0.078	-0.011	1		
PC1 for Immunity	-0.045	**0.187**	0.067	0.065	-0.177	0.075	1	
PC2 for Immunity	**0.485**	0.014	0.152	**-0.283**	**-0.194**	-0.101	-0.137	1
PC3 for Immunity	-0.105	0.095	-0.060	-0.099	**0.235**	**0.385**	0.148	0.026

Statistical significant correlations ($p < 0.05$) are shown in bold.

this study. The abundance of cestode infection can be connected with the temporal presence of intermediate hosts. The highest cestode abundance was recorded in late summer. Similar seasonal changes in the abundance of *A. huronensis* were previously reported for German pond-farmed carp [76]. Moreover, this cestode species was the only parasite affected weakly by host sex. Reimchen and Nosil [77], who monitored the level of parasitism in a population of threespined stickleback (*Gasterosteus aculeatus*), showed that females were more likely to be parasitized by the cestode *Schistocephalus solidus* (Cestoda) and suggested that this sex-biased infection could be connected with a dietary niche variation, which may result in differential exposure to infected intermediate hosts.

Digeneans parasitizing common carp were represented mainly by the larval stage (metacercaria) of *Diplostomum* species, which live in the eyes of this second intermediate fish host. This ubiquitous parasite causes cataracts, reduces fish vision, and may even induce total blindness [78]. A marginal effect of seasonality on the abundance of this species was observed. The highest abundance values were found in early summer and autumn. Similar findings have been documented in several studies (e.g. [79,80]), and Burrough [81] suggested that the first peak of infection (early summer) may probably come from the snails that survived through the winter. A second peak of infection may occur in autumn from snails that hatched during the spring period.

Crustacea were represented by an abundant species, *Argulus foliaceus*, which is considered to be an obligate branchiuran ectoparasite infecting many freshwater fish species. Some *Argulus* species are able to tolerate a wide range of water temperatures (e.g. [82]). *Argulus foliaceus*, a common species in Europe, is known to reach high abundance on their hosts during late summer and early autumn (e.g. [83,84]), which was also found here. However, no infection was observed in winter. Hakalahti and Valtonen [85] showed a low abundance of *Argulus coregoni* in fish during winter suggesting that this

Table 4 GLM analyses on the relationship between parasite abundance, immunity and physiology

Dependent variable	Independent variables	SS	Df	F	p	Total F (p)
Monogenea	Condition factor	1.653	1	11.144	0.001	
	Haemoglobin	0.588	1	3.966	0.049	
	Sampling	13.619	4	22.958	0.000	22.209 (< 0.0001)
Crustacea	Sampling	1248.215	4	7.114	0.000	5.826 (< 0.0001)
Cestoda	Respiratory burst	5.357	1	5.639	0.019	
	Phagocytes	7.865	1	8.278	0.005	
	Sampling	56.846	4	14.958	0.000	8.841 (< 0.0001)
Digenea	SSI	174.815	1	5.648	0.019	1.683 (0.074)

GLM analyses on the relationship between parasite abundance and immune or physiological variables, taking into account sampling period.

Table 5 GLM analyses on the relationship between host immunity and physiology

Dependent variable	Independent variables	SS	Df	F	p	Total F (p)
Condition factor	GSI	0.018	1	4.628	0.034	
	HSI	0.059	1	15.24	0.000	
	Haemoglobin	0.019	1	5.039	0.027	
	Sampling	0.131	4	8.505	0.000	6.629 (< 0.0001)
GSI	Condition factor	1.275	1	4.628	0.034	
	HSI	3.948	1	14.329	0.000	
	Haemoglobin	2.321	1	8.423	0.004	
	IgM	4.237	1	15.378	0.000	
	Sampling	5.853	4	5.311	0.001	11.34 (< 0.0001)
HSI	Condition factor	0.152	1	15.24	0.000	
	GSI	0.143	1	14.329	0.000	
	Sampling	0.514	4	12.916	0.000	20.054 (< 0.001)
Haemoglobin	GSI	0.043	1	7.681	0.007	
	Sampling	0.351	4	15.763	0.000	
	Sex	0.129	1	23.191	0.000	11.384 (< 0.001)
SSI	Sampling	0.44	4	6.446	0.000	3.691 (0.0001)
Phagocytes	Respiratory burst	2.924	1	35.009	0.000	
	Sampling	1.871	4	5.602	0.000	10.771 (< 0.0001)
Respiratory burst	Phagocytes	402.309	1	35.009	0.000	
	Sampling	242.144	4	5.268	0.001	9.922 (< 0.0001)
IgM concentration	Sampling	378.458	4	4.372	0.003	
	Sex	504.633	1	23.316	0.000	
	Sampling_Sex	597.857	4	6.906	0.000	7.773 (< 0.0001)
Complement	Sampling	1.972	4	19.414	0.000	9.189 (< 0.0001)
11-ketotestosterone	GSI	6586.91	1	25.556	0.000	
	Respiratory burst	1791.337	1	6.95	0.011	
	Complement	3611.305	1	14.011	0.000	6.524 (< 0.0001)

GLM analyses on the relationship between immune and physiological parameters, taking into account sampling period and sex effect.

species can survive winter due to overwintering egg stages laid in autumn.

The link between host immunity and physiology

We hypothesized that fish investing more in immunity should show less investment in other physiological functions. Moreover, we also hypothesized that seasonality acts as an important factor determining the levels of fish physiology and immunological activity. Using PCA, we revealed that one PC for physiology and two PCs for immunity (from three PCs analyzed) were correlated with seasonality suggesting that the majority of measured variables are under seasonal changes. The different PCs for immunity and physiology were correlated with different variables (mainly when comparing PC1 and 2 to PC3). This fact together with the significant correlations between PCs of immunity and physiology may suggest the potential relationships between different immunity and physiology variables.

We conducted analyses among selected measures of host status related to condition and reproduction and of immunity taking into account the sampling period. Fish store energy in muscle tissues or in the liver (glycogen) during periods of high food and energy intake [86]. Therefore, both condition factor and the relative size of liver (HSI) are recommended as an indirect indicator of energy status [86]. The gonado-somatic index (GSI) represents an accurate assessment of reproductive maturity. Negative relationships were found between fish energy status (measured either by the condition factor or HSI) and GSI, suggesting the existence of a trade-off, with a decreasing fish condition in the period of gonad formation and reproduction. A significant relationship was also observed between haemoglobin concentration and GSI. The seasonal variation in haemoglobin concentration is potentially related to variation in water temperature and variation in water oxygen concentration. Fish adapt via increases in total haemoglobin concentration or by other mechanisms such as changes in red cell nucleoside triphosphate concentration [87,88]. The spawning process may affect haematological parameters [89], which may explain the observed relationship between haemoglobin concentration and GSI. Moreover, the concentration of

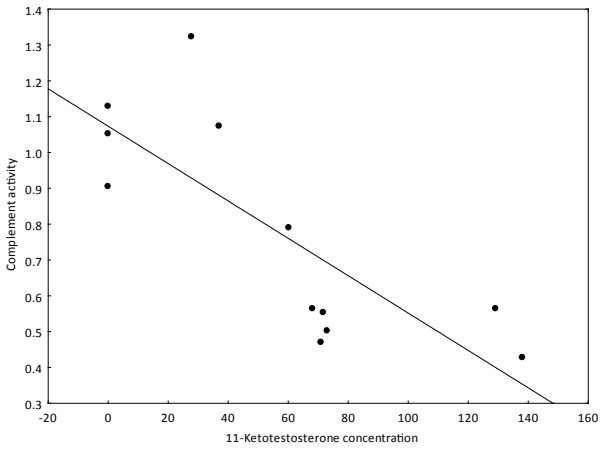

Figure 5 The negative relationship between the level of 11-ketotestosterone and the activity of complement in spring.

haemoglobin was the single physiological parameter that differs between females and males, which has already been observed in other fish species [51].

Although significant relationships were found between PCs of physiology and immunity suggesting the potential trade-off associations between physiology and phagocyte activity or SSI (using PC2 for immunity and PCs 1 and 2 for physiology) and between physiology and complement activity or IgM (using PC3 for immunity and PCs 2 and 3 for physiology), no associations between individual immune variables and physiological variables were found using GLM. However, host immunity was strongly dependent on the sampling period, which confirms that seasonality is the driving force of immune variation in fish.

We hypothesized that higher investments in somatic condition could be associated with lower investments in immune defence. Although, our analyses revealed no significant relationship between condition factor and spleen size, both these variables showed similar seasonal dynamics. The highest values of SSI were found in early summer followed by decreasing values in late summer. Our findings are not in agreement with previous studies in roach *Rutilus rutilus* [90] or in Arctic charr, *Salvelinus alpinus* [91], where spleen size was shown to decrease in the breeding season.

Concentration levels of IgM were also dependent on the sampling period with low IgM values recorded in winter and spring. Previous studies on IgM concentration showed a decrease in IgM level during the winter period, probably related to water temperature, in rainbow trout *Oncorhynchus mykiss* [46] and goldfish *Carassius auratus* [92]. According to Avtalion [93] and Stolen et al. [94], low water temperature causes a selective suppression of in vitro T cell responses and antibody synthesis. Suzuki et al. [95] followed the annual changes of IgM in three strains of rainbow trout under constant water temperature and natural day length, and showed that the IgM concentration level varied due to the immunosuppression effect induced by sex hormones. Here, we did not find any clear association between seasonal changes (potentially related to water temperature) and IgM concentration level. The lowest water temperature was recorded in autumn and winter whilst the lowest concentration in IgM level was observed in winter and spring. Moreover, IgM concentration was the only immune variable affected by host sex, with a significantly lower IgM concentration in males. Although, the immunosuppressive effect of testosterone and/or gonad maturation on IgM has already been demonstrated in rainbow trout [96], this effect has not been investigated in common carp before our study. Other hormones like cortisol may also reduce the IgM secretion, as shown in salmonid fish [97].

The annual fluctuation in complement activity was investigated in gilthead sea bream, *Sparus aurata* by Hernández et al. [7]. These authors showed increasing complement activity in warm months probably in relation to higher metabolic activity at higher temperatures in poikilothermic organisms. In this study, the total complement activity decreased in spring but not in winter at the coldest temperature. Seasonal changes (potentially related to water temperature) does not seem to affect the total complement activity and its decrease in spring could be related to reproduction (see below its relationship with 11-ketotestosterone).

Phagocyte counts and phagocyte activity (measured by the respiratory burst) were both affected by seasonality, although these variables were highly correlated. Seasonal variability in phagocyte activity has been investigated in several fish species with inconsistent conclusions. An immunosuppressive effect of water temperature on the innate immune response in catfish *Ictalurus punctatus* [11] and in tench, *Tinca tinca* [98,99] has been observed. In accordance with these studies, we recorded low values of respiratory burst in late summer, where water temperature is higher comparing to other period investigated. High values of respiratory burst were recorded from autumn to spring. Phagocyte activity seems not to be suppressed by water temperature in several fish species e.g. rainbow trout under laboratory conditions [35].

11-ketotestosterone is considered to be a major androgen hormone in teleost fish (see review by Borg [50]). This hormone influences spermatogenesis and effects the expression of secondary sexual characters and reproductive behavior. It also suppresses several immune functions. Steroid hormones have dualistic functions: they increase the expression of elaborated sexual

ornamentation but decrease the immunocompetence of an individual. This observed mechanism is at the basis of the immune-handicap hypothesis [28]. We found a positive association between gonad size (measured by GSI) and 11-ketotestosterone concentration. Moreover, we found support for the immunosuppressive role of 11-ketotestosterone, with negative relationships observed between the concentration of 11-ketotestosterone and two immune parameters - total complement activity and respiratory burst (taking into account the sampling period). Using linear regression (not included in the results), only complement activity was negatively correlated with concentration of 11-ketotestosterone. A significant immunosuppressive effect of 11-ketotestosterone on complement activity was also found when using only spring data, i.e. where concentrations of 11-ketotestosterone were the highest and correlated with gonad development.

The role of the season: host immunity and physiology versus total parasite load

One aim of our study was to investigate the potential link between parasitism and host immunity or physiology on two different scales. First, using the computed PCs we showed the significant role of seasonality on each of parasitism, immunity and physiology.

After correcting for sampling period, the negative relationships between parasitism and physiology were found, which suggests that a "good" physiological status reflects a host's ability to escape from parasitism (especially concerning Monogenea, Crustacea and Cestoda). Moreover, parasitism was positively related to immunity, which could indicate that despite of strong effect of seasonality on fish immunity, this system (or at least some immune pathways) is activated by increasing level of parasite infection.

Parasitism versus host immunity and physiology: effect of season or a real association?

Effects of seasonal variability on the parasite abundance of several parasite groups that differ according to life strategy were observed. A significant relationship between monogeneans, the most abundant ectoparasite group, and the condition of fish was found, taking into account the sampling period. The opposite seasonal patterns in fish condition and in the abundance of monogeneans (Figures 1A and 2A) suggest that high infection by these parasites should be detrimental to fish.

A negative relationship between haemoglobin concentration and the same parasites was also observed. The presence of *Eudiplozoon nipponicum*, a haematophagous monogenean species with intracellular blood digestion [100] and a body size 25-60 times higher than the average body size of *Dactylogyrus* and *Gyrodactylus* species, may contribute to the decrease in haemoglobin concentration. Sitja-Bobadilla and Alvarez-Pellitero [101] suggested that even a low intensity of infection by a monogenean species (*Sparicotyle chrysophrii*) may induce fish anaemia, and activation of fish haematopoesis leading to an increase in immature erythrocytes with lower amounts of haemoglobin.

We found a significant relationship between the abundance of Cestoda and counts of peripheral blood phagocytes. Moreover, the immunological activity of phagocytes measured by the respiratory burst was also found to be associated with cestode infection (taking into account the sampling period). The abundance of cestodes (*A. huronensis* was the dominant species) was negatively related to both these measures of immunity using linear regression (not shown in the results section). We could hypothesize that, during a period of low parasite infection, phagocytes are present in peripheral blood ready to react quickly to an entering antigen derived from parasites. However, during high parasite infection, the phagocytes and other blood cells colonize the tissues surrounding the attachment organs of *A. huronensis* [102]. A low activity of phagocytes, measured by respiratory burst, was also demonstrated in fish experimentally infected by the cestode *Schistocephalus solidus* [103] or in gynogenetic form of triploid *Carassius auratus* infected by metacercaria of *Metagonnimus* sp. [104]. Other examples demonstrating the depression of oxidative burst and/or the impairment of phagocyte activity induced by parasites are given by Alvarez-Pellitero [19]. In addition, some parasites are able to exploit the host immune reaction in order to improve their attachment to the host tissue. Alvarez-Pellitero [19] documented that attachment of the cestode *Cyathocephalus truncatus* in the fish pyloric caeca was facilitated by an inflammatory reaction. However, Vainikka et al. [72] observed the positive correlation between parasite loads and the relative proportion of phagocytes in blood of roach (*Rutilus rutilus*). They also showed that functional characteristics of these cells were positively related to the proportion of dead *Rhipidocotyle campanula* (Digenea) which may indicate that the chemiluminescence method is a suitable measure to estimate functional immunocompetence in fish.

A significant relationship was observed between the abundance of digeneans and SSI. However, using simple linear regression (not shown in the results section), a positive relationship was found between larval digeneans of *Diplostomum* species and relative spleen size. Skarstein et al. [90] suggested that large spleen in fish may reflect the ability to respond to parasite infection or may indicate high immunological activity against already established infection. The associations between spleen size and parasitism by metazoan parasites have been

tested in many intraspecific studies (e.g. [29,59]), but a significant relationship is rarely reported [e.g. [30,61]]. Vainikka et al. [72] did not find any associations between spleen size and parasite counts in roach suggesting that spleen size might not represent the measure of immunocompetence in roach and thus this variable should be interpret with caution in immunoecological studies. Moreover, they suggest that it is difficult to interpret causal relationships when using only correlation study to analyze the associations between immune variables and parasite load and therefore, they propose that the experimental studies are needed.

Conclusions

Our study showed that host immunity and physiology, as well as parasite infection, are highly dependent on seasonal variability (i.e. temporal variation) potentially related to the changes of water temperature (one of the principal cues of seasonality), although several other abiotic characteristics of the water environment may play a part. Nevertheless, we confirmed the associations between parasitism and both host physiology and immunity after correction for temporal variability. When considering parasites with different life strategies, and taking into account the effects of seasonality, fish in a worse physiological condition suffer from a higher level of infection by the abundant ectoparasitic monogeneans. The infection by cestodes seems to activate several mechanisms of the immune system and particularly phagocyte activity. Seasonal variability affects host immunity and physiology through energy allocation in a trade-off between important functions, i.e. reproduction and fish condition. However, the measures of host immunity were not found to be in a direct trade-off with the investigated physiological traits or functions, but the immunosuppressive role of 11-ketotestosterone was observed.

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Authors' contributions
AŠ designed this study. KR and AŠ drafted the manuscript. SM significantly contributed in drafting the statistical part of manuscript. SM, MF and PH involved in revising for important content and discussing the results. All authors contributed to acquisition of data, data analysis or data interpretation. All authors read and approved the final version of the manuscript.

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The authors declare that they have no competing interests.

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PŘÍLOHA Č. 24

POISOT Timothée, ŠIMKOVÁ Andrea, HYRŠL Pavel, MORAND Serge

Interactions between immunocompetence, somatic condition, and parasitism in the chub in early spring. Journal of Fish Biology, 75, 1667-1682, 2009.

Charakteristika:

Popis vztahu mezi imunokompetencí, kondicí a parazitismem u jelce tlouště (*Leuciscus cephalus*). Pozitivní vztah byl nalezen mezi parazitací a oxidačním vzplanutí fagocytů.
IF=1,658; citováno 10 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Ondrackova, Marketa; Valova, Zdenka; Kortan, Jiri; et al.: Consequent effects of the great cormorant (*Phalacrocorax carbo sinensis*) predation on parasite infection and body condition of common carp (*Cyprinus carpio*), Parasitology Research: 110, 4, 1487-1493, 2012.

PŘÍLOHA Č. 25

WENGER Michael, ONDRAČKOVÁ Markéta, MACHALA Miroslav, NEČA Jiří, HYRŠL Pavel, ŠIMKOVÁ Andrea, JURAJDA Pavel, von der OHE Peter, SEGNER Helmut

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Charakteristika:

Komplexní studie populace jelce tlouště (*L. cephalus*) na řece Bílině, kde byly sledovány parametry přirozené imunity ve vztahu k chemické zátěži, parazitaci a celkovému zdraví ryb.

IF=3,225; citováno 6 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Marcogliese, David J.; Pietrock, Michael: Combined effects of parasites and contaminants on animal health: parasites do matter, Trends in Parasitology: 27, 3, 123-130, 2011.

PŘÍLOHA Č. 26

JAVŮRKOVÁ Veronika, KRKAVCOVÁ Eva, KREISINGER Jakub, HYRŠL Pavel, HYÁNKOVÁ Ludmila

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Charakteristika:

Studie zabývající se antibakteriální ochranou zárodku ve vejcích. Manipulací s hladinou lysozymu v bílku vajec křepelek japonských (*Coturnix japonica*) byl prokázán vliv na imunitní funkce a také na regulaci růstu během embryonálního vývoje.

IF=1,440; citováno 0 x (údaje k 31.1.2016).

PŘÍLOHA Č. 27

KOTLÍK Petr, MARKOVÁ Silvia, VOJTEK Libor, STRATIL Antonín, ŠLECHTA Vlastimil, HYRŠL Pavel, SEARLE Jeremy B.

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Charakteristika:

Studie zabývající se rozšířením norníka rudého (*Clethrionomys glareolus*) na základě rozdílu v molekule hemoglobinu u dvou populací. Měření celkové antioxidační kapacity plasmy norníků pomohlo popsat funkční rozdíly v mutaci hemoglobinu a tím následný postup kolonizace Evropy na konci doby ledové.

IF=5,051; citováno 2 x (údaje k 31.1.2016).

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Filipi, Karolina; Markova, Silvia; Searle, Jeremy B.; et al.: Mitogenomic phylogenetics of the bank vole *Clethrionomys glareolus*, a model system for studying end-glacial colonization of Europe, Molecular Phylogenetics and Evolution: 82, 245-257, část A, 2015.

PŘÍLOHA Č. 28

DENEV Petko, KRATCHANOVA Maria, ČÍŽ Milan, LOJEK Antonín, VAŠÍČEK Ondřej, BLAZHEVA Denitsa, NEDELCHEVA Plamena, VOJTEK Libor, HYRŠL Pavel Antioxidant, antimicrobial and neutrophil-modulating activities of herb extracts. Acta Biochimica Polonica, Polish Academy of Sciences, Committee of Biochemistry, 61(2), 359-367, 2014.

Charakteristika:

Testování rostlinných extraktů na izolovaných lidských fagocytech, které přispělo k determinaci biologicky aktivních polyfenolických látek přítomných v různých plodech. IF=1,153; citováno 1 x (údaje k 31.1.2016).

Nejvýznamnější citace této práce:

Krivokuca, Marija; Niketic, Marjan; Milenkovic, Marina; et al.: Anti-*Helicobacter pylori* Activity of Four *Alchemilla Species* (Rosaceae), Natural Product Communications: 10, 8, 1369-1371, 2015.

PŘÍLOHA Č. 29

DENEV Petko, KRATCHANOVA Maria, ČÍŽ Milan, LOJEK Antonín, VAŠÍČEK Ondřej, NEDELCHEVA Plamena, BLAZHEVA Denitsa, TOSHKOVA Reneta, GARDEVA Elena, YOSSIFOVA Liliya, HYRŠL Pavel, VOJTEK Libor

Biological activities of selected polyphenol-1 rich fruits related to immunity and gastrointestinal health. Food Chemistry, Elsevier Science, 2014 (157), 37-44, 2014.

Charakteristika:

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Nejvýznamnější citace této práce:

Kratchanova, M. G.; Denev, P. N.; Kratchanov, C. G.: Rose hip extract synergistically increase antioxidant activity of fruit and herb extracts, Bulgarian Chemical Communications: 46A, 59-64, 2014.