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Effects of Toxoplasmosis on Reaction Times and Prepulse Inhibition of Startle Reaction in Humans

Vliv toxoplasmosy na reakční časy a prepulsní inhibici úlekových reakcí u člověka

Dizertační práce
Vedoucí práce: prof. RNDr. Jaroslav Flegr, CSc.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 28. 7. 2019

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Lenka Příplatová

Poděkování

Děkuji svému vedoucímu práce prof. RNDr. Jaroslavu Flegrovi, CSc. za vedení, a především za nesmírnou trpělivost. Kdyby mi neustále nestál za krkem, nikdy bych to nedopsala. Dále děkuji všem svým kolegům, s nimiž jsme na výzkumech spolupracovali a nadále spolupracujeme. Vzhledem k objemu vložené práce by bez nich žádná z přiložených studií nebyla možná. Velmi děkuji i personálu spolupracujících institucí, a jmenovitě pak Blance Širocké a RNDr. Petru Kodymovi, CSc. z Národní referenční laboratoře pro toxoplasmosu (SZÚ) za tisíce serologických analýz našich vzorků, neustálé zlepšování diagnostických metod, a pořádání Konzultačního dne pro toxoplasmosu, kde člověk pokaždé načerpá novou inspiraci a chuť do výzkumu.

Nakonec bych chtěla poděkovat svým přátelům, jejichž nasolená masa a společně připravované domácí klobásky dávají tušit, že se mě navzdory dřívějším negativním testům bude téma této dizertační práce brzy dotýkat osobně, jakož i svým dvěma oblíbeným hnízdním parazitům Tamince a Kryštofovi, kteří mi svými neustálými pokusy narvat se na klávesnici, nechat se hladit a přist u toho na celé kolo nikdy nepřestali dávat najevo, že na světě jsou podstatně důležitější věci než nějaká toxoplasmóza nebo dokonce dizertace, například spokojené kočičky. A samozřejmě mámě. Že mě ještě nezabila.

Poznámka k autorským právům

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Abstrakt

Toxoplasma gondii, jednobuněčná kokcidie z téměř výhradně parazitického kmene Apicomplexa, zřídka působí u lidí akutní potíže. Ty se týkají převážně imunodeficientních jedinců a těhotných žen, respektive vyvíjejích se plodů. Ve své latentní fázi mohou bradyzoiti z tkáňových cyst umístěných zejména v nervové a svalové soustavě vyvíjet mírný, avšak dlouhodobý tlak na hostitelský organismus, a to v podobě normálních průvodních jevů přítomnosti parazitického organismu v tkáních hostitele, ale pravděpodobně i jako následek adaptivní evoluce parazita směřující ke zvýšení pravděpodobnosti trofického přenosu do definitivního hostitele, kočkovité šelmy. Následkem mohou být v případě člověka mírné změny v osobnostním profilu hostitele, zhoršení psychomotorických i kognitivních funkcí, až rozvoj závažných duševních poruch.

Práce se zabývá převážně jedním z aspektů těchto změn – efektem latentní toxoplasmózy na zpracování úlekových signálů samotných a modifikovaných prostřednictvím slabého signálu předcházejícího samotný signál úlekový, neboť právě tento aspekt by mohl souviset s rozvojem schizofrenie u predisponovaných jedinců. Studie provedené v rámci projektu zjistily změny v rychlosti zpracování signálu u ne-psychiatrické populace seropositivní na toxoplasmu jakož i rozdílné výsledky ve výkonnostních testech, a znovu ukázaly na změny v osobnostním profilu nakažených. Studie prováděné na populaci pacientů se schizofrenií ukázaly výrazně zvýšenou prevalenci toxoplasmozy u mužských pacientů oproti běžné populaci, a nalezly rozdíly v hladinách imunomodulačních steroidů, hormonů a tuků v krvi *Toxoplasma*-positivních a *Toxoplasma*-negativních pacientů. Vyhodnocení dalších dat získaných v rámci studia vlivu latentní toxoplasmozy na lidské chování a reakce přineslo objev „Justinina efektu“ v experimentálních hrách a souvislost mezi hladinami steroidních a pohlavních hormonů a výsledky výkonnostních a kognitivních testů.

Klíčová slova

Toxoplasma gondii – prepulsní inhibice – úleková reakce – reakční časy – manipulační hypotéza – schizofrenie

Abstract

Toxoplasma gondii, a single-cell coccidia from almost exclusively parasitic phylum Apicomplexa, does not typically cause acute health issues in humans with most exceptions among immunodeficient individuals and pregnant mothers or, more precisely, their offspring. In the latent phase, the bradyzoites in tissue cysts placed most often in neural and muscle tissues can evolve pressure on the host's body both as a collateral effect of the presence of the parasitic organism in host's tissues and as a consequence of adaptive evolution leading to increase in probability of trophic transmission to the final host, a felid. In humans, this can result in slight changes in personality profiles, deterioration of psychomotor and cognitive functions, and development of serious mental disorders.

The thesis focuses predominantly on one of the aspects of the changes, namely the effect of latent toxoplasmosis on the processing of startle signals themselves and when modified by a preceding low-intensity signal; this processing may be connected with the development of schizophrenia in predisposed individuals. Studies conducted within the project framework found changes int the speed of signal processing in *Toxoplasma*-seropositive non-psychiatric population and differences in performance tests results and brought new confirmation of changes in personality profiles of infected individuals. Studies conducted on a population of schizophrenic patients shown distinctively increased the prevalence of latent toxoplasmosis in male patients in comparison with standard population, and found differences in levels of immunomodulatory steroids, hormones and lipids in the blood of *Toxoplasma*-positive and *Toxoplasma*-negative patients. Evaluation of data collected within the framework of studies of the effects of latent toxoplasmosis on human behavior and reactions brought the discovery of "Justina effect" in experimental games and found an association between levels of steroid and sex hormones and results of performance and cognitive tests.

Keywords

Toxoplasma gondii – prepulse inhibition – startle reaction – reaction times – manipulation hypothesis – schizophrenia

Table of Content

List of Abbreviations

- 16PF. Cattell's 16 Personality Factors.
- 7-α-OH-DHEA / 7-β-OH-DHEA. 7-α-Hydroxydehydroepiandrosterone, 7-β-Hydroxydehydroepiandrosterone.
- AIDS. Acquired immunodeficiency syndrome.
- BALB/c. Bagg Albino. An inbred strain of laboratory mice.
- BDI. Beck Depression Inventory.
- BPRS. Brief Psychiatric Rating Scale.
- CFR. Complement Fixation Test.
- CI. Confidence Intervals.
- CMV. Cytomegalovirus.
- CZK. Czech Koruna.
- DHEA/S. Dehydroepiandrosterone and its sulfate.
- DNA. Deoxyribonucleic acid.
- ECDC. European Centre for Disease Prevention and Control.
- ECLIA. Electro-Chemiluminescence Immunoassay.
- EEG. Electroencephalography.
- ELISA. Enzyme-Linked Immunosorbent Assay.
- FIV. Feline Immunodeficiency Virus.
- GLM. General Linear Model.
- HIV. Human Immunodeficiency Virus.
- IgG. Immunoglobulin G.
- IQ. Intelligence Quotient.
- iTOL. Interactive Tree of Life.
- LDL. Low-density lipoprotein.
- MATLAB. Matrix Laboratory. Numerical computing environment and proprietary programming language by MathWorks.
- MUH. Military University Hospital Prague.
- N-70. Questionnaire for assessment of anxiety, depression, phobia, hysteria, hypochondria, psychosomatic symptoms, and psychastenia.
- NCBI. National Center for Biotechnology Information.
- NEO-PI-R. The Revised Neuroticism-Extraversion-Openness Personality Inventory.
- NIMH. National Institute of Mental Health.
- NIPH. National Institute of Public Health.
- OD-1. Questionnaire for assessment of emotional characteristics of an individual.
- OR. Odds Ratio.
- PAM. Primary Amoebic Meningoencephalitis.

- PCP. Psychiatric Centre Prague,
- PPI. Prepulse inhibition of startle response.
- RhD. Rhesus factor D antigen.
- RNA. Ribonucleic acid.
- S.D. Standard deviation.
- SPSS. Statistical Package for the Social Sciences.
- SR. Startle response.
- TAG. Triglyceride (Triacylglycerol).
- TCI. Cloninger's Temperament and Character Inventory.
- TSH. Thyroid Stimulating Hormone.
- UV, UV light. Ultraviolet light.
- VCM. Voluntary Contribution Mechanism.
- WHO. World Health Organization.
- WMT. The Wiener Matrizen-Test of nonverbal intelligence.
- WOS. Web of Science.

Note: Besides the given list, standard measurement units (such as ms, dB), statistical variables (such as p, eta$^{2)}$ as well as standard abbreviations of countries (EU, US) were used without explanation. Certain questionnaire names (such as OTIS), though customarily written in upper case, are derived from author's names and are not thus considered as abbreviations.

List of Figures and Tables

List of Included Publications

Publ. 1. Kuběna, A.A., Houdek, P., Lindová, J., Příplatová, L., and Flegr, J: 2014: **Justine effect: punishment of the unduly self-sacrificing cooperative individuals**. PLoS ONE, 9 (3): e92336. doi:10.1371/journal.pone.0092336 (Kuběna, Houdek, Lindová, Příplatová, & Flegr, 2014)

Publ. 2. Flegr, J., Hampl, R., Černochová, D., Preiss, M., Bičíková, M., Sieger, Příplatová, L., Kaňková, Š., Klose, J. 2012: **The relation of cortisol and sex hormone levels to results of psychological, performance, IQ and memory tests in military men and women**. Neuroendocrinology Letters, 33, 224-235 (Jaroslav Flegr et al., 2012)

Publ. 3. Lindová J., Příplatová L., Flegr J. 2012: **Higher extraversion and lower conscientiousness in humans infected with *Toxoplasma***. European Journal of Personality, 26: 285–291, DOI: 10.1002/per.838 (Lindová, Příplatová, & Flegr, 2012)

Publ. 4. Flegr, J., Příplatová, L., Hampl, R., Bicíková, M., Ripova, D., Mohr, P. 2014: **Difference of neuro- and immunomodulatory steroids and selected hormone and lipid concentrations between *Toxoplasma*-free and *Toxoplasma*-infected schizophrenia patients**. Neuroendocrinology Letters 35: 20-27 (Jaroslav Flegr, Příplatová, et al., 2014)

Publ. 5. Příplatová, L., Šebánková, B., Flegr, J. 2014: **Contrasting effect of prepulse signals on performance of *Toxoplasma*-infected and *Toxoplasma*-free subjects in an acoustic reaction times test**. PLoS ONE, 9 (11): e112771 (Příplatová, Šebánková, & Flegr, 2014)

Publ. 6. Flegr, J., Šebánková, B., Příplatová, L., Chvátalová, V. and Kaňková, Š., 2018. **Lower performance of *Toxoplasma*-infected, Rh-negative subjects in the weight holding and hand-grip tests.** PLoS

ONE, 13 (7), p.e0200346. (Jaroslav Flegr, Šebánková, Příplatová, Chvátalová, & Kaňková, 2018)

Student's Contribution

Publication 1: I lead most of the on-site experiments, collected data from the experimental software, and participated in the publication process.

Publication 2: I have participated in the publication process with a major part in converting the N-70 questionnaire into English.

Publication 3: I lead most of the on-site experiments, participated in the organization of the experiments and blood sampling of university students, and participated in digitalization and preprocessing of the questionnaire data.

Publication 4: The data on the experimental population were gained and analyzed in concurrence with a study of the effects of toxoplasmosis on performance tests' results. I have participated in data pre-processing and analysis of both studies with a more substantial role in the second study, which has not been published yet.

Publication 5: I came up with the idea of prepulse experiments in *Toxoplasma*-infected subjects, helped with the experimental design, assisted with blood sampling, lead all the on-site experimental sessions, and participated in the data preprocessing, analysis and publication process.

Publication 6: I have participated in the experimental sessions, assisted with the blood sampling, data collection, and the publication process.

1 Introduction

Toxoplasma gondii, an apicomplexan protist first identified (Nicolle & Manceaux, 1908) and named (Nicolle & Manceaux, 1909) in a desert rodent *Ctenodactylus gundi* by Charles Nicolle and Louis Manceaux, and later identified as a human parasite by a Czech physician Josef Janků (Janků, 1923) and Romanian physician and microbiologist Constantin Levaditi [1] (Levaditi, 1928) has undergone an exciting development in views of both the scientific community and the general public.

Figure 1 *Ctenodactylus gundi* (c) Jaroslav Flegr, Tunisia 2019.

It is this very development from a rather harmless symbiont gently shifting our behavior into medically and agriculturally significant and potentially murderous pathogen which, beside direct pathogenicity during

pregnancy in humans and individual animals alike, positioning its victims in ways of various harms, be it a quickly approaching vehicle or a danger of mental illness, that caught my attention during my studies, and manipulated me, though uninfected myself, into devoting most of my research activities to this strange organism.

During the years, we have conducted various experiments and studies leading us deeper into understanding the ways of the parasite, and in this thesis, I would like to introduce the ones I have participated in the most. Two of the resulting publications (publications 1 and 2) do not, ultimately, bring results on T. *gondii* and its potentially manipulative activity while in a human host, the experiments were, however, conducted as a part of the studies of latent toxoplasmosis and were published as side products of the research. The other four are focused solely on various effects of the parasite on human behavior and physiology and closely relate with the thesis' topic.

Besides the enclosed studies, I have also participated in two publications (Jaroslav Flegr & Příplatová, 2010; Kleisner, Příplatová, Frost, & Flegr, 2013) which are not included in this thesis, since most of the experiments did not relate to the *Toxoplasma* research and were also partly conducted on a different population. My current h-index, according to the Web of Science, is 6. I have participated in multiple other experiments, but I am scarcely participating in the publication of the collected data, which leads to my publication activity being appalling. I am aware of this shortcoming and plan on overcoming it after this thesis is finally out of my sight.

I have structured the thesis in the following manner. First, I am introducing state of the art in this area of science in the chapter **Theoretical Background**. I will shortly describe the manipulation hypothesis (part 2.1) with a particular focus on latent toxoplasmosis in both animal and human experiments (part 2.2.2, 0). Increased attention will be paid to latent toxoplasmosis in context with changes in mental health and brain function of the infected individuals (part 2.3), especially in relation to schizophrenia (part 2.4). Last but not least, the fifth section of the Theoretical Background will

introduce the prepulse inhibition of startle reaction in its context and history (part 2.5). The names of the subchapters in the theoretical background, being it the literary part of the thesis, are referencing some of my favorite books. Sorry, I could not help myself.

The next chapter, **Aims and General Methods,** focuses on how we proceeded with experiments on latent toxoplasmosis and its effect on human reaction times and prepulse inhibition of startle reaction, why we chose such experiments, where were our experimental subjects recruited from, and I give other general information that concern all the presented publications.

The fourth chapter, **On Publications,** consists of subchapters based on individual articles. Each subchapter contains an explanation of why the study was chosen for inclusion in the thesis, and a brief presentation of the article itself (including theoretical background, materials and methods, results and discussion, and limitations) followed by the attached copy of the publication. My participation in each research is stated at the beginning of the thesis in the Student's Contribution part.

Chapter five, **Summary and Foreseeable Future Development** describes the current state of experiments in our laboratory, potential problems and limitations, and further questions that should be answered either by other researchers all over the world or by us.

The thesis itself is brought to a close with **Conclusion** and **References** as its last chapters, while I am confident that as the studies of *T. gondii* and other pathogens affecting human health and behavior are concerned, we are yet at the very beginning of the journey.

Subchapters of the Theoretical Background dedicated to *Toxoplasma gondii* are somewhat problematic in that on one hand, the parasite itself, its biology, prevalence, and clinical symptoms in animals or economic losses connected with congenital toxoplasmosis in domestic animals aren't directly connected to the thesis' topic and could be thus omitted altogether with a sentence or two of generalizing characteristic.

On the other hand, without understanding of how closely is the parasite tied to our ecosystems, food chains, and human efforts in medicine, agriculture or wildlife preservation, it is hard to imagine why is it even worth studying in every aspect of its existence, even as a psychiatric threat instead of just a curiosity at the fringe of neurobiology and psychiatry. As shall be explained in the appropriate sections, we can get infected by *T. gondii* while eating seafood, drinking goat milk, or using modern techniques of meat preparations. We can find it in zoos as well as in the wilderness. We are confronted with it in almost all the aspects of human medicine, from birth (gynecology and neonatal care) to death (HIV patients) and in many fields in between (immunology, ophthalmology, neurobiology, psychiatry to name a few). And we stumble upon it even in such distant areas as urbanism and municipal policies (areas with an overpopulation of feral cats in proximities to playgrounds, for example) or conservational efforts (health problems in offspring of endangered species bred in captivity). So, perhaps more detailed information is needed, after all.

Ay, there's the rub.

Precisely because of the above-implied scope of the topic, detailed information would produce several books rather than one thesis. I have thus chosen a route somewhere in the middle: I will outline the topic more broadly than it would be perhaps required, yet it will keep the character of an outline mentioning multiple connected subtopics with examples of what have been already found here and there, of course, equipped with appropriate citations for anyone interested in broader details. I will do my best not to stray into a detailed review of any of these subtopics.

That being said, I have intentionally omitted most information regarding molecular biology, genetics, diagnostic methods, and other mostly molecular, biochemical, and technical topics connected with the discussed parasite. Connections of these areas to the thesis topic are loose and indirect, and I do not think it would be of interest to the potential reader. If it is, I can recommend using books "*Toxoplasma* molecular and cellular biology" by Ajioka, J. W., and D. Soldati (2007, Norfolk, UK: Horizon Bioscience) and "

Toxoplasma gondii: the model apicomplexan. Perspectives and methods" by Weiss, Louis M., and Kami Kim, eds. (2011, Elsevier) as starting points for further research.

While reading through piles of previous research, I came across various captivating facts just outside the scope of even these loosely connected areas. I have not included them in the main text of the thesis since they might cloud the intended message and impair fluency of the text. I did, however, included the most intriguing ones as footnotes at the end of each chapter, for they might be of interest to some readers and because I believe they help to settle the presented research into a richer context.

1.1 Aims of the Thesis

Previously, the studies concerning toxoplasmosis were, at least in our laboratory, conducted both on mice and men; later, the research focused mostly on the effects latent toxoplasmosis has on humans, which is what ultimately became my focus as well.

The main topic of my doctoral project was to study changes in reaction times, startle reactions, and prepulse modifications of startle reaction possibly caused by the presence of a protist *Toxoplasma gondii* in brains of infected subjects, and to further investigate connections between latent toxoplasmosis and schizophrenia. I have primarily focused on these questions:

1. What are the effects of latent toxoplasmosis on human psychological profile and performance?
2. Are there any effects of latent toxoplasmosis on startle reaction in humans?
3. Are there any effects of latent toxoplasmosis on prepulse inhibition of startle reaction in humans?
4. Are the possible effects in healthy individuals similar to those observed in schizophrenia patients?
5. Moreover, if the answer to the previous question is yes, how can it help us discover more about the toxoplasmosis – schizophrenia relation? This question is, indeed, more a topic for discussion than for the experimental part itself.

While this thesis can be but one shard to the mosaic of our understanding of the parasitic role in origin and onset of mental diseases, it shall bring us at least one step closer to the mastery of this complex, engrossing and medically valuable topic.

1.2 Footnotes

1 Usually, solely Josef Janků is stated as the first person to describe *T. gondii* infection in humans. Although I am somewhat restrained to steal part of the pioneering effort from a person of our nationality, a meticulously substantiated text of an American specialist on infectious diseases Jack S. Remington (Jack S. Remington, Jerome O. Klein, Christopher B. Wilson, 2011) nudged me to research further into the history. Apparently, Josef Janků wasn't sure about the exact nature of the parasite, while famous Romanian physician and microbiologist Constantin Levaditi presented Janků's finding to the Societé de biologi in Paris and suggested the parasite to be *Toxoplasma*. On the other hand, one of the world's foremost expert on *Toxoplasma,* an American scientist of Indian origin Jitender P. Dubey forgets Josef Janků altogether in his 2008's article commemorating a hundred years of *Toxoplasma* research (Jitender P. Dubey, 2008), so I think it is a good balance to present the actual history here, mentioning the share on the discovery of both Josef Janků and Constantin Levaditi.

2 Theoretical Background

Toxoplasma gondii and its effects on human behavior and mental health are rather than a topic in itself more of a red thread beginning somewhere in the realm of host-manipulation hypothesis and ending wrapped around a schizophrenic patient's bed, entwining together seemingly distant topics of infectious diseases and measurement tools for mental health diagnosis. With this in mind, I am piecing the thesis together from distinct chapters on all the main topics around this cotton's road.

The first stop of the journey brings general information on host-manipulation hypothesis, which will take us to *Toxoplasma gondii*'s interactions with warm-blooded hosts as a model for studying this phenomenon. Then, we will temporarily draw back from specific models to the general understanding of infectious diseases as causes for mental health issues, to focus a while later again on latent toxoplasmosis and its possible relation to schizophrenia and other health challenges.

I will cap the theoretical background for studied topics by a theoretical background for my chosen measurement methods that are a measurement of reaction times, startle reactions, start-go reactions, and prepulse inhibition of startle reaction.

I will be briefly talking about *Toxoplasma gondii* as an organism, and about schizophrenia as the mental illness most connected with our studies, but I will not cover these topics in depth since that would far exceed both scope and volume of this thesis.

2.1 The Art of Manipulation[1] (Manipulation Hypothesis in Parasite-Host Coevolution)

The idea of a change in host's behavior being adaptive for the parasite, causing it has been first suggested in Holmes' and Bethel's field-changing article (Bethel & Holmes, 1972) in the early 1970s. Up till then, changes in host's behavior were undoubtedly well known, but they were considered mere side-effects of infection – just like when sore joints, fever, headache and other symptoms of flu prevents you from doing your normal activities. They were

certainly not seen as something favorable for and "intentionally" (the word is here used strictly in an evolutionary sense, of course, not in the sense of a conscious intention of a particular pathogen) caused by the pathogen, or, in biological terms, adaptive for the pathogen. Reviews of such pathological changes affecting host behavior were presented for example by Holmes and Zohar (Holmes & Zohar, 1990), Thompson (Thompson, 1990), or Thompson and Kavaliers (Thompson & Kavaliers, 1994). The last article is especially interesting because it pays attention also to subclinical effects of parasitism shifting the host's behavior over a more extended period, an effect we have seen in our studies of *Toxoplasma*-human interactions.

Furthermore, the side-effects explanation is not the only one competing with the adaptive-for-parasite account for changes in the host's behavior. While an alternation toward an increased dispersal[2] of an infected animal could be seen as adaptive for the germ causing the infection (it can now infect more distant populations), it could also be advantageous for the animal's very own genes in at least two ways:

(1) it is going further from his relatives preventing them thus from infection,

(2) also, it might be displaying a type of "high-risk – high-gain strategy" using its very last bits of energy to increase its chances for reproduction by leaving a highly parasitized natal habitat.

Examples of the possible adaptive changes in the host's behavior implemented by the host himself scatter through scientific literature in various forms. Typical cases range from recollections of observed suspicious behavior of Harris's checkerspot butterfly (*Chlosyne harrisii*) larvae parasitized by a braconid wasp and following thought process by Arthur Shapiro (Shapiro, 1976) to an experimental demonstration of behavioral shifts in bumblebee (*Bombus terrestris*) workers parasitized by larvae of thick-headed flies (Conopid family). The parasitized bumblebees spend the night in the field rather than in the nest which, due to the colder environments, retards the parasite's ontogeny and lowers chances for successful development while also

enabling the bumblebees to work for the colony for a longer time (C. B. Müller & Schmid-Hempel, 1993).

Of course, the chance for finding the appropriate explanation for such behavior or even such expected behavior – since often the infected animals disperse less rather than more as is the case with, for example, the common chub (*Squalius cephalus*) from the carp family Cyprinidae infected by a duck mussel's (*Anodonta anatina*) larvae stage. Although higher dispersal rate of infected fish could be advantageous for the freshwater bivalve, the changes found in the host's behavior were toward less activity in both laboratory and field conditions. The parasitized fish displayed lower dispersal upstream in comparison with non-parasitized individuals (Horký, Douda, Maciak, Závorka, & Slavík, 2014) – thorough, rigorous experimental work is not always research-friendly, and for a long time, the scientific mainstream was quite unsure of whether there really is something as parasite-induced behavioral change adaptive for the perpetrator.

Let's look at the expected adaptiveness using a study (Chow & Mackauer, 1999) examining effects of parasitoid wasps of the Aphidiinae subfamily on a "green dolphin" pea aphid (*Acyrthosiphon pisum*), a model organism of the Aphididae family as well as common worldwide pest of forage crops such as pea (*Pisum sativum*), clover (*Trifolium*), alfalfa (*Medicago sativa*), or broad bean (*Vicia faba*). Earlier studies preceding this one suggested profound changes in the dispersal of parasitized aphids under the influence of various parasitoids. For example, Brodeur and McNeil (Brodeur & McNeil, 1989) found a difference in dispersion not only between potato aphids (*Macrosiphum euphorbiae*) parasitized and unparasitized by a parasitoid wasp (*Aphidius nigripes*), but also between those parasitized by diapausing (these left the host plant and mummified in concealed sites) and nondiapausing (these left the colony and mummified on upper surfaces of the leaves) parasitoids. The difference between the effects of parasitoids in a different physiological state would be indicative of for-the-parasite adaptive changes in host behavior.

Back to the pea aphids; in the study, Chow and Mackauer used five parasitoid species (*Aphidius ervi, A. pisivorus, Ephedrus californicus, Monoctonus paulensis,* and *Praon pequodorum*) of four different genera from within the Braconidae family, all with slightly different ontogeny and developmental rates. Should the change in host dispersal be controlled by the parasitoid larvae rather than by the host, the observed dispersion will be different for the individual wasp species. Also, for a behavioral change to be considered adaptive for the parasite, the four requirements defined by Poulin in his 1995 article (Robert Poulin, 1995) should apply:

1. **The complexity** of the change or preferably of the mechanism behind the change in host's behavior needs to be high enough to be suggestive of an organizing principle (e. g., selection) instead of mere chance.
2. **Purposiveness of the design** should resemble one that an engineer would come up if confronted with a similar task, again in an indication of a selection behind its development.[3]
3. **Convergence** in these traits should occur independently within different lineages under similar conditions to demonstrate it is not a product of chance.
4. **Fitness effects** on the parasite as measured usually by an effect on a specific function such as transmission is an essential criterion for assessment of the trait's adaptiveness.

Although the dispersal behavior of parasitized aphids differed a little bit – namely the ones parasitized by *E. californicus* often dropped from their plant and mummified on the ground unable to return to their colony while hosts of all of the other used species mummified directly on the bean plant –, the researchers found no convincing evidence the changes fulfilled any of Poulin's criteria for adaptiveness of the trait.

A finding like this is no exception. Poulin himself found almost no studies that would imply the existence of the adaptive parasitic manipulation, at least not at the time of his 1995 article. In fact, he found only 3 studies (Bethel & Holmes, 1973, 1977; Hindsbo, 1972) that he categorized as fulfilling

his complexity, purposiveness, and fitness criteria (all describing an acanthocephalan (parasite) – amphipod (host) interactions, and 1 digenea (parasite) – amphipod (host) study (Helluy, 1984) where he coded the latter two criteria as fulfilled and the complexity as "with some ambiguous cases". Paulin's conclusions spoke of the necessity to apply strict criteria and rigor in the research of parasitic modifications of host behavior (and well as in other fields of parasitology (R Poulin, 2018)). Although the beforementioned findings paint a somewhat skeptical picture of the field of adaptive changes, he stayed confident the adaptiveness is there, and future research will undoubtedly uncover it – and this approach stayed with Poulin up to these days. Fast forward 20 years, and he is still suggesting the proper ways of doing research in the area and finding possible biases in modern studies (Robert Poulin & Maure, 2015), but I will discuss this later in the Summary and Foreseeable Future Development part of the thesis.

Before the end of this subchapter, I would like to complicate the topic even further by mentioning that parasites can manipulate even such improbable phenomena as memetic characteristics of their hosts[4]. The parasitic manipulation also sometimes opens a new niche for various other organisms to use for their own purposes, so that the parasitic manipulation might, in the end, serve not to the parasite himself but to another species. All in all, there still lies many findings – as well as many mysteries especially in the area of manipulative mechanisms – and it would take many more pages to cover them all, but I will sharpen my attention closer to the one example of a manipulative parasite our laboratory is dealing for many years, *Toxoplasma gondii*.

2.2 Lord of the Parasites (*Toxoplasma gondii* as the Model Manipulator)

As previously mentioned, *Toxoplasma gondii* was first discovered at the beginning of the 20th century by a famous[5] French bacteriologist Charles Nicolle and his colleague, French parasitologist Louis Manceaux, and by the end of the 1920s, we already knew it as a human parasite. By now, the thematic search for the parasite on Web of Science counts over 22,000 publications from various research areas (see Figure 2) and although 2018 was

a little less plentiful than the previous year, a simple look at the numbers of articles being published each year (see Figure 3) suggests, the theme has not experienced its definitive peak, yet.

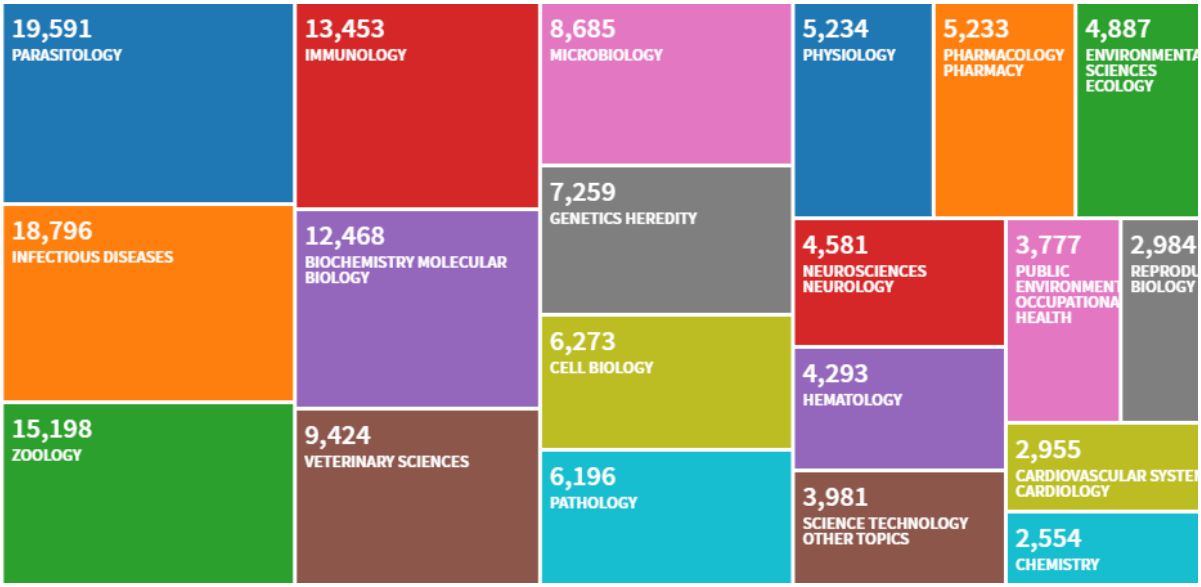

Figure 2 *Toxoplasma gondii* on WOS.
WOS-generated representation of research areas with a substantial share of work on *Toxoplasma gondii,* showing the first 20 categories. (http://apps.webofknowledge.com, accessed 24th June 2019.)

The importance of *Toxoplasma* research is quite apparent in human medicine, where it infiltrates fields as distant as immunology and psychiatry, the second of which will be discussed in subchapters 2.3 and 2.4 of this thesis, while other areas I will briefly review in part 0. However, there are other reasons for why this particular parasite enjoys such an interest of various researchers.

Right outside of the medical field lies the area of behavioral sciences and especially human psychology. Several laboratories are currently working on research concerning the effects of latent toxoplasmosis on human behavior outside the realm of psychopathology. This topic, too, will be discussed later in part 0.

Perhaps the key circumstance of *Toxoplasma* studies beyond the realm of human (and veterinary) medicine and behavioral sciences is its position in the phylogenetic tree of Alveolates together with non-parasitic groups of Ciliates, Dinoflagellates and several other smaller groups of eukaryotic organisms. 21st-century research seeks to uncover specific lineages within the apicomplexan parasites e.g., (Xavier, Santos, & Veríssimo, 2018) as well as within the broader group of Alveolates e. g., (Moore et al., 2008).

One of the areas of interest from which we can devise a robust model of apicomplexan evolution is the study of glycolytic enzymes. For example, a 1999 French study found two plant-like glycolytic enzymes glucose-6-phosphate isomerase (G6-PI) and enolase (Dzierszinski et al., 1999) in the *T. gondii* genome. Both of the enzymes are also present in another apicomplexan parasite, the malaria-causing *Plasmodium falciparum* (Kaslow & Hill, 1990; Read, Hicks, Sims, & Hyde, 1994), which suggested a possibility to place Apicomplexa closer to plants than to animals and other eukaryotic groups. However, other studies, such as that of Björn Canback (Canback, Andersson, & Kurland, 2002), brought slightly different results for enolase. Evolution aside, a more profound study of glycolytic genes might help us in other areas such as medicine. For example, the very enolase has been recently discussed as a potential target for cures of multiple vector-borne diseases, including malaria, Lyme disease, dengue, or even certain helminthiases (Mangalam P, Balasubramaniyan R, & Vasuki V, 2016).

Adding to the evolutionary perspective, there is the evolutionary route with felines into and then from South America[19] as well as complex life cycle worth studying for both parasitologists and evolutionary biologists, especially considering its vast differences (ecologically speaking) from its close relative another parasitic apicomplexan *Neospora caninum*[6] (Dubey, Carpenter, Speer, Topper, & Uggla, 1988).

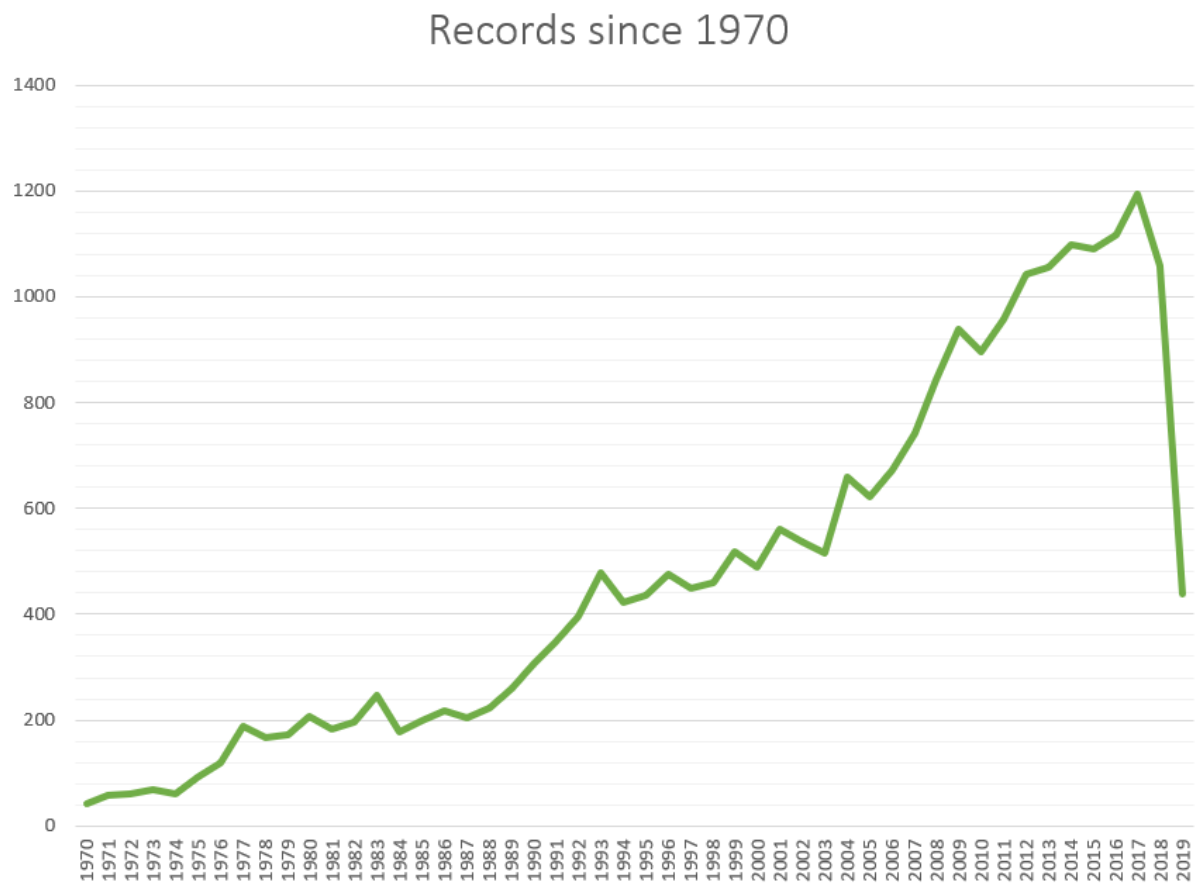

Figure 3 *Toxoplasma gondii* by Year.
Last 50 years of publications with *T. gondii* as their topic. Based on data from Web of Science, All Databases, http://apps.webofknowledge.com, accessed 24th June 2019. The drop in 2019 and partially in 2018 is due to the date the data were accessed and due to WOS having access to some publications many months after they are published. I have kept the unfinished year there to show over 400 publication were out and recorded in the database before half of the year was over.

2.2.1 What is *Toxoplasma gondii*

Now we know *T. gondii* is studied, but what do we know about it? As previously mentioned, it is a single-cell eukaryote intracellular parasite nested in the Sarcocystidae family in the phylum Apicomplexa (see Figure 4 *Toxoplasma*'s position in Alveolata kingdom.).

The phylum, much of which biology has been understood using *T. gondii* as a model organism (K. Kim & Weiss, 2004), comprises almost exclusively of obligately parasitic species with rare exceptions such as the genus *Nephromyces*, a mutualistic symbiont of so-called “sea grapes”, marine

tunicates of genus *Mogula* (Saffo, McCoy, Rieken, & Slamovits, 2010), and two relict apicomplexans (Votýpka, Modrý, Oborník, Šlapeta, & Lukeš, 2017) lineages of free-living organisms, the predatory Colpodellids, and photosynthetic Chromeridas – these are, as of now, not considered core apicomplexans.

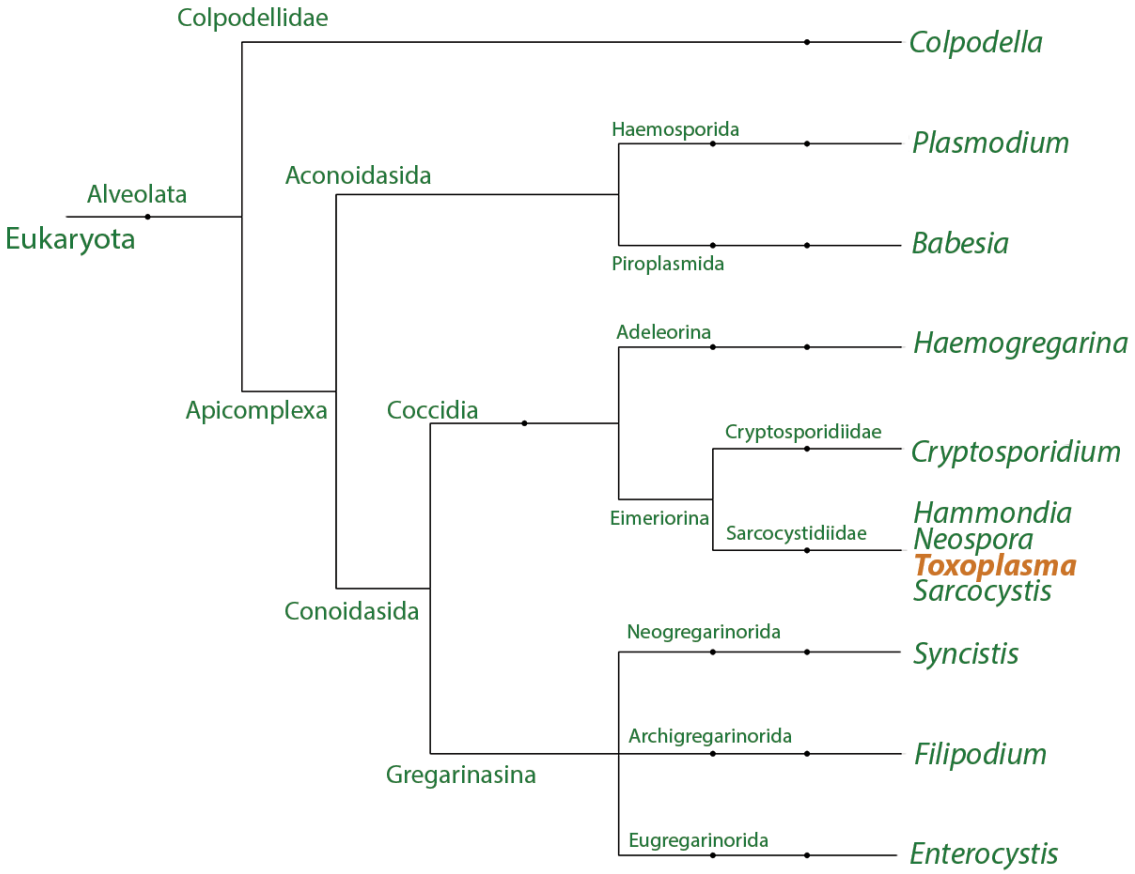

Figure 4 *Toxoplasma*'s position in Alveolata kingdom.
Note: The phylogenetic tree is based on a dataset from the NCBI taxonomy database ("Taxonomy browser (Sarcocystidae)," n.d.) and is presented for better idea of how is *Toxoplasma* related to other pathogens in the kingdom; however, DNA analyses bring new information about the actual evolution of protists on a daily basis, and the database is not necessarily up to date with the current knowledge – note, for example, *Cryptosporidium* species in the Eimeriorina suborder where it was supposed to belong toward the end of 20th century (Current & Garcia, 1991). Today's knowledge puts the order Cryptogregarinorida, in which it belongs, into the subclass Gregarinasina alongside orders Neogregarinorida, Archigregarinorida, and Eugregarinorida. The figure was generated using the iTOL software (Letunic & Bork, 2019), the typo is, however, upon me. I apologize for that.

In 1988, Apicomplexa were mapped in detail (naming all then-known species) in a two-volume monography (Levine, 2018) by a zoologist Norman Dion Levine of the University of Illinois. Current knowledge and characteristics were summarized (Votýpka et al., 2017) by the Czech team consisting of Jan Votýpka, David Modrý, Miroslav Oborník, Jan Šlapeta, and Julius Lukeš in the Handbook of Protists (Archibald, Simpson, & Slamovits, 2017). Classification, basic characteristics, and some the representatives of the phylum are given in Table 1 Classification and characteristics of Apicomplexa. since many are known as important parasites of animals and men. Although such a table would not be probably necessary for the presented thesis, I wanted to include the digest of the phylum not just because of the medical and economic importance of the listed organisms, but also because the research is rapidly changing our understanding of the group, and some of the information (such as the newest recognized phylogeny and existence of non-parasitic genera) might be new and of use for the reader.

Phylum: **Apicomplexa** Levine, 1970 emend. Adl et al., 2005		
Banana-shaped single-cell organisms with a highly polarized cell, a derived non-photosynthetic plastid called apicoplast, subpellicular microtubules, and an apical complex, which usually comprises of a conoid, one or more microtubular polar rings, and unique secretory organelles (rhoptries and micronemes) (Morrissette & Sibley, 2002). Note: the characteristics may be present only in certain stages of a life cycle or might be, in rare cases, omitted altogether.		
	Core apicomplexans (obligatory parasites of various invertebrate and vertebrate species including humans and domestic animals)	
		Incertae sedis **Agamococcidiorida** Levine 1979
		Parasitic genera of marine invertebrates (Caribbean scleractinian corals (Upton & Peters, 1986) and polychaetes (LEANDER & RAMEY, 2006)).
		Incertae sedis **Protococcidiorida** Kheisin 1956
		Poorly understood phylum of the basal lineage of apicomplexan parasites of invertebrates. Lacking merogony, with gamogony and

				sporogony, are extracellular. Taxa within the phylum lack DNA sequence information. Biology of several species such as *Eleutheroschizon duboscqi* (Valigurová, Paskerova, Diakin, Kováčiková, & Simdyanov, 2015) and their interactions with host closely studied in the 2010s, while others such as *Sawayella* are still mostly unknown.
				Incertae sedis various genera such as **Aggregata** Frenzel 1885, **Christalloidophora** Dehorne, 1934, **Dobellia** Ikeda, 1914, **Echinococcidium** Porchet 1978, **Globidiellum** Brumpt 1913, **Joyeuxella** Brasil 1902, **Rhabdospora** Laguesse 1906, **Spermatobium** Eisen 1895, **Spiriopsis** Arvy and Peters 1972, **Spirogregarina** Wood and Herman 1943, **Toxocystis** Leger and Duboscq 1910, and **Trophosphaera** Le Calvez 1939.
				Many of the listed taxa still lack DNA sequence information to enable proper positioning in a phylogenetic tree.
				Subphylum: **Conoidasida** Levine, 1988
				As of 2019, not considered monophyletic, with artificial and unclear subdivisions. Characteristics: No cilia except for microgametes. Closed conoid in most (or all) motile stages. Complete apical complex. Motility usually via gliding
				Gregarinasina, Dufour, 1928
				Mature gamonts often with extracellular development, often syzygy of gamonts producing similar numbers of microgametes and macrogametes in the gametocyst; syngamy leads to gametocyst with oocysts containing sporozoites. Merogony only in some species. *Incertae sedis* Gregarinisida: **Digyalum** Koura et al. 1990, **Exoschizon** Hukui 1939.
				Archigregarinorida Grassé 1953
				Parasites of marine invertebrates such as *Scolelepis squamata* (Annelida), *Themiste pyroides* (Sipuncula), *Phascolosoma agassizii* (Sipuncula), *Siphonosoma cumanense* (Sipuncula), or *Aspidosiphon clavatus* (Sipuncula) (Rueckert

				& Leander, 2009). Aseptate trophozoites. Oocysts contain 4-8 sporozoites.
				Eugregarinorida Leger 1900
				Diverse taxon of both septate and aseptate gregarines. Parasites of marine and terrestrial invertebrates, most with a single-host life-cycles. Oocysts with 8 sporozoites. Some species considered as biological pest control agents (R. B. Lopes & Alves, 2005; Rueckert & Devetak, 2017).
				Neogregarinorida Grassé 1953
				The taxon contains both parasites of economically important insects such as *Apis mellifera* and *Bombus terrestris* (Plischuk, Meeus, Smagghe, & Lange, 2011), and parasites useful in biological pest control. For example, *Mattesia grandis* has been mass-produced and used against cotton-eating boll weevils (*Anthonomus grandis*) since the 1960s (McLaughlin, 1966).
				Cryptogregarinorida Cavalier-Smith, 2014, emend. Adl et al. 2019
				Cryptosporidium parvum and *C. parvum*-like organisms were reported to infect at least 155 species of mammals including humans. Many of its species are zoonotic, primarily infecting non-human animals but capable of infecting humans. The primary source of outbreaks is contaminated water, in which *Cryptosporidium* can remain infective for months. The infection causes watery diarrhea and is the most severe in immunocompromised individuals (Fayer, 2004). While studying its epidemiology and biology, it is often compared with another cause of diarrhea in humans, *Giardia* (Cacciò, Thompson, McLauchlin, & Smith, 2005). It has been found that *Cryptosporidium* is, in comparison with *Giardia*, more resistant to treatments of drinking water with chlorine dioxide, ozone (Korich, Mead, Madore, Sinclair, & Sterling2,

				1990) and UV light, with the UV being the most effective for inactivating both (Adeyemo, Singh, Reddy, Bux, & Stenström, 2019). Effective water treatment is needed because *Cryptosporidium* is highly infective, with a low dose of oocysts capable of causing infection in healthy adults (Chappell, 1995).
			Coccidia, Leuckart, 1879	
			Usually, immotile zygote, no syzygy, mature gametes develop intracellularly over 3500 named species of parasites of both vertebrates and invertebrates.	
				Adeleorina Léger 1911
				Parasites of invertebrates (mostly Arthropoda and Oligochaeta) and vertebrates (fish, frogs, snakes, and others).
				Eimeriorina Léger 1911
				Contrains many important parasites of humans and domestic animals as well as of other vertebrates and invertebrates. Includes genera such as *Eimeria*, a parasite of poultry, cattle, or ruminants, *Sarcocystis*, species of which parasitize on reptiles, birds and mainly mammals with rare infections in humans, as well as related genera of *Hammondia, Neospora*, and *Toxoplasma*.
			Blastogregarines Chatton and Villeneuve 1936, emend. Simdyanov et al. 2018	
			Relatively poorly studied parasites of bristle worms (Polychaeta) with characteristics resembling both Coccidia and Gregarinasina; might be even a separate class of Apicomplexa (Simdyanov et al., 2018).	
		Subphylum: **Aconoidasida** Mehlhorn, Peters and Haberkorn, 1980		
		Heteroxenous parasites including some of the most dangerous single-cell parasites of humans. In asexual motile stages, apical complex lacks conoid. Ookinetes sometimes with conoid. Microgametes and macrogametes form independently.		

			Class: **Haemosporidia** Danielewsky, 1885
			The class includes parasites such as *Haemoproteus*, which affects reproduction and survival in wild birds (Puente et al., 2010), *Hepatocystis*, a parasite of bats (Olival, Stiner, & Perkins, 2007; Schaer et al., 2017), squirrels (Canning, Sinden, Landau, & Miltgen, 1976), primates (Thurber et al., 2013), and hippopotami (Garnham, 1958), *Leucocytozoon* which uses black flies (of the Simuliidae family, which also spread *Onchocerca volvulus*, a nematode causing river blindness) as its vector and parasitize birds including human-bred species (Siccardi, Rutherford, & Derieux, 1974), a bats' parasite *Nycteria* (Rosskopf et al., 2019; Schaer et al., 2015), and most importantly one of the greatest single-cell killers (Cowman, Healer, Marapana, & Marsh, 2016), blood parasites from the genus *Plasmodium* (De Niz et al., 2017). Despite the effort and finances going into the fight with the parasite, as of 2015, there were still around 214 million cases of malaria (88 % in WHO African Region, 10 % in WHO South-East Asia Region, and 2 % in WHO Eastern Mediterranean Region) and it was killing around 300,000 children under 5 years of age per year (Cibulskis et al., 2016).
			Class: **Piroplasmida** Wenyon, 1926
			Parent class of parasite of horse and cattle *Theileria* (Dolan, 1989) as well as of the genus *Babesia* (described in 1888 by a Romanian bacteriologist Victor Babeş (Babes, 1888)), which infects many wild and domestic animals as well as humans (especially the species using *Ixodes* tick as its vector) (Dantas-Torres, Alves, & Uilenberg, 2017).
			Class: **Nephromycida** Cavalier-Smith 1993, emend. Adl et al. 2019
			An example par excellence of scientific development in a century and a half of protozoan research: *Nephromyces,* the endosymbiotic parasite of Molgulid tunicates (Saffo et al., 2010),

			which became an unexpected exception in the almost entirely parasitic phylum of Apicomplexa, was upon its discovery believed to be a parasitic chytrid fungus (Giard, 1888). Now it is also believed to be one of a very few apicomplexans with its own hereditarily transmitted bacterial symbiont (Saffo, 1990). After being identified as an apicomplexan and spending years hidden among those of *incertae sedis* (Adl et al., 2012), it is now classified as aconoidasid of the class Nephromycida together with parasitic *Cardiosporidium* observed in ascidian tunicate *Ciona intestinalis* (Scippa, Ciancio, & Vincentiis, 2000).
	Relict apicomplexans (autotrophic, heterotrophic, and sometimes mixotrophic alveolates related to both core apicomplexans and dinoflagellate algae)		
		Order: **Colpodellida** Cavalier-Smith 1993, emend. Adl et al. 2005, 2019	
		Mostly free-living predatory and photosynthetic "proto-apicomplexan" alveolates with apical complex and rostrum; biciliate, with microspores, mitochondrion with tubular cristae, cortical alveoli flattened; usually with complex plastids with four membranes. Together with core alveolates related to photosynthetic dinoflagellates – core apicomplexans and some colpodellids probably lost photosynthesis in separate evolutionary events, as suggested by Oborník et al. 2012 (Oborník et al., 2012).	
			Vitrellaceae Oborník & Lukeš 2012
			Photosynthetic immotile vegetative cells with laminated cell walls, motile biciliate zoospores, and immotile autospores. Plastids with chlorophyll a, violaxanthin, vaucheria xanthin, and β-carotene. Capable of producing zoosporangium in a budding-like process hypothesized to represent the ancestral form of reproduction in Apicomplexa (Füssy, Masařová, Kručinská, Esson, & Oborník, 2017).
			Colpodellaceae Adl et al. 2019

			Ciliated predators of other protists. Case studies of patients with symptoms reminiscent of either core-apicomplexan *Babesia* infection or even wholly unrelated tick-borne encephalitic virus revealed possible infection with parasitic strains of *Colpodella* spp., suggesting both the existence of intracellular parasites within this assumedly free-living taxon and possible transmission through a tick vector *Ixodes persulcatus* (Jiang et al., 2018).
			Chromeraceae Oborník & Lukeš 2012
			Photosynthetic organisms with immotile coccoid cells reproducing by binary division. Plastids with chlorophyll a, violaxanthin, osifucoxanthin, and β-carotene. Previously considered a mutualistic symbiont of corals, further studies of the transcriptomic response of coral larvae to *Chromera* infection suggest it might be rather a parasite, commensal, or an accidental bystander (Mohamed et al., 2018).
			Alphamonaceae Adl et al. 2019
			Non-photosynthetic ciliated predators of other protists.

Table 1 Classification and characteristics of Apicomplexa.
It is adapted and summarized from Apicomplexa (Votýpka et al., 2017) in Handbook of the Protists (Archibald et al., 2017), a Journal of Eukaryotic Microbiology article (Adl et al., 2018) (information without citations). I have kept the Handbook's division into "core" and "relic" apicomplexans, since it shows phylogenetic relations of the phylum to its' probable closest relative, colpodellids, but I had adjusted it based on the system presented in the recent 2019 article and supplemented with specific mostly ecological characteristics from individual articles (citations included with the respective supplemented information).

Morphologically, *T. gondii*'s cell is about 3.5 to 7 µ long and 1.2 to 4.5 µ long and has a crescentic shape both in fresh and fixed state. The individual strains are indistinguishable on a morphological basis. (HARBOE & ERICHSEN, 1955) There is a conoid with a polar ring and an interior cone in the anterior end, with twelve to sixteen radial "ribs" running from the conoid toward the middle of the cell where the nucleus is located (Ludvik, 1958). The anterior surface of the nucleus hosts Golgi apparatus and long tubes of the

endoplasmic reticulum, while in the posterior part of the cell we find mitochondria (Ludvik, 1958) indispensable for intracellular growth of the parasite (MacRae et al., 2012). The cell uses three forms of motility (circular gliding, upright twirling, and helical rotation) which are all dependent on actinomyosin motors; the gliding form of motion is probably necessary for penetration of the host cell and might also be important in dissemination (Håkansson, Morisaki, Heuser, & David Sibley, 1999). More detailed information of *Toxoplasma gondii* morphology can be found in Hogan et al. article containing multiple photographs of the parasitic cells (Hogan, Yoneda, Feeney, Zweigart, & Lewis, 1960).

Within the host organisms, it forms either intracellular pseudocysts (in the acute phase) or intracellular cysts (in the chronic phase) containing multiple parasites: The pseudocysts are contained in host's own membrane and usually comprise of up to 20 individual parasites, while the true cysts are confined in highly elastic membrane and can, over months, develop to host thousands of individuals (Lainson, 1958).

The life cycle of *T. gondii* is as follows: A felid host eats an infected animal containing cysts of the parasite. The cysts rupture after being exposed to gastric enzymes and the freed bradyzoites transform into trophozoites undergo schizogony in intestinal cells of the definitive host; the schizogony also follows after ingestion of sporulated oocysts from the external environment. Schizonts continue into gamogony again in the intestinal cells, where the male micro and female macro gametes form oocysts. These are excreted into the external environment where the sporogeny proceeds after which a warm-blooded animal consumes sporulated oocysts with 8 sporozoites divided into two sporoblasts. Sporozoites infect intestinal cells of the non-felid, tachyzoites form and invade monocytes where they undergo endodyogeny. The invaded monocytes are disseminated in tissues through the bloodstream and turn into parasitic cysts containing thousands of bradyzoites. If the infected animal is preyed upon by other non-felids, the cycle repeats similarly as upon ingesting an oocyst, while after consummation by a felid, the whole process repeats (Frenkel, Dubey, & Miller, 1970; Robert-Gangneux & Darde, 2012).

Beside house cat, *T. gondii* was found to undergo sexual reproduction in african wild cat (*Felis lybica)* (Polomoshnov, 1979), Amur leopard cat *(Felis euptilurus)* (Lukešová & Literák, 1998), Asian leopard (*Felis bengalensis*) (Janitschke & Werner, 1972) (N. L. Miller, Frenkel, & Dubey, 1972), bobcat (*Lynx rufus*) (N. L. Miller et al., 1972) (Marchiondo, Duszynski, & Maupin, 1976), cheetah (*Acinonyx jubatus*) (Marchiondo et al., 1976) (Polomoshnov, 1979), cougar (*Felis concolor*) (Marchiondo et al., 1976; N. L. Miller et al., 1972), cougar (*Felis concolor vancouverensis*) (Aramini, Stephen, & Dubey, 1998), Geoffroy's cat (*Oncifelis geoffroyi*) (Pizzi, Rico, & Pessat, 1978) (Lukešová & Literák, 1998), Iriomote cat (*Felis iriomotensis*) (Akuzawa, Mochizuki, & Yasuda, 1987), jaguarundi (*Felis yagouaroundi*) (Ruiz, Reed, Frenkel, Johnson, & Jewell, 1972), lion (*Panthera leo*) (Ocholi, Kalejaiye, & Okewole, 1989; Polomoshnov, 1979), mountain lion (*Felis concolar*) (Marchiondo et al., 1976), ocelot (*Felis pardalis*) (Patton, Rabinowitz, Randolph, & Johnson, 1986; Ruiz et al., 1972), Pallas cat (*Felis manul*) (Basso Et Al., 2005; J. P. Dubey, Gendron-Fitzpatrick, Lenhard, & Bowman, 1988; Polomoshnov, 1979), pampas cat (*Oncifelis colocolo*) (Pizzi et al., 1978), Siberian tiger (*Panthera tigris altaica)* (Dorny & Fransen, 1989), wild cat (*Felis silvestris)* (Lukešová & Literák, 1998). The list is adapted from Dubey's article on the history of the discovery of *Toxoplasma gondii* life-cycle (Dubey, 2009a). In feces sample of a leopard (*Panthera pardus*), *Toxoplasma*-like parasite was found; however, it hasn't be confirmed (Patton & Rabinowitz, 1994). *Toxoplasma*-seropositivity is routinely found in zoo felids (de Camps, Dubey, & Saville, 2008; J. C. R. Silva, Ogassawara, Marvulo, Ferreira-Neto, & Dubey, 2001) as well as in the wild ones (Cañón-Franco et al., 2013; A. P. Lopes, Sargo, Rodrigues, & Cardoso, 2011), but some species are still awaiting confirmation of shedding oocysts in their feces.

Prevalence of toxoplasmosis in domestic cats is often said to be between 30 and 40 percent worldwide (e. g., Elmore et al., 2010), due to extreme variability in local prevalences, Dubey argues against such generalizations (Frenkel, Dubey, & Beattie, 2006). In other felid species, prevalence differs across populations, species, geological locations and between wild animals and those in captivity.

The parasite is truly widespread: from Alaska to Australia (D. E. Hill, Chirukandoth, & Dubey, 2005) – although south-north gradient is visible in animals with lower numbers of infected species in the north (Jokelainen et al., 2010), from aardvark (*Orycteropus afer*) to various zebra species (Bártová et al., 2018), from *Accipiter gentilis* (northern goshawk) to *Tyto alba* (barn owl): indeed, we may even find it where eagle owls dare, since *Bubo bubo* seems to be the species with the highest prevalence of anti-*Toxoplasma* antibodies (Cabezón et al., 2011). Actually, the ability to infect such a wide range on warm-blooded vertebrates is quite unique among parasitic species in general as well as within the family of apicomplexans, and as such, it could not stay unnoticed by researchers. It led some biologists to believe that not only was the wide range of intermediate host a driving force of *Toxoplasma gondii* evolution especially in the gene families connected with invading hosts' cells and escaping their immune mechanisms, but also that this ability might perhaps enable it to widen the range of its final hosts as well – and that, perhaps, we might be overseeing something by repeating the "cats only" mantra (Boothroyd, 2009).

Apart for this speculation and changing environmental factors which shifts routes of infection to previously scarcely encountered sources such as water organisms and even contaminated water itself (Dubey, 2004), it seems that we are well acquainted with the rough outlines of *Toxoplasma*'s life cycles. The predatory route of infection has been confirmed for example by comparison of carnivorous animals from the same environment who, however, consume varying amounts of mammal meat: When comparing adult spotted hyenas (*Crocuta Crocuta*), striped hyenas (*Hyaena hyaena*) and lions (*Panthera leo*) (who gain most of their energy from meat), young spotted hyenas (being predominantly fed by mother's milk, and insectivorous bat-eared foxes (*Otocyon megalotis*), the seroprevalence of anti-*Toxoplasma* antibodies was, as expected, most frequent in adult hyenas and lions, less frequent in young hyenas, and not found at all in the foxes (although the n=4 for foxes in this particular experiment is far from impressive) (Ferreira et al., 2019).

In the zoo environment, particular species which can be blamed for being the predominant source of infection of the captive felids are still studied. For example in Sydney, the common brushtail possums (*Trichosurus vulpecula*) was suspected, but the study 2008 study brought surprising results suggesting that carnivory is not a particularly effective way of transmission in zoo environment and that the suspected culprit pose minimal threat if any at all, at least where *T. gondii* transmission is concerned (N. J. Hill, Dubey, Vogelnest, Power, & Deane, 2008).

As for the genetic diversity of *T. gondii*, strains I-III are usually encountered in human infections, with the type II being responsible for most diseases in Europe and North America (Sibley, Khan, Ajioka, & Rosenthal, 2009), however recent genetic analyses revealed fourth lineage in North America (Khan et al., 2011), and analyses of the strains that are considered "atypical" or "exotic" suggest more complex population structure originating from a mix of clonal and sexual propagation (Ajzenberg et al., 2004). Genetical diversity is the highest in South America (Dubey et al., 2007), with more distant genetic lineages causing different clinical symptoms in infected hosts (Khan et al., 2006). A 2012 study compared genotypes of 956 previously described and genotyped strains of *T. gondii* and found 138 unique genotypes in 15 haplogroups falling into 6 major clades. Based on the analysis of gene flow, the researchers hypothesized that a small number of ancestral lineages started off current diversity through limited admixture (Su et al., 2012).

We know what *Toxoplasma* is, to what family it belongs, where is it found, and how is it transmitted, now why is that important? Because it is not nearly as harmless as previously thought.

2.2.2 *Toxoplasma gondii* in Warm Blooded Vertebrae

Toxoplasma in cats. Starting with the final host, infections in felids including domestic cats are usually asymptomatic, though sometimes an acute infection can manifest in cats, especially in cases of congenital infection (Dubey & Carpenter, 1993b, 1993a). Cats with FIV can even encounter similar problems to human AIDS patients –severe generalized toxoplasmosis

(Davidson, Rottman, English, Lappin, & Tompkinst, 1993), although such cases (Heidel et al., 1990) seem to be rare.

The "usually asymptomatic" rule has, however, one grave exception concerning an endangered species, the Pallas cat (*Otocolobus manul*), which is highly susceptible to fatal toxoplasmosis infection in newborns. The mechanisms of pathogenicity are not yet known, except for the ecological aspect of the situation: While in the wild, Pallas cat is hardly ever infected with toxoplasmosis, the risk of infection raises quickly in captivity due to a high prevalence of toxoplasmosis in felids kept in zoos (Basso et al., 2005; M. Brown, Lappin, Brown, Munkhtsog, & Swanson, 2005). The problem lies, of course, in that the number of feasible ecosystems for the Pallas cats to live in is rapidly diminishing, and we thus need to breed it in captivity to avoid complete extinction of the species (Ross, 2009).

Death of offspring exposed to toxoplasmosis was also observed in sand cats (*Felis margarita*) (Dubey et al., 2010; Pas & Dubey, 2008), but further research is needed for confirmation of the same mechanisms affecting the populations of Pallas and sand cats in captivity.

Toxoplasma in intermediate hosts excluding humans. In other animals which can serve as intermediate hosts, the infections are of interest for three main reasons: (1) transmission to humans and clinical symptoms in economically important food animals, (2) clinical symptoms in human pets, wild animals and endangered species, and (3) manipulative effects of the parasite on its host.

1) Transmission to humans and clinical effects in food animals. Congenital toxoplasmosis causes high losses in domestic animals such as sheep (Buxton et al., 2007), goats (Dubey, 1981; Munday & Mason, 1979) or pigs (Dubey, 2009b). Economical damage arises mainly due to spontaneous abortions and stillborns in gravid animals and high percentage of neonatal death in the offspring, while from the epidemiological point of view, possible transmission to human population must be considered not only through meat, but perhaps surprisingly also with milk of the infected goats (Riemann, Meyer,

Theis, Kelso, & Behymer, 1975; Sacks, Roberto, & Brooks, 1982; Skinner, Timperley, Wightman, Chatterton, & Ho-Yen, 1990).

In cattle, toxoplasmosis doesn't plan an important role in abortions or stillborns, it seems to be quickly cleared from bovine tissues, and milk of the infected cows doesn't seem to pose a risk for human consumers (Dubey, 1986). The meat, however, is somewhat problematic. Although it is hard to find infective cysts in edible tissues (Dubey, 1983), an increased risk related to consumption of undercooked (medium rare) beef was, however, found in several studies (e. g., A. J. C. Cook, 2000; MacKnight & Robinson, 1992) – and not found in others including a two-year-long prospective study on 37,000 women in Norway, which found no significant increase of risk of infection with *T. gondii* after ingestion of raw beef (Kapperud et al., 1997). There are reports of humans being infected by undercooked beef; however, it is not always clear whether a piece of added pork or other contaminant was not, in fact, responsible for the infection (Kean, Kimball, & Christenson, 1969).

Symptoms of avian toxoplasmosis differ with avian species and dosage of infective oocysts. While experimental pigeons developed severe symptoms when inoculated by 500 infective oocysts (at 50, no symptoms besides the presence of the parasite in tissues are manifested), experimental hens infected with 5000 oocysts showed no symptoms. A group infected with 50,000 oocysts had fewer eggs in comparison with non-infected controls and showed increased mortality in embryonated eggs (Biancifiori, Rondini, Grelloni, & Frescura, 1986). Case reports and experiments with turkeys show that the birds only scarcely manifest symptoms of acute toxoplasmosis (Dubey et al., 1993), although rare reported cases of fatal toxoplasmosis exist (Quiet, Dubey, Luttrell, & Davidson, 1995). Risk of *Toxoplasma* infection arises especially with consumption of poultry from organic farms; the prevalence of toxoplasmosis in chicken (Dubey et al., 2004; Guo et al., 2015) and ducks and geese (Maksimov et al., 2011) is much lower in animals raised exclusively indoors.

Among other sources of infection, *T. gondii* was isolated from horse tissues (Al-Khalidi & Dubey, 1979) which, of course, pose a risk, especially in

areas with increased consumption of horse meat. Undercooked venison (Sacks, Delgado, Lobel, & Parker, 1983) and game in general (A. J. C. Cook, 2000) has been linked with increased risks of toxoplasmosis, and data from the Czech republic suggest the seroprevalence in game to be around 15 % for wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*) (Karel Hejlíček, Literák, & Nezval, 1997) – the prevalence in fallow deer was, actually, 100 % in the cited study, however, only three specimens of this species were tested. Brown hares (*Lepus europaeus*) are quite susceptible to toxoplasmosis which is often fatal (Sedlák, Literák, Faldyna, Toman, & Benák, 2000), biological prevalence has been, nonetheless found to be about 4 % (Karel Hejlíček et al., 1997) in the Czech republic suggesting that transmission through meat is indeed possible, albeit infrequently due to low prevalence. This is in contrast to the situation with rabbits, or specifically with the domestic rabbit (*Oryctolagus cuniculus*), in which severe manifestation of acute toxoplasmosis is existent (Dubey, Brown, Carpenter, & Moore, 1992), but the prevalence is really high: in the Czech Republic, a study from South Bohemia reported seropositive rabbits in 36 of 36 home-bred flocks, the prevalence in 366 rabbits was 53 % (K. Hejlíček & Literák, 1994). Domestic breeding of rabbits thus poses, at least in the Czech Republic, high risk of toxoplasmosis.

Due to the presence of *T. gondii* in water environments, infection with *Toxoplasma* can be found in coypu (*Myocastor coypus*) (Literák, Rychlík, Svobodová, & Pospíšil, 1998), a study from Italy found prevalence 59.4 % in local populations of wetlands in Central Italy (Nardoni, Angelici, Mugnaini, & Mancianti, 2011). Since nutrias (coypus) are, at least in Europe, now not considered as prominent sources of meat, risk of infection from this source is not too high. However, presence of *T. gondii* in both freshwater and saltwater bodies throughout the world means increased risk of infection not only from fish and mussels (Aksoy et al., 2014; Massie, Ware, Villegas, & Black, 2010; M. A. Miller et al., 2008; Palos Ladeiro, Bigot-Clivot, Aubert, Villena, & Geffard, 2015; Putignani et al., 2011; Zhang et al., 2014) but from the water itself (ARAMINI et al., 1999; Bahia-Oliveira et al., 2003; Isaac-Renton et al., 1998; J. L. Jones & Dubey, 2010; Sroka, Wójcik-Fatla, & Dutkiewicz, 2006).

Besides the efforts to reduce transmission among food animals, which could be done for example through vaccination of farm cats (Mateus-Pinilla, Dubey, Choromanski, & Weigel, 1999), researchers are looking into, for example, meal preparation in a human environment with surprising findings such as that not all the means of heat preparations are not created equal. As presented on the 2018 conference "XXIV. jednodenní konference (Konzultační den) "Problémy toxoplasmózy"" (XXIV Single-Day Conference "Problems of Toxoplasmosis") and published in a proceedings from another conference (Koudela, Dámek, & Kameník, 2018), Czech experiments with sous-vide preparation of pork meat shown that tissue cysts of *T. gondii* survive 24 hours of sous-vide cooking at 50, 53 a 56 °C, and 12 hours of sous-vide cooking at 60 °C.

This is in contrast with an experiment with slow cooking without the utilization of the sous-vide vacuum method, where the *Toxoplasma* cysts showed relatively low resistance to heat in comparison to another human parasite transmitted through raw pork meat, *Trichinella spiralis* (Purslow, 2016). The temperatures over 50 °C were shown to almost zero the risks of transmission if used for several minutes (a specific number of minutes depends on specific temperature) and even at 49 °C, 24 minutes of cooking was enough: of 45 mice inoculated with samples of heat-treated infected meat, 0 turned *T. gondii*-positive (Dubey, Kotula, Sharar, Andrews, & Lindsay, 1990).

Of course, pork products prepared without heat pose the highest risks of transmission. *T. gondii* cysts are very resistant toward changes in pH levels, they are, however, susceptible to salt and even more so to nitrate-enriched curing salt; low sodium fermented pork product pose, thus, the highest risk of transmission (Pott et al., 2013).

2) Toxoplasmosis in pets and wild animals. Beside rare manifestation of acute infection in cats, *T. gondii* causes occasional severe to lethal infections in dogs (M. Brown et al., 2005). In fact, toxoplasmosis in a dog was one of the first observed cases of the disease in animals, if not the very first (Mello, 1910). According to most studies, acute toxoplasmosis with

symptoms usually only occur in puppies (Jacobs, Melton, & Cook, 1955) and immunosuppressed animals (Webb et al., 2005) – as shown, for example, on dogs dying from toxoplasmosis following a renal transplant (Bernsteen, Gregory, Aronson, Lirtzman, & Brummer, 1999), although cases of severe disease in previously healthy adult animals are not unheard of (Pimenta, Piza, Cardoso, & Dubey, 1993).

Prevalence of toxoplasmosis in dogs varies in studies from 3.98 % in a recent UK study on dogs with suspected meningoencephalitis (Coelho et al., 2019) through 11.1 % and 20.56 % in mainland (Gao, Ding, Lamberton, & Lu, 2016) and Southwest (Li et al., 2015) China respectively or 23 % in Sweden (Uggla, Mattson, & Juntti, 1990) to 25.9 % or even 39.3 % in Czech dogs (Sedlak & Bartova, 2012) and Czech and Slovak army dogs (Hejlícek, Literák, & Lhoták, 1995) respectively. There are indications that toxoplasmosis in dogs play a role in upkeeping the cycle of transmissions to human hosts (Jacobs, 1957), and in turn, wild canids and felids probably keep the cycle going between wild and pet animals (Otranto et al., 2015).

Studies on toxoplasmosis in small pet mammals are rare, however, toxoplasmosis can infect *Agapornis* (Cooper, Šlapeta, Donahoe, & Phalen, 2015) or other pet birds (Cong et al., 2014), often with serious (up to deadly) consequences (Parenti, Sola, Turilli, & Corazzola, 1986), chinchillas (*Chinchilla lanigera*), in which the acute infection seems to be particularly severe (R. A. McAllister, 1964) (Rakich, Dubey, & Contarino, 1992), degu (*Octodon degu*) (Christen & Thiermann, 1953), ferrets (*Mustela nigripes* (Burns, Williams, O'Toole, & Dubey, 2003), *Mustela putorius furo* (Thornton & Cook, 1986)), various foxes (Dubey & Pas, 2008; Dubey, Storandt, Kwok, Thulliez, & Kazacos, 1999; Prestrud et al., 2007, 2008)guinea pigs (*Cavia porcellus*) (de Rodaniche & de Pinzon, 1949), hamsters (*Mesocricetus auratus* in case of the cited study, but applicable to other species) (Pavesio, Chiappino, Gormley, Setzer, & Nichols, 1995), hedgehogs (*Erinaceus europaeus* (Orlandella, Alosi, Campagna, Ilacqua, & Coppola, 1972) (Hofmannová & Juránková, 2019), *Erinaceus roumanicus* (Hofmannová & Juránková, 2019)), as well as in food animals (as mentioned in previous sections) and mice and

rats, where most of the studies on manipulative effects of the parasite were conducted.

Many species of wild animals are susceptible to toxoplasmosis (Wendte, Gibson, & Grigg, 2011); among the most threatened are wallabies (*Macropus rufogriseus*) (Basso et al., 2007) and other marsupials (Attwood, Woolley, & Rickard, 1975; Canfield, Hartley, & Dubey, 1990; Pan et al., 2012; Parameswaran, O'Handley, Grigg, Wayne, & Thompson, 2009; Parameswaran et al., 2010) (although a thorough 2016 study suggested there is yet not enough evidence to conclude, whether toxoplasmosis represents a threat to the conservation efforts related to Australian marsupials (Hillman, Lymbery, & Thompson, 2016)) as well as various marine mammals (Dubey et al., 2009; Mikaelian, Boisclair, Dubey, Kennedy, & Martineau, 2000; Verma et al., 2018), and we find it in many other wild and domestic species of mammals (Costa et al., 2012; Gauss et al., 2005; Karel Hejlíček et al., 1997; Kapperud, 1978) and birds (Dubey, 2002) species all over the world. The problem is, of course, that – just as with many other diseases (Frölich, Thiede, Kozikowski, & Jakob, 2002) – wild populations serve as reservoirs of the infection for livestock (Gortázar, Ferroglio, Höfle, Frölich, & Vicente, 2007) and humans (Meng & Lindsay, 2009). This, together with clinical syndromes in our food animals, pets and humans as well, leads to experiments with various kinds of vaccination against toxoplasmosis and its effects (Faridnia, Daryani, Sarvi, Sharif, & Kalani, 2018; Innes, Bartley, Maley, Katzer, & Buxton, 2009; Jongert, Roberts, Gargano, Förster-Waldl, & Petersen, 2009) as well as new kinds of medication (Montazeri et al., 2017).

3) Manipulative activity. For our laboratory, the most interesting phenomena related to *T. gondii* are not, however, the clinical effects and its prevalence, although we will revisit a few of these topics again later on. It is the way it seems to be driving its unsuspecting host into the maw of a felid predator – or a car, in case of humans. Or is it?

In a 2013 article (Worth, Lymbery, & Thompson, 2013), Amanda R. Worth and her colleagues from Murdoch University in Australia argue, that manipulative activity of *T. gondii* in its non-felid host isn't as clear as formerly

suspected. The argument has two main points, that (a) the behavioral changes may not increase transmission rate to cats, and that (b) the increased transmission to cats may not increase parasite's fitness.

Now, what behavioral changes are we talking about? Effects of *Toxoplasma* infection seems to encompass a wide range of phenomena including but not limited to, as shown in rat studies, impairment of learning and memory (Daniels, Sestito, & Rouse, 2015), reduction in predator aversion (Hari Dass & Vyas, 2014), increased tolerance for risk of reward forfeiture (Tan & Vyas, 2016) (in this study, it was also experimentally shown that the change in testosterone synthesis itself is sufficient to explain the observed change), or specific changes in regard to odor of cats (Berdoy, Webster, & Macdonald, 2000; Vyas, Kim, Giacomini, Boothroyd, & Sapolsky, 2007) done, perhaps, through epigenetic engineering (Jaroslav Flegr & Markoš, 2014). In mice, there were found symptoms such as deterioration of cognitive skills as well as causing anxiety-like symptoms (Mahmoudvand et al., 2015), impairment in long-term fear memory consolidation (associated with increased levels of dopamine metabolites in cortex of infected mice in contrast with the uninfected controls) (Ihara et al., 2016), worsening symptoms of simulated Alzheimer disease (mice intrahippocampally injected with amyloid-beta peptide ($A\beta_{1–42}$) because of neuroinflammation promoted through cytokine networks (Mahmoudvand et al., 2016). Perhaps it is worth mentioning that observed changes are often dependent on variables such as sex of the infected host (Hegazy, Elmehankar, Azab, El-Tantawy, & Abdel-Aziz, 2019) or strain of the parasite (Kannan et al., 2010).

For the full range of effects, I would recommend reviews by Joanne Webster (Joanne P. Webster, 2001) (J. P. Webster, 2007), Glenn McConkey, et al. (McConkey, Martin, Bristow, & Webster, 2013), Manuel Berdoy at al. (Berdoy, Webster, & Macdonald, 1995), and Rodrigo Costa da Silva and Helio Langoni (R. C. da Silva & Langoni, 2009), the last one including also brief information on host-parasite interaction such as immunological response to parasitism and effects of toxoplasmosis in the brain.

Back to the rather skeptical article – why do we think that the effects do indeed increase predation of the vector/intermediate host by the felids rather than being simple side-effects of a parasitical infection? Up to date, I haven't been able to find a study on predation of *Toxoplasma*-infected and uninfected prey – to do such studies is one of the main recommendations given by Amanda Worth at al. in another article for us to be able to confirm manipulation hypothesis in *Toxoplasma*-rodent scenario (Worth, Andrew Thompson, & Lymbery, 2014) –, however, we now have four reasons to believe the manipulation hypothesis is, in this case, quite correct.

(1) We know that infection is transmitted to cats through predation since infection rates in cats differ with both the amount of predation the cat needs to do to sustain itself and with the type of prey (and its *Toxoplasma*-prevalence) in a given region – not all prey is indeed created equal (Afonso, Thulliez, Pontier, & Gilot-Fromont, 2007).

(2) We have studies on different predator-parasitized prey systems showing that manipulation of the intermediate host by its parasite can indeed increase the predation rate (Bethel & Holmes, 1977) (Perrot-Minnot, Kaldonski, & Cézilly, 2007), although this can be sometimes exploited by other predators (Mouritsen & Poulin, 2003). In some cases, the parasite seem to even shift the behavior toward being an easy prey in conditions where predation by the final host is probable, while shifting it the other way when the intermediate host is likely to be preyed upon by a non-host species, as has been reviewed in 2011 article by Vincent Médoc and Jean-Nicolas Beisel (Médoc & Beisel, 2011).

(3) At least some of the changes in intermediate host's behavior seems to specifically increase the risk of predation by final hosts in contrast to other predators, as shown for example in rat experiments with cats vs. mink odor (Lamberton, Donnelly, & Webster, 2008). Moreover, this effect seems to be connected not with the cats in general, but with the specific felid species present in the environment and posing risk for the infected animals. This was shown in infected chimpanzees (*Pan troglodytes troglodytes*), which lost their

aversion toward the smell of a leopard (*Panthera pardus*) but not to odors of other felids which are not preying on them (Poirotte et al., 2016).

(4) In humans, infection by *Toxoplasma gondii* does not lead to increased predation by felids, we are, however, being preyed upon, metaphorically speaking, by cars. Studies from the Czech Republic (Jaroslav Flegr, Havlícek, Kodym, Malý, & Smahel, 2002) (Jaroslav Flegr, Klose, Novotná, Berenreitterová, & Havlíček, 2009), Mexico (Galván-Ramírez et al., 2013), Russia (Stepanova et al., 2017) or Turkey (Yereli, Balcioğlu, & Özbilgin, 2006) suggest that latent toxoplasmosis plays an important role in the risk of traffic accidents in infected individuals, probably due to prolonged reaction times (Havlícek, Gasová, Smith, Zvára, & Flegr, 2001; Novotná et al., 2008).

The second argument against truly manipulative activity performed by the parasite suggested that transmission through cats does not have to be necessary for upkeeping the parasite's fitness. This is, beyond general remark that asexuality in eukaryotic parasites, let alone apicomplexans, is incredibly rare (Weedall & Hall, 2014), usually requires evolution of other mechanisms to increase genomic plasticity, as is the case in, for example, asexual root-knot nematodes (*Meloidogyne* spp.) (Castagnone-Sereno & Danchin, 2014), and that ways to make sexual reproduction in final hosts even more effective could pose another completely separate selection pressure toward development and maintenance of complex life cycles (Rauch, Kalbe, & Reusch, 2005), probably not the case. First, both models and field experiments with cat vaccines against toxoplasmosis result in a significant decrease of the parasite in intermediate hosts, thus effectively reducing its fitness (Mateus-Pinilla et al., 1999; Mateus-Pinilla, Hannon, & Weigel, 2002). And second, observations from isolated islands suggest that where there are no cats, there is no toxoplasmosis (Wallace, 1969), leading us to believe that transmission to a felid host is indispensable for the survival of the parasite in nature.

In the previous sections, I have tried to address the following points:

- Manipulation hypothesis proposes that there is a phenomenon in nature where a parasite with complex life cycle shifts the behavior of

its host in a way that facilitates its advancement into the next step of that particular life cycle.

- *Toxoplasma gondii* is a parasite with a complex life cycle that affects the survival, health, and behavior of its intermediate hosts to advance into a felid to enter the sexual stage of its reproductive cycle.
- We have enough evidence to believe that felid host is crucial for the sustenance of the parasitic population and that at least some of the changes in behavior in the affected host are adaptive and serve as a mechanism facilitating the transmission to these felid hosts.

Let us shift our attention now toward humans and answer the questions of what does the presence of *Toxoplasma gondii* in the human body, and specific tissues mean for our (especially mental) health, and whether some of the observed effects relate to manipulative activities of the parasite.

2.2.3 *Toxoplasma gondii* in Humans

As is the case with mammals and birds, the effects of *Toxoplasma* on human hoses can be divided into two groups: there are the clinically important symptoms of congenitally acquired, acute or reactivated disease, and there are the long-term effects of latent infection.

Toxoplasmosis as a clinically important disease. For gynecologists, pediatrics, neurologists and ophthalmologists, toxoplasmosis is a salient congenitally transmissible disease with severe consequences varying from ocular manifestations including total loss of vision (e. g., Mets et al., 1997) through degree of neurological damage (e. g., Cortina-Borja et al., 2010) to miscarriage or abortion for medical reasons (although research suggests induced abortions are scarcely needed in cases of acute toxoplasmosis if antiparasitic treatment is used (Berrebi et al., 1994)).

The question whether we should implement a mandatory screening in pregnancy examination is currently under debate with some research groups not recommending prenatal toxoplasmosis screening and the subsequent antiparasitic treatment of primarily infected pregnant women due to the low prevalence of congenital toxoplasmosis and thus inefficiently used medical

expenses (e. g., Wallon, Liou, Garner, & Peyron, 1999). In many countries, there is a consensus on testing mostly pregnant women at higher risk of infections, e. g., the gynecological guidelines for Canada (Yudin et al., 2013), report of the ECDC for EU (European Centre for Disease Prevention and Control, 2016). In the Czech Republic, the usual prenatal screenings do not currently include laboratory tests for toxoplasmosis. Specialists on congenital toxoplasmosis recommend the tests to be done at regular intervals for pregnant women without latent toxoplasmosis (e. g., Petr Kodym & Geleneky, 2012), and the recommendation is also included in Recommendations for the Diagnosis and Treatment of Toxoplasmosis published by the Society of Infectious Diseases of Czech Medical Association of J. E. Purkyně (Geleneky, Prášil, & Kodym, 2017).

The immunodeficient population is at significant risk of developing toxoplasmic encephalitis and other complications. The complications are of importance for people with acquired immunodeficiency syndrome (AIDS) e. g., (Luft et al., 1993), and both solid-organs and bone marrow transplant recipients. In the case of organ transplants, the danger lies mainly in transmission via the transplanted organ of an infected donor e. g., (Fernandez-Sabe et al., 2012), while for the bone marrow transplant recipients the complications are caused by reactivation of their own latent infection (e. g., Slavin, Meyers, Remington, & Hackman, 1994). Additionally, problematic are also various hereditary forms of immunodeficiency, especially since it is not always apparent from early childhood, which might interfere with differential diagnostics of an adult patient (Yong et al., 2008).

Acute illness in immunocompetent adults is notable, especially in local outbreaks, where multiple cases can be observed together; this is usually when a source of drinking water for a bigger population is contaminated with parasitic cysts. For example, in an outbreak in the Greater Victoria area of British Columbia, 100 individuals showed symptoms of acute toxoplasmosis, some developing complications such as *Toxoplasma* retinitis or lymphadenopathy (Bowie et al., 1997). An atypical strain of *Toxoplasma* probably endemic to the region of French Guiana has been noted to cause

severe acquired toxoplasmosis in immunocompetent adults. Symptoms varied in between patients and included cases of fever, headaches, diarrhea, enlarged lymph nodes, splenomegaly, hepatomegaly, and elevated liver enzymes, and diarrhea (Carme et al., 2002).

The history of discoveries of *Toxoplasma*-related clinically important issues has been listed in Weiss's and Dubey's review Toxoplasmosis: A history of clinical observation (Weiss & Dubey, 2009), and the topic is thoroughly covered from the medical point of view in various medical textbooks and monographies. I have found the best coverage, including information on histological samples, laboratory tests, clinical symptom, diagnosis, and treatment in Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Tigertt, 2018).

Latent toxoplasmosis in humans. Latent *Toxoplasma* infection has been shown to affect pregnancy and early motherhood of infected women in areas such as fertility (Kankova, Flegr, & Calda, 2015),length of pregnancy and fetal development (Šárka Kaňková & Flegr, 2007), body weight and weight gain of pregnant women (Jaroslav Flegr, Hrdá, & Kodym, 2005; Kaňková, Šulc, & Flegr, 2010), blood glucose levels and gestational diabetes (Šárka Kaňková, Flegr, & Calda, 2015), production of thyroid hormone during pregnancy (Šárka Kaňková et al., 2014), secondary sex ratio (Š. Kaňková et al., 2007), or postnatal motor development of children during the first 18 months of life (Šárka Kaňková, Šulc, Křivohlavá, Kuběna, & Flegr, 2012).

A study searching for relationship between prevalence of toxoplasmosis in 88 countries and specific disease burden of 128 different diseases estimated with age-standardized Disability-Adjusted Life Year (DALY) or with mortality used GLM analyses with GDP, latitude, and humidity as covariates showed positive correlation of *Toxoplasma* prevalence and 18 diseases and diseases categories, 5 correlations were negative, and further 12 diseases showed positive trends ($p<0.1$) (Jaroslav Flegr, Prandota, et al., 2014).

Studies with healthy adults show relationship between latent toxoplasmosis and changes in performance (Havlícek et al., 2001), personality profiles (T. B. Cook et al., 2015; J Flegr & Hrdy, 1994; Jaroslav Flegr, Kodym,

& Tolarová, 2000; Jaroslav Flegr, Preiss, & Klose, 2013; Khademvatan, Khajeddin, Saki, & Izadi-Mazidi, 2013; Lindová et al., 2006) as well as with cognitive functions such as intelligence levels (Jaroslav Flegr et al., 2013, 2003) or impairment of memory (Gajewski, Falkenstein, Hengstler, & Golka, 2014), which, together with some of the diseases from previous paragraph, brings us to the topic of the effects of infectious diseases and especially toxoplasmosis on human mental health.

2.3 Toxo Visible: A Memoir of Madness (Effects of Infections on Mental Health of an Individual)

Throughout the ages, European medicine believed the mental illness to have either biological or behavioral basis, or to be caused by external phenomena, namely demonic possessions. According to historical reviews such as the 1987 article by a psychologist Simon Kemp and his colleague Kevin Williams (Kemp & Williams, 1987), confusing mental illnesses with witchcraft (Spanos, 1978) or punishment for sin (Kroll & Bachrach, 1984) was not as usual as was previously thought. However, the demonic possession and black magic is believed by some to be responsible for mental health issues even today and not only in originally animistic cultures (Razali, Khan, & Hasanah, 1996) and Muslim communities (Haque, 2004) where belief in jinns (although not jinns-caused insanity (Islam & Campbell, 2014)) is encoded in their holy book of Qur'an, but also in, for example, Pentecostal and some other Christian charismatics (Mercer, 2013).

This matters, because religious-magical beliefs concerning causation of mental health issues, which belong among the most prevalent causes of death worldwide (Walker, McGee, & Druss, 2015), might deepen negative attitudes and stigmatization of affected individuals (Gureje, Olley, Olusola, & Kola, 2006) or even complicate the diagnosis and treatment of mental illness (Alonso & Jeffrey, 1988). Thankfully, current psychiatry knows a perfectly non-supernatural external cause of mental illness, and that is an infectious disease[7].

2.3.1 Infections in General

Perhaps the most commonly studied cause of either neurological or mental health issues are infections in pregnancy, although our understanding of the mechanisms is still in the beginnings. It is possible that the maternal immune system is causing the damage instead of the pathogen itself. In animal models, we have seen reductions of the gray matter in the whole cortex and of the white matter in the parietal cortex in rhesus monkey (*Macaca mulatta*) offspring after the mother got infected with influenza during pregnancy (Short et al., 2010). The experiments in the BALB/c strain of a house mouse (*Mus musculus*) showed a mice model of schizophrenia in the offspring after pregnant mice were injected not with a virus, but with a synthetic double-strand RNA polyriboinosinic-polyribocytidylic acid (poly I:C) to mimic an infection. The poly I:C mice showed increased subcortical dopamine function, changes in sensorimotor gating, and impaired cognitive functions, suggesting the role of an immune system rather than a virus in the developmental damage (Ozawa et al., 2006). Two recent articles contain a similar effect, suggesting the role of immune reactions in the development of autism, or, again, an animal model of autism (S. Kim et al., 2017; Shin Yim et al., 2017).

In humans, multiple infections have been found to impair brain functions and cause mental health issue if the mother gets infected during pregnancy. For example, while non-specific bacterial, respiratory, and genital infections shown mixed results while searching for associations of a prenatal infection with schizophrenia (reviewed in Khandaker, Zimbron, Lewis, & Jones, 2013), viral infections such as influenza showed an association with more severe consequences after an infection in earlier stages of pregnancies, and an infection with *Toxoplasma gondii* in pregnancy shown increased risk of schizophrenia in later life (A. S. Brown et al., 2005). However, what is the cause? In a 2013 article, Philip Nielsen, Thomas Laursen, and Preben Mortensen showed that while the risk of schizophrenia is indeed associated with the mother being treated in hospital for an infectious disease during pregnancy, the same is true for both parents. The authors speculated that perhaps schizophrenia might be associated with a genetic cause of worsened

immunity, both traits being thus hereditarily transmitted from either of the parents (Nielsen, Laursen, & Mortensen, 2013).

The effects of congenital toxoplasmosis on the health of a child are a well-studied area with a steady increase in knowledge. For example, a 2018 article of Brazilian researchers found a 5-fold higher risk of an infant of 1 to 3 months of age with congenital toxoplasmosis to have altered brainstem auditory evoked potentials in comparison with a healthy child (Fontes et al., 2018). Perhaps more interestingly, also the children born to mothers with latent toxoplasmosis display changes in development, or specifically delayed motoric development in comparison with children of *Toxoplasma*-free mothers (Šárka Kaňková et al., 2012).

Beside infections in pregnancy, many infectious diseases are known to interfere with brain functions, or more specifically with cognitive functions and mental health issues. Brain malaria caused by an apicomplexan parasite *Plasmodium falciparum* is leading cause of neuro-related disabilities and deaths in tropical countries (Snow, Guerra, Noor, Myint, & Hay, 2005), and ongoing research currently aims at improving the neurocognitive outcome of the infection (Idro, Marsh, John, & Newton, 2010). Neurotoxoplasmosis in immunosuppressed patients causes encephalitis, psychiatric symptoms, and coma with case-studies of *Toxoplasma*-infected patients showing schizophrenia-like symptoms being reported since the 1950s (Minto & Roberts, 1959). Primary amoebic meningoencephalitis (PAM) caused by a perlocozoan (Excavata) protist *Naegleria fowleri* leads to death in rapid succession after symptoms such as confusion, irritability, and visual hallucionations (John, 1982). Despite an intensive effort to find a cure (Debnath et al., 2018), only 3 of 130 cases of the infection in the US did not end by the death of the patient (Pugh & Levy, 2016). As the multicellular parasites are concerned, neurocysticercosis caused by a pork tapeworm (*Taenia solium*) is a leading cause of late-onset epilepsy (i.e., epilepsy in patients who first encountered seizures in their mid-twenties or later (Dam, Fuglsang-Frederiksen, Svarre-Olsen, & Dam, 1985)) in many developing

countries and is not unheard of even in the US, Canada, or Japan (Singh, Burneo, & Sander, 2013).

A viral infection is often found to be associated with cognitive malfunctions. For example the HIV is associated with subcortical dementia, impaired ability to focus, impaired motoric abilities, learning abilities, and information processing, while language skills, basic attention, and executive functions stay mostly unharmed; there is also an impairment of neurobehavioral functions, rapid movements, walk, fine motor skills, and change in social behavior (irritability, apathy, emotional lability) (Lipton & Gendelman, 1995). Associations have also been found with mania (Gabel, Barnard, Norko, & O'Connell, 1986) and depression (Lyketsos et al., 1996). Statistically significant correlation has been found between schizophrenia and herpes simplex virus 2, Borna virus, and human endogenous retrovirus (Arias et al., 2012), although in other studies no role in etiology of schizophrenia, bipolar disorder, and clinical depression was found for the Borna virus (Hornig et al., 2012).

2.3.2 Latent Toxoplasmosis in Development of Mental Health Issues

Toxoplasma gondii invades brains of mice and men and other animals by hijacking leukocytes (Courret et al., 2006) or dendritic cells and inducing their hypermotility (Lambert, Hitziger, Dellacasa, Svensson, & Barragan, 2006). In the brain, it invades mostly astrocytes and neurons, where both types of cells are used to accommodate cyst stages while mostly astrocytes are affected in case of reactivation of the disease (Halonen, Lyman, & Chiu, 1996). The brain, in which the cysts are distributed without particular preference for regions of specific functionality (Berenreiterová, Flegr, Kuběna, & Němec, 2011) (Evans, Strassmann, Lee, & Sapolsky, 2014), reacts with inflammatory cytokine response which is more pronounced when the parasite is in the tachyzoite rather than bradyzoite stage (Blader, Manger, & Boothroyd, 2001). Immune reaction of the host plays an important role in keeping toxoplasmosis in chronic (latent) stage and preventing reactivation of the disease, as reviewed in Vern Carruthers' and Yasuhiro Suzuki's article (Carruthers & Suzuki, 2007).

The infection in brain is accompanied not only by the reaction of the immune system, but also by manipulating even the non-invaded cells by rhoptry proteins produced by the parasite (Koshy et al., 2012), changes in the morphology of infected neurons (Parlog, Schlüter, & Dunay, 2015), and epigenetic modulation of gene expression (Hari Dass & Vyas, 2014)[8]. Last but not least, changes are seen in levels of particular neurotransmitters, especially the dopamine.

The genome of *Toxoplasma gondii* contains two active genes (TgAaaH1 and TgAaaH2) encoding tyrosine hydroxylase, an enzyme which is involved in production dopamine precursor L-DOPA (Gaskell, Smith, Pinney, Westhead, & McConkey, 2009). Previous behavioral studies were consistent with hypothesized changes in dopaminergic neuromodulatory systems in infected hosts (A. Skallová, Kodym, Frynta, & Flegr, 2006; Anna Skallová et al., 2005), and mice study showed both an increase in the concentration of dopamine and its metabolites in infected mice and changes in the regulation of dopamine receptor pathways (J. Xiao et al., 2014). Another study in human neuroepithelioma cells infected with type I strain (but not type II and III strains) of *Toxoplasma* found abnormalities in three neurotransmitters (dopamine, glutamate, and serotonin) and two neuropeptides ($PROK_2$ and TAC_1) systems (Jianchun Xiao, Li, Jones-Brando, & Yolken, 2013). These findings were also supported by the study showing significant *Toxoplasma*-orchestrated increase in dopamine metabolism in neural cells (Prandovszky et al., 2011). However, not all of the published studies show the same results: an experimental study with parasitic strains without a functional AAH2 gene suggested the effect of the parasite on the hosts' dopamine levels to be very limited if any at all (Wang, Harmon, O'malley, & David Sibley, 2015). Still, considering all of the previous studies as well as the relationship between toxoplasmosis and schizophrenia, which will be discussed below, the role of dopamine in the *T. gondii* infection and modulation of host behavior seems probable, if not fairly certain.

Other studies also suggested changes in the activity of acetylcholinesterase and glutathione reductase and in nitrite and nitrate levels in infected mice (Tonin et al., 2014). A study conducted on human and

mouse myeloid dendritic cells brought result indicating that intracellular pathogens such as *T. gondii* can hijack GABA's activation functions in the immune system and facilitate parasitic dissemination in targeted organs (Fuks et al., 2012), while a study in mice confirmed changes in GABAergic signaling in *Toxoplasma*-infected mice. Such changes may be clinically important and lead to the development of seizures (Brooks et al., 2015).

Stacked together, these studies recount a coercive story of *Toxoplasma*-induced changes in brain physiology and chemistry, which may consecutively explain why we find so many effects of latent toxoplasmosis on human mental health and cognitive capacities. Latent toxoplasmosis was, for example, linked with changes in management in auditory distraction leading to impairment in goal-oriented behavior of elderly people (Beste, Getzmann, Gajewski, Golka, & Falkenstein, 2014). A study in psychiatric patient suggested modulation of human behavior and personality traits in *Toxoplasma*-positive population and shown that psychiatric patients diagnosed with personality disorders are more likely to be seropositive to *Toxoplasma* (Hinze-Selch, Daubener, Erdag, & Wilms, 2010). A recent meta-analysis on 3771 epileptic patients and 4026 healthy controls distributed across 16 separate studies showed an increased risk of epilepsy in subjects infected with *Toxoplasma gondii* with the association stronger in children and also in cryptogenic epilepsy as compared with active convulsive epilepsy (Sadeghi et al., 2019).

Of course, not all results are indicative of any effects of toxoplasmosis on human behavior. A study on the population-representative birth cohort (N=837) from Dunedin, New Zealand reported a suggestive link between latent toxoplasmosis and suicide attempt and poorer memory performance, however, many other variables including increased susceptibility to neuropsychiatric disorders, criminal behavior, poor impulse control, personality changes, or impaired neurocognitive ability (Sugden et al., 2016). 115 patients with Parkinson's disease from Imam Reza Psychiatric hospital of Khorramabad in Iran were screened for latent toxoplasmosis revealing no significant increase of risk for Parkinson's disease in *Toxoplasma*-infected population (Fallahi et al., 2017), although, on the other hand, an older study in the United Kingdom

showed a higher prevalence of latent toxoplasmosis in patients with Parkinson's disease (Miman, Kusbeci, Aktepe, & Cetinkaya, 2010)

Some studies even found a positive effect of latent toxoplasmosis on action control in stop-change experimental paradigm, probably due to the increased dopamine levels (Stock, Heintschel von Heinegg, Köhling, & Beste, 2014). A recent study with Amish population (N=833) showed significantly fewer sleep problems and fewer daytime problems due to poor sleep in seropositive subjects, as well as a significant and positive association of *T. gondii* titers and longer duration of sleep, earlier bedtime, and earlier mid-sleep times. Possible adaptive-for-the-parasite explanation for these positive effects could be decreased the chance of death of the host by other means than predation, but alternative explanations such as the effect of microbial "Old Friends" of certain strains of the parasite leading to the mutually beneficial relationship between the parasite and its host are also possible (Corona et al., 2019).

Beside the studies linking toxoplasmosis with problems (or benefits) related to individual problems such as epilepsy, quality of sleep, and such, there is an increasing amount of evidence of toxoplasmosis being related to severe psychiatric disorders such as bipolar disorder (Chen et al., 2019; Del Grande, Contini, et al., 2017; Del Grande, Galli, et al., 2017; Jaroslav Flegr, Prandota, et al., 2014; A. L. Sutterland et al., 2015), addiction (A. L. Sutterland et al., 2015), obsessive-compulsive disorder (A. L. Sutterland et al., 2015) (Jaroslav Flegr, Prandota, et al., 2014) (Nayeri Chegeni et al., 2019), as well as self-directed violence (Jaroslav Flegr, Prandota, et al., 2014) (Arjen L. Sutterland et al., 2019) (Bak et al., 2018).

The biggest case of evidence we have is, however, related to the relationship between toxoplasmosis and schizophrenia.

2.4 A Beautiful Infected Mind (The Role of Toxoplasmosis in Development and Symptoms of Schizophrenia)

Besides the significantly increased odds ratio found in a meta-analysis by Sutterland et al. (Arjen L. Sutterland et al., 2019) or Torrey et al. (E. F.

Torrey, Bartko, & Yolken, 2012) as well as in a recent large-scale study on 81,912 blood donors from Denmark (Burgdorf et al., 2019), schizophrenia patients who test seropositive for toxoplasmosis show different severity of symptoms, different age of the onset of the disease, as well as other abnormalities including different density of gray matter in comparison with uninfected controls and in case of gray matter density also in comparison with non-schizophrenic population (Horacek et al., 2012).

For example, a 2013 study on 251 schizophrenic individuals admitted to Prague Psychiatric Centre's diagnostic and treatment programs during the years 2000 to 2010 found that schizophrenic patients with toxoplasmosis experienced significantly more severe (positive) symptoms of schizophrenia and stayed in a hospital for significantly longer period of time by the time of their last hospitalization (the stay differed by about 33 days) (Holub et al., 2013). It is, however, worth noting that another 100 admitted patients were excluded from the study due to refusal to participate (67 individuals), refusal to give blood sample (25 individuals), fulfillment of exclusion criteria (5 individuals), and uncertain seropositivity (6 individuals), meaning the study can be biased by selection effect.

Schizophrenia patients of the Psychiatry Department of Monastir Hospital in Tunisia (N=246, 93 diagnosed with paranoid schizophrenia, 98 with undifferentiated schizophrenia, and 45 with a disorganized type of schizophrenia) plus 117 healthy controls recruited from blood donors with neither personal no family history of any serious illness were recruited to a retrospective study. Blood samples were collected after the treatment. The analysis showed highly significant increased odds ratio ($p=0.00001$; OR=2.55; 95% IC =1.59–4.09) for the development of schizophrenia in comparison with healthy controls after statistical control for age. *Toxoplasma*-positive males showed significantly higher age of onset of schizophrenia than in seronegative patients as well as significantly higher BPRS score, which indicates the more severe clinical manifestation of schizophrenia in seropositive patients; however, these effects were observed in male patients only (Esshili et al., 2016).

A Turkish study comparing continuously ill schizophrenia patients with those in partial remission showed a significantly higher prevalence of toxoplasmosis in the continuously ill patients. The probability for a continuous course of illness was 15 times higher for *Toxoplasma*-positive patients (Çelik et al., 2015). A French study showed three times higher prevalence of *Toxoplasma*-positive subjects among schizophrenia patients in comparison with the general French population. *Toxoplasma*-positive schizophrenia patients also differ in several specific psychotic features in comparison with seronegative schizophrenics (Fond et al., 2018).

Such results suggesting role of infective diseases and specifically toxoplasmosis together with, as is now believed, formerly exaggerated hereditary character of schizophrenia constitute, in fact, positive news for future psychiatric patients, since, as discussed in 2019 review by Torrey and Yolken, infectious diseases are generally much easier to treat than those hidden in genes (E. Fuller Torrey & Yolken, 2019). Unfortunately, current progress in the development of cure for latent toxoplasmosis is pretty much nonexistent, with very few and methodologically highly problematic randomized controlled trials evaluating antiparasitic medication in schizophrenic patients behind us, and probably none under the way (Chorlton, 2017). Consistent effort to uncover mechanisms of *Toxoplasma* manipulation in the human brain can, thus, help steer attention to this topic and perhaps suggest possible targets for drug development and testing. Among those mechanisms, there we may also find a modification of reaction times, startle reactions, and sensorimotor gating in general.

2.5 Response, Fast and Slow (The Startle Reflex and Its Modifications)

2.5.1 A Tale of Four Discoveries

Ivan Sechenov: The Russian Approach. If there is the one most surprising thing about the prepulse modification of startle response, it just might be that the discovery of this phenomenon precedes the discovery of *Toxoplasma gondii*. Moreover, most of the studies you will find would talk about it being first used far into the second half of 20th century by Hoffman

and Flesher (Hoffman & Fleshler, 1963), mere two years before the book with the original discovery was first published in English translation by M.I.T. Press and about 100 years after its first publication.

19th century and especially its second half was interested in human perception, reaction to stimuli, and learning. Franciscus Donders used testing of reaction times for the first time to measure cognitive abilities in humans[9][34, 35], Hermann von Helmholtz led his pioneering studies in human vision and auditory perception, and a little later, Ivan Pavlov began his famous experiments with dogs[10]. Right in the middle of these efforts, Pavlov's teacher and "the father of Russian physiology" (as named by his pupil Pavlov) Ivan Sechenov during his work with frogs noticed that responses to painful tactile stimuli could be decreased by either chemically or electrically stimulating the brain (Sečenov, 1965).

Warren P. Lombard: Here Come Humans. After Sechenov, the research concerning reflex modifications initiated in the US among neurologists. They were now for some time intrigued by patellar reflex, a simple[11] yet mighty diagnostic tool first described simultaneously by an internist Wilhelm Heinrich Erb (Erb, 1875) and a psychiatrist Carl Friedrich Otto Westphal[12] (in Fearing, 1927; Westphal, 1875), although there are indices of the reflexes being studied by others prior to that year (Louis, 2002). The new research built on works by a Hungarian physician Ernő Jendrassik best known for the Jendrassik maneuver (or "Jendrassik's Handgriff"), a method which is still (Ertuglu, Karacan, Yilmaz, & Türker, 2018) being used for reinforcement of stretch reflexes in neurological diagnostics and comprised of not-widely-accepted 1886 studies by Mitchell and Lewis (Mitchell & Lewis, 1886) and subsequent work of University of Michigan's Professor of Physiology Warren Plympton Lombard. Professor Lombard (Lombard, 2006) complimented Mitchell and Lewis on their keen power of observation and exposed his own 6,639 experimental trials done by his wife on himself using his device for testing knee-jerk reactions (see part of his recorded trial in Figure 5).

No. 1, Series I—April 1st, 1887.

Knee-Jerk.					Extracts From Journal.	U. S. A. Weather Observations.					
Time of Examination.	No. of Experiments.	Average Movement in Millimetres.	Extremes.	Lightest Effective Blow.	Well and Vigorous.	Time.	Barometer.	Thermometer.	Relative Humidity.	Wind.	Weather.
8.30 a.m.	25	36	20–48	27°	Just out of bed and half asleep.	7 a.m.	30.150	31°	84	n.	lt. snow
9.45 "	18	88	55–120	20°	Just after breakfast.						
1.15 p.m.	12	111	90–130	20°	Morning spent writing.						
2.15 "	20	68	29–93	23°	Just after lunch.	3 p.m.	30.089	36°	87	n.e.	lt. snow
6.15 "	23	49	10–78	26°	Afternoon spent writing, head tired.						
8.15 "	26	44	16–75	29°	Just after dinner.						
10.30 "	25	45	22–60	30°	Evening spent reading and writing.	10 p.m.	30.036	34°	88	n.	lt. snow
	149	63	10–130	25°		mean.	30.092	34°			

Figure 5 Lombard's Table of Experiments.
This is an example of Lombard's meticulously controlled experimental conditions during experiments (Lombard, 2006).

Lombard's experiments were expanded by a dean of Harvard Medical School Henry Pickering Bowditch [13] and Harvard's physiologist Joseph Weatherhead Warren. The duo used a new version of an experimental device for testing knee-jerks (see Figure 6) and penned a detailed article important not only for attentive descriptions of the methodology but also for noticing the importance of the length of the interval between a modification event and the main stimuli. They have found that the modification might either increase or decrease the response to the main stimuli with increase caused by a motor activity happening if the main stimuli occur within a narrow window of for about 400 ms (Bowditch & Warren, 1890).

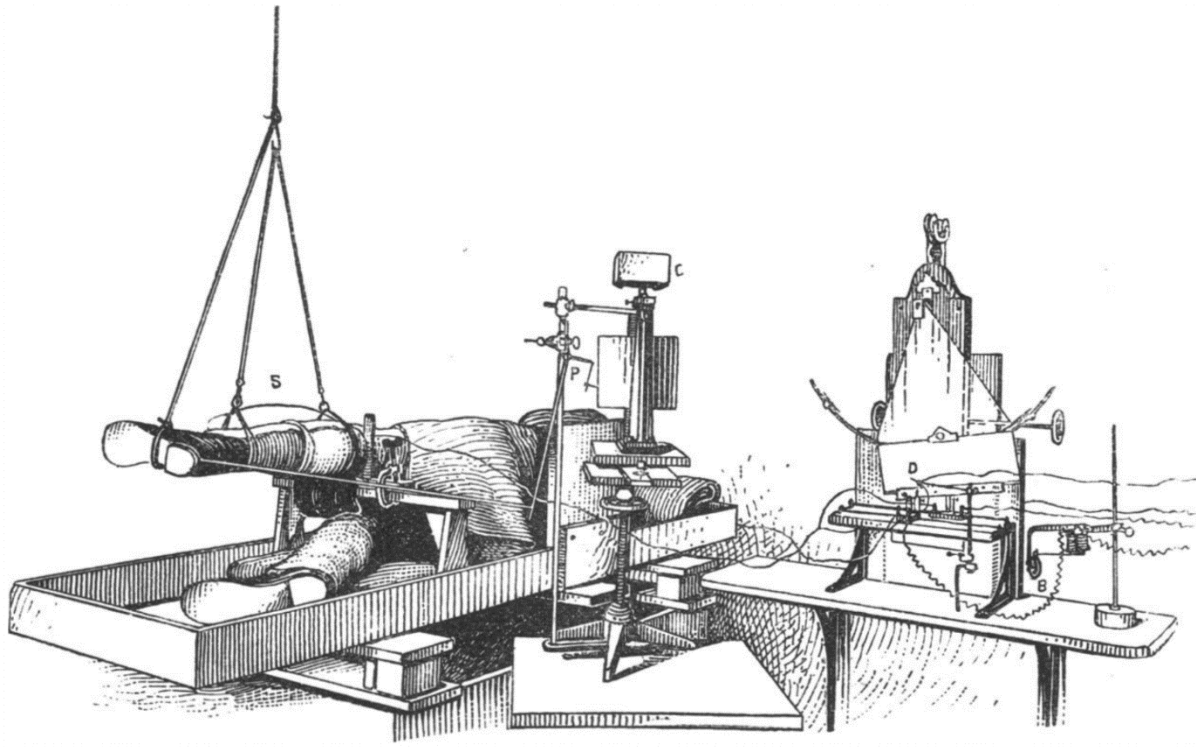

Figure 6 Testing Knee-Jerks in Humans.
An experimental subject fixed in Bowditch and Warren's testing apparatus (Bowditch & Warren, 1890).

Robert Yerkes: Back to Frogs. In the year of Sechenov's death, young Yerkes, a future President of the American Psychological Association, though then just three years after getting his Ph.D. found a similar phenomenon in his frog experiment (see Figure 7), using electric shocks and acoustic signaling as the response modifier (Yerkes, 1905). Yerkes's experiments are essential since he managed to repeat Bowditch and Warren's findings of the inhibitory and facilitatory characteristics of the first signal in dependence on the length of the time interval between the first and the second stimuli, but this time on frogs instead of humans.

Unfortunately, there is no evidence on whether Yerkes knew about the Sechenov's discoveries. As for the later studies of patellar reflexes, Ison and Hoffman speculate that he was unaware of them at the beginning of his research, while in later works, he is comparing his findings to those of Bowditch and Warren. Most of the previous experiments were already described in English and German literature[14] before Yerkes begun his studies,

so there is also the possibility he read about them before his work (Ison & Hoffman, 1983).

Yerkes experiments were unique and original in several aspects. In words of Ison and Hoffman's 1983 article: „*They provided an early example of how a phenomenon available to naturalistic observation could be brought into the psychological laboratory for systematic analysis. They showed that the inhibitory effects of exteroceptive stimuli on "voluntary" spontaneous responses could also be obtained with "forced" reflex movements. They showed that the phenomenon of reflex modification had wide generality, in that the temporal functions for facilitation and inhibition obtained in the frog were almost identical to those obtained earlier with human subjects. Finally, Yerkes' experiments provided the first example of reflex modification being used to solve a difficult research problem in psychology, which was, namely, how to assess the sensory abilities of nonarticulate organisms.*" (Ison & Hoffman, 1983)

Howard Hoffman: The Real Beginning. In 1965, Howard Hoffman and John Searle [15] (Hoffman & Searle, 1965) published a study of acoustic modifications of startle in rats (scheme of an apparatus they used was published two years later, see Figure 8) using background noise of various intensity and weak stimulus preceding the startle signal by 100 ms. They showed that while the former seemed to increase the startle reaction, the later functioned as its inhibitor. Finally, just as with the patellar reflex some eight decades earlier, the whole field of prepulse modifications begins to flourish with Howard Hoffman being the prominent figure. Over several decades, Hoffman pretty much paves the field of prepulse modification study, by defining the term *reflex modification* (Hoffman & Ison, 1980), using the term *prepulse* for the first time to describe the weak stimulus preceding main signals [16] (Powers & Leitner, 2007), and executing and publishing several dozens of his experiments.

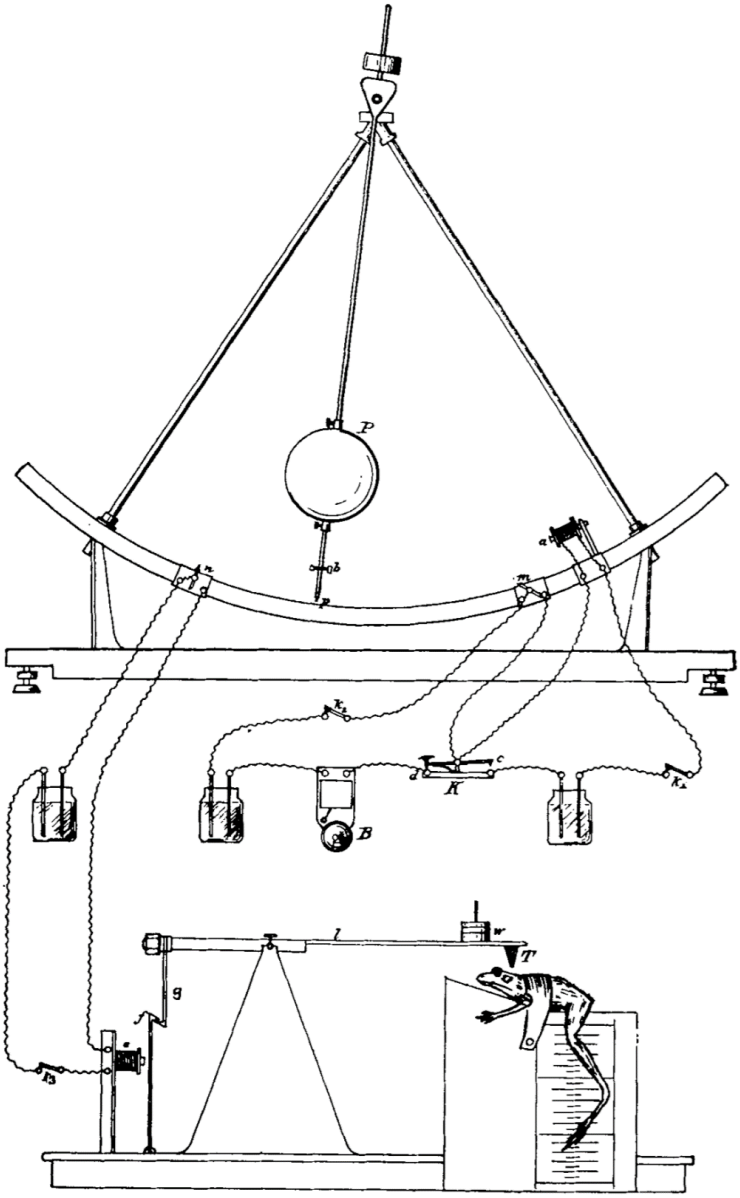

Figure 7 Yerkes' Auditory-Tactual Apparatus.
William Ernest Hocking produced this drawing of the actual device used by Robert Yerkes in his experiments with hearing in frogs and published in his article (in Yerkes, 1905). The device comprised of a pendulum (P) with a contact point (p) and an attachment (b) for an electromagnet (a), electric bell (B) with a key for its electric circuit (m), a key for a magnet circuit of a touch apparatus (n), a hand-key for release of the pendulum and temporary closing of electric bell circuit (K), other keys in the circuit (k1, k2, k3), magnetic release for touch apparatus (e, f, g), a pivoted lever (l) bearing rubber cone (T), weights (w), and one poor unlabelled frog.

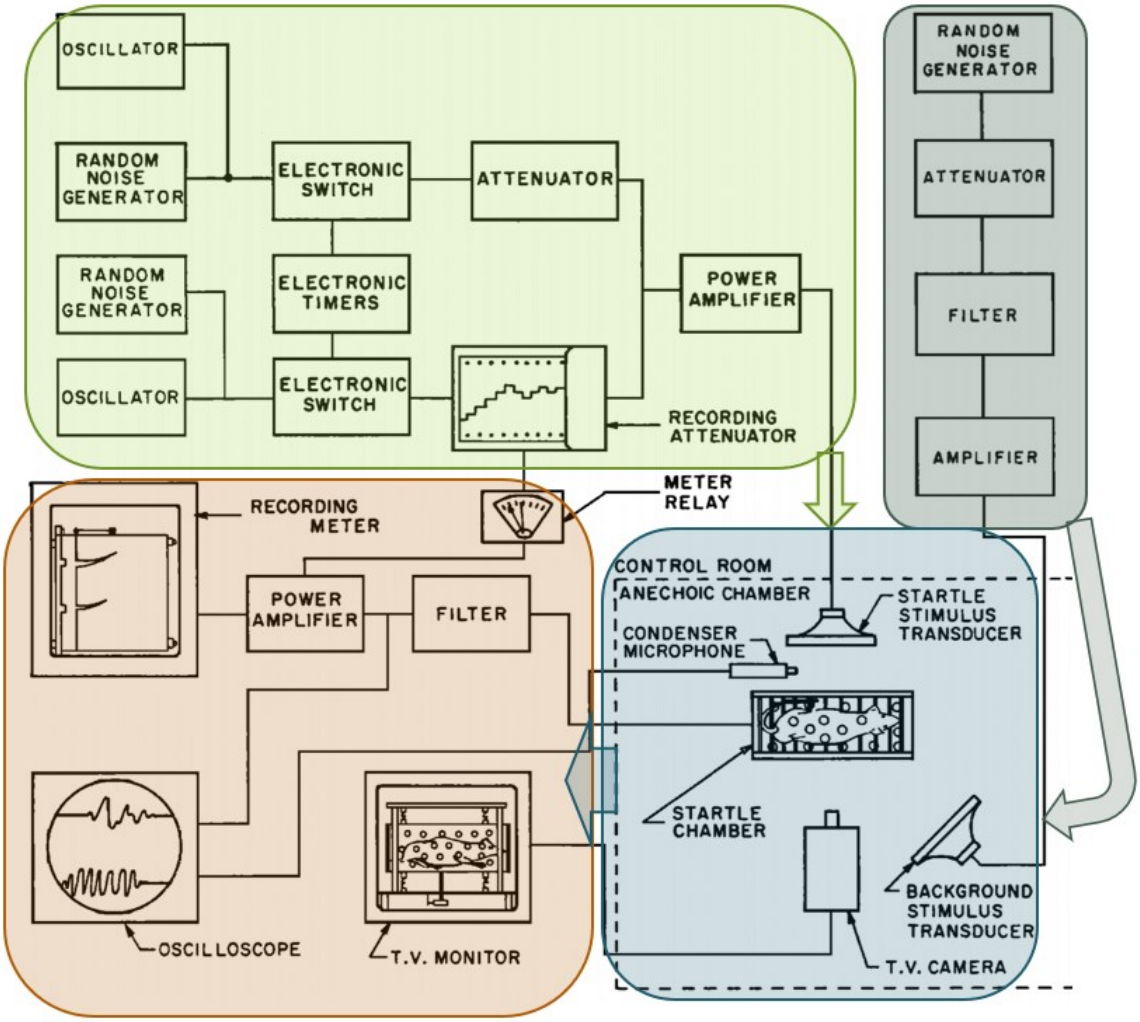

Figure 8 Hoffman & Searl's Rat-Startling Device.
The figure shows a schematic drawing of an experimental device used by Hoffman and Searl in their rat experiments as published in (Hoffman & Searle, 1968). **1. Top-left. Generation of startle signals. 2. Top-right. Generation on background noise. 3. Bottom-right. Chamber with the experimental subject. 4. Bottom-left. Outputs and monitoring.**

Of course, Hoffman and Searle were not building their experiments on thin air. Previously mentioned Hoffman's own experiments preceded them with background noise in startle trials (Hoffman & Fleshler, 1963) as well as with various studies of startle (in animal as well as human subjects), which occur throughout the previous decades of the 20th century. For example, Frances Clarke (Clarke, 1939) studied reactions to a startle in human infants, Lars-Erik Larsson (Larsson, 1960) correlated blink responses to startle stimuli

and EEG in human males under specific conditions, and Kenneth Moyer and Bradford Bunnell (Moyer & Bunnell, 1960) investigated the role of adrenalin in startle reaction in rats. The methodological background stands on, for example, a study published by Frank Jones and John Kennedy (F. P. Jones & Kennedy, 1951). It is the prepulse modification part that is re-invented or at least re-introduced and further developed by Hoffman and his collaborators.

An experimental psychologist turned a professor of audiology Levi A. Reiter and a neurobiologist specialized on the brain, cognitive sciences, and otolaryngology James R. Ison managed to tie up on where Yerkes left, but yet again on human subjects. They tested a rather small group of 12 college students and shown that both visual (a flash of light) and acoustic (a burst of noise) signals inhibit an eyeblink reflex (a startle reaction to an air puff) (Reiter & Ison, 1977).

At the same time (though publishing their results a year later), a trio of researchers consisting of a pediatric otolaryngologist Roger R. Marsh, an experimental psychologist Howard Hoffman, and an experimental psychologist (and later a systems engineer) Christopher L. Stitch, worked on experiments with infants as young as 6 weeks after birth. They showed that an eyeblink reaction to tactile stimuli is inhibited by an acoustic signal presented 100 ms before the tactile one, thus showing a diagnostic power of response modifications for young children prior to the development of speech (Marsh, Hoffman, & Stitt, 1978).

2.5.2 Instrument of Choice

The second half of the 20th century as well as the beginning of 3rd millennium sees uses of startle modification in cognitive research pretty much on every corner due to its high sensitivity, beginning with fear-potentiated modifications in humans (Grillon & Davis, 1997) as well as other animals, for example rhesus monkeys (Winslow, Parr, & Davis, 2002), through shock sensitization that could be responsible for rapid conditioning to the context (Richardson, 2000) or aversive stimulation with threat signals or darkness (Grillon et al., 1999), and ending with combination of several modification phenomena such as emotional state (Globisch, Hamm, Esteves, & Öhman,

1999) or conscious attention and prepulse signal (Filion, Dawson, & Schell, 1993).

In psychiatry, increased usage of PPI as a measure for impairments of sensorimotor gating started with 1978 study by Braff et al. in 20 healthy volunteers and 12 schizophrenic patients. The study confirmed that blink reflex amplitude and latency changes in the presence of weak preceding stimuli in particular time intervals from the main stimulus, that this modification of blink reflex was reliable within-subject, and that schizophrenia was correlated with decreased blink amplitude inhibition and latency facilitation (D. Braff et al., 1978).

Following this study, prepulse inhibition of blink reaction on startle signal has been used on patients with various psychiatric and neurological disorders, and also effects of medication and illegal drugs on sensorimotor gating was studied this way, unfortunately often with varied results. PPI was found to be negatively associated with all anxiety spectrum constructs except for obsessive-compulsive personality disorder in one study (Franklin, Bowker, & Blumenthal, 2009), while another one has linked the reduction in PPI to OCD patients as well (Hoenig, Hochrein, Quednow, Maier, & Wagner, 2005). One study has shown significantly reduced PPI in war veterans with PTSD (Grillon, Morgan, Southwick, Davis, & Charney, 1996), while later study in adolescent girls with PTSD found no such a thing (Lipschitz et al., 2005) – perhaps because PPI is, in general, less pronounced in women (Swerdlow et al., 1993) and tend to vary across the menstrual cycle (Kumari, 2011) and with sexual orientation of women (Rahman, Kumari, & Wilson, 2003) (although surprisingly not in between pre-menopausal and post-menopausal age (Kumari et al., 2008)), which makes search for significant changes in prepulse modification in women all the much harder.

Altogether, thousands of studies on prepulse inhibition of startle reaction have been done up till today, most of them are still related to schizophrenia. In schizophrenic patients, the prepulse modification seems to be diminished both in prepulse inhibition and prepulse facilitation (this depends on the interval between the weak and the main stimuli) as well as in

startle habituation (Ludewig, Geyer, & Vollenweider, 2003), which effect seem to stay as a good biomarker for schizophrenia even with medication, since the impairment in sensorimotor gating (but not in startle habituation) seems to be present even months after discharge (Mena et al., 2016). In recent studies, four single nucleotide polymorphism on genes associated with schizophrenia has been correlated with expression of PPI in humans; however further analysis indicates that PPI is a highly polygenetic similarly to schizophrenia itself (Quednow et al., 2018).

This, of course, could be a problem for our research as well. The idea beyond using endophenotypes (i. e. stable behavioral symptoms, measurable biomarkers connected with an illness, usually with genetic causes, as demonstrated, for example, by presence in healthy family members of patients) is being able to identify risks of development of a mental disease in healthy individual as well as having a basis for discovering new treatment targets. If, as it seems to be the case here, the endophenotype is of the same polygenetic complexity as the disease itself, it cannot be of much use. One the other hand, perhaps this is exactly the reason for us to approach it from another side – what if, as suggested in the abovementioned Torrey's and Yolken's review (E. Fuller Torrey & Yolken, 2019), schizophrenia really as more infection based than genetic-based and perhaps so is the impairment of sensorimotor gating connected with it? In that case, we might be still able to use it both to study risk factors for the development of schizophrenia in previously healthy individuals, as well as to use it as a target for future treatment therapies.

2.5.3 So, Can Wen Now Return to Toxoplasmosis?

Yes, I believe so, though shortly: Although a study on mice showed no significant difference in sensorimotor gating of infected and uninfected subjects (Kannan et al., 2010), first human studies employing acoustic startle in association with toxoplasmosis has shown higher magnitude of acoustic startle in seropositive individuals (Massa et al., 2017) and significantly slowed latency of the acoustic startle response in seropositive versus seronegative

individuals among both schizophrenic patients and non-schizophrenic controls (Pearce et al., 2013). That's all that is known now.

However, besides first attempts to measure startle reflex and its modifications in *Toxoplasma*-positive individuals, the experimental methods are also developing. There is a growing amount of literature on the interaction between startle and voluntary reactions in humans (Valls-Solé, Kumru, & Kofler, 2008), and some of the studies are focused on completely new effects. For example, when the signal in reaction time task takes place at the same time as auditory startle signal; the reaction time is shortened; this effect has been called StartReact effect, first described in 1998 by Valldeoriola et al. (Valldeoriola et al., 1998) and now also used in prepulse-modified version (Kumru, Valls-Solé, Kofler, Castellote, & Sanegre, 2006). Published methodology as this allows us to take upon our previous reaction time tests in which we have shown connections between latent toxoplasmosis as changes in the performance of tested subject (Havlícek et al., 2001; Novotná et al., 2008) and employ the new methods in researching scope of the parasitic effects on human behavior.

2.6 Footnotes

[1] Books referenced by the subchapters' titles:

The Art of War by **Sun Tzu**: because manipulation is a war, and parasite-host interactions are one of the greatest examples of arms races in nature.

Lord of Flies by **William Golding**: because *Toxoplasma gondii* is one of the greatest models for studying manipulation hypothesis – we can actually ask its vector some questions – and from this vintage point, it really is a king of them all. Also, when considering all of the possible effects appearing in our data, one sometimes has a hard time telling between the reality and the dream, i.e. between what is really there and what is but an experimental artefact or a chance-driven statistics. And the "Parasite Rex" title was already taken.

Darkness Visible: A Memoir of Madness by **William Styron:** because mental illness is a tough topic to tackle professionally and personally. It's wide, complex, emotional, and sometimes seemingly hopeless. Forwarding the studies on parasite-caused mental health issues can lead to development of better diagnostic and treatment methods thus creating hope for affected individuals. Thus, symbolically, I see the Styron's book as better fit than also considered Bell Jar Full of Toxo.

A Beautiful Mind by **Sylvia Nasar:** schizophrenia is not the only reason for choosing this book. The first publication enclosed in this thesis also deals with economy, mathematics, and game theory, so it is only appropriate to reference one of the fields' giant on whose shoulders we are all standing.

Thinking, Fast and Slow by **Daniel Kahneman:** because the reaction times, startles, and sensorimotor gating employed in prepulse inhibition of startle reaction are among the simplest examples of Kahneman's System 1 reactions – they are quick, automatic, unconscious. And bloody important for our survival.

[2] Effects of infectious agents on movement and dispersal of their hosts is well known and works both ways: sometimes the infection causes decrease in movement and dispersion (for example, a ciliate *Paramecium caudatum* reduces its dispersion while infected by a bacterium *Holospora undulata* (Fellous, Quillery, Duncan, & Kaltz, 2011)), sometimes it increases it. Unsurprisingly, making the host go to hardly typical places makes the parasite actually go places: Into the „increased dispersal and mobility" box fit all those peculiar manipulative activities such as insects trying to

get grazed on – and there are a whole lot of these cases, as can be seen for example in a recent review in Myrmecological News, where the authors listed ant hosts and their manipulative parasites. Of 21 listed cases, 17 included seeking an untypical environment (water, elevation, microclimate) (Bekker, Will, Das, & Adams, 2018).

[3] The obvious usefullness of the parasite-induced modification was often used as evidence for creation. One example could be a short pamphlet (Smith, 1984) by a zoologist turned creationist E. Norbert Smith published in a lovely "science journal" Creation Research Society Quarterly (you can find and read it in full here: https://creationresearch.org/crsq-archive/). Of course, biologists sometimes contribute to the confusion by their use of figurative language, which is why the teleological expressions and possible ways of their remedy are often contemplated in literature on the borderline of biology and philosophy (Bekoff & Allen, 1995).

[4] The familiar example of sporocysts of *Leucochloridium paradoxum* pulsating in eye stalks of its snail vector *Succinea putris* while also manipulating its behavior toward seeking higher and better illuminated positions (Wesołowska & Wesołowski, 2013) is far from being the only example. Perhaps even more interesting is the first described case of a parasite causing fruit-like mimicry in ants. The newly described tetradonematid nematode *Myrmeconema neotropicum* (Poinar & Yanoviak, 2007) in a tropical ant *Cephalotes atratus* changes the color of its host's gaster from black to red so it resembles ripening fruit of the sympatric tree *Hyeronima alchorneoides*, making the ant more appetizing to fruit-eating birds (the effect was experimentally tested). The infection also weakens the postpetiole-gaster junction, so it is easier to pluck the gaster from the ant without removing the ant from the surface. This isn't possible in uninfected ants of the species. (Yanoviak, Kaspari, Dudley, & Poinar, 2008)

[5] Charles Nicolle was awarded a Nobel Prize in Physiology or Medicine in1928 for his work on typhus, or more specifically for discovering the key role of mice in transmission of epidemic typhus.

[6] Of course, on some level it makes sense that while there are cat people and dog people (Gosling, Sandy, & Potter, 2010), there is also a cat's *Toxoplasma* and a "dog's *Toxoplasma*" (i.e. *Neospora caninum*). This protist was long considered just another form of *Toxoplasma*, since it is similar in both appearance and the problems it causes (lost gestations in cattle). However, in 1988, no one other than the main specialist on toxoplasmosis Jitender P. Dubey identified and named it as a separate species. Later,

it was found that *Neospora* is related to *Toxoplasma*, but it's definite hosts are canines instead of felines (it was first discovered only in domestic dogs (Lindsay, Dubey, & Duncan, 1999; M. M. McAllister et al., 1998), but later other canines such as coyotes (Gondim, McAllister, Pitt, & Zemlicka, 2004), dingoes (King et al., 2010), and grey wolves (Dubey et al., 2011) were identified as definite hosts as well). In contrast with *Toxoplasma, Neospora* doesn't use such a wide range of vectors, and mostly infect bovines. For more information on this parasite, I would recommend Dubey's history on *Neospora*-related discoveries (Dubey & Lindsay, 1996) and his later review of *Neospora* in animals (Dubey & Dubey, 2003), while the latest research can be found in 2010s reviews by Goodswen (Goodswen, Kennedy, & Ellis, 2013) and Donahoe (Donahoe, Lindsay, Krockenberger, Phalen, & Šlapeta, 2015).

[7] In this point, I am reducing the much wider field of causes of mental diseases to the one aspect relevant for the topic of the thesis. I do not under any circumstances suggest that the infectious agents are all that is to it. The epidemiology of mental diseases works with broader array of various internal (such as genetics) and external (such as socioenvironmental influences) causes, a compendium of which might be found for example in the Textbook of Psychiatric Epidemiology (Tsuang, Tohen, & Jones, 2011).

[8] For more information on the changes caused by toxoplasmic cysts in brain of the infected host I can recommend the review by Ellen Tedford and Glenn McConkey (Tedford, McConkey, Tedford, & McConkey, 2017).

[9] Donders' simple reaction times and choice reaction times in an adapted computerized form are used even in modern research including that done in our laboratory, see for example (Jaroslav Flegr, Novotná, et al., 2008) and Publ. 5 included in this thesis.

[10] The 19th century is an immensely interesting time for psychology in general. By the beginning of the century, the prevailing opinion was that it is impossible to study the inner workings of mind. By the end of the century, first departments of what would be now called cognitive psychology were already founded, while during the century's turmoil we had already glimpsed the inner workings of our brain through studying one of the most famous patients in history – Phineas Gage –, or through the works of Paul Broca, a discoverer of one of the centres of speech (Goldstein, 2008). Unfortunately, most of the discoveries lie beyond the scope of the thesis.

[11] "Simple" might actually not be a proper term here. True, the knee-jerk is a monosynaptic stretch reflex involving no interneurons in its arc, however, it is affected by multiple voluntary and involuntary processes in the body. In the first half of the 20th century, experts were already aware that there is nothing simple about simple reflexes, as demonstrated for example in the words of an American psychologist Harold Schlosberg: "At present, the position that the knee jerk is a simple reflex is being abandoned, along with other so-called simple reflexes. The work of Sherrington, Liddell and Fulton, and of Travis especially, and the innumerable studies of the effect of facilitation and inhibition on the patellar reflex, make it apparent that the supposedly simple reflex not only involves a simple path from receptor to effector, with one synapse in the lumbar region of the cord, but also calls forth the activity of a fairly large portion of the central nervous system. The initial contraction of the quadriceps seems to be the result of a simple spinal reflex, but the curve of contraction of the muscle is soon complicated by discharges from higher centers." (Schlosberg, 1928)

[12] Interestingly, it took only 5 to 10 years for the patellar reflex to become a widespread diagnostic practice for various forms of paralyses and other disorders (Louis, 2002), making a huge contrast with various forms of response modifications, that went out of sight & out of mind for several decades, and completely so, at least according to Ison & Hoffman (1983). I might not be that strict, though, since for example (Varnum, 1934) presents both a review of older literature including works of Bowditch & Warren and even Mitchell & Lewis, and his own experiments measuring extent and latency of knee-jerks in university students using Jendrassik's Handgriffs and other methods facilitating the response.

[13] One of the founders of the American Physiological Society.

[14] Interestingly enough, descriptions of Sechenov's experiments included in the first edition of an influential psychological textbook Elements of Physiological Psychology (Pillsbury, Ladd, & Woodworth, 2006) were later dropped out and cannot be found in the second edition (Pillsbury, Ladd, & Woodworth, 1912).

[15] Just to be clear, this is John L. Searle from Pennsylvania State University, not the renown philosopher John R. Searle specialized in the philosophy of language and mind and in intentionality.

[16] Beside his experimental work, he also thoroughly explored the history of the discovery, beginning with Sechenov's theoretical speculations about the existence of purely inhibitory neural processes (Ison & Hoffman, 1983). In this interesting and well written article, the authors not only detailed a hundred of years of development of this methodology, but also attempted to explain why did such an important and useful experimental tool needed to be discovered and rediscovered at least four times before entering the realm of mainstream science. The authors came up with three possible explanations, one of them being possible confusion with Pavlovian habituation. As the second reason, the authors are mentioning probable incompatibility of Sechenov's observations with mainstream beliefs and theoretical concepts of his time; scientists by than considered seemingly suppressive neural effects to be but experimental artifacts. The third suggested reason is that psychology was far more concerned with search for a general theory than with simple individual behavioral phenomena, even though these might actually help constructing the very theory they were searching for. For more details on history of startle modification discoveries, see the cited article; for more information on inhibitory neural processes in general, see the 1963 book *Inhibition and Choice* by Solomon Diamond, Richard Balvin and Florence Diamond (Singer, Diamond, Balvin, & Diamond, 2006).

3 Materials and Methods

3.1 Experimental subjects

We recruited our experimental subjects from three independent sources. Since we conduct most of our studies on our workplace at the Faculty of Sciences, Charles University, undergraduate students of our faculty (the first population) volunteered as experimental subjects in four of the studies (publications 1, 3, 5, and 6).

The cortisol and sex hormones levels and their effect on performance and other measures were studied on military personnel (the second population) in cooperation with the Military University Hospital (MUH) in Prague (publication 2). The data of schizophrenia patients (the third population) for the last study were obtained in cooperation with Psychiatric Centre Prague (PCP), later National Institute of Mental Health (NIMH) and Institute of Endocrinology, Prague (publication 3).

Each experiment conducted at the Faculty of Science was conducted with the permission of our Ethical committee, and all our experimental subjects signed an informed consent prior to undergoing any experiments or blood sampling. The experiments conducted in the Military University Hospital Prague and Psychiatric Centre Prague were conducted in compliance with the ethical code of the respective institutions.

3.2 Laboratory tests

The National Laboratory for Toxoplasmosis of the National Institute of Public Health (NIPH) conducted all immunological tests for **toxoplasmosis**. Two methods were used to establish anti-*Toxoplasma* antibodies in sera of experimental subjects in all four publications dealing with toxoplasmosis (publications 3, 4, 5, and 6). Enzyme-Linked Immunosorbent Assay (ELISA) was used to establish levels of anti-*Toxoplasma* antibodies IgG and IgM, while the Complement Fixation Test (CFR) was used as a complementary method which allows us to detect older *Toxoplasma* infections (P. Kodym, Machala, Roháčová, Širocká, & Malý, 2007).

RhD phenotype of probands in Publication 6 was tested using a standard agglutination method (anti-D serum - human monoclonal antiD reagent; SeracloneH, Immucor Gamma Inc.) directly during the blood sampling.

The serological diagnosis of **cytomegalovirus** (CMV) infection for Publication 4 was conducted in the National Reference Laboratory for Herpes Viruses of NIMH using quantitative ELISA (Cappel, de Cuyper, & de Braekeleer, 1978).

Steroid concentrations for Publications 2 and 4 were established in the Institute of Endocrinology using commercial kits and in-house radioimmunoassay methods (Lapčík, Hampl, Hill, Bičíková, & Stárka, 1998; Lapčík, Hampl, Hill, & Stárka, 1999); detailed descriptions of serological methods are included in both publications as well as in (Flegr et al., 2008).

Publication 4 included also using (ECLIA) test for determination of **Thyrotropin** or Thyroid-Stimulating Hormone (TSH), Glucose analyzer for measuring fasting blood **glucose levels**, and commercially available kits (CHOL2 HiCo T 400, HDL-C III 200, LDL-D Gen 2 200, and TRIGL 250, respectively (Roche Diagnostics GmbH) with a Cobas 6000 module C analyser) for establishing various **lipid parameters** (total serum cholesterol, high and low-density lipoproteins, and triacylglycerides).

3.3 Experimental methods

We have used various experimental approaches during the period of the whole doctoral project including

- Experimental games, namely the Public Good Games with and without punishment (publication 1),
- Questionnaires, namely N-70, OD-1, and BDI (publication 2), NEO-PI-R (publication 3),
- Intelligence tests, namely WMT and Otis test for verbal intelligence (publication 2),
- Memory and performance tests, namely Meili selective memory test, Test of attention and short memory (both used in publication 2), newly

developed acoustic reaction time test (publication 5); grip test and weight-holding test were used to test performance in university students for the publication 6.

All tests but the publication 6 performance tests, which were conducted individually in order to ensure the same social environment for each proband, were administered in group-setting scenarios and all except for the NEO-PI-R questionnaire, which was printed on paper forms, were computer-administered.

All experiments conducted for purposes of publication 2 took place in Military University Hospital in Prague during regular acceptance examinations for military personnel, while experiments for publications 1, 3, and 5 were conducted on Faculty of Science, Charles University.

3.4 Statistical tests

Programs Statistica, SPSS, and MS Excel (with a customized sheet for partial Kendall correlation, which could be downloaded here https://web.natur.cuni.cz/flegr/programy.php) were used for all analyses.

Most of the data of enclosed studies were analyzed using General linear models, t-tests, one-tailed test, partial correlation Kendall test and non-parametric tests (Mann Whitney U test, Spearman rank correlation, and Kruskal Wallis test).

Statistical tests used in each study are mentioned in the Material and Methods sections of the next chapter.

3.5 The Project Timeline

My doctoral project began long before I have finished my master degree, or perhaps even before I have started it. During my bachelor studies, I became interested in the manipulation hypothesis paradigm with *Toxoplasma* – human interactions as model organisms. We have been conducting experiments using psychological tests both general (namely the Cattell's 16 Personality Factors (Cattell's 16PF), Cloninger's Temperament and Character Inventory (Cloninger's TCI), and the Revised Neuroticism-Extraversion-Openness Personality Inventory (NEO-PI-R) and specifically developed to

study possible changes in fear and several other traits in *Toxoplasma*-positive human subjects.

While attending lessons on behavioral pharmacology, I have learned about an experimental method used in psychology and psychiatry to detect even the slightest changes in the way brain processes simple startle responses (SR) and their modification by weak stimuli closely preceding main signals. The method called prepulse inhibition (or modification in more general usage) of startle reaction (PPI) seemed both applicable for our research at hand and feasible to conduct in conditions of our laboratory. We have decided to try the idea and started preparing experimental designs adapted to our needs and possibilities. It could get us some points for originality since, by that time, no one had used this method in *Toxoplasma*-related studies. It is one of my few regrets that we were unable to conduct the experiments in time to grab that first place with our publications.

While our PPI and acoustic startle programs were being developed, we have conducted studies using experimental games, and while our experimental subjects were waiting for next experiment within an all-morning experimental session to be conducted, they have filled in NEO-PI-R questionnaires.

In the meantime, data for publications 2 and 4 were being collected and analyzed in our cooperating institutions, Military University Hospital in Prague and Psychiatric Centre Prague.

After the data of the experimental games were collected, experimental sessions were changed to include acoustic and visual reaction times tests with prepulse signal modifications and customized computer-administered Stroop's tests, and both visual and acoustic reaction times were also included in tests administered to military personnel. Data collected from the acoustic reaction time test administered to our students were analyzed, and the following publication is included in this thesis, while other data are still waiting to be analyzed and published, see Chapter 5 for further details. The data for performance tests (grip test and weight holding tests) for publication 6 were collected together with computer-administered acoustic and visual

reaction times within the same sessions, but individually, with probands being sent to the experimental site one by one.

In the years 2013 to 2016, we have also been collecting data in individual experimental sessions using our custom-created prepulse inhibition device. We have tested several hundreds of experimental subjects, mostly undergraduate students of biology, using this device, and by the time of this thesis' preparation, we are developing a program (MATLAB script) in collaboration with a researcher from the Institute of Thermodynamics of The Czech Academy of Sciences, to help us convert collected biosignals into analyzable form. As of now, we are trying to overcome electronic noise found in some of the experimental records.

Beside publications included in this thesis, my colleagues published multiple studies based on data collected during the same experimental sessions which I have helped organized and lead, for example, a study associating perceived and measured intelligence in male and female university students (Kleisner, Chvátalová, & Flegr, 2014), piles of data, however, remain unprocessed.

4 Publications

4.1 Justine Effect: Punishment of the Unduly Self-Sacrificing Cooperative Individuals

4.1.1 Reasons for Inclusion

The reason for the inclusion of this particular publication into a *Toxoplasma*-related thesis is as follows. Since most of our previous experiments with *Toxoplasma*-infected population and uninfected controls focused on differences in personality as measured by standardized psychological questionnaires, and on differences in simple reaction times, we searched for an experimental tool that would enable us to study also the expected difference in our subjects' behavior. For this reason, we have used experimental games on our studied population of university students. While analyzing the data, we had found one rather exciting and unexpected effect (at least in the experimental settings) of human behavior – the punishment of those, who are sacrificing their own gain for the gain of the whole group. And that is what we had published.

It is worth mentioning, that although the data from this particular study did not, in the end, contributed to the studies of human-*Toxoplasma* relationship, the tool was chosen correctly. Our colleagues have shown changes in the behavior of *Toxoplasma*-infected subjects in other studies using experimental games – specifically the Trust Game and the Dictator Game. Infected individuals acted less generously in the Dictator Game; in the Trust Game, the effect of latent toxoplasmosis was present as well, although it was modified by the subjects' gender (Lindova et al., 2010).

4.1.2 Introduction

In an experimental economy, the so-called "Public Good Games" or Voluntary Contribution Mechanism (VCM) allow us to model and study human cooperative behavior and factors leading to its sustenance. Although—as speculated, for example, by Fehr and Gächter (Fehr & Gächter, 2000)—in entirely selfish individuals (the standard reduction of humans to so-called *Homo oeconomicus*), no change in cooperation maintenance should occur in

experimental settings with punishment opportunities since punishing is costly to the punishing individual. The purely selfish individuals would not waste their resources on maintaining cooperation, yet each experiment shows human behavior to be different from the idealized model. While it is true that some subjects show lesser and some higher tendency toward punishment of the uncooperative individuals (the so-called free riders) and that the willingness to punish follow punishment costs[1], the experimental subjects punish the free riders and the more so, the lesser the contribution of the particular free rider (Anderson & Putterman, 2006).

It is clear that a punishment added to an experimental scenario serves as a tool for sustaining cooperation in an experimental situation. However, as with all the tools, there is a hitch: Tools can serve their purpose, or they can be used mischievously, in this case, the possibility of punishment of uncooperative individual can be exploited by the very free riders to punish antisocially. This is, indeed, the case in both real-life situations and experimental settings. In real life, antisocial punishment in the form of social ostracism was observed, for example, during strikes of British miners in 1984, when the strike-breakers faced complete expulsion from their former communities (F.J. Roethlisberger, Dickson, & Thompson, 2003); hostility is often directed on highly effective workers in workplaces with quota settings. While the productivity of the whole team could be better and each worker could often gain higher salary, workers tend to limit their effectivity in order not to exceed the minimum quota by much, as well as to dislike those who refuse to slow down (Fritz Jules Roethlisberger & Dickson, 2003).

An example is given in an interview with a worker talking about his relative who: *"… gets in here early and goes ahead and make up a lot of parts so that when the rest of the girls start in she's already got a whole lot stacked up. In that way she turns out a great deal of work. She's money greedy. That's what's the matter with her, and they shouldn't allow that. All she does is spoil the rate for the rest of the girls"* [in 104].

Of course, the findings mostly come from the beginning and middle of the 20th century[2], surely these could be exceptions, and antisocial punishment

does not work in most environments the modern days. It turns out it does – all over the world. The antisocial behavior occurred in 16 out of 16 experimental populations from all over the world, though in varying amounts from very low antisocial punishment to such levels where the antisocial punishment almost extinguished pro-cooperative effect of punishment aimed at free riders (Herrmann, Thoni, & Gachter, 2008).

In our experiment, we tried to uncover reasons for antisocial punishment by focusing on typical victims (is maliciousness blind or attracted by virtue?) as well as on typical culprits (are the antisocially punishing individuals recruited from contributing players, free riders, or hypocrites contributing in the "with punishment" scenario but not in the "without punishment" one?

4.1.3 Materials and Methods

77 females and 41 male students (mean age 21.46, S.D. 1.60) of Faculty of Science were tested using a computer-administered public good game scenario. Twelve subjects participated in each session (in two sessions, one missing experimental subject was substituted by a *virtual player*, a person with instruction to never punish, and to always contribute median of what contributed the other players; we have not included the virtual players in analyses of punishment strategies).

Each session consisted of six rounds of the Public Goods Game with and six rounds of the game without punishment; in half of the experimental session experimental subjects were presented first with the game without punishment, and in another half of the sessions they started with the game with punishment. The amount given to each player at the beginning of each round was 20 CZK, the maximal amount a player could gain was thus 12 rounds x 20 x 2, that is 480 CZK.

The twelve experimental subjects were each provided an experimental notebook and a workplace separated from other by a cardboard partition to ensure anonymity. All within-game interactions were conveyed through a web application which attributed each player with a random number that differed for each round so other players could not connect any player with his or her

contributions, nor was it possible for players to know how much each player contributed in previous rounds.

We used general linear models with repeated measures for primary analyses, cooperativeness and individual characteristics of the culprits as well as analyses of contributing players were tested separately using standard linear regression as well as non-parametric methods (Mann Whitney U test, Spearman rank correlation, and Kruskal Wallis test).

4.1.4 Results and Discussion

As expected, contributions in scenario without punishment decreased significantly during the six rounds (1st round 10.98 CZK, 6th round 2.98 CZK, GLM rep. measures, linear contrast, $p<0.001$; avg. contributions 6.11 CZK) and slightly increased in the scenario with punishment (1st round 11.06 CZK, 6th round 12.38 CZK, GLM rep. measures, linear contrast, $p=0.022$; avg. contributions 11.86 CZK). Penalties decreased significantly from the first round (1.72 penalty marks per player) to the sixth round (1.61 penalty marks per player; GLM repeated measures, linear contrast, $p=0.007$) with average penalty marks at 1.64 per player.

We have found negative correlation between the contribution and level of punishment (Spearman $\rho= -0.665$ for number of obtained penalty marks, $p<0.001$, and $\rho= -0.651$ for rank of the punishment within round, $p<0.001$), however, the actual graph is U-shaped since, for contributions in upper quartile, the correlations were positive ($N=206$, $\rho=0.254$, $p<0.01$, $\rho=0.141$, $p=0.043$). Furthermore, within the anti-social punishment, players contributing the most were also punished the most.

4.1.5 Limitations

In real life, both pro-social and anti-social punishment often takes the form of social ostracization rather than direct financial penalties. It would be interesting to come up with an experiment closely following the real-life scenarios where, the punishment would take the form of, for example, showing photographs of the punished individuals to other players or society. However, most telling scenarios would be unethical and thus highly unrecommendable.

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PLOS ONE

Justine Effect: Punishment of the Unduly Self-Sacrificing Cooperative Individuals

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Abstract

Background: Allowing players to punish their opponents in Public Goods Game sustains cooperation within a group and thus brings advantage to the cooperative individuals. However, the possibility of punishment of the co-players can result in antisocial punishment, the punishment of those players who contribute the most in the group. To better understand why antisocial punishment exists, it must be determined who are the anti-social punishers and who are their primary targets.

Methods: For resolving these questions we increased the number of players in a group from usual four to twelve. Each group played six rounds of the standard Public Goods Game and six rounds of the Public Goods Game with punishment. Each player in each round received 20 CZK ($ 1.25). Players (N = 118) were rematched after each round so that they would not take into consideration opponents' past behavior.

Results: The amount of the punishment received correlated negatively with the contribution ($\rho = -0.665$, $p<0.001$). However, this correlation was positive for players in the highest contributors-quartile ($\rho = 0.254$, $p<0.001$). Therefore, the graph of relation between the contribution given and punishment obtained was U-shaped ($R^2 = 0.678$, $p<0.001$) with the inflection point near the left boarder of the upper quartile. The antisocial punishment was present in all groups, and in eight out of ten groups the Justine Effect (the positive correlation between the contribution to the public pool and the risk of suffering punishment in the subpopulation of altruistic players) emerged. In our sample, 22.5% subjects, all of them Free riders and low contributors, punished the altruistic players.

Conclusions: The results of our experimental game-study revealed the existence of the Justine effect – the positive correlation between the contribution to the public pool by a subpopulation of the most altruistic players, and the amount of punishment these players obtained from free-riders.

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Introduction

Most of the published results of experimental studies concerning Voluntary Contribution Mechanism (VCM or "Public Goods Game") propose that allowing players to punish their opponents sustains cooperation within a group and thus brings advantage to the cooperative individuals. Experimental subjects tend to use punishment even in cases where punishment is relatively expensive. This might seem to be nonsensical for the self-interested individual: a rational assessment of the direct impact of punishment reveals that it is costly for both parties. The income of the punisher decreases with the cost of the punishment; the income of the punished decreases with the fines, and the income of the group decreases by the sum of both the punishment's costs and the fines. Nevertheless, punishment is shown to be a great benefit for the group in studies comparing VCM with (VCM-P) and without (VCM) punishment [1–3]. The benefits arise from better group discipline, cohesion, and elimination of free riders which outweighs the losses of punishment costs and fines [4–7]. Yet in some of the experiments, e. g. in [8], the authors in particular conditions reported the opposite result: punishment was unfavorable due to the amount of fines outweighing the benefits of better discipline.

In contrast to the punishment of free riders, the voluntary choice of the players of whether and how to punish the co-players can result in so-called antisocial (or perverted) punishment [9–11]. Some players will sacrifice part of their income to punish players contributing more than sufficiently or even the most in the group. Cinyabuguma, Page, & Putterman [12] claim "typically 20% or more" of the punishments are misused in this way. A cross-cultural experiment [13] conducted in 16 cities of the world reports a range of 6 (Melbourne) to 48 (Muscat) per cent. This figure is too large for the antisocial punishment to be considered a marginal phenomenon. But on the other side—and in contrast to the pro-social punishment—this phenomenon is unstable; the willingness to punish antisocially changed with a small modification in

conditions detrimental to the antisocial punishers. Moreover, the behavior of players in social games is generally sensitive to a number of subtle factors, including gender of players [14,15], health status [14] and details of an experimental setup [16].

The VCM-P experiment conducted by [17], tested the effectiveness of punishment, i.e. the ratio f = (financial loss of the punished)/(punishment cost). In conditions of "High sanction treatment" the ratio was set to $f_C = 3.3$ for punishment of a cooperating player and $f_D = 2.5$ for punishment of a free rider in conditions of "Low sanction treatment" f = 1 in all cases meaning that the cost of every punishment equals the amount of loss the punished player suffers. The antisocial punishing in "High sanction treatment" comprised 22% of the resources expended on punishing and 46% of the free riders' punishing resources (the cooperators punished exclusively pro-socially); in "Low sanction treatment" not a single antisocial punishment was rendered. Rate of punishment given by cooperators to defectors was almost the same in both treatments.

The experiments of Nikiforakis [9] and Cinyabugma et al. [12] enabled reactions to the punishment by a counter-punishment; the difference between the two designs was as follows: "*In Nikiforakis's design, if you are first-order punished you learn who punished you and by what amount. This makes targeted revenge easy. In [Cinyabuguma et al.'s] design, if you are first-order punished you don't learn who punished you, only that you were punished in an identified aggregate amount*" [12], p. 267. The possibility of secondary counter-punishment substantially decreased the percentage and the absolute number of antisocial punishments given during the first phase of punishing but the counter-punishing phase evened up the difference. In the counter-punishment phase the antisocial punishing could be explained as revenge, blindly targeted and with risk of punishing the innocent.

The interpretation of economic or evolutionary motivation for the antisocial punishment is a bit more difficult than interpretation of the motivation for usual pro-social punishment [18]. To better understand why antisocial punishment exists, it must be determined who this punishment primarily targets. In this, there is much disagreement in the literature. As [12], p. 267 put it: "*But a substantial amount of punishment was directed at high contributors… typically 20% or more of all punishment events are directed at the highest contributor in the group.*" Publications allege the existence of antisocial punishment but do not explicate the differences between targets. They are also mostly based on experiments with groups of four, which cannot distinguish between high and the highest contributions from the punisher's point of view:. The authors of the article do not know about any experiment dealing with antisocial punishment in a larger cooperative group.

Our experiment deals with the difference between high and the highest contributions as a target of punishment in conditions of VCM-P. The experiment was designed to discriminate between the following two hypotheses:

1. A player deciding to punish antisocially would randomly choose a victim. If the victim is a free rider, i.e. a player contributing little or not at all, the punishment would not be recognized as antisocial by the experimenter.
2. A player deciding to punish antisocially would target those who contribute more with higher probability.

When trying to differentiate between the former (maliciousness is blind) and the latter (maliciousness is attracted by virtue) hypotheses we focused on the following questions:

1. "Victims." Who is the typical victim of antisocial punishment? Our null hypothesis is: while antisocially motivated, the punisher chooses his target impartially; the portion of the punishments given to non-cooperative players stays unrecognizable to the experimenter. The alternative hypothesis is that after some threshold of group cooperativeness antisocial punishment will be observed and correlation between contribution and punishment received would be positive and significant.

   The hypothesis "maliciousness is attracted by virtue" assumes an unpleasant position for the player contributing the most, as he is exposed to more punishments than average or slightly above-average players. In hyperbole, we have called the phenomenon The Justine Effect in honor of the unusually altruistic character of the well-known 1791's novel of de Sade [19].
2. "Culprits." Which of the strategies exercised in Public Goods is typically connected with antisocial punishment or punishment of the extraordinary altruistic individuals? Is it to be anticipated from socially responsible players, parasitic free riders or from hypocrites cooperating in the *Public Goods with Punishment Game* and free riding in the *Public Goods Game*?

We showed that the victims of antisocial punishment are the most altruistic players. The probability of being a target of antisocial punishments was positively correlated with the contribution in subpopulation of the altruistic players. Antisocial punishers were free riders and low contributors.

Results

A) General

In the standard *Public Goods Game* the average contribution to the fund was 6.11 CZK and it decreased significantly from 10.98 CZK in the first round to 2.98 CZK in the sixth round (GLM measures, linear contrast, $p<0.001$). In the *Public Goods Game with punishment* players almost doubled their contribution as they invested 11.86 CZK on average in the public fund. The contribution in the first round was similar to the *Public Goods Game* (11.06 CZK) and significantly increased over the course of the game (GLM repeated measures, linear contrast, $p = 0.022$) to 12.38 CZK in the last round. The number of penalty marks granted during the game significantly decreased (GLM repeated measures, linear contrast, $p = 0.007$) from 1.72 penalty marks per player to 1.61 penalty marks per player in the last round. On average players granted 1.64 penalty marks. These results exclude three virtual players, who because of their strategy, do not influence the average contribution, but decrease slightly (117/120 times) the average number of granted penalty points.

B) Victims

The behavioral pattern present in our data is consistent with fairness theory: the lower the contribution, the higher the punishment. Correlation between the rank of the player's contribution (within the specific round and group) and level of the punishment obtained was strongly negative and significant (*Spearman correlation*: $\rho = -0.665$ for number of obtained penalty marks and, $\rho = -0.651$ for rank of the punishment within round, for both $p<0.001$). In case of limitations of insight into the contributions where malicious punishment begins to outweigh prosocial punishment we obtained not a neutral, but an inversed result: For 206 contributions higher or equal to the outline of the upper quartile within the corresponding group, the correlations were positive $\rho = 0.254$, $p<0.001$ and $\rho = 0.141$, $p = 0.043$. In addition, the players with highest contributions were punished most. For further analysis we used our sub sample consisting of the higher than median contributions. We divided this sample in two

groups. The first group with 107 observations consisted of the highest contributions. The second group with 182 observations was the contributions between median and the highest contributions. We found that the players in the group with the highest contributions received significantly higher levels of punishment (0.31) compared to those in the latter group (0.16). (Mann Whitney U, $p = 0.001$ for the number of obtained punishments, $p = 0.064$ for the rank of the obtained punishment within the corresponding round). When dividing the group by a stricter rule in which the highest contribution must be radically higher than other contributions of the corresponding round, 30 highest contributions "attracted" significantly more severe punishments than the rest 259 contributions (Mann Whitney U, $p = 0.010$ for the number of obtained punishments and $p = 0.009$ for the rank of the obtained punishment within the corresponding round; on average, 0.47 versus 0.19 of the obtained penalty marks). This kind of malicious behavior was not frequent but it was targeted those who behaved most prosaically.

Both types of regression enabled us to reject the hypothesis about the "just shape" of the punishment curve (i.e. the dependency of the obtained punishment on the height of the contribution given as its rank within the corresponding round). Straight line approximation of the dependency of the contribution (relative rank) on the obtained punishment (relative rank), explained significantly smaller fraction of the variance in the comparison with the parabolic approximation ($R^2_{linear} = 0.492$, $R^2_{quadratic} = 0.678$, $p_{quad>\mathrm{lin}} = 0.002$). On the contrary, the cubic approximation explained virtually as much variation as quadratic one ($R^2_{cubic} = 0.679$, $p_{cub>quad} = 0.433$).

We found that the relationship between obtained punishment rank and the rank of the contribution can be approximated with the following quadratic polynomial $p(x) = 0.122x^2 - 2.215x + 14.348$; this enabled us to find the critical point that is the contribution that was punished the least. We found that $x_{opt} = 9.078 \in <9,10> \sim$ upper quartile which is above the average level of cooperativeness, though not exceedingly high.

Regression analysis proved the superiority of U shape curve which predicted steep decrease of obtained punishment from minimum contribution to around median contribution, then continuing with small changes up to the point of absolute minimum. This occurred around the limitations of top quadrant $x_{opt} = 9.5$ (see Fig. 1). If the player contributed more than that, he could expect a higher risk of punishment. This U-shape curve explained data significantly better ($p<0.001$, likelihood comparison) than optimal *L-shaped* curve decreasing up to a specific point (median of the contribution in this experiment) and then continuing as a constant.

A small number of players were willing to punish maliciously in the sample (about 22%, see "the culprits"). Their concurrence in punishment of the most altruistic players, however, caused discomfort in the position of the maximal altruist. The Justine effect does not work as an absolute law - in some groups the malicious punisher need not even be present. In our experiment the malicious (severe and unjust) punishment was present in all ten experimental groups and in eight of the groups we observed the Justine effect, i.e. increasing dependency between the rank of contribution and the obtained punishment for the contributions higher or equal to upper quartile.

Punishment strategies did not differ significantly among groups with different orders of games.

C) Culprits

Six players out of 118 total players didn't punish at all and 68 players punished always justly and appropriately ($Sev^{1-6} \leq 0.5$). 19 players were occasionally unfair but never punished unduly. 25 players manifested both unjust and undue punishing in *Public Goods Game with punishment* and 15 of them punished the highest contributor at least once. There was no player who punished severely but justly half or more than half of the players. Unjust punishing was thus always caused by another motivation of the punisher than simply excessive severity; in the text bellow these players will be referred to as malicious. As the majority of them punished the highest contributors, they can be also labeled as the culprits of above mentioned Justine effect.

In both variants of the game, the players that punish unjustly were significantly less cooperative than other players (Mann Whitney U test comparing average ranks of the contributions of 68 just players and other players: $p = 0.003$ for *Public Goods Game*, $p < 0.001$ for *Public Goods with Punishment Game*). As for the excessive severity, relative contribution within the group in both games correlated negatively with the level of severity Sev^{1-6}_i of the player i ($\rho = -0.249$, $p = 0.007$ for average relative ranks of contributions in standard *Public Goods Game*, $\rho = -0.232$, $p = 0.012$ for the same in *Public Goods Game with punishment*).

In comparison of the three groups of punishing players (just and adequate, sometimes unjust but always adequate, malicious; non-punishing players were excluded) from the point of view of the relative level of contribution into the *Public Goods* fund, the malicious players had the lowest contribution in both games, while the justly and adequately punishing players had the highest contributions (Kruskall Wallis, $p = 0.004$ in test of the contributions position in standard *Public Goods Game*, $p<0.001$ in contributions position in *Public Goods Game with punishment*). When considering only the 25 *malicious* players into the correlation, we obtained a significant correlation for selfishness and severity of contributions in standard *Public Goods Game* (though markedly stronger: $\rho = -0.432$, $p = 0.031$). The correlation in *Public Goods Game with punishment* stayed strong as in the first method, but was not significant ($\rho = -0.257$, $p = 0.214$).

This all suggests the conclusion that the culprits of Justine Effect are more likely free riders than cooperative players.

A submatrix of the contingent table (see Table 1) characterizes the *punishment behavior* of malicious players in each round. In 6×25 possibilities, 25 malicious players did not punish at all in 31 rounds, punished exclusively pro-socially in 56 rounds, and punished both prosocially and antisocially in 42 rounds. In total, only 5 cases punishment can be characterized as "severe but just", meaning that it affected players contributing median or more and also all the players below median.

In the table we can see the two most important characteristics of punishing behavior:

1. The "severe but just" punishing is present only in marginal number of cases and only in players that acted maliciously in other rounds. This suggests that, punishing of a player who contributes median or more within the round, doesn't occur as a result of unfulfilled expectations of the punisher, but almost in all cases as a result of a desire to punish antisocially. The goal of these punishments was almost certainly not meant to motivate higher levels of cooperation within the group.
2. The players who punished antisocially also punished non-cooperative players.

Discussion

The discussion is trying to understand the motivation of antisocially punishing players and bring the Justine Effect into the context of other results in VCM-P. Existence of the Justine

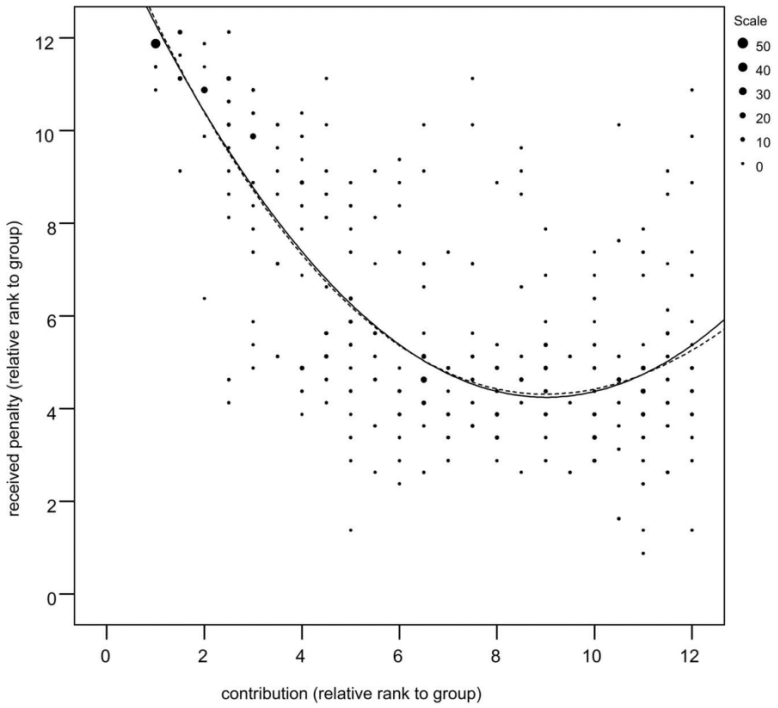

Figure 1. Relation between contribution to the public pool and punishment suffered in particular round of game. Solid and dotted line shows fitted quadratic and cubic functions, respectively.
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effect extended the list of two usual questions about the motivation of antisocially punishing players:

1. What are the reasons for the PGG players to be willing to sacrifice their own costs to punish the others?
2. What possible reasons are there for the decision to punish antisocially?

upon the third question:

3. If a player decides to punish antisocially what is the reason for directing this punishment intentionally and primarily to the most altruistic players?

Kollock [20] offers to distinguish among the participants of collective action with the task of choosing between individual and collective rationality according to four possible approaches:

1. Cooperation – maximizing joint outcome.
2. Competitive orientation –maximizing a relative difference between self and partner.
3. Altruism – maximizing partners' outcome without regard for one's own outcome.
4. Individualism – maximizing one's own outcome without any concern for the partners' outcome.

Table 1. The frequencies of particular types of punishers and types of punishments by malicious players.

All 118 players			
punished	**No. of players**	**always fair**	**sometimes unfair**
never	6		
always adequately	87	68	19
sometimes unduly	25	0	25
25 malicious players			
punishments	**No. of punishments**	**fair**	**unfair**
no (in particular round)	31		
adequately	72	56	16
unduly	47	5	42

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In a footnote he mentions three further possibilities which he, however, excludes from further research, namely:

5. Martyrdom
6. Sadism.
7. Egalitarianism – minimizing the difference between own and partners' outcomes (as absolute value; the name isn't used in the original).

The VPM is also a type of collective action and as such includes the contradiction between individual and collective rationality. This suggests the necessity of discussing Kollock's approaches in relation to the anti-social punishment and the Justine Effect.

From the point of view of the final outcomes, no costly punishment is cooperative, individualistic or altruistic. It lowers the income of the punishing party by the cost of punishment (i.e. no individualism), the income of the punished party by the fine (i.e. no altruism) and as a result also the total income of the group (i.e.no cooperation). On the other hand it shows accordance with egalitarianism and competitive orientation. A necessary (but not sufficient) condition for the validity of any of these explanations is the punishment effectiveness $f>1$ – where the punishment is more costly for the punished than for the punisher. In this case egalitarianism is able to explain the punishment handed out by the player with low income to the player with higher income. Competitive orientation accounts for the behavior of the punisher, who achieves maximum punishment effectiveness, that is, the ratio of the punishment suffered by punished player to the cost of punishment is highest. The fact that egalitarianism, and envy respectively, motivated by the competitive orientation of a subject, can be sufficient for creating a model which predicts that strategy profile which includes punishment to be a Nash Equilibrium [21], and experimentally [22].

However, *egalitarianism* does not come into consideration as possible motivation for perverse punishment (and especially the Justinian one) in the first line; the perverse punishment and its consequences do not support equality. Inequality aversion predicts sacrificing of one's own means to lower the gain of others under the assumption that the affected will be those with the highest gain. The perverse punishment, however, strengthens the inequality of income distribution: each player received an equal share and only those less than $20-X_j^k$ of saved money are subject to fine.

A competitive approach action need not contradict the immediate (first line) consequences of perverse punishment. Justinian punishment, on the other hand, stands in contradiction to the competitive approach. The *competitive* approach leads to the decision of no punishment or to spending the means for punishment with maximum efficiency, depending on what is more advantageous from perspective of the punisher. The observed data, however, did not support efficient punishment, as malicious punishment of the largest contributions is the least effective strategy of punishment. The cost of distributing penal points is equal but each penal point deducts the punished 10% (off the saved amount+fund share). The rate f_{social}/f_{Just} between the maximum and minimum possible punishment efficiency oscillated between 1.11 and 2.0, with the average of 1.54. The Justine Effect offenders are paying for their spite 1.54 times more expensively than they would pay for the punishment of a lower contributor.

The fact that the punisher sacrifices punishment effectiveness to punish the most contributing player contravenes with the punishment motivation hypothesis (pro-social and partly perverse). According to this interpretation, the benefit from gaining resources in group action is measured both as absolute gain and as relative share of the gain of the whole group. It would explain the distribution of voluntary payment for punishment as far as it significantly lowers the denominator – gain of the whole group. This motivation implies that the punishers will try to deduct as much as possible group money in the cheapest possible way. However, the selection of the most contributing players as the targets of punishment violates the second part of this rule. In a second view, i. e., from the perspective that the punishment can motivate the other player to change his behavior, the pro-social punishment can be cooperative, altruistic and individualistic. It motivates the punished to increase his contribution, which is advantageous for the individual and the group in the following rounds. This advantage compensates for the costs of punishment (individualism), the fine deducted as punishment (altruism) or the sum of both (cooperation). Even under such conditions anti-social punishment is not in accordance with egalitarianism or competitive orientation. Whichever way the punisher motivates his victim – to increase, to decrease or keep the contribution the same – the possible impact of the change in his behavior will be equal to all players. All disparities created by the anti-social punishment will thus be preserved.

As the first possible approach we can thus consider that the Justinian, eventually each anti-social punishment, as described in [20], is *exotic*. The acceptance of this is, however, contradicted – besides the null testifying value of this listing – by the not quite marginal spread of such behavior. The above mentioned source [12] found anti-social behavior, though with significantly different frequency in all observed cultural milieus.

The result of the experiment contradicts the explanation of the Justine Effect as manifestation of solidarity of the "gallows guild" – namely as a contribution to collective action of all free riders. This hypothesis is supported by the typical view of the victim as a player who contributes most and the punisher who contributes the least, but is contradicted by the behavior of the antisocially punishing individuals. They were characterized by their "benevolence to the middle"; apart from the victims in the group of the overly altruistic players, they also typically punished their *free riding* colleagues. Validity of the "*strong reciprocity inverted hypothesis*" is therefore unlikely.

The explanation that cannot be excluded in the framework of this experimental design is "blindly targeted revenge". This explanation is based on the *ad hoc* assumptions that the antisocially punishing non-cooperating players consider manifestation of exaggerated altruism as "*strong reciprocity*" [7,23] and they look to the altruists as possible initiators of their punishment.

Another possibility of explaining perverse punishment in relation to Justine Effect is the naturally additional rule to the competitive orientation approach - the ensuing Envy concept. The addition is the prerequisite that Envy is not fully unbiased and impartial. It is quite plausible to assume that the rate of benefit increase from the financial damage (or on the other hand profit) of the other will depend also on the identity of the other person. Evolutionary psychology claims that an important element of such benefit is the subjectively viewed similarity between the other person with oneself. One study [24], showed that the willingness to take part in another participant's gain or loss depends on similarity of the person to the research subject. At first, our explanation might not seem to be applicable under the conditions of experimental anonymity; however, this very explanation predicts the Justine Effect. If the only available identifiable characteristic of others is the amount of their contribution and at the same time the offenders significantly differentiate among the victims, then they must choose their victims based on this feature alone. Both main observed facts fit into the hypothesis of envy resulting from behavioral dissimilarity: the prevalence of spiteful punishment

victims in the overly altruistic players and the origination of the unjustly and unduly severe punishing in the group of parasitical players. The envy of a free rider is better appeased by damage caused to the most self-sacrificing individual, not damage caused to a player of average cooperation.

An additional question can be raised here whether simple pro-social punishment can also be explained by the following phenomenon: socially conscious players punish free riders not because they behave *badly* but because they behave *differently*. This explanation could explain the behavior but only partially. In support of this possibility, Shinada et al. [25] published a complementary Justine Effect result, in which the cooperating players punished non-cooperative more than the free riders. However, the "benevolence to the middle" rule, typical of those who punish spitefully (see Table 2), does not support the envy resulting from behavioral dissimilarity. Also, the *Utilitaristic* view - convincing others to higher contributions – was stronger motivation for prosocial punishment than Envy.

Conclusions

The results of this experiment show that overly self-sacrificing individuals are favorite subjects of perverse punishment. They are targets for punishers significantly more often than individuals who contribute slightly above the median, and their typical punishers are those who contribute less. No economical or evolutionary interpretation of this data can be accepted (nor excluded) without additional assumptions. The most likely interpretation is based on the biased envy: the malicious player punishes his counterpart more when he finds him less similar to himself. As he has no avenue to acquire additional information, the contribution level is the only feature available to him to assess (dis)similarity of a co-player.

Methods

Ethics statement

All participants provided their written informed consent. The recruitment of study subjects and data handling were performed in compliance with the Czech legislation in force and were approved by the Institutional Review Board of the Faculty of Science, Charles University.

Experimental setup

The experimental setup reflects the theoretical background of the present study:

Antisocial punishment is characterized by the two criteria: injustice and undue severity. We define the punishment strategy as **unjust** when the punisher in one round punishes players with higher contributions more and **unduly severe** if punished players' contribution is more than median contribution level. This definition of antisocial or unjust punishment does not take the contribution of the punishing player into account, therefore the free riders too could punish justly. Severity of a player j in round i where $Sev^i(j) \in \langle 0, 1 \rangle$ is defined as a rank of the highest contribution of the player that was punished by another player j in round i, divided by 11 (groupsize-1). Contribution of the player j is not a matter of interest for us. If $Sev^i(j) > 0.5$, the punishment is considered to be unduly severe; The Justine Effect is associated with $Sev^i(j)$ being in near proximity of one. The player who, at the same time, punishes unjustly and severely is considered to be punishing antisocially.

Our definition of antisocial punishment by conjunction of the two characteristics is theoretically in contrast that is commonly used in the literature, e.g. [13], that considers every unsuitable punishment as antisocial (punishment of a contribution above median or average). Results of this experiment, however, proved the validity of the commonly used definition, as the occurrence of unsuitable punishment always implied occurrence of an unjust punishment. We have modified the definition because of the theoretical possibility of "severe but just" punishment, i.e. punishment by a player with normative assessment of what others should contribute.

In our experiment we had a group of 12 instead of 4 as it is in practice, in order to differentiate between different forms (impartial versus targeted) of antisocial punishment. In this scenario only it is possible for a player to differentiate those who contribute most and contribute near group average and thus decide the punishment level accordingly. Larger number of players in the group also increases the mean value of antisocially punishing players thus increasing the statistical strength of the comparison between punishments received by those two types of players.

The subject pool (118 in total with 77 females and 41 males) mostly consisted of students of the Faculty of Science, Charles University in Prague (mean age 21.46, S.D. 1.60). The participants were divided into ten groups of twelve players. Two of the groups with eleven players only were completed by two "virtual" players, the students with instruction to contribute always the median of contributions of others and never punish. Participants didn't know about the existence of virtual players. Presence of the virtual players doesn't debase results of the experiment, because our hypothesis is not about the level of contributions but about the players' reactions on the contributions. Virtual players' results are not included in the analysis of punishment strategies (namely in the Results – Culprits section).

Players of every group played six rounds of standard *Public Goods Game* and six rounds of *Public Goods Game with punishment*. We focused on *Public Goods with punishment*; behavior of players' behavior in *Public Goods Game* was analyzed to complete the findings about typical behavior of the **culprits**. In five groups, the players started with the standard *Public Goods Game*, in another five groups the order of games was reversed. Each player in each round received 20 CZK (about $ 1.25) on average; Maximum amount a player could earn was 480 CZK ($ 30).

The experiment was conducted under the charter of absolute anonymity. All players were sitting in the same room, however, the room was divided by partitions into separate cubes. The players interacted (contributed and punished) through a web application. Information about other players' contributions were administered

Table 2. The function *c(p)* of the punishment costs.

p	0	1	2	3	4	5	6	7	8	9	10
c(p)	0	1	2	4	6	9	12	16	20	25	30

p is the number of punishments submitted by a player to another player. *c(p) is a function* of the punishment costs is adopted from [1].
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to each player through the web application after each round – the information couldn't be used to identify the other 11 players or to determine the total contributions of individual players submitted in preceding rounds. Players were rematched after each round, so that the player, contemplating punishment, would not take into account opponents past behavior [26]. For details of the experimental setup, including screenshots, see File S1.

Detailed rules of Public Goods Game design used in the experiment

If player i in round k invested sum $X^k_i \in <0.20>$ CZK, his total payout was

$$y^k_i = (20 - X^k_i) + \alpha \bar{X}^k$$

Where $\alpha = 2\ldots$ in our experiment, that is, the fund of Public Goods doubled the total contribution. $\bar{X}^k$ is an average of group contributions. The contributions were made public, though without possibility to identify the contributor.

Public Goods Game then continued to the next round; in the *Public Goods Game with punishment* the players were offered the possibility to punish other players after observing their contributions. The total amount of punishment received was subtracted from player's final payoff:

$$Y^k_i = \max(0, 1 - \gamma \sum_j p^k_{j \to i}) y^k_i - \sum_j c(p^k_{i \to j})$$

where Y^k_i is a final payoff, y^k_i — basic payout — counted by the same formula as in "without punishment", $p^k_{ij} \in <0, 10>$ is the number of punishments submitted by player i to player j in round k. $\gamma = 0.1$ is the coefficient which maps punishment received to actual monetary loss. $c(p)$ is the cost of punishment. The function c of the punishment costs is adopted from [1] and it is superlinear, that is higher level punishments are more costly to the player (see Table 2).The player is never punished by an amount bigger than his basic payout for the corresponding round. However, punishment costs could decrease his income below zero.

Data analysis

General. We used General linear model with repeat measures for basic analyses of the game process, amounts of contribution, and dynamics of the punishment.

Victims. The generosity of player in each round was characterized by their relative ranks (1–12) of contribution $r^{kl}(X^{kl}_i) \in <1,12>$, i.e. the relative positions of an individual player's contribution compared to other players within the corresponding group and round; in case of equal contribution amounts these were assigned average rank of players with the same contribution. The obtained punishment level was characterized by relative ranks of punishment in exactly the same way. As for the obtained punishment level, we specify the results both for absolute amount of obtained penalty marks and for the relative rank in the corresponding round alongside the simpler analyses. In more complex analyses and in graphs we specify in scales of relative punishment, though we conducted both also for absolute amounts of punishment.

The hypothesis regarding nonmonotonic correlation between individual contribution and punishment received, was at first tested by non-parametric methods: *Spearman's rank correlation* was used for general comparison, and *Mann Whitney U test* was used for comparison of the summed punishments obtained by the player (or players) with the maximum contribution, with the punishments obtained by the rest of above median-contributing players.

In the next step we used general regression analyses to test the relationship between contribution and obtained punishment. For our purposes it was necessary to determine whether the dependence of obtained punishment level on cooperation level is better estimated by "fair" (monotonically non-increasing) curve or "Justinian" curve decreasing up to specific point of maximal acceptable level of relative contribution and increasing from this point. "Fair" shape doesn't exclude existence of the individuals who punish maliciously; it does, however, assume that those choose their victims randomly. We used two following regression models:

1. Standard linear regression analysis: dependence of the punishment on the contribution approximated by linear, quadratic and cubical curves, respectively. The cubical curve has to be taken into account also in case the quadratic dependence has statistical significance; quadratic dependence interleaves the "fair" dependence in L-shape (the curve is monotonically decreasing up to the point of maximal punishments; then it is constant) significantly better than the linear one. The cubical curve is thus suitable to differentiate between the desired "U-shape" and "L-shape" matching the null hypothesis of fairness. In the ideal case (that we also observed in the experiment – see below) the optimal cubical curve does not differ from the quadratic curve either optically or by the level of explained variance.
2. Analysis was specifically constructed to resolve the question of whether the monotonous or U-shape is more appropriate. Variance explained by the optimal continuous L-shaped curve of arbitrary monotonically non-increasing shape was compared with the optimal U-shaped continuous curve with two monotonic intervals. The first is a generalization of a line, the latter of a parabola. Besides, the point of optimum (= the least punished behavior), which is estimated by the vertex of parabola in the previous model could yield a more plausible interval here.

Culprits. We measured the level of manifested cooperativeness of individual players in standard *Public Goods game* and *Public Goods Game with punishment* by average ranks of their contributions within each of the 6 rounds. Relation of hereby quantified cooperativeness and individual characteristics of the culprits of the Justine effect were tested by non-parametric methods: The Mann Whitney test was used for comparing cooperativeness of individuals punishing justly and unjustly, Spearman rank correlation was used to test the relationship between severity of punishment and the cooperativeness of the player. Kruskall Wallis statistics was used to compare contributions of groups of players according to their punishing strategy.

Supporting Information

File S1 Details of the experimental setup, including screenshots.
(PDF)

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Author Contributions

Conceived and designed the experiments: JF JL. Performed the experiments: JL LP. Analyzed the data: AAK. Contributed reagents/materials/analysis tools: JF. Wrote the paper: JF PH AAK LP.

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4.2 The relation of cortisol and sex hormone levels to results of psychological, performance, IQ and memory tests in military men and women

4.2.1 Reasons for Inclusion

Research in hormonal levels of the studied population is a necessary step in understanding *Toxoplasma*-human interactions. In previous studies, our laboratory has already shown that *Toxoplasma* increases levels of serum testosterone in infected mice (Šárka Kaňková, Kodym, & Flegr, 2011) and brought evidence for *Toxoplasma*-induced changes of testosterone hormones in humans (Jaroslav Flegr, Lindová, Pivoňková, & Havlíček, 2008). These changes seem to be gender dependent (J. Flegr, Lindová, & Kodym, 2008) and play a role in observed personality changes in studied individuals (Hodková, Kolbeková, Skallová, Lindová, & Flegr, 2007).

Hormonal levels are important also in context in fear, startle reactions and prepulse modification of startle reactions, as has been shown to affect startles, PPI, and fear in mice (Charitidi, Meltser, & Canlon, 2012), rats (Van den Buuse & Eikelis, 2001), non-human primates (Morris et al., 2010; Sánchez et al., 2005), and various populations of humans (Grillon et al., 2006; Jovanovic et al., 2004; Kumari et al., 2008; Rahman et al., 2003; Richter et al., 2011) in dependence on gender, age (e. g., pre-menopausal vs. post-menopausal women), time (in a circadian cycle), and in context of the menstrual cycle.

All the experimental subjects were serologically screened for *Toxoplasma.* However, we haven't managed to secure adequate funding for hormonal evaluation in a population large enough to analyze the data in regard to latent toxoplasmosis, which is why the resulting study does not work with the latent toxoplasmosis status of the experimental subjects. Due to the abovementioned connection between hormonal levels and both toxoplasmosis and PPI, we consider the study an integral part of our toxoplasmosis research.

4.2.2 Introduction

Cortisol and other hormones of hypothalamic-pituitary-adrenal axis belong to main factors influencing psychological and pathognomic factors,

intelligence, and memory due to their role in fear and anxiety regulations (Korte, 2001) and human responses are affected by their biological gender, or more specifically by the level of their sexual hormones (Kajantie & Phillips, 2006). According to previous studies, *Toxoplasma gondii* affects fear in its intermediate hosts (e. g., Berdoy et al., 2000; Jaroslav Flegr & Kuba, 2016) and its effects on behavioral changes are affected by biological gender of the infected person (J. Flegr et al., 2008; Lindová et al., 2006). In this study, we have analyzed data collected on military personnel undergoing regular entrance psychological examination not with regard to toxoplasmosis, but with focus on hormonal levels and their association with various performance and intelligence measures as tested by a large battery of psychological, performance, IQ, and memory tests.

4.2.3 Materials and Methods

100 male and 93 female subjects (mean age 27.9 years, S.D. 7.9 in men; 29.2 years, S.D. 7.3 in women) signed an informed consent with their data being used for research purposes, provided 5 ml of their blood for steroid hormone assays. Levels of cortisol, testosterone, and estradiol were tested in Institute of Endocrinology, Prague using radioimmunoassay kits Spectria from Orion, Finland for cortisol and estradiol, and kits from Immunotech for testosterone.

The battery of tests used to measure various performance and personal characteristics included:

- **Meili selective memory test**, an electronic version using 30 simple pictures (four of which had aggressive and four sadomasochistic themes) arranged in a six by five grid.
- **TOPP, Test of attention and short memory**, where a participant is trained to find dictated symbols arranged in a specific pattern and followingly tested for a number of correct, wrong, and skipped reactions in three repetitions of the test.
- **N-70 questionnaire** used to determine scores in seven areas, namely anxiety, depression, phobia, hysteria, hypochondria, psychosomatic symptoms, and psychasthenia.

- **OD-1 questionnaire** used to determine the emotional characteristics of an individual in areas of emotions, regulation, impulsivity, adaptability, risking, and enthusiasm with an added scale for psychopathology.
- **BDI, Buss-Dürker Inventory** used to determine physical, verbal and indirect aggressivity, irritability, negativism, resentment, guilt, and paranoia.
- **WMT, Wiener Matrizen Test** used to measure general, non-verbal intelligence by testing an individual's reasoning ability.
- **OTIS test of verbal intelligence** derived from original Otis quick-scoring mental ability tests (Otis, 1954) by using 7 types of items from the original test (term or object definition by choosing the most suitable characteristics, term or object definition by choosing the most suitable description, the choice of an object based on common attributes, the choice of the opposite, the identifying of "foreign" (unrelated) term, the logical resp. the ethical solution of the situations, and the interpretation of the adage).

Frequency tables, logistic regression, general linear model, and partial correlation Kendall test were used for statistical analysis using software Statistica 6.1, SPSS 16.0, and MS Excel.

4.2.4 Results and Discussion

We have found various significant correlations between hormonal levels and measured variables, and the results were different for male and female experimental subjects.

In **men**, we have found positive correlation between **cortisol** and emotion area (experience of emotions during interactions and situational changes; τ=0.151, p=0.032) of OD-1 test, and negative correlation between cortisol and impulsivity area (impulsivity of falling for immediate impressions when values are high, and self-control and judicious behaviour when values are low; τ=-0.162, p=0.021) of OD-1 test. A positive correlation was found between **testosterone** and psychopathology scale added to the OD-1 test

(τ=0.233, p=0.001), and testosterone and paranoia scale of BDI (τ=0.148, p=0.049). Another positive correlation was found between **estradiol** and psychopathology scale (added to OD-1; τ=0.159, p=0.024).

In **women**, **cortisol** correlated negatively with hypochondria (N-70; τ=-0.141, p=0.049) and psychopathology (added to OD-1; τ=-0.198, p=0.006), and positively with aggression (Meili; τ=0.301, p=0.025). We have found negative correlations between **testosterone** and hypochondria (N-70; τ=-0.142, p=0.049), psychastenia (N-70; τ=-0.144, p=0.045), indirect aggression (BDI; τ=-0.319, p=0.017), irritability (BDI; τ=-0.358, p=0.007), and paranoia (BDI; τ=-0.334, p=0.013). Negative correlation was also found between **estradiol** and phobia (N-70; τ=-0.142, p=0.047), while correlation between estradiol and negativism (BDI; τ=0.266, p=0.047) was positive.

Significant statistical differences established by t-test were found between men in women in 13 characteristics including (as expected) hormonal levels of all three hormones (**cortisol** (nmol/l) n_f=93, $mean_f$=728, $S.D._f$=121; n_m=100, $mean_m$=505, $S.D._m$=353; t=-5.953, p<0.0005; **testosterone** (nmol/l) n_f=93, $mean_f$=1.10, $S.D._f$=3.868; n_m=100, $mean_m$=15.53, $S.D._m$=0.594; t=34.59, p<0.0005; **estradiol** (nmol/l) n_f=93, $mean_f$=0.29, $S.D._f$=0.029; n_m=100, $mean_m$=0.11, $S.D._m$=0.342; t=-5.28, p<0.0005). Significant differences between men and women were also found in anxiety (N70; t=-2.107, p=0,037), risking (OD-1; t=4,085, p<0.0005), psychopathology (added to OD-1; t=4.571, p=<0.0005), physical aggression (BDI; t=3.679, p<0.0005), sadomasochism (Meili; t=2.005, p=0.048), Otis raw intelligence (t=5.546, p<0.0005), WMT raw intelligence (t=-10.341, p<0.0005) and all three parts of test of attention and short memory (t_1=-3.581, p_1=0.000; t_2=-3.156, p_2=0.002; t_3=-3.538, p_3=0.001).

While some of our results, e. g., differences between men and women in such areas as risk-taking or aggressiveness corresponded well with other psychological studies (Korte, 2001), other results, specifically high cortisol levels in women, were more surprising. There are several possible explanations for this result, such as hormonal contraceptives or even chronic stress in military women. Based on our previously published research (Jaroslav Flegr

& Příplatová, 2010) it would be possible to contribute higher measured levels of cortisol in similar experiments to good feeling from well-accomplished tests, it is not the case in our scenario, though, since the blood samples were collected before and not after the tests were presented.

4.2.5 Limitations

There are two important limitations of this study, the first being a small number of experimental subjects. The second limitation is that almost no found effect would survive correction for multiple tests. This is, of course, also related to the small number of participants and the full analysis should be thus repeated with a much larger population sample.

ORIGINAL ARTICLE

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The relation of cortisol and sex hormone levels to results of psychological, performance, IQ and memory tests in military men and women

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Abstract

BACKGROUND: Cortisol, along with other hormones of hypothalamo-pituitary-adrenal axis, belongs to one of the main factors influencing psychological and pathognomic factors, intelligence, and memory.
METHODS: The aim of our study was to review a large battery of psychological, performance, IQ and memory tests as to their relation with cortisol, testosterone and estrogen levels in groups of 100 men and 93 women who attended the Central Military Hospital in Prague for regular entrance psychological examinations for military personnel.
RESULTS: In men, we detected positive correlations between cortisol and emotional lability, and negative correlations with impulsivity, while in women hypochondria and psychopathology were negatively correlated, and aggression measured with the Meili selective memory test had a positive relation to cortisol level. Testosterone correlated positively with emotional liability and negatively with impulsivity in men, and negatively with hypochondria and psychasteny, indirect aggression, irritability and paranoia in women. Estradiol correlated positively with psychopathology in men, and negatively with phobia. It was positively correlated with negativism in women. No clear correlation was observed between the concentration of steroid hormones and psychomotor performance or intelligence.
CONCLUSIONS: Concentrations of steroid hormones correlate with results of several psychological tests, the sign and magnitude of these correlations, however, very often differ in military men and women.

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INTRODUCTION

Cortisol, as one of principal stress hormones which, along with other hormones of hypothalamo-pituitary-adrenal axis, belongs to one of the main factors influencing psychological and pathognomic factors, intelligence, and memory. Though corticosteroids do not directly regulate behavior, they play crucial roles in fear and anxiety regulation. Glucocorticoids as well as mineralocorticoids induce chemical changes in particular sets of neurons via their receptors, thus contributing to certain behavioral outcomes. Both corticosteroids act in a cooperative way, influencing different aspects of fear at different phases of the stress response (Korte 2001). The reaction to stressful conditions and, more generally, to psychology, pathopsychology, intelligence and memory differ in males and females, which suggests the potential effects of sex hormones (Kajantie & Phillips 2006). Evidence has accumulated that the hypothalamo-pituitary-adrenal (HPA) axis may function differently in borderline personality disorder (BPD), a psychiatric diagnosis characterized by high exposure to stress, reactivity, and vulnerability to stress (Zimmerman & Choi-Kain 2009). Meta analyses have been undertaken recently of 66 journal articles that directly manipulated social stress or emotions and measured subsequent cortisol and also immune responses, taking into account various psychobehavioral aspects such as cognitive appraisals, basic emotions, rumination and worry, social threat, and global mood states (Denson *et al.* 2009). The conclusions were not unequivocal and were criticized as oversimplified due to their omission of certain outcomes (Miller 2009). Indeed, it is difficult to establish an integrated hypothesis from experiments with animals and psychological or behavioral tests in humans. On the other hand, psychological an performance tests and measurements of hormonal status may be useful in the selection of candidates for physically and psychologically extremely demanding occupations such as soldiers, pilots etc. (Taverniers *et al.* 2010; Taylor *et al.* 2007). The aim of our study was to review a large battery of psychological, performance, IQ and memory tests as to their relation with cortisol and steroid sex hormone levels in groups of healthy military men and women.

MATERIALS AND METHODS

Subjects

The study population consisted of 100 male and 93 female military personnel (mean age 27.9 years, s.d. 7.9 in men; 29.2 years, s.d. 7.3 in women) who attended the Central Military Hospital in Prague for regular entrance psychological examinations between 2009 and 2010 and consented to participate in the research project. The subjects were tested for RhD phenotype during the health examination and also provided 5 ml of blood for the steroid hormone essay. All study subjects were screened for health status prior to their enrolment in the study. In the informed consent form, the general aim of the project (a study of the influence of physiological traits on human psychology and psychomotor performance) and the need for obtaining their consent to using the results of their psychological and clinical examinations was explained to the subjects. About 65 % of the military personnel consented to the use of their test results for research purposes and provided 5 ml of blood for laboratory testing. The recruitment of study subjects and handling of data was performed in compliance with Czech law and approved by the Institutional Review Board of the Faculty of Science at Charles University.

Meili selective memory test

For the purposes of the present study, a computerized version of a selective memory test based on the Meili test (Meili 1961) was developed and used. The participants were asked to memorize the location of thirty simple pictures in a 6 × 5 grid. After sixty seconds (as indicated by the progress bar), the pictures disappeared from the grid and individual pictures began to be displayed for four seconds in the left corner of the monitor. The participants were requested to point out the cell of the grid in which the picture had originally been displayed (see Figure 1). Of the thirty pictures (stimuli), four items (9, 21, 24, 25) represented objects with aggressive and four (6, 7, 18, 29) with sadomasochistic themes (Jozífková & Flegr 2006). The output of the test was the number of correctly localized pictures and the fraction of aggressive and fraction of sadomasochistic items among correctly localized pictures.

TOPP – Test of attention and short term memory

This test is used to evaluate attention and short-term memory of operational character under time pressure. The touch screen is used during testing. The stimulation field is represented by columns of symbols compounded from numbers and letters that are spread across the screen according to a specific system which the participant is made aware of prior to the testing. The task of the participant is to look for the dictated symbol and mark it with the touch pen. After training, where the participant gets to know the pattern, 50 symbols are dictated. The speed of dictation is constant. The test is repeated three times. During each set the number of correct, wrong and skipped reactions is observed. The main output of the test is the number of correct reactions in three parts of the test, TCI-1, TCI-2 and TCI-3. It is therefore possible to find out the quality and dynamics of efficiency by comparing the performance during a single set and in all three sets in between. For the participants tested for the purposes of choice of profession, which also means participants with higher motivation to achieve better performance, this test can show not only the level of attention and short-term memory, but also the negative influence of emotion on performance.

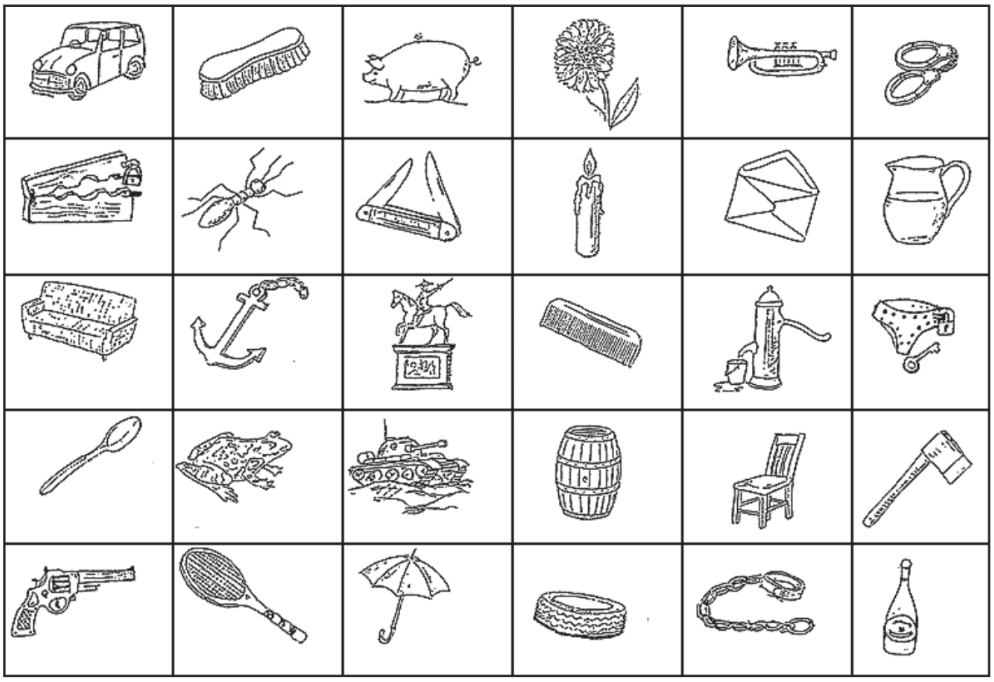

Fig. 1. Stimuli used in the Meili selective memory test. Of the thirty pictures, four items (9, 21, 24, 25) represented objects with aggressive and four (6, 7, 18, 29) with sadomasochistic themes.

N-70

The N-70 is a questionnaire constructed for the assessment of 7 areas of clusters – anxiety, depression, phobia, hysteria, hypochondria, psychosomatics symptoms and psychastenia. The purpose of this method is to detect individuals who may be too sensitive for military operations (Vacíř 1973). Subjects are asked to answer 70 questions using a 3-point agreement scale. Scores in each cluster range from 0–30. The total N-70 score is a sum of all clusters. The English translation of this questionnaire is provided in Appendix 1.

OD-1

This questionnaire covers the dimensions of the SPIDO questionnaire (Mikšík *et al.* 1991) that describes emotional characteristics of the individual, namely: EM, RG, ER, AD, KR and EA. Emotion (EM) measures the experiencing of emotions during interactions and situational changes. The dynamic of emotions and their consequences are considered. On the positive pole there is high excitability and a tendency to experience situational tension. On the negative pole there is emotional stability up to lowered emotivity. Regulation (RG) refers to behavioral regulation and activity control. High values mean low self-control and low consideration of possible consequences. The opposite values represent anticipation of behavior. Impulsivity (ER) characterizes impulsivity of falling for immediate impressions when values are high, and self-control and judicious behavior when values are low. A high value of the next trait, the Adaptability (AD), is characteristic of subjects who easily and willingly adapt to conditions, up to submissive stands in extremes. The individual scoring on the opposite pole has, on the contrary, a tendency to persist in his behavioral schematics and to request adaptation of surroundings. Risking (KR) reflects the tendency towards risk. High values represent immediate action coming from a sudden idea, without considering the impact of possible failure. Low values mean persistent consideration of consequences up to almost inactive, very careful and restrained behavior. Enthusiasm (EA) reflects position on the optimism-pessimism axis. On the positive pole this means satisfaction, optimism, enthusiasm; on the negative pole it means pessimism, disappointment, and low self-confidence. Psychopathology (PP) scale was added to the OD-1 questionnaire; it is not contained in the SPIDO questionnaire. PP scale was composed of items that measure various personality psychopathology characteristics, see Appendix 2.

BDI (Buss-Dürker Inventory)

For a measurement of physical, verbal and indirect aggressivity, irritability, negativism, resentment, guilt and paranoia, we used the Buss-Dürker Inventory (Buss & Durkee 1957). In the Czech version of the questionnaire (Svoboda 1999), we replaced a categorical way of answering the items (yes/no), with a 5-grade scale

from "absolutely agree" to "absolutely disagree", which is better suited to testing subjects who are highly motivated to successfully pass psychological examinations.

WMT

The Wiener Matrizen-Test WMT, (Formann & Piswanger 1979), a non-verbal intelligence test, is an adapted version of the Raven progressive matrices which conforms to the Rasch model (Rasch 1960). The WMT assesses general intelligence by measuring reasoning ability. The test requires the completion of 24 matrices with increasing task difficulty and was administered without an explicit time limit. The intention and conceptualization of the WMT are largely based on Raven's Matrices (Raven 1947; 1958a;b). The correlation between the WMT and Standard Progressive Matrices is about r = 0.92 (Formann & Piswanger 1979). Construction and item selection, however, follow the standards of Rasch scaling. For these reasons, and due to the fact that the WMT showed comparable validity characteristics but had a considerably higher administration economy, we prefer the WMT to the Raven matrices in clinical practice. The split-half reliability of the WMT is 0.83(Formann & Piswanger 1979). The 1993 Czech adopted version (Klose *et al.* 2002), distributed by Psychodiagnostika (Brno), was used in the present study. Both the raw score and the IQ (adjusted for age of the participant based on results of 2007 survey performed in Central Military Hospital: age 15–20, N=1 232, mean=16.33, S.D.=4.33; 21–30, N= 4 551, mean=15.99, S.D.=4.32; 31–40, N=2 482, mean= 15.66, S.D.=4.38; 41–50, N=569, mean=15.02, S.D.=4.46; higher than 50, N=31, mean=11.81, S.D.=4.89) were compared in statistical tests.

OTIS

The test of verbal intelligence which was derived from the original test (Otis 1954). Seven types of items were taken from the original test:

- term or object definition by choosing the most suitable characteristics
- term or object definition by choosing the most suitable description
- the choice of an object based on common attributes
- the choice of the opposite
- the identifying of "foreign" (unrelated) term
- logical resp. ethical solution of the situations
- the interpretation of the adage

The test contains 32 items (0–32). The maximum score is therefore 32 points. Norms for computing IQ values were created in the Central Military Hospital in Prague in a separate large scale study for the Czech population. This study included 1 470 subjects with elementary education (mean score=19.47, S.D.=4.92), 1 225 subjects with secondary school education (mean score=24.15, S.D.=4.21), and 403 subjects with higher school education (mean score=26.62, S.D.=3.09). Both the raw score and the IQ (adjusted for the achieved educational level of a proband) were compared in statistical tests.

Steroid hormone determination

Cortisol: Serum samples, 20 µl, were diluted with 0.1 M sodium phosphate-citrate buffer, pH 4 containing BSA and sodium azide (0.1% each) to final volume 400 µl in plastic eppendorf tubes, and measured by radioimmunoassay kits Spectria from Orion (Finland). The range of physiological levels from morning sampling for both males and females, determined in the author´s laboratory, were 138–607 nmol/l.

Testosterone was determined by radioimmunoassay after the extraction of 100 µl serum samples with diethyl ether, using kits from Immunotech (Beckman Coulter, Czech Division, Prague). The range of physiological levels from morning sampling for the age groups studied, as determined in the author´s laboratory, were 10–34 nmol/l and 0.40–3.00 nmol/l for males and females, respectively.

Estradiol was determined in 100 µl serum samples without extraction, using radioimmunoassay kits Spectria from Orion (Finland). The range of physiological levels from morning sampling, determined in the author´s laboratory were 0.01–0.23 nmol/l and 0.09–1.29 nmol/l for males and females, respectively. The levels of estradiol in women varied considerably during the menstrual cycle.

In all cases where an analyzer, Stratec (Immunotech, Prague), was used for analyses, the analytical criteria (sensitivity, precision, accuracy) agreed with those reported by the manufacturers. For other details of the laboratory procedures also see (Flegr *et al.* 2008).

Statistical analysis

The Statistica 6.1 and SPSS 16.0 programs were used for statistical testing (frequency tables, logistic regression and the Generalized linear model) and to check statistical test assumptions. Partial correlation Kenadall test was used for nonparametric analyses (Kaňková *et al.* 2010) ; the Excel sheet for this analysis is available at http://web.natur.cuni.cz/flegr/programy.php. All variables including the covariates entered in the respective analyses are specified in the Results section.

RESULTS

The total experimental set contained 93 women and 100 men; however, only some subjects were tested with all tests (see Table 1). Women and men differed in some psychological and pathognomic factors; for results of t-tests and descriptive statistics see Table 1. Since the concentrations of hormones differ between men and women, both sexes were analyzed in separate tests. Most of the monitored psychological traits changed with the age of the subjects. Therefore, we controlled the data for the effects of age using partial correlation Kendall tests with the age of the subjects as a covariate.

Tab. 1. Descriptive statistics of population and differences between men and women.

Test / parameter	WOMEN			MEN			SEX DIFFERENCES	
	n	Mean	S.D.	n	Mean	S.D.	t	*p*-value
Age	100	29.25	7.87	100	27.95	7.95	–1.181	0.239
Total N70 score	90	19.92	9.56	93	18.76	10.9	–0.797	0.426
Anxiety N70	90	5.36	2.45	93	4.6	2.39	–2.107	**0.037**
Depression N70	90	2.44	2.19	93	2.16	1.96	–0.922	0.358
Phobia N70	90	2.71	1.84	93	2.68	1.92	–0.121	0.904
Hysteria N70	90	2.44	1.6	93	2.85	1.73	1.641	0.102
Hypochondria N70	90	2.13	1.97	93	2.29	1.82	0.56	0.576
Vegetative lability N70	90	3.69	2.4	93	3.24	2.61	–1.305	0.193
Psychasteny N70	90	2.29	2.49	93	2.13	2.16	–0.464	0.643
Emotions OD-1	88	4.82	4.51	93	5.15	4.64	0.488	0.626
Regulation OD-1	88	7.11	1.1	93	6.26	2.37	1.684	0.094
Adaptability OD-1	88	14.91	2.35	93	15.44	2.76	1.391	0.166
Risking OD-1	88	3.10	1.76	93	4.22	2.17	4.085	**0.000**
Enthusiasms OD-1	88	3.68	2.24	93	3.81	2.45	0.356	0.722
Impulsivity OD-1	88	10.14	1.37	93	10.15	1.44	0.068	0.946
Psychopathology OD-1	88	1.57	1.56	93	3.28	3.17	4.571	**0.000**
Physical aggression BDI	28	18.11	7.37	82	24.1	7.32	3.679	**0.000**
Indirect aggression BDI	28	25.14	7.69	82	23.99	7.55	–0.697	0.487
Irritability BDI	28	22.52	7.27	82	20.64	7.16	–1.196	0.234
Negativism BDI	28	21.5	7.88	82	22.1	9.18	0.308	0.759
Resentiments BDI	28	20.0	7.93	82	21.39	8.94	0.729	0.468
Paranoia BDI	28	24.75	1.6	82	27.17	9.28	1.276	0.205
Verbal aggression BDI	28	33.88	7.35	82	33.56	5.66	–0.238	0.812
guilty feelings BDI	28	30.72	8.7	82	29.85	9.60	–0.445	0.657
total memory Meili score	28	10.25	3.88	73	9.25	4.40	–1.13	0.261
aggression Meili score	28	0.13	0.08	73	0.16	0.11	1.349	0.181
SM Meili score	28	0.14	0.07	73	0.18	0.10	2.005	**0.048**
Otis raw intelligence	88	18.22	6.17	93	22.94	5.27	5.546	**0.000**
Otis IQ	88	101.67	13.4	93	100.72	16.26	–0.432	0.666
WMT raw intelligence	86	21.88	5.64	93	14.13	4.35	–10.341	**0.000**
WMT IQ	86	103.21	11.69	93	100.24	15.84	–1.419	0.158
TCI-1	89	40.27	9.661	93	35.8	1.60	–3.581	**0.000**
TCI-2	89	41.94	8.208	93	38.43	1.60	–3.156	**0.002**
TCI-3	89	43.94	8.073	93	40.16	6.18	–3.538	**0.001**
cortisol (nmol/l)	93	728	121	100	505	353	–5.953	**0.000**
testosterone (nmol/l)	93	1.10	3.868	100	15.53	0.594	34.59	**0.000**
estradiol (nmol/l)	93	0.29	0.029	100	0.11	0.342	–5.28	**0.000**

Significant differences ($p<0.05$) between men and women are printed in bold.

Cortisol and sex hormone levels

The differences in psychological traits between men and women are shown in Table 1. In men, higher scores than in women were found in anxiety, propensity to risk taking, overall aggressivity, risk, psychopathology, physical aggression, SM Meili score and Otis intelligence. Significantly higher scores in women were recorded in anxiety, WMT intelligence, TCI-1, TCI-2 and TCI-3. The levels of sex hormones corresponded to known values for both sexes in the studied age groups, while the level of cortisol was higher in women.

Table 2 shows the correlations between traits measured with psychological tests and steroid hormone concentrations. In women, significant negative correlations with cortisol were found in hypochondria (N-70 test) and psychopathology in OD-1 test, while a positive correlation was found with the aggression score in the Meili test. Negative correlations with testosterone were recorded with hypochondria and psychasteny in N-70 test and in three traits of BDI test, namely indirect aggression, irritability and suspiciousness. Estra-

Tab. 2. Correlation between results of psychological tests and hormone concentrations.

	WOMEN						MEN					
	cortisol		testosterone		estradiol		cortisol		testosterone		estradiol	
	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value
N70 test												
total score	–0.079	0.271	–0.082	0.252	0.013	0.853	0.023	0.747	–0.007	0.915	–0.017	0.810
anxiety	–0.071	0.323	–0.063	0.378	0.081	0.261	0.036	0.613	–0.018	0.800	–0.026	0.717
depression	–0.082	0.253	–0.074	0.303	0.083	0.244	0.050	0.477	0.059	0.403	–0.107	0.130
phobia	0.072	0.316	0.072	0.314	–0.142	**0.047**	0.078	0.269	0.098	0.164	0.058	0.407
hysteria	–0.083	0.245	0.037	0.604	0.018	0.801	–0.068	0.333	0.007	0.926	0.002	0.973
hypochondria	–0.141	**0.049**	–0.142	**0.048**	0.060	0.401	0.037	0.598	–0.043	0.546	–0.059	0.405
vegetative lability	–0.139	0.053	–0.091	0.206	0.036	0.614	0.044	0.528	–0.015	0.829	0.069	0.327
psychasteny	–0.057	0.430	–0.144	**0.045**	–0.005	0.941	–0.010	0.886	–0.017	0.807	–0.072	0.309
OD1 test												
emotion	–0.031	0.669	–0.006	0.934	0.016	0.822	0.151	**0.032**	0.025	0.725	0.112	0.111
regulation	0.012	0.873	–0.022	0.758	–0.015	0.833	–0.018	0.794	0.090	0.201	0.026	0.710
adaptability	0.099	0.173	0.031	0.672	–0.053	0.465	–0.050	0.477	–0.006	0.937	–0.052	0.459
risking	0.038	0.599	0.071	0.326	–0.023	0.746	0.046	0.512	0.052	0.462	0.099	0.159
enthusiasm	–0.002	0.974	–0.016	0.830	0.041	0.573	0.090	0.200	0.112	0.111	0.019	0.790
impulsivity	0.126	0.082	0.049	0.498	–0.076	0.297	–0.162	**0.021**	–0.063	0.371	–0.025	0.723
psychopathology	–0.198	**0.006**	–0.022	0.765	0.107	0.140	0.122	0.084	0.233	**0.001**	0.159	**0.024**
BDI test												
physical aggression	0.068	0.612	–0.211	0.114	–0.049	0.717	0.061	0.416	0.065	0.388	0.041	0.589
indirect aggression	–0.179	0.181	–0.319	**0.017**	0.038	0.779	0.013	0.858	–0.023	0.755	0.052	0.486
irritability	–0.255	0.057	–0.358	**0.007**	0.159	0.234	0.028	0.707	0.036	0.634	0.019	0.799
negativism	–0.244	0.068	–0.102	0.447	0.266	**0.047**	–0.086	0.252	–0.006	0.934	–0.024	0.750
resentiments	–0.243	0.069	–0.139	0.300	0.195	0.146	0.091	0.225	0.110	0.145	0.112	0.135
paranoia	–0.054	0.685	–0.334	**0.013**	–0.094	0.480	0.081	0.284	0.148	**0.049**	0.127	0.091
verbal aggression	–0.079	0.554	–0.172	0.200	0.000	0.998	0.120	0.112	–0.014	0.849	–0.054	0.475
guilty feelings	–0.139	0.298	–0.056	0.673	0.031	0.815	0.104	0.167	–0.008	0.919	0.030	0.692
Meili test												
total memory score	0.238	0.075	0.145	0.277	–0.258	0.054	–0.062	0.435	0.001	0.992	0.005	0.946
aggression score	0.301	**0.025**	–0.031	0.819	–0.234	0.081	0.051	0.526	0.105	0.187	0.015	0.846
SM score	0.065	0.628	–0.018	0.892	–0.182	0.173	–0.156	0.052	–0.137	0.086	–0.095	0.234

Significant correlations and trends ($p<0.1$) between psychological factors and performance and hormone concentrations are printed in bold. Positive and negative Tau (reflecting the effects size) means positive and negative correlation, respectively.

diol correlated negatively with phobia (N-70 test) and positively with negativism in the BDI test. In men, cortisol correlated positively with emotional liability and negatively with impulsivity (both measured with OD-1 test). Testosterone correlated positively with psychopathology (OD-1 test) and with paranoia in the BDI test. Only one trait was positively correlated with estradiol, namely psychopathology in OD-1 test. No clear correlation between the concentration of hormones and the performance of subjects measured with the TOP test and intelligence tests was observed. Furthermore, performances in the 1st, 2nd and 3rd minute of the TOPP test were analysed by repeat measurement of GLM with a logarithm of hormone concentration and age of subject as independent variables. No correlation was observed between results of TOPP and concentration of cortisol or estradiol, however, the interaction between minute of the test and testosterone concentration was significant both for men (p=0.018, η^2=0.044) and women (p=0.043, η^2=0.036). In women, only an insignificant negative correlation between testosterone concentration and number of correctly localized targets was observed in the first part of the TOPP test and no correlation was observed in the second and third minute of the test. In men, no correlation between testosterone concentration and number of correctly localized targets was observed in the first minute of the TOPP test and only insignificant negative correlation in the second and third minute of the test. No association was observed between intelligence and level of hormones, except a negative association between intelligence and testosterone concentration in men quantified with repeatedly measured GLM, using IQ estimated with OTIS and WMT tests as repeated measures and a logarithm of testosterone and age of subjects as independent variables (p=0.047, η^2=0.043).

DISCUSSION

Sex differences in psychobehavioral traits such as a higher tendency towards risk taking, aggressiveness, and propensity to personality psychopathology in men correspond to known differences in male and female psychology (Korte 2001). Of interest is the higher anxiety score in women, which was recently reported as one of the paradoxical effects of steroid modulation of $GABA_A$ receptors (Andreen *et al.* 2009). Differences in sex hormone levels between males and females are well known, as well as the fact that these levels are strongly influenced by the phase of the menstrual cycle in women. Rather surprising were the higher levels of cortisol in women, which could be ascribed to oral contraceptives or, in later age, to hormone replacement therapy, the information of which was lacking in our records. Higher cortisol levels could thus be a consequence of the impact of estrogen on transcortin formation and, more generally, on hypothalamo-pituitary control of steroid secretion. However, it can be speculated that the higher level of cortisol in women may be caused by higher levels of chronic stress in female military personnel (see below).

In this paper a large battery of psychological tests was performed and the data were correlated with actual cortisol and sex steroid levels. The aim was, among others, to evaluate the usefulness of cortisol and sex steroid hormone determination in the selection of candidates for professional military service. The results differed considerably between men and women. While in men the only significant correlations with cortisol were recorded in the test expressing emotional lability and impulsivity, there were three traits (hypochondria in the N-70 test, psychopathology in OD-1 test and aggression in Meili test), which were related to cortisol in women.

A high concentration of cortisol is characteristic of subjects under chronic stress. It was recently shown (Flegr & Příplatová 2010) that the concentration of cortisol (as well as testosterone) increases in female and male university students that achieved good results in a written exam. At face validity, the nature of the psychological traits of a female with a higher concentration of cortisol, i.e., decreased N-70 hypochondria score and OD-1 psychopathology score, and increased Meili aggressivity score, suggest that good feelings from the absolved tests, rather than chronic stress, were responsible for the observed association. It must be considered, however, that the level of cortisol was measured before the psychological and psychomotor performance testing in the present study. Therefore, chronic stress rather than positive feelings after the test was responsible for the observed associations. In contrast to men, who seem to use more individualistic and antisocial (e.g. aggressive, hostile) forms of coping with stress (Carver *et al.* 1989; Hobfoll *et al.* 1994), women are more likely to seek and provide social support (Carver *et al.* 1989; Stone & Neale 1984; Rosario *et al.* 1988), join with others (Hobfoll et al 1994), and verbalize towards others or the self (Tamres *et al.* 2002). This can result in seemingly more desirable personality traits observed in high-cortisol (chronically stressed) women (Lindová *et al.* 2010).

Concerning testosterone, of interest may be the highly significant positive correlation with inclination to psychopathology and related paranoia in men, while in women there were as many as five traits (N-70 hypochondria and psychastenia, BDI indirect aggressivity, irritability and suspiciousness) negatively correlated with testosterone. Of those, paranoia correlated with testosterone in both sexes, but negatively in women and positively in men. Generally, the psychological profile of high-testosterone women expressed more desirable character traits than that of low-testosterone women. With one exception (psychopathology), no trait correlated with estradiol in men. The former corresponds to the fact that estradiol in males is synthesized from testosterone. The data on estradiol association with psychological traits in women couldn't be evaluated in

the present study due to their being influenced by the menstrual cycle and the possible use of contraceptives.

In contrast with previous results (Flegr *et al.* 2008c), we did not detect a strong positive effect of testosterone concentration in the results of the psychomotor performance test in men. However, the simple reaction test was used in the original study while a more complex TOPP test was used in the present study. It is possible that the higher competitiveness of high testosterone men (Archer 2006), rather than higher psychomotor performance, is responsible for their better results in the simple reaction time test. It is, however, also possible that differences between older and newer studies resulted from the statistical control of some important confounding variables, such as Rh blood group factor and toxoplasmosis infection, which are known to strongly influence the results of psychomotor performance tests (Flegr *et al.* 2008c; Havlíček *et al.* 2001; Novotná *et al.* 2008) or levels of testosterone in the human organism (Flegr *et al.* 2008a;b; Hodková *et al.* 2007).

The first studies of the relation of psychological tests to cortisol and testosterone appeared as early as the 1980s (Francis 1981), and confirmed that men under psychological stress, as reflected by high cortisol, had significantly lower testosterone levels. Later studies have shown that personality traits were (also) associated with different cortisol responses to stress due to altered function of the HPA axis (Oswald *et al.* 2006). Various personality measures of negative affectivity have been associated with high cortisol levels, but so have measures of positive social adaptation and agreeableness (Tops *et al.* 2006). Our results are in agreement with some of the data reported and, in any case, confirm the importance of cortisol and eventually testosterone measurement along with psychological tests. On the other hand, the determination of estradiol in men is of much less importance and in women its importance is strongly limited by estradiol fluctuation during the menstrual cycle phase and also by use of contraceptives. An important limitation of the present study is the moderate number of subjects. Despite the relatively strong effect size of some correlation (estimated on the basis of Kendall Tau), the statistical significance was relatively low because of the high variability of hormone concentrations and personality traits in the normal human population. With the exception of a correlation between psychopathology (OD-1) and concentration of testosterone in men, no other significance would survive correction for multiple tests. Therefore, the present study must be considered an exploratory study and the observed phenomena should be confirmed in other populations in the future.

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Appendix 1

The N-70 questionnaire constructed for the assessment of 7 areas of clusters – anxiety (1–10), depression (11–20), phobia (21–30), hysteria (31–40), hypochondria (41–50), psychosomatics symptoms (51–60) and psychastenia (61–70). Subjects are asked to answer 70 questions using a 3-point agreement scale (often- 2, sometimes- 1, never- 0). Scores in each cluster range from 0–30. The total N-70 score is a sum of all clusters.

1. Do you feel anxious when your superior calls you and you don't know why?
2. Do you experience uncomfortable inner tension such as if something bad is going to happen?
3. In demanding situations, do you feel butterflies in your belly or chest; do you need to go to the toilet more often?
4. Do you suffer from stage fright?
5. Do you suffer from fear that is unproportional to the situation from which it originates?
6. Do you feel like vomiting when expecting trouble?
7. Do you comfort eat when you are sad or worried more than other people?
8. Do you suffer from indeterminate fear despite having no reason for such worries?
9. Do you feel unpleasant thumping of your heart when confronted with a distressing situation?
10. Do you suffer from any agitated states when you cannot hold still, you must keep moving or do something without purpose, e. g. chain smoking?
11. Have you been lately easily depressed?
12. Do you ever feel like you have lost your ability to have fun, revel, or look joyfully to the future?
13. Do you think that you are an unhappy person?
14. Do you have problems controling tears in harming situations?
15. Do you have black thoughts; are you unable to get rid of them?
16. Do you feel that your interest in things that you were earlier interested in has decreased because of sad moods?
17. Does sadness or joyless mood decrease your working performance?
18. Do you have problems to get asleep in the evening because you are unable to get rid of distressing thoughts?
19. Do you feel that your friends avoid you though you haven't done them anything bad?
20. Have you been experiencing suicidal thoughts lately?
21. Do you often have thoughts about you suffering from some severe illness or the possibility of acquiring one?
22. Do you suffer from irrational fear in closed spaces?
23. Do you have strong fear of heights, are you afraid of fall or – when high above – is something inside tempting you to jump down?
24. Do you suffer from strong anxiety in crowded places?
25. Do you suffer from an uneasy feeling that you forgot some of your domestic tasks (such as closing windows, locking doors, switching off lights)?
26. Do you often have nonsensical ideas such as to count windows, to walk only on specific cobblestones, or to say inappropriate words in stresful situations?
27. Do you often have inappropriate thoughts that you don't agree with and that are difficult to get rid of?
28. Do you often have to double check your previous tasks to get calm and to be reassured that you did them right?
29. Do you get severely out of balance when your daily habits are disturbed?
30. Do you think that you are a perfectionist? Do you hate when something is done imprecisely or if there is not absolute order around you?
31. Do you feel like fainting when you are strongly keyed up?
32. Do you feel good when you are the center of attention?
33. Do you feel pins and needles or loss of sensitivity in some places when you are strongly keyed up?
34. Do you have problems to control your limbs because of strong excitement?
35. Do you feel that you can't stop yourself when you are upset despite unconsciously feeling that you are acting wrongly?
36. Are you ever unable to talk as if your tongue is numb in unpleasant situations?
37. Do you like dramatic situations when you have a chance to show off?
38. Do you have spasms in your limbs during conflict situations?
39. Do you ever feel that you have tendencies to sham and pretend?
40. Do you ever deceive other people to achieve your own goals?
41. Do you often feel ill?

Appendix 1 *cont.*

42. Do you obsess over health problems?
43. Do you often visit a physician even with minor problems?
44. Do you check your body temperature, pulse, face in mirror etc. when feeling sick?
45. Do you try to educate yourself when diagnosed with a health problem (by reading popular medical articles or books)?
46. Are you always well aware of the hidden propaganda in things you read?
47. Do you suffer from indigestion?
48. Do you have diarrhea or constipation?
49. Do you ever suffer from a general feeling of pain or discomfort in your muscles or joints?
50. Do you confide your problems to your acquaintances and/or friends?
51. Do you suffer from full body excess sweating or sweating of your hands and/or feet?
52. Do you suffer from headaches?
53. Do you feel like your heart sometimes skips a beat?
54. Does your heart flutter or start to race easily in demanding situations?
55. Do you blush easily?
56. Do you always feel cold?
57. Do you feel that you have difficulties with breathing even when relaxing?
58. Do you suffer from vertigo or dizziness?
59. Do you feel like vomiting when you see something disgusting or if you hear someone talking about detestable things?
60. Do you feel nauseous before common dental or medical interventions and minor surgical procedures?
61. Do you suffer from inner disquiet, tension, or restlessness?
62. Do you think that your memory isn't as good as it was?
63. Do you think that you are less tolerant to noise and rush in your surroundings lately?
64. Do you feel weariness and exhaustion that doesn't correspond with your working load?
65. Do you feel very weak (like after suffering longterm ilness)?
66. Do you feel that your sexual appetite is diminishing, do you have problems in sexual intercourse that you didn't have before?
67. Do you have a short fuse, do nonessential things that get you out of balance?
68. Do you feel that you don't work as effectively as before and that your performance is worsening despite all your efforts?
69. Do you tire more quickly than before?
70. Do you sleep badly; do you wake up feeling that sleep didn't refresh you?

Appendix 2

Psychopathology (PP) scale of the OD-1 questionnaire was composed of following items (the numbers mean the position of particular item in the questionnaire):

3 - I do a lot of things that I lately regret to have done and I do them a lot and probably more often than other people.
6 - I would be far more successful in my life if it wasn't for people who crimped me.
7 - I think that my family life is not that pleasant as that of the most of the people I know.
9 - Sometimes I really wanted to leave home.
11 - One of the best ways of dealing with problems is not to think about them.
14 - One may work as hard as he wants to but he will not reach his goals anyway.
17 - In comparison with other families there is not as much of love and coherence in mine.
19 - I think there's a multitude of people who try to avoid me.
21 - I never mind being often involved in arguments.
23 - My friendships tend to break often and it's not my fault.
25 - I act in a way that people easily misunderstand my actions.
27 - My parents and my family criticize me more than it's necessary.
28 - I often feel that I did something wrong or bad.
36 - There are people who want to disserve me.
38 - I have a desire to break, crush and destroy things and it's an inherent part of myself.
45 - I'm used to think of or need to hit, harm or injure someone.
50 - I can easily bear to witness someone hitting a child or abusing an animal.
55 - It makes me feel good to hurt even those people that I like.
57 - I haven't lived a rightful life.
59 - Sometimes I like to annoy people that hurt me or didn't do what I wanted.
61 - I don't abide rules because they misguide me in a way.
68 - In childhood I wanted to be a member of a group or a gang that goes through various adventures.

4.3 Higher Extraversion and Lower Conscientiousness in Humans Infected with *Toxoplasma*

4.3.1 Reasons for Inclusion

Studies of changes in personality profiles of men and women with latent toxoplasmosis stood at the very beginning of the latent toxoplasmosis studies of our laboratory. That by itself would not, of course, explain the inclusion of one of these studies in the doctoral project. The need for testing whether the now popular NEO-PI-R questionnaire is feasible for our purposes of studying the effects of toxoplasmosis on human behavior and cognition is, however, directly connected with the study of prepulse modification of startle reaction in human subjects.

One of the reasons for using the PPI tests in our experiments is the high sensitivity of the test toward all kind of circumstances. This advantage could, however, easily become an obstacle if we as experimenters were not aware of the kinds of circumstances that could affect the results of our experiments. For example, some of the Big 5 personality traits affect performance in both physical (Roberts & Woodman, 2017) and cognitive disciplines (Curtis, Windsor, & Soubelet, 2015). Personality traits were shown to affect driving accidents (Loo, 1979), reaction times of human experimental subjects in cases of, for example, introverts and extroverts (Rammsayer, Netter, & Vogel, 1993), and in case of extreme personalities ("psychosis-prone", as defined in the article) sensorimotor gating and cognitive and visual interference in general (Swerdlow, Filion, Geyer, & Braff, 1995). As for the PPI specifically, in some experiments the personality traits were not found to be associated with changes in PPI (while at the same time the differences were found in the amplitude of auditory startle reaction) (De Pascalis, Cozzuto, & Russo, 2013); other studies found differences in PPI associated with personality traits such as neuroticism as profound, that they recommend routine examination of personality together with tests of PPI – especially, if we are pursuing the research of changes in sensorimotor gating in context of schizophrenia development (Corr, Tynan, & Kumari, 2002).

4.3.2 Introduction

Differences in personality traits between *Toxoplasma*-infected and *Toxoplasma*-free individuals were previously found in our laboratory using now somewhat outdated Cattell's 16 Personality Factors questionnaire (J. Flegr, Zitková, Kodym, & Frynta, 1996; Jaroslav Flegr, 2007; Jaroslav Flegr & Havlíček, 1999) as well as Cloninger's Temperament and Character Inventory (Anna Skallová et al., 2005) on various population samples. In this study, we wanted to find out whether some of the previously shown differences in personality traits between *Toxoplasma*-infected and uninfected individuals will also be detectable by now more used Neuroticism—Extraversion—Openness Personality Inventory—Revised (NEO-PI-R, also commonly called "Big 5"). Specifically, we were interested in agreeableness, since this factor has been repeatedly linked to better performance in various tests (Bell, 2007; Burke-Smalley & Mount, 2002; Schippers, 2014).

4.3.3 Materials and Methods

Three hundred and twenty-three undergraduate biology students (mean age=21.53, SD=2.40) of the Faculty of Science, Charles University were tested for anti-*Toxoplasma* antibodies in blood sera by ELISA and the complement fixation test (both tests were conducted in the National Reference Laboratory for Toxoplasmosis of National Institute of Public Health, Prague). All of the subjects underwent the same battery of tests, including filling up the NEO-PI-R questionnaire in Czech translation (Hřebíčková et al., 2002) and experimental games (the very same as in Publication 1). The experimental subjects were rewarded with 400 – 600 CZK for their participation in the experiment based on the actual amount of money won during the experimental games. T-tests, general linear models, one-tailed test, and partial Kendall correlation with age as a covariate were used in the statistical analysis of the collected data.

4.3.4 Results and Discussion

Of 211 female subjects, 181 turned out to be *Toxoplasma*-negative and 30 *Toxoplasma*-positive, while among the 117 male students, 95 were *Toxoplasma*-negative and 17 *Toxoplasma*-positive. No significant mean age difference was found between neither male (21.48) and female (21.56; t=0.283,

p=0.778) nor *T.*-negative (21.53) and *T.*-positive (21.51; t=0.059, p=0.953) experimental subjects.

Using General linear models with independent variables sex and toxoplasmosis, we have found associations between extraversion (F=5.23, p=0.023, eta^2=0.016) and conscientiousness (F=5.38, p=0.021, eta^2=0.017) and *Toxoplasma* infection. No significant *Toxoplasma*-sex interactions were found in any of the 5 factors of the NEO-PI-R questionnaire, while one-tailed tests of association between toxoplasmosis and 5 personality factors conducted separately for men and women uncovered positive association for agreeableness in women (p=0.021, eta^2 =0.020), and positive association for extraversion (p=0.036, eta^2 =0.029) and negative conscientiousness (p=0.035, eta^2 =0.030) in men.

Using partial Kendall correlation with age as a covariate performed separately for *Toxoplasma*-positive men and women we have discovered significant negative correlation between levels of anti-*Toxoplasma* antibodies as determined by the complement fixation test and conscientiousness (partial τ=0.519, p=0.005), which would indicate cumulative changes in personality with length of the latent infection, results for other traits in men and women stayed non-significant.

4.3.5 Limitations

The main limitation of the study was an uneven representation of male and female, and *Toxoplasma*-positive and *Toxoplasma*-negative experimental subjects, especially considering the low percentage of latent toxoplasmosis prevalence (14.2% in women and 15.2% in men). Only 17 men and 30 women of the subjects included in the analysis were infected with the small number of male subjects being caused by standard sex ratio of biology students enrolled in undergraduate studies on our faculty. A study conducted on a population sample with closer-to-even sex ratio and perhaps higher prevalence of toxoplasmosis could bring different results.

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Higher Extraversion and Lower Conscientiousness in Humans Infected with *Toxoplasma*

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Abstract: Toxoplasmosis is associated with specific differences in the personality of infected subjects relative to non-infected subjects. These differences are usually considered to be a side effect of the manipulative activity of the parasite aimed to increase the probability of its transmission from the intermediate host to the definitive host by predation. The personality of infected subjects was studied mostly using the Cattell's questionnaire. However, this questionnaire is now considered outdated and has been mostly substituted with the Neuroticism–Extraversion–Openness Personality Inventory—Revised (NEO-PI-R) questionnaire in clinical practice. Here, we searched for the association between toxoplasmosis and the personality by screening a population of students with the NEO-PI-R questionnaire. We found that Toxoplasma-infected male and female students had significantly higher extraversion and lower conscientiousness. The conscientiousness negatively correlated with the length of infection in men, which suggested that the toxoplasmosis associated differences were more probably the result of slow cumulative changes induced by latent toxoplasmosis, rather than transient side effect of acute Toxoplasma infection. The existence of this correlation also supported (but of course not proved) the hypothesis that Toxoplasma infection influenced the personality, rather than the hypothesis that the personality influenced the probability of the infection. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: Big Five; Cattell 16PF; *Toxoplasma*; parasite; manipulation hypothesis

INTRODUCTION

Toxoplasma gondii is a coccidian parasite that uses practically any warm-blooded animals including humans as secondary hosts and felids as definitive hosts. In humans, the prevalence of infection reaches about 30% in the Czech Republic and up to 60% in several European countries. Mostly, infected people are unaware of the fact that they are infected. After a short phase of acute infection, the parasitosis turns to the latent stage, which in medical terms is considered asymptomatic and is life-long.

Experimental infection with *Toxoplasma* is known to influence the behaviour of mice and rats (for a review, see Webster, 2001, 2007). Humans with latent toxoplasmosis differ from *Toxoplasma*-free subjects in behaviour in ethological experiments including experimental games (Lindová et al., 2006, 2010) and in personality profile measured with personality questionnaires (Flegr, Novotná, Fialová, Kolbeková, & Gašová, 2010). Most psychological data in toxoplasmosis research were collected using Cattell's 16PF questionnaire, fourth edition (Cattell, 1970), translated and standardized for the Czech population by Říčan (1975) in the 1990s (Flegr, 2007). Specifically, Flegr et al. (1996) studied 443 biology students and professors (69 men and 44 women infected by *Toxoplasma*) and found higher affectothymia (factor A, warmth) and lower protension (factor L, low vigilance) in seropositive women and lower superego strength (factor G, low conscientiousness), higher protension (factor L, vigilance) and higher guilt proneness (factor O, apprehension) in infected men, all relative to seronegative subjects. Factors A (affectothymia, warmth), G (superego strength, conscientiousness), L (protension, vigilance), N (shrewdness, privateness) and Q_3 (high strength of self-sentiment, perfectionism) showed a gender-different pattern of *Toxoplasma*-associated alterations (women had higher A, G and Q_3 whereas men exhibited higher L and N relative to uninfected controls; for results of the enlarged sample, see Flegr, Hrdá, & Havlíček, 1999). Although a later study in 191 women (55 infected) failed to confirm some of these differences (Flegr & Havlíček, 1999), the authors found a positive association of toxoplasmosis with B (intelligence) and O (guilt proneness) and higher variance in factors L and Q_3 in *Toxoplasma*-infected relative to *Toxoplasma*-free subjects. Another study (Flegr, Kodym, & Tolarová, 2000) found a positive correlation between the length of toxoplasmosis and factors G and Q_3 in 230 women diagnosed with acute toxoplasmosis 2–13 years before the study and a negative correlation between the anti-*Toxoplasma* antibodies titre (proxy for the length of toxoplasmosis) and factors A, F (surgency, liveliness), G, H (parmia, social boldness) and L in *Toxoplasma*-infected women. This effect was relatively

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strong—the effect size estimated with the tau of Kendall non-parametric correlation test was 0.21–0.27 for particular factors.

In addition to the 16PF questionnaire, Cloninger's Temperament and Character Inventory has been used to study the association between toxoplasmosis and personality. Skallová et al. (2005) found lower levels of novelty seeking in *Toxoplasma*-infected women compared with *Toxoplasma*-free women. In terms of the subscales, this was caused mainly by lower extravagance and disorderliness. Lower novelty seeking in infected subjects has also been reported in a large sample of men (military conscripts; Flegr et al., 2003; Novotná et al., 2005). Flegr et al. (2010) have confirmed lower novelty seeking in female blood donors. Their study also found a significant effect of the RhD phenotype–toxoplasmosis interaction on the factors harm avoidance, reward dependence, self-directedness and cooperativeness in men.

The mechanism underlying a possible association between personality, behaviour and toxoplasmosis is unknown. In animals, artificial infection experiments proved that the behavioural alterations associated with toxoplasmosis are induced by the infection. Artificial infection of humans cannot be carried out, and therefore, we have to rely on indirect evidence. It suggests that the observed behavioural differences are induced by toxoplasmosis rather than being responsible for an increased risk of infection in some subjects (for details, see the DISCUSSION section). Although behavioural changes elicited by toxoplasmosis in rodents and other animals have been explained by the manipulatory activity of the parasite aimed to increase the probability of transmission to the definitive host by predation (Holmes & Bethel, 1972), such an assumption does not hold in humans who, of course, are not preyed on by felids at the present time (Webster, 2001). Therefore, other theories have been proposed, which consider psychological and behavioural changes in toxoplasmosis-infected humans as non-specific behavioural responses to toxoplasmosis-induced neurological or endocrinological changes. However, the explanation of the observed behavioural changes by the adaptive manipulation activity of the parasite cannot be completely ruled out because (i) man's animal ancestors were probably hunted by large cats (Zuberbuhler & Jenny, 2002) and (ii) *Toxoplasma* is not aware of the host where it is located in.

The major aims of the present cross-sectional study were to test whether the personality factor differences associated with latent toxoplasmosis can also be detected by a more standardly used personality questionnaire, the Neuroticism–Extraversion–Openness Personality Inventory—Revised (NEO-PI-R), and to show which of the five personality factors are associated with toxoplasmosis. We also evaluated the correlation between the length of toxoplasmosis (estimated from the concentration of anti-*Toxoplasma* antibodies) and the amount of the personality factor differences relative to uninfected subjects, which in our view, could indicate that the personality differences between infected and non-infected subjects were rather induced by toxoplasmosis than that pre-existing personality differences influence the probability of infection.

METHOD

Participants

Undergraduate biology students of the Faculty of Science, Charles University, Prague, were addressed during regular biology lectures and were invited to participate in the study on a voluntary basis. Three hundred and twenty-three students (mean age=21.53, SD=2.40) were enrolled in the study and signed an informed consent form. All participants provided 2 ml of blood for serological testing. During a 5-hour testing session, all participants performed the same panel of psychological and behavioural tests, including the NEO-PI-R (Costa & McCrae, 1992) Czech version by Hřebíčková (2001). The students were paid CZK200–600 ($10–30) for participation in the study, depending on their results (how much they won) in experimental games. The recruitment of the study subjects and data-handling practices complied with the Czech legislation in force, and the study was approved by the IRB Faculty of Science, Charles University.

Immunological tests for toxoplasmosis

All serological tests were carried out in the National Reference Laboratory for Toxoplasmosis, National Institute of Public Health, Prague. All study subjects were screened for specific anti-*Toxoplasma* IgG antibodies, and those with high IgG levels were tested for IgM antibodies by ELISA (IgG: SEVAC, Prague, Czech Republic; IgM: TestLine, Brno, Czech Republic; optimized for early detection of acute toxoplasmosis) and the complement fixation test (CFT; SEVAC, Prague, Czech Republic), which is more reliable in established (old) *T. gondii* infections as decrease in CFT titres is more regular (Kodym et al., 2007). *Toxoplasma* antibody titres in the sera were measured at dilutions between 1:4 and 1:1024. The subjects testing IgM negative by ELISA (positivity index <0.9) and having CFT titres higher than 1:8 were considered latent toxoplasmosis positive. None of the study subjects had a CFT titre higher than 1:128 or a positivity index for IgM higher than 0.9.

RESULTS

The final sample consisted of 181 *Toxoplasma*-negative and 30 *Toxoplasma*-positive female students and 95 *Toxoplasma*-negative and 17 *Toxoplasma*-positive male students. The seroprevalence rates of latent toxoplasmosis were 14.2% in women and 15.2% in men. No significant difference was found in the mean age between women (21.56) and men (21.48) ($t=0.283$, $p=0.778$) or between *Toxoplasma*-negative (21.53) and *Toxoplasma*-positive subjects (21.51) ($t=0.059$, $p=0.953$). Neither the CFT titres nor the concentration of anti-*Toxoplasma* IgG antibodies significantly correlated with the age of male or female subjects (results not shown).

General linear model (GLM) analyses with sex and toxoplasmosis as independent (binary) variables showed that two of the five personality factors, that is, extraversion and conscientiousness, were associated with *Toxoplasma* infection

(Table 1). The results were approximately the same when the age of subjects was included into the model as a continuous variable (results not shown). The *Toxoplasma*–sex interactions were not significant for any of the five factors and were only significant for two of 30 facets (Table 2), values (p=0.018) and straightforwardness (p=0.012).

To compare the toxoplasmosis–personality association in men and women, we performed separate one-tailed test analyses for male and female students. These analyses showed a positive association of toxoplasmosis and agreeableness (p=0.021, eta^2=0.020) for women and a positive association of toxoplasmosis and extraversion (p=0.036, eta^2=0.029) and a negative association of toxoplasmosis on conscientiousness (p=0.035, eta^2=0.030) for men.

The length of the infection in particular subjects is mostly unknown. However, statistically, the concentration of anti-*Toxoplasma* antibodies decreases with the length of the infection (see Figure 2 in Kodym et al., 2007). Therefore, it is possible to study the correlation between the length of the infection and personality factors by an evaluation of the correlation

Table 1. Mean Neuroticism–Extraversion–Openness Personality Inventory—Revised personality factors in female and male students and the effect of latent toxoplasmosis

Factor	$F_{1,319}$	p	Eta^2	F, $T-$	F, $T+$	M, $T-$	M, $T+$
Neuroticism	0.49	0.483	0.002	96.1, *19.29*	99.1, *21.54*	91.6, *17.93*	93.0, *20.09*
Extraversion	5.23	**0.023**	0.016	99.49, *16.27*	103.7, *14.66*	101.5, *15.87*	109.5, *19.39*
Openness	1.94	0.164	0.006	112.6, *17.78*	115.6, *18.23*	113.7, *16.62*	118.9, *23.08*
Agreeableness	0.66	0.417	0.002	107.7, *15.46*	114.0, *16.81*	104.9, *15.04*	102.8, *18.99*
Conscientiousness	5.38	**0.021**	0.017	105.4, *16.38*	101.3, *19.40*	105.1, *20.19*	95.6, *17.89*

The results of general linear model analyses, that is, F-values, significance and the effect sizes, are given in columns 2–4, and the mean factors and standard deviations (in italics) for 181 *Toxoplasma*-negative women, 30 *Toxoplasma*-infected women, 95 *Toxoplasma*-negative men and 17 *Toxoplasma*-infected men are given in columns 5–8. Internal reliabilities of domain scales were 0.84, 0.78, 0.82, 0.78 and 0.83 for neuroticism, extraversion, openness, agreeableness and conscientiousness, respectively. The significant effects of toxoplasmosis are printed in bold.

Table 2. Mean Neuroticism–Extraversion–Openness Personality Inventory—Revised personality facets in female and male students and the effect of latent toxoplasmosis

Facets	$F_{1,319}$	p	Eta^2	F, $T-$	F, $T+$	M, $T-$	M, $T+$
Anxiety	0.33	0.564	0.001	17.5	17.9	16.0	14.6
Angry hostility	0.00	0.958	0.000	15.9	15.9	15.4	15.5
Depression	1.62	0.205	0.005	15.1	16.5	14.4	15.1
Self-(non)consciousness	1.26	0.263	0.004	16.2	16.6	15.4	16.6
Impulsiveness	2.83	0.094	0.009	16.1	16.8	16.3	18.4
Vulnerability	0.53	0.468	0.002	15.3	15.4	14.1	12.8
Warmth	3.94	**0.048**	0.012	20.3	21.6	19.9	21.6
Gregariousness	2.86	0.092	0.009	14.6	14.7	14.5	17.1
Assertiveness	2.53	0.112	0.008	12.5	13.5	14.2	15.4
Activity	0.39	0.533	0.001	16.3	16.8	16.2	16.5
Excitement seeking	4.98	**0.026**	0.015	15.0	16.6	16.5	18.1
Positive emotions	0.03	0.859	0.000	20.8	20.5	20.2	20.9
Fantasy	1.03	0.311	0.003	19.1	19.1	19.5	21.1
Aesthetics	0.28	0.595	0.001	20.0	19.3	19.1	19.1
Feelings	0.83	0.363	0.003	19.9	21.0	19.8	20.3
Actions	2.02	0.156	0.006	17.0	18.8	16.9	17.1
Ideas	1.12	0.291	0.003	16.2	17.4	18.8	19.6
Values*	3.03	0.083	0.009	20.4	20.0	19.6	21.8
Trust	0.26	0.611	0.001	16.4	17.1	16.4	16.5
Straightforwardness*	2.26	0.134	0.007	20.2	21.0	19.9	16.9
Altruism	1.11	0.293	0.003	20.3	21.3	19.5	20.0
Compliance	0.65	0.421	0.002	14.7	15.8	14.7	14.8
Modesty	0.20	0.659	0.001	18.0	19.5	16.5	15.6
Tender mindedness	3.96	**0.048**	0.012	18.0	19.3	17.9	19.0
Competence	0.73	0.394	0.002	18.6	17.3	19.1	19.4
Order	11.20	**0.001**	0.034	17.9	16.5	17.6	13.7
Dutifulness	1.03	0.312	0.003	19.2	19.2	18.5	16.9
Achievement	2.39	0.123	0.007	17.0	16.2	17.2	15.8
Self-discipline	2.58	0.109	0.008	16.7	16.5	16.0	13.8
Deliberation	0.57	0.451	0.002	16.0	15.6	16.7	15.9

The results of general linear model analyses, that is, F-values, significance and the effect sizes, are given in columns 2–4, and the mean facets for 181 *Toxoplasma*-negative females, 30 *Toxoplasma*-infected females, 95 *Toxoplasma*-negative males and 17 *Toxoplasma*-infected males are given in columns 5–8. The significant effects of toxoplasmosis are printed in bold, and the significant effects of toxoplasmosis–sex interaction are marked with asterisks.

between the concentration of antibodies and the levels of those personality factors that differ between *Toxoplasma*-infected and *Toxoplasma*-free subjects. Partial Kendall correlation with age as a covariate performed separately for men and women showed that the concentration of anti-*Toxoplasma* antibodies measured with CFT correlated negatively with conscientiousness (partial tau=0.519, p=0.005) in men. Partial correlations for extraversion in *Toxoplasma*-infected men and for extraversion and conscientiousness in *Toxoplasma*-infected women were non-significant (extraversion: partial tau=−0.121, p=0.366; conscientiousness: partial tau=−0.129, p=0.337).

DISCUSSION

Toxoplasma-infected subjects had higher extraversion and lower conscientiousness than *Toxoplasma*-free subjects. In *Toxoplasma*-infected men, the length of infection estimated on the basis of CFT titres correlated with low conscientiousness.

In some aspects, the present results are compatible with the reported data obtained using Cattell's 16PF questionnaire in different population samples. Higher extraversion could be considered as corresponding to higher Cattell's factor A (affectothymia, warmth) found previously for women, as Cattell's factor A has been previously found to correlate with several NEO extraversion scales (Gerbing & Tuley, 1991). Indeed, one of the facets that the NEO-PI-R extraversion can be decomposed into is warmth, and this facet is significantly higher in infected individuals in our study (but note that this result would be non-significant after Bonferroni correction for multiple testing). However, Cattell's factor A was found to load rather weakly on the NEO-PI-R extraversion in contrast to factors F (liveliness) and H (social boldness; Rossier et al., 2004). Moreover, the NEO-PI-R facet warmth was found to load comparably strongly, for example, on agreeableness and extraversion (Cattell, 1996; Child, 1998; Conn & Rieke, 1994). Therefore, it is unlikely that the positive association of extraversion with toxoplasmosis might be caused by increased warmth only. In fact, our results show at least one more positive association of latent toxoplasmosis with the extraversion facet excitement seeking (non-significant after Bonferroni correction).

At face value, lower conscientiousness, observed especially in men, corresponds well with lower factor G (conscientiousness) and factor Q_3 (perfectionism), sometimes observed in men. However, factor G was found to correlate only moderately with the NEO-PI-R conscientiousness, in contrast to, for example, low factor M (low abstractedness; Rossier et al., 2004). Also, the significantly lower score for infected subjects found in the facet order (p=0.001) in this study indicates the relatively stronger connection of perfectionism (Q_3) with the NEO-PI-R conscientiousness.

The important difference between the present NEO-PI-R personality profile and 16PF personality profiles of infected subjects was that the toxoplasmosis-associated differences relative to *Toxoplasma*-free subjects in Cattell's personality factors were mostly in opposite directions for men and women, whereas the differences in the NEO-PI-R factors were generally in the same direction for both genders. Specifically, the NEO-PI-R extraversion differs in the same direction (relative to *Toxoplasma*-free subjects) in both genders in contrast to Cattell's factor A proposed to be comparable with extraversion (see preceding discussion), which was different only in women. It was speculated (Lindová et al., 2006) that these observed differences in the directions of the 16PF factor shifts between men and women might be an artefact of the personality assessment method used (Cattell's 16PF), which made it possible that the subjects conventionalized, endeavoured to appear better than they really were and hid their 'weaker' personality traits, where one of the genders (women as proposed) was more prone to such behaviour. Moreover, the 16PF questionnaire, based on 16 primary factors discovered by the factor analysis, faced a variety of criticisms regarding the ability to verify the primary factor level across gender, age and method (Digman, 1990; Eysenck & Eysenck, 1969; Eysenck, 1972). This may also be responsible for the inconsistencies in the toxoplasmosis–personality associations between men and women and between different population samples. More specifically, gender difference concerning Cattell's factor A may be at least partly caused by the relatively lower internal consistency and reliability of the 16PF factor A (Rossier et al., 2004; Lindová et al., 2008). We have to note, however, that there seems to be not a strong theoretical ground for the proposed interpretation of Cattell's 16PF allowing stylization of the subjects in contrast to the NEO-PI-R, because the NEO-PI-R items seem to require at least as much self-assessment as the 16PF (Cattell & Mead, 2008).

The 16PF questionnaire is still widely used in clinical practice in many countries, including the Czech Republic. For experimental purposes, however, it has been mostly abandoned in favour of the questionnaires based on the Big Five model measuring five basic personality traits, namely neuroticism, extraversion, openness, agreeableness and conscientiousness, in the past two decades (Goldberg, 1992; Costa & McCrae, 1992). These five basic dimensions appear repeatedly in broad cross-cultural studies, and even the 16PF itself was modified towards a five-factor model on the second-order level in its fifth edition (Cattell, Cattell, & Cattell, 1993). The higher popularity of the Big Five model is also very likely based on the fact that dealing with five factors is more straightforward than dealing with 16 factors. However, Cattell (Cattell & Mead, 2008) points out that the validity of the Big Five model, which was in fact originally obtained in a factor analysis of 16PF primary factors, can be seriously limited by the statistical methods used to generate it, namely the induced orthogonality of factors by varimax rotation (see also Cattell, 1996). For instance, because of the induced orthogonality, valid personality concepts such as warmth and dominance are being given little credit in the Big Five model. Interestingly also, Depue and Collins (1999) show in their review on extraversion the relative independence of the affiliation (warmth) and agency (social dominance) components of extraversion (and further regard impulsivity as a third component whose association with extraversion they consider as questionable). However, our finding of an association of the biological factor toxoplasmosis with the whole factor extraversion rather than only with specific extraversion facets indicate that extraversion could be viewed as homogeneous with a possible common biological underpinning.

On the other hand, the gender differences found with the use of Cattell's 16PF have been basically confirmed by two behavioural studies. Lindová et al. (2006) have observed a lower tendency to maintain close relationships, a lower tendency towards orderly and responsible behaviour, and lower clothes tidiness in infected men compared with uninfected men. In contrast, infected women were found to behave more orderly and responsible than uninfected women. Moreover, Lindová et al. (2010), using the experimental trust game, have reported infected men compared with uninfected men to repay less money from the amount invested by the opponent back to him or her. The same was not observed for women. Gender difference was also found in the levels of testosterone of infected compared with uninfected subjects, with infected women having lower and infected men having higher levels compared with uninfected controls (Flegr, Lindová, & Kodym, 2008).

Secondly, neither the results obtained with the NEO-PI-R questionnaire revealed identical differences between infected and non-infected subjects in both genders when analysed separately. Interestingly, the higher agreeableness seen in our study for *Toxoplasma*-infected women only corresponds to lower 16PF factor L (higher trust; Rossier et al., 2004), which was also shown in previous studies for women but not for men (see preceding discussion). In spite of the fact that the main five personality dimensions are associated with toxoplasmosis identically for both genders, the divergences found on lower levels in both the NEO-PI-R and 16PF support the existence of some gender differences in toxoplasmosis-associated personality traits possibly reflecting the variation in biological (e.g., endocrinological) effects of *Toxoplasma*.

The possible mechanisms of toxoplasmosis-associated differences observed in humans and animals are still unknown. It is highly probable that an increase of dopamine in certain parts of the brain is involved (Flegr et al., 2003; Skallová, Kodym, Frynta, & Flegr, 2006; Hodková, Kodym, & Flegr, 2007; Webster & McConkey, 2010; Vyas & Sapolsky, 2010), which could also explain the strong association between latent toxoplasmosis and schizophrenia (Torrey, Bartko, Lun, & Yolken, 2007; Fekadu, Shibre, & Cleare, 2010). There are, however, also some indices suggesting that toxoplasmosis-associated differences in steroid hormone concentration could play a role in the observed phenomena (Flegr, Hrušková, Hodný, Novotná, & Hanušová, 2005; Flegr, Lindová, & Kodym, 2008; Hodková, Kolbeková, Skallová, Lindová, & Flegr, 2007). The concentration of some steroid hormones (e.g., oestrogen) highly fluctuates during the menstrual cycle. The higher variance of hormone concentration in women relative to men could explain the difficulties in demonstrating the existence of a significant correlation between the length of toxoplasmosis and the level of personality factors in women observed in the present as well as in some other studies (Flegr & Hrdý, 1994).

Theoretically, the association between toxoplasmosis and personality traits could be caused either by an increased probability of *Toxoplasma* infection in subjects with certain personality profile or by induction of personality changes by toxoplasmosis. However, the former hypothesis has been rejected by several studies showing that the personality alterations increase with the duration of infection (not with age of patients; for a review, see Flegr, 2007). Moreover, it has been shown that Rh-positive subjects are protected against the toxoplasmosis-associated alterations (Flegr et al., 2010). Last but not the least, behavioural differences between infected and non-infected individuals closely related to specific personality differences between infected and non-infected individuals observed in humans, namely the higher scores of novelty seeking, have been proven to exist by experiments on animals where a laboratory infection of rodents was performed (Webster, 2001; Skallová et al., 2006). For obvious reasons, such experimental infection cannot be carried out with humans. A longitudinal prospective study in humans is out of the reach of normal a researcher—due to a low incidence of toxoplasmosis in adult subjects, many thousands of subjects would have to be involved into such a study. Moreover, even a longitudinal study cannot resolve whether the association between toxoplasmosis and the personality profile differences is the result of the influence of toxoplasmosis on the personality profile or the result of the influence of some unknown factor both on the personality profile and on the risk of the *Toxoplasma* infection. The present cross-sectional study again supports the hypothesis that the observed personality differences were induced by *Toxoplasma* infection. It was shown that the CFT titres (in contrast to the concentration of specific IgG antibodies measured with ELISA) decreased relatively regularly with the length of latent toxoplasmosis (Kodym et al., 2007). Therefore, the length of *Toxoplasma* infection in a particular subject can be estimated on the basis of the CFT titres. In the present study, we demonstrated that the score of the personality factor conscientiousness in men was correlated with the length of latent toxoplasmosis. Therefore, the observed personality differences between *Toxoplasma*-infected and *Toxoplasma*-free subjects were more probably a result of the cumulative effect of the latent infection rather than some kind of a carryout effect of acute toxoplasmosis. The existence of such correlation in models where the age of subjects is controlled also suggests that the opposite causality direction (increased probability of *Toxoplasma* infection in, for example, subjects with lower conscientiousness) is less probable. It must be stressed, however, that using cross-sectional observational studies, the question of what is the cause and what is the effect in the *Toxoplasma*-human system cannot be definitely solved.

The strength of the observed effects (effect size estimated with eta^2 in the GLM test and Kandal tau in Kendall correlation test) was relatively low. In the GLM tests, toxoplasmosis explained 2–3% of the variability in particular personality traits. On the other hand, the effect of the length of infection on the score of conscientiousness in men is rather strong: Kendall tau of 0.519 indicates that the probability that among two randomly chosen male students the one with the lower concentration of anti-*Toxoplasma* antibodies (with probably longer infection) will have a lower conscientiousness score is about 76%. It should be, however, reminded that the real effect size of the associations and correlation is probably stronger as any source of stochastic errors (e.g., any inaccuracies in measurement of the concentration of anti-*Toxoplasma* antibodies or in measurement of the personality traits) has a negative influence on the estimated strength of the effect. Similarly, the

obtained significances are negatively influenced by the fact that the prevalence of toxoplasmosis among Czech students (and in the Czech population in general) is relatively low (Schachter & Chow, 1995). A similar study performed on a population of the same size in some high-prevalence country, for example, in France, in Spain or in any South American or African country, would probably give more significant result.

In 2006, Lafferty demonstrated that differences in the prevalence of latent toxoplasmosis could explain a statistically significant portion of the variance in aggregate neuroticism among populations of various countries as well as in the 'neurotic' cultural dimensions of sex roles and uncertainty avoidance (Lafferty, 2005, 2006). It would be interesting to repeat this correlational study to find out whether the international differences in the Big Five personality traits extraversion and conscientiousness could be partly explained by the variation in the prevalence of toxoplasmosis in different parts of the world.

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4.4 Difference of neuro- and immunomodulatory steroids and selected hormone and lipid concentrations between *Toxoplasma*-free and *Toxoplasma*-infected but not CMV-free and CMV-infected schizophrenia patients

4.4.1 Reasons for Inclusion

It is well known that maternal immune response during pregnancy might lead to permanent changes in the brain of development of the offspring, thus leading to animal models of schizophrenia in rats manifested beside other symptoms through impairment of sensorimotor gating as tested by prepulse modification of startle reaction experiments (Borrell, Vela, Arévalo-Martin, Molina-Holgado, & Guaza, 2002; Romero, Guaza, Castellano, & Borrell, 2010; Wolff & Bilkey, 2008). Less known due to relatively sparse publications related to interactions between modification of sensorimotor gating and physiological phenomena such as modulation of immunity, metabolism of lipids, or concentration of steroids and hormones in blood, is perhaps, the fact that sensorimotor gating can be influenced by a lot of seemingly unrelated phenomena even in youngsters and adults.

For example, lipopolysaccharide which stimulates innate immune system can, if injected intraperitoneally in rats, decrease startle response magnitude while at the same time not modulating normal levels of PPI, suggesting highly specific effects on sensorimotor processing (Lockey, Kavaliers, & Ossenkopp, 2009). Deficiency in n-3 fatty acids in the diet of experimental mice leads to substantial deficits in PPI of the acoustic startle in comparison with mice with a standardized diet (Fedorova, Alvheim, Hussein, Salem, & Jr, 2009). Rats treated with ketamine manifested significant PPI deficits while treatment with immunomodulatory drugs and risperidone were able to reverse these ketamine-induced PPI deficits (da Silva Araújo et al., 2017). Testosterone and other sex steroids regulate brain development, and lack of it can affect (attenuate) prepulse inhibition of startle reaction as demonstrated, for example, in adolescent rhesus monkeys (Morris et al., 2010). Fear potentiated startle in humans is positively associated with cortisol

and negatively with sulfate ester of dehydroepiandrosterone, both of which have effects on stress and anxiety (Grillon et al., 2006).

When studying relations between PPI, schizophrenia, toxoplasmosis, and all the above-mentioned phenomena such as immunomodulatory lipids, steroid hormones or human metabolism, we must be aware that connections between all of these do not create a list of simple relations, but rather an intricately entwined network, which we, as of now, only begin to understand. To test the effects immunomodulation on schizophrenia and/or toxoplasmosis-related changes in the modulation of prepulse inhibition, we need to research each relationship between the phenomena. Here we started by studying *Toxoplasma*-related differences in steroids, hormones, and lipid concentrations in schizophrenia patients.

4.4.2 Introduction

Immunological disorders are sometimes suggested as possible causational link between toxoplasmosis and schizophrenia (Bhadra, Cobb, Weiss, & Khan, 2013; Hinze-Selch et al., 2007; N. Müller & Schwarz, 2006), and possible neuroprotective function of 7-hydroxylated derivatives of dehydroepiandrosterone have been discovered in the last decades (R. Morfin et al., 2000; Robert Morfin & Stárka, 2001) making differences in neuro- and immunomodulatory steroids, hormones and lipid concentrations between *Toxoplasma*-infected individuals and uninfected controls among schizophrenia patients an interesting and potentially clinically important topic to study.

4.4.3 Materials and Methods

We have tested 94 female (mean age: 33.9, S.D.: 6.92) and 79 male (mean age: 35.4, S.D.: 8.93) schizophrenia patients using serological tests for *Toxoplasma gondii* and *Cytomegalovirus.* Hormones (Cortisol, dehydroepiandrosterone and its sulfate (DHEA/S), prolactin, 7-α-Hydroxydehydroepiandrosterone (7-α-OH-DHEA) and its 7-β-hydroxy isomer (7-β-OH-DHEA)) determined from blood collected at 8:00 a.m. after overnight fasting. Thyrotropin levels were tested using Electro-Chemiluminescence Immunoassay (ECLIA), blood glucose levels were established after overnight

fasting using glucose analyzer, and commercially available kits were used for measuring various lipid parameters (total serum cholesterol, high- and low-density lipoproteins, and triacylglycerides).

Assumption tests, t-test, logistic regression, and the general linear model were computed using Statistica v.9, while partial Kendall correlation test was accomplished using our MS Excel Sheet.

4.4.4 Results and Discussion

Logistic regression of the biochemical and serological data with sex and age as independent variables showed a significantly lower prevalence of toxoplasmosis in female (29.8%) than in male (53.2%) schizophrenia patients (Wald's Chi^2=8.57, p=0.003, OR=2.57, C.I.95=1.36–4.85). Prevalence of cytomegalovirus infection was approximately the same in female (85.6%) and male (83.3%) patients (Chi^2=0.20, p=0.654, OR=0.82, C.I.95=0.35–1.92), and no association between toxoplasmosis and cytomegalovirus infection were detected (Wald's Chi^2=2.27, p=0.132, OR=2.05, C.I.95=0.80–5.27).

We have found several significant or borderline-significant (marked by an asterisk) effect of toxoplasmosis, toxoplasmosis-sex interaction, CMV and CMV-sex interaction on biochemical parameters, namely of toxoplasmosis* (p=0.053, eta^2=0.002) and CMV-sex interaction (p=0.031, eta^2=0.053) on 7-β-OH-DHEA, CMV* on DHEAS (p=0.056, eta^2=0.022), *Toxoplasma* (p=0.034, eta^2=0.027) and *Toxoplasma*-sex interaction (p=0.026, eta^2=0.029) on glucose, *Toxoplasma* on cholesterol (p=0.048, eta^2=0.023), and *Toxoplasma* on LDL* (p=0.089, eta^2=0.018).

Partial Kendall correlation between the concentration of studied molecules and binary variable toxoplasmosis and anti-*Toxoplasma* IgG antibodies in serum of infected patients, both with age as the controlled-for variable showed many significant results. While correlations between 7-α-OH-DHEA and 7-β-OH-DHEA and anti-*Toxoplasma* IgG antibodies was only significant in men (τ_α=-0.26, p_α=0.044, τ_β=-0.229, p_β=0.032), there were significant and borderline significant (marked with asterisk) correlations between the binary variable toxoplasmosis (0/*Toxoplasma*-negative, 1/*Toxoplasma*-positive) and 7-α-OH-DHEA (τ=-0.108, p=0.026), 7-β-OH-

DHEA (τ=-0.155, p=0.001), DHEA (τ=-0.097, p=0.047), and DHEAS* (τ=-0.082, p=0.092) in both sexes analysed together, 7-β-OH-DHEA (τ=-0.167, p=0.017), DHEA* (τ=-0.129, p=0.066), DHEAS* (τ=-0.135, p=0.054), and glucose (τ=0.172, p=0.014) in women, and TSH* (τ=0.134, p=0.081), TAG* (τ=0.131, p=0.087), Cholesterol (τ=0.187, p=0.015), and LDL (τ=0.198, p=0.012) in men.

Since the duration of the infection negatively correlates with the concentration of anti-*Toxoplasma* IgG antibodies, the decreased levels of DHEA metabolites in the *Toxoplasma*-infected patients were most probably fading effects of acute toxoplasmosis, while increased levels of cholesterol and LDL-cholesterol in sera may represent a cumulative effect of latent toxoplasmosis, potentially accelerating the development of atherosclerosis. The found correlation could explain the positive correlation between toxoplasmosis prevalence and incidence of cardiovascular diseases in some European countries (Jaroslav Flegr, 2013).

Perhaps the most interesting result of the study is the more than 50% prevalence of toxoplasmosis in schizophrenic males. We are suggesting a possible explanation: Blood samples were originally gained for clinical use, and only secondarily were they anonymously serologically tested for antibodies against *Toxoplasma*. Because of this, also the samples of severely ill schizophrenic patients with would be probably hesitant to join the study were included (under the permission given by the ethical board of PCP

4.4.5 Limitations

No healthy (i.e., *Toxoplasma*-positive and *Toxoplasma*-negative non-schizophrenic individuals) were included in the study. Because of that, it is impossible to decide whether the found effect of toxoplasmosis are only present in the schizophrenic population or whether they could be found in the general population.

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Difference of neuro- and immunomodulatory steroids and selected hormone and lipid concentrations between *Toxoplasma*-free and *Toxoplasma*-infected but not CMV-free and CMV-infected schizophrenia patients

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Abstract

OBJECTIVES: *Toxoplasma gondii*, the protozoan parasite infecting about 30% population worldwide, is suspected to be the etiological agent of certain form of schizophrenia disease. *Toxoplasma* is known to change levels of certain neurotransmitters, cytokines and several hormones in both infected animals and humans. A common feature of toxoplasmosis and schizophrenia is a disorder of immune system.
METHODS: Here we studied the levels of five neuro- and immunomodulatory steroids, selected hormones and lipids in sera of 173 schizophrenia patients.
RESULTS: *Toxoplasma* infected schizophrenia patients expressed only insignificantly lower concentration of neuro- and immunomodulatory DHEA metabolites. Infected women had higher concentration of glucose while infected men had higher concentration of cholesterol and LDL cholesterol. No significant effect of human cytomegalovirus infection on the concentration of the above parameters was observed. The difference in the concentration of DHEA metabolites faded with the decrease of the concentration of anti-*Toxoplasma* IgG antibodies (i.e. with the duration of *Toxoplasma* infection) while the difference in the concentration of cholesterol and LDL-cholesterol increased with the decrease of the concentration of anti-*Toxoplasma* IgG antibodies. The prevalence of toxoplasmosis in male (53.2%) but not female (29.8%) schizophrenia patients was unusually high in comparison with prevalence of toxoplasmosis in a general population.
CONCLUSION: Our results provided an explanation for seemingly decreasing prevalence of toxoplasmosis in schizophrenia patients observed in current studies

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(increased concerns about the rights of patients resulting in absence of non-cooperative *Toxoplasma*-positive patients in the study population) and suggest possible explanation for reported positive correlation between prevalence of toxoplasmosis and incidence of cardiovascular diseases (accelerated atherosclerotic development due to increased level of cholesterol and LDL in *Toxoplasma* infected humans).

INTRODUCTION

Intracellular parasite *Toxoplasma gondii* is believed to infect about 30% of population worldwide (Tenter *et al.* 2000). Congenital toxoplasmosis acquired in the first trimester of gestation, the result of transmission of parasites from mother with acute infection to fetus, has very serious impacts on health of children and could even result in spontaneous abortion. Also, ocular forms of toxoplasmosis, the frequent result of the congenital toxoplasmosis or of postnatal infection with virulent strains of *Toxoplasma*, has a very serious impact on public health (Jones *et al.* 2007; Scallan *et al.* 2011). For a long time, the most common form of *Toxoplasma* infection, latent toxoplasmosis, was considered more or less harmless in immunocompetent subjects. However, results of many case-control, cohort as well as correlation studies suggest that latent toxoplasmosis is associated with various disorders and probably even plays an etiological role in certain diseases (Flegr 2013a). For example, a strong statistical association exists between toxoplasmosis and schizophrenia (Torrey *et al.* 2007), obsessive compulsory disease (Miman *et al.* 2010), epilepsy (Yazar *et al.* 2003) or risk of suicide (Pedersen *et al.* 2012). From nonpsychiatric diseases, the toxoplasmosis is suspected to play a role in etiology of some tumor diseases (Thomas *et al.* 2012; Vittecoq *et al.* 2012; Yazar *et al.* 2004) and possibly even in certain cardiovascular diseases (Flegr 2013a; Yazar *et al.* 2006). Subjects with latent toxoplasmosis differ in concentration of free testosterone (Flegr *et al.* 2008a; Flegr *et al.* 2008b), dopamine (Skallová *et al.* 2005), total leukocyte, monocytes, NK-cells and B-cells counts (Flegr *et al.* 2011). Most of these effects have been observed also in experimentally infected rodents suggesting that the *Toxoplasma* infection is the cause rather than the effect of observed changes in physiology of the infected hosts – for recent reviews see (Flegr 2013b; McConkey *et al.* 2013, Vyas 2013).

Very close relation exists between latent toxoplasmosis and schizophrenia disease, probably the most important psychiatric disorder with incidence about 1% (McGrath *et al.* 2004). Prevalence of toxoplasmosis is usually higher in schizophrenia patients than in controls (Torrey *et al.* 2007; 2012; Zhou *et al.* 2011). Longitudinal cohort study performed on US soldiers showed that anti-*Toxoplasma* antibodies appear in serum of individuals between 6–18 months before onset of schizophrenia (Niebuhr *et al.* 2007). *Toxoplasma*-infected patients express more severe positive symptoms of schizophrenia (hallucinations, delusions) than *Toxoplasma*-free schizophrenics (Holub *et al.* 2013; Wang *et al.* 2006). This is probably related to increased concentration of dopamine that is synthetized with help of unique *Toxoplasma*-coded enzymes (Gaskell *et al.* 2009) and which is released from *Toxoplasma* cysts in the host brain (Prandovszky *et al.* 2011). Certain endophenotypes that were originally attributed to schizophrenia are probably typical only for *Toxoplasma*-infected subpopulation of schizophrenia patients. For example, the decreased density of gray matter in certain parts of brain (reduction of GM volume bilaterally in the caudate, median cingulate, thalamus, and occipital cortex and in the left cerebellar hemisphere) probably occurs only in *Toxoplasma*-positive schizophrenia patients, not in *Toxoplasma*-free patients or in *Toxoplasma*-infected controls (Horacek *et al.* 2012). Similarly, the reported earlier onset of schizophrenia in male than in female patients (Hafner 2003; Howard *et al.* 2000) occurs only in *Toxoplasma*-infected subjects (Holub *et al.* 2013).

A common feature of toxoplasmosis and schizophrenia is a disorder of immune system. Indeed, immunity disorders may be one of the connecting links between schizophrenia and toxoplasmosis infection (Bhadra *et al.* 2013; Hinze-Selch 2002; Muller *et al.* 2006). Recent reports demonstrated that some metabolites of dehydroepiandrosterone, namely its 7-hydroxylated derivatives, abundant in brain tissues, possess neuroprotective and neuromodulatory properties (Morfin *et al.* 2000; Morfin *et al.* 2001).

Therefore, in the present study we searched for differences in concentration of these DHEA metabolites, in *Toxoplasma*-free and *Toxoplasma*-infected schizophrenia patients in samples of sera collected by clinics for various reasons in the same psychiatric clinic during the period of 5 years.

METHODS

Subjects

The patient group consisted of 94 (54%) female patients, and 79 (46%) male patients with diagnosed schizophrenia made according to the Structured Clinical Interview for DSM-IV. Out of them there were 8 females and 13 males with first episode (drug naive) schizophrenia, the other patients were treated at least for six months with olanzapine or 'non-olanzapine' type of antipsychotic drugs. Antipsychotic treatment was prescribed in a flexible dosing schedule, adjusted according to the treating physician's discretion. The whole male and female groups were considered for statistical evaluation irrespective of the treatment, in order to avoid atomization of the data. Blood was collected at 08:00h after overnight fasting. The rests of sera from other study were used for analyses with approval of the physicians and the study was approved by the Local Ethical Committee of the Institute of Endocrinology, Prague.

Immunological tests for toxoplasmosis and human cytomegalovirus infection

The serological diagnosis of toxoplasmosis was performed in the National Reference Laboratory for Toxoplasmosis of the Czech Republic by two methods: ELISA (Enzyme-Linked Immunosorbent Assay; IgG: SEVAC, Prague, IgM: TestLine, Brno) and the Complement Fixation Test (CFT; SEVAC, Prague). The decrease in CFT titres compared with ELISA method detects more reliably the 'old' *Toxoplasma* infection (Kodym *et al.* 2007). CFT titres of antibodies to *Toxoplasma* were measured at dilutions between 1 : 4 and 1 : 1 024. All subjects testing IgG positive by IgG ELISA (positivity index >0.9) and those with CFT titres equal or higher than 1:8 were considered toxoplasmosis positive. Whenever more than one sample from the same patients were available, we repeated the test to detect possible seroconversion of patients. Only the patients with clear result of diagnosis were included into our study. The diagnosis of the cytomegalovirus CMV infection was performed in the National Reference Laboratory for herpes viruses of the National Institute of Public Health, Prague. Specific anti-CMV IgG antibodies were measured by quantitative ELISA (ETI-CYTOK-G plus, DiaSorin). Antibody concentration was expressed in arbitrary units (AU). Individuals with AU <40 were considered seronegative for CMV.

Steroid determination

Cortisol, dehydroepaiandrosterone and its sulfate (DHEA/S), and prolactin were determined by commercial kits from Beckman Coulter (previously Immunotech,Marseille,France).7-α-Hydroxydehydroepiandrosterone (7-α-OH-DHEA) and its 7-β-hydroxyisomer (7-β-OH-DHEA) were determined using an in-house radioimmunoassay as previously described (Lapcik *et al.* 1998, Lapcik *et al.* 1999).

Other biochemical tests

Thyrotropin (TSH) was measured by ECLIA (obtained from Roche Diagnostics GmBH, Mannheim, Germany) using a commercial Elecsys System 2010. Fasting blood glucose levels were measured with a Glucose analyser (Beckman, Fullerton, CA) using the glucoso-oxidase method. Lipid parameters, namely total serum cholesterol, high- and low density lipoproteins, and triacylglycerides, were measured using commercially available kits CHOL2 HiCo T 400, HDL-C III 200, LDL-D Gen 2 200, and TRIGL 250, respectively (Roche Diagnostics GmbH) with a Cobas 6000 module C analyser.

Statistics

The statistical analyses (statistical methods assumption tests, t-test, Logistic regression and General Linear Model (GLM) tests) were performed using the programe Statistica v. 9. (Stat Soft Inc.). The partial Kendall correlation test suggested by Siegel and Castellan (Siegel *et al.* 1988) based on Taus computed with standard Kendall correlations was used for nonparametric analyses (Kaňková *et al.* 2011); the Excel sheet for this analysis is available at http://web.natur.cuni.cz/flegr/programy.php.

RESULTS

We obtained both biochemical and serological data of 94 (54%) female patients (mean age: 33.9, S.D.: 6.92) and 79 (46%) male patients (mean age: 35.4, S.D.: 8.93), t=−1.33, p=0.186. Logistic regression of the biochemical and serological data, with sex and age as independent variables showed that the prevalence of toxoplasmosis in female patients was significantly lower (29.8%) than in male patients (53.2%), Wald's Chi^2=8.57, p=0.003, OR=2.57 (C.I.95=1.36–4.85). Prevalence of CMV was approximately the same in female patients (85.6%) and in male patients (83.3%), Wald's Chi^2=0.20, p=0.654, OR=0.82 (C.I.95=0.35–1.92). No association between toxoplasmosis and CMV was detected, Wald's Chi^2=2.27, p=0.132, OR=2.05 (C.I.95=0.80–5.27).

The GLM analyses suggested the existence of several significant or borderline effects of toxoplasmosis or toxoplasmosis-sex interaction on biochemical parameters, as demonstrated in the Table 1 and Figure 1. Separate nonparametric analyses for men and women showed that the effects of toxoplasmosis were mostly stronger in male than in female patients, see the Table 2. As may be seen in the Table 1, no significant effect of CMV or CMV-sex interaction on biochemical parameters was observed.

Statistically, the duration of the infection negatively correlates with concentration of anti-*Toxoplasma* IgG antibodies. Therefore, we can estimate whether observed effects are more probably the vanishing aftereffects of acute infection or the cumulative effects of latent toxoplasmosis. The partial Kendall analyses with concentration of anti-*Toxoplasma* IgG showed that the decreased level of DHEA metabolites in the *Toxoplasma*-infected patients were more probably only the aftereffect of acute toxoplasmosis, while increased level of cholesterol and LDL-cholesterol in sera of infected men more probably represented the cumulative effect of latent toxoplasmosis; see the Table 2 and Figures 2–3. No significant effect of CMV or CMV-sex interaction on biochemical parameters was observed, see the Table 1.

DISCUSSION

The aim of the present study was to find out whether schizophrenia patients with toxoplasmosis differ from those without serologically diagnosed toxoplasmosis in hormonal parameters, reflecting function of immune system. The subjects with toxoplasmosis had lower serum levels of DHEA and its sulfate, as well as its 7-hydroxylated metabolites, believed now to act as immune- and neuroprotective agents (Bicikova *et al.*

Neuro- and immunomodulatory steroids and selected hormone and lipid concentrations in infected schizophrenia patients

Tab. 1. Effects of toxoplasmosis, human cytomegalovirus infection, and sex on hormones and lipids in serum in schizophrenia patients.

	Mean				toxo		toxo-sex		CMV		CMV-sex	
	women Toxo-	women Toxo+	men Toxo-	men Toxo+	*p*-value	eta^2	*p*-value	eta^2	*p*-value	eta^2	*p*-value	eta^2
7-α-OH-DHEA	0.751	0.629	0.577	0.531	0.371	0.005	0.833	0.000	0.724	0.001	0.136	0.014
7-β-OH-DHEA	1.073	0.812	0.841	0.783	**0.056**	0.022	0.529	0.002	0.861	0.000	**0.031**	0.053
DHEA	25.723	21.570	25.729	22.276	0.315	0.006	0.745	0.001	0.861	0.000	0.357	0.005
DHEAS	5.450	4.462	5.917	5.036	0.346	0.005	0.897	0.000	**0.056**	0.022	0.904	0.000
cortisol	462.880	514.176	480.454	500.303	0.205	0.011	0.614	0.002	0.983	0.000	0.984	0.000
TSH	1.885	1.938	1.408	1.840	0.163	0.012	0.371	0.005	0.322	0.006	0.868	0.000
PRL	759.120	838.725	404.406	472.673	0.370	0.005	0.596	0.002	0.501	0.003	0.124	0.014
Glucose	4.131	4.977	4.338	4.294	**0.034**	0.027	**0.026**	0.029	0.754	0.001	0.419	0.004
TAG	1.364	1.489	1.462	1.843	0.129	0.014	0.370	0.005	0.622	0.001	0.460	0.003
Cholesterol	5.170	5.431	4.619	5.167	**0.048**	0.023	0.385	0.004	0.273	0.007	0.557	0.002
HDL	1.477	1.462	1.248	1.313	0.690	0.001	0.428	0.004	0.567	0.002	0.203	0.010
LDL	3.122	3.275	2.779	3.258	**0.089**	0.018	0.341	0.006	0.317	0.006	0.478	0.003

The table shows mean for *Toxoplasma*-infected and *Toxoplasma*-free male and female patients and results of GLM analyses, effect sizes (Eta^2), and significances (p-values) with toxoplasmosis (or CMV infection), sex, and toxoplasmosis-sex (or CMV infection-sex) interaction as independent variables and age of a patients as covariate. The trends (p<0.1) are printed in bold. No formal correction for multiple tests has been performed.

Tab. 2. Correlation of toxoplasmosis with hormones and lipids in serum in schizophrenia patients.

	toxoplasmosis						anti-Toxo IgG					
	All		women		men		All		women		men	
	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value	Tau	*p*-value
7-α-OH-DHEA	–0.108	**0.026**	–0.083	0.234	–0.046	0.547	–0.112	0.160	–0.055	0.680	–0.216	**0.044**
7-β-OH-DHEA	–0.155	**0.001**	–0.167	**0.017**	–0.060	0.434	–0.018	0.826	0.127	0.343	–0.229	**0.032**
DHEA	–0.097	**0.047**	–0.129	**0.066**	–0.102	0.185	–0.031	0.694	–0.093	0.486	–0.025	0.817
DHEAS	–0.082	**0.092**	–0.135	**0.054**	–0.123	0.109	0.002	0.977	0.043	0.747	–0.065	0.543
cortisol	0.027	0.597	0.105	0.153	0.065	0.422	–0.039	0.653	–0.052	0.713	–0.027	0.818
TSH	–0.020	0.674	–0.053	0.452	0.134	**0.081**	–0.110	0.168	–0.175	0.191	–0.075	0.485
PRL	–0.023	0.631	0.075	0.285	0.001	0.986	–0.062	0.440	–0.213	0.111	0.117	0.275
Glucose	0.046	0.341	0.172	**0.014**	–0.022	0.771	–0.116	0.146	–0.117	0.383	–0.078	0.467
TAG	0.073	0.134	0.031	0.659	0.131	**0.087**	–0.045	0.576	0.139	0.298	–0.070	0.512
Cholesterol	0.071	0.143	0.074	0.293	0.187	**0.015**	–0.083	0.301	0.077	0.567	–0.102	0.342
HDL	–0.029	0.552	–0.050	0.478	0.105	0.170	–0.026	0.741	–0.071	0.594	0.026	0.805
LDL	0.053	0.282	0.047	0.511	0.198	**0.012**	–0.072	0.383	0.126	0.356	–0.158	0.157

The left part of the table shows results of partial Kendall correlation (age controlled) between concentration of particular molecules in serum of a patient and binary variable toxoplasmosis for all patients, the right part of the table shows results of partial Kendall correlation (age controlled) between concentration of particular molecules in serum of a patient and concentration of anti-*Toxoplasma* IgG antibodies for a subpopulation of *Toxoplasma*-infected patients. *Toxoplasma*-infected patients were coded as 1 and *Toxoplasma*-free patients as 0, therefore, positive partial Tau corresponds to positive correlation between the *Toxoplasma* infection and the concentration of particular molecules. The positive Tau in the right part of the table reflects positive correlation between concentration of particular molecules and concentration of specific antibodies in serum, i.e. the negative correlation between concentration of particular molecules and duration of the *Toxoplasma* infection. No formal correction for multiple tests has been performed.

2013; Morfin *et al.* 2000; 2001; Muller *et al.* 2006). This shift was observed in both sexes, however it was much stronger in women than in men. The generally worsened spectrum of biochemical parameters in schizophrenic patients with toxoplasmosis reflects also their higher glucose levels in female patients and less favor-

able lipid spectrum in male patients. The decreased levels of DHEA and its 7-hydroxylates metabolites return to norm with the decrease of concentration of anti-*Toxoplasma* antibodies and therefore, most probably, with duration of the *Toxoplasma* infection. That means that these decreases could by possibly just transient after-effect of acute *Toxoplasma* infection. On the other hand, no correlation with the anti-*Toxoplasma* antibodies concentration was observed for lipids or glucose, suggesting that these shifts are stable during the infection.

Toxoplasma infected patients, especially the men, had increased level of cholesterol and LDL. No such data have been published for humans, however, a similar effect, namely the increased level of LDL was observed in outbred mice infected in laboratory. *Toxoplasma* imports LDL for synthesis of its membrane lipids from the host organism. It was observed that *T. gondii* diverts cholesterol from low-density lipoproteins for cholesteryl ester synthesis and storage in lipid bodies (Nishikawa *et al.* 2005). Acute phase of infection is characterized by decreased levels of cholesterol and LDL in normal (Milovanovic *et al.* 2009) and ApoE-deficient (Portugal *et al.* 2004) mice and postacute phase (42 days after the infection) by an increased concentration of LDL (Milovanovic *et al.* 2009). The increase of LDL correlated with cyst counts in 44% of mice with more than 300 cysts per brain. The authors suggested that *Toxoplasma* induces serum lipoprotein changes by influencing on lipid receptors and apolipoproteins (Milovanovic *et al.* 2009).

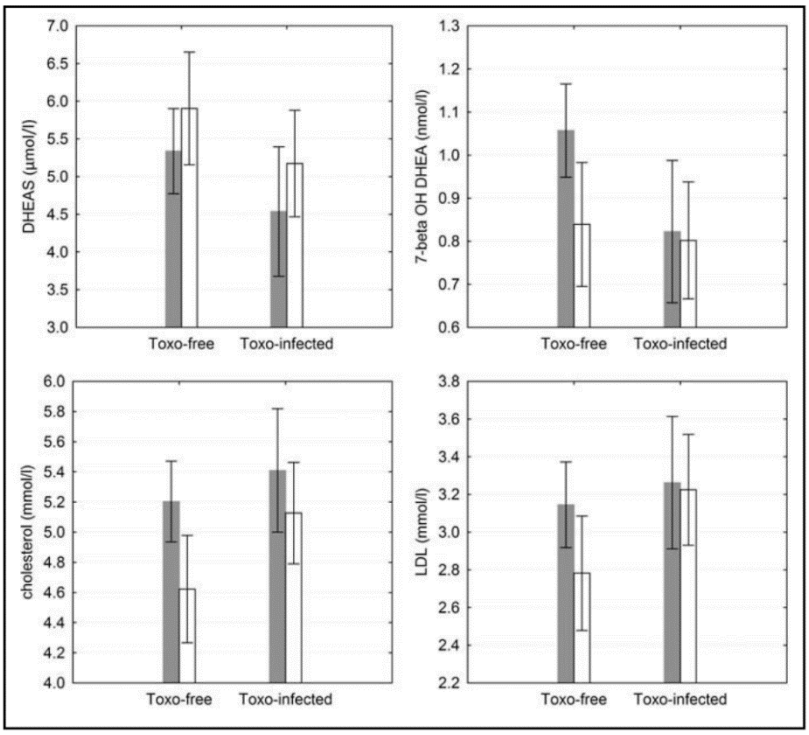

Fig. 1. Effect of *Toxoplasma* infection on DHEA metabolites, cholesterol and LDL-cholesterol in female (dark columns) and male (white columns) patients.

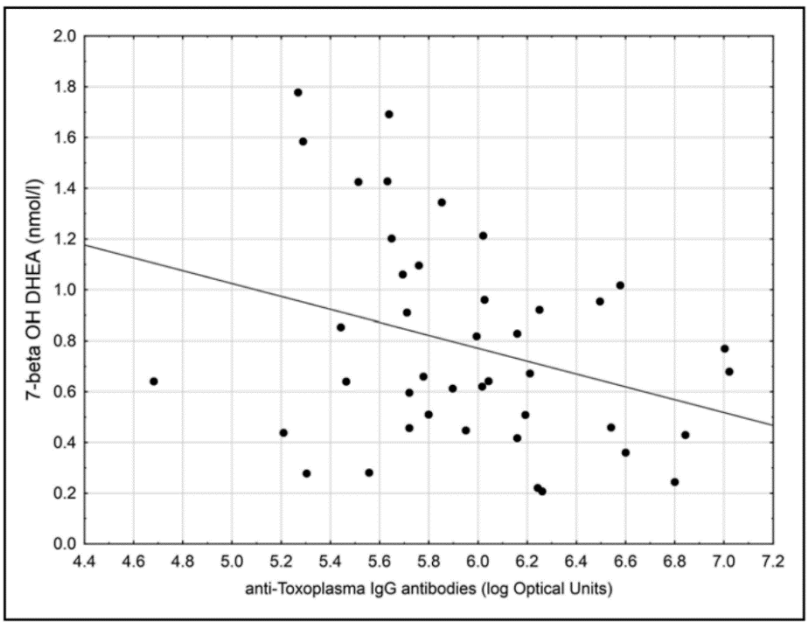

Fig. 2. Correlation between the concentration of 7-beta DHEAS and the level of anti-*Toxoplasma* antibodies in *Toxoplasma*-infected male patients.

Infection with *Toxoplasma gondii* increased atherosclerotic lesion in ApoE-deficient mice, which was considered to be a result of increased interferon production in chronic phase of *T. gondii* infection. Our results suggest that *Toxoplasma* infection could accelerate atherosclerotic development not only by continu-

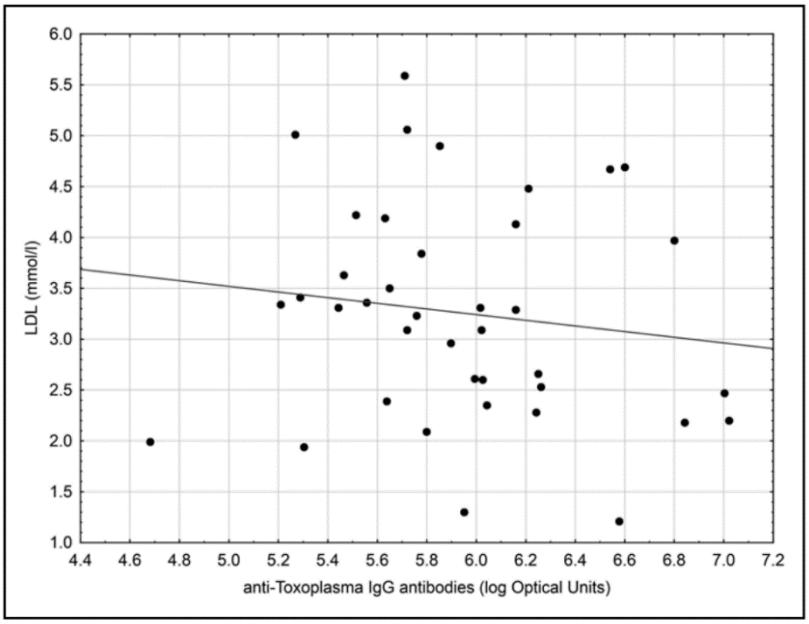

Fig. 3. Correlation between concentration of LDH and level of anti-*Toxoplasma* antibodies in *Toxoplasma*-infected male patients.

ous stimulation of synthesis of lymphokines (Flegr *et al.* 2011; Gazzinelli *et al.* 1991;1992; Milovanovic *et al.* 2009) but also by increasing levels of cholesterol and LDL in latently infected humans and in mice in postacute phase of the infection. This can explain the observed positive correlation between prevalence of toxoplasmosis and incidence of cardiovascular diseases in particular European countries (Flegr 2013a).

Though insignificantly, *Toxoplasma*-infected subjects had higher cortisol levels, with cortisol being known to act as an immunosuppressive hormone. Concentration of cortisol is known to decrease after victory in a competition events (Salvador 2005). However, in some situations, an opposite shift in concentration of cortisol after the victory, or for example, after success in written university examination was observed (Flegr *et al.* 2010; Kirschbaum *et al.* 1995; Suay *et al.* 1999; Wirth *et al.* 2006). Generally, an increased level of cortisol is associated with various types of acute and especially chronic stress, including physical and mental stress (Fukuda *et al.* 2001a,b; Russell *et al.* 2012; Staufenbiel *et al.* 2013). Indirect evidence suggest that, in fact, so called "asymptomatic" latent toxoplasmosis represents a mild but long term stressor (Lindová *et al.* 2006; 2010). It can be speculated that the latent toxoplasmosis-associated chronic stress accompanied with increased level of an immunosuppressor cortisol could be the proximal cause of impaired immunoreactivity of *Toxoplasma*-infected subjects. Of course, it is also possible that observed toxoplasmosis-associated immunosuppression represents the result of adaptive manipulative activity of the parasite, aimed to increase its chance for survival in a host organism.

The most striking finding, in fact accidental, was the higher prevalence of toxoplasmosis in schizophrenic men than in women, irrespectively to age and even to presence of anti-*Toxoplasma* antibodies (IgG) in serum, in contrast to common population. This may be influenced by the fact that samples from all the patients were evaluated, irrespective to the severity of their schizophrenia disease and associated willingness to participate in basic research. It has been already suggested that absence of differences in prevalence of toxoplasmosis between schizophrenia patients and general population observed in certain recent studies performed in developed countries (Hinze-Selch *et al.* 2007; Horacek *et al.* 2012) is the result of increased concerns about the rights of patients (Flegr 2013b). In the past, all patients of a particular hospital were automatically included in the study. Currently, only the patients who are able and willing to sign the informed consent document participate in the studies. It is known that *Toxoplasma*-infected men are more suspecting (Flegr *et al.* 1994; 1996) and both men and women are less cooperative and conscientious than their *Toxoplasma*-free peers (Lindová *et al.* 2010; 2012). Also, *Toxoplasma*-infected schizophrenia patients express more severe symptoms of psychosis than *Toxoplasma*-free patients (Amminger *et al.* 2007; Holub *et al.* 2013; Wang *et al.* 2006; Yolken *et al.* 2009). Higher suspiciousness and lower cooperativeness and conscientiousness of *Toxoplasma*-infected subjects as well as their more severe positive symptoms of schizophrenia increase the probability of rejecting the participation in research study.

Limitation of the study. The major limitation of the presented study is the absence of control populations of *Toxoplasma*-free and *Toxoplasma*-infected subjects without any psychiatric diseases. Without such data we cannot decide whether observed differences between *Toxoplasma*-free and *Toxoplasma*-infected subjects can be observed only in schizophrenia patients or whether they can be detected also in a general population. It is therefore highly desirable to repeat this study also with non-psychiatric subjects matched for age and gender with our schizophrenia patients.

Our study (accidently) provided possible explanation for seemingly decreasing prevalence of toxoplasmosis in schizophrenia patients observed in current

studies. It also suggests that latent toxoplasmosis could play a role in the development of atherosclerosis, which offered a possible explanation for reported positive correlation between prevalence of toxoplasmosis and incidence of cardiovascular diseases.

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4.5 Contrasting Effect of Prepulse Signals on Performance of *Toxoplasma*-Infected and *Toxoplasma*-Free Subjects in an Acoustic Reaction Times Test

4.5.1 Reasons for Inclusion

This is the pivotal part of the *Toxoplasma* studies in the context of prepulse inhibition of startle reaction. We have conducted several related experiments: A standard individual tests of prepulse inhibition of startle reaction was used on a population of student volunteers. We are currently developing a script for analysis of the collected biosignals. A modified computer-administered Stroop test with additional prepulse stimuli was distributed in student volunteer test group and military personnel. The data from these experiments are currently awaiting analysis. Computer-Administered tests of prepulse modification of startle reaction in an acoustic and a visual form were used on our student volunteers and military personnel. The data from military personnel are being analyzed and prepared for publication, while the data from the student population are presented in the presented study.

4.5.2 Introduction

Nonexperimental observational studies of traffic accidents participants (Jaroslav Flegr et al., 2002) as well as questionnaire studies (although those were not published until a few years ago (J Flegr, 2010)) suggested a possible deficiency in reaction times in humans with latent toxoplasmosis. This lead to research in experimental settings using reaction times measuring methods (namely the simple reaction times test) to evaluate *T. gondii*'s effects on human behavior with satisfactory results (Jaroslav Flegr, Novotná, Lindová, & Havlíček, 2008; Havlícek et al., 2001) and consequently even to the hypothesis of possible toxoplasmosis-associated preservation and geographical distribution of Rh blood group polymorphism (Novotná et al., 2008).

Based on previously observed association between latent toxoplasmosis and impaired startle gating in schizophrenia patients (David L. Braff, Swerdlow, & Geyer, 1999; Grillon, Ameli, Charney, Krystal, & Braff, 1992) as well as on remarkable sensitivity of the experimental method of the prepulse

inhibition of the startle reaction ((D. L. Braff, Geyer, & Swerdlow, 2001), we have decided to incorporate startle signal and prepulse modifications into the previously used simple reaction time test.

4.5.3 Materials and Methods

We have tried to bring the experimental settings nearer to the natural scenario where the predator-caused signals both startles the potential prey and serves as the go signal. The newly developed computer test used stimuli of the intensity of 107 dB and duration of 40 ms as both the startle and the go signal. The white noise used as a background has an intensity of 57 dB and the prepulse stimuli 67 dB. The intensity in the real experimental settings was tested using a calibrated digital storage oscilloscope Agilent DSO 5054A. Both the duration of the prepulse signal and the interval between the prepulse signal and the main stimuli were set at 20 ms. Signals with and without the prepulse stimuli as well as standalone prepulses were presented in a pseudorandom order.

The experimental computer environment consisted of a single button in the middle of a grey screen. Each experimental subject was seated in front of an experimental notebook, fitted with a pair of headphones (Philips SHO1900) and a mouse, and asked to click the left mouse button each time they hear an acoustic signal. We have used the same type of experimental notebook (with the same program settings), headphones and mouse for every proband in order to minimize differences in delays caused by the used hardware and software. Experiments were conducted in group settings with 10 to 12 subjects seated in one room; the same person always told the same set of instruction, and 2 to 3 experimenters were present to answer potential questions or to help with potential problems.

4.5.4 Results and Discussion

We tested 170 females (age 21.8, range 19-30, S.D. 2.12) and 66 male (age 22.2, range 19-31, S.D. 2.74) subjects; the *Toxoplasma*-infected subjects (44, age 22.7) were older than the *Toxoplasma*-free subjects (192, age 21.7, $t_{234} = 2.60$, $p = 0.01$). We used repeated measure GLM with two dependent (mean reaction time with and without prepulse signal) variables and latent

toxoplasmosis, sex and age were used as independent variables. There were no main effects of the standalone variables (age: $p = 0.77$, $\mu^2 < 0.001$; toxoplasmosis: $p = 0.128$, $\mu^2 = 0.010$; sex: $p = 0.308$, $\mu^2 = 0.004$; prepulse $p = 0.262$, $\mu^2 = 0.005$), we have, however, found significant effects of prepulse-sex ($p = 0.035$, $\mu^2 = 0.020$) and prepulse-toxoplasmosis-sex ($p = 0.020$, $\mu^2 = 0.23$) interactions. Reaction time was shorter in the presence of the prepulse signal with an even stronger effect in *Toxoplasma*-positive male subjects.

Our previous experience with simple reaction times test where the *Toxoplasma*-infected subjects differ from the *Toxoplasma*-free controls mainly in the later parts of the experiment suggested the *Toxoplasma*-positive become tired more quickly and possibly to have worsened ability of long-term concentration (Havlícek et al., 2001), thus we have decided to perform analyses separately for the first, second and third part of the test. While no-effect of the time-toxoplasmosis-prepulse-sex interaction was found ($p = 0.095$, $\mu^2 = 0.010$), significant effects were shown for time-toxoplasmosis-prepulse ($p = 0.10$, $\mu^2 = 0.020$), time-toxoplasmosis-sex ($p = 0.013$, $\mu^2 = 0.019$), and time-prepulse-sex ($p = 0.026$, $\mu^2 = 0.016$) interactions. The third, but not the first and the second, part of the test have also suggested significant effects of toxoplasmosis ($p = 0.026$, $\mu^2 = 0.022$) and toxoplasmosis-sex ($p = 0.028$, $\mu^2 = 0.021$), prepulse-toxoplasmosis ($p = 0.009$, $\mu^2 = 0.029$), prepulse-sex ($p = 0.006$, $\mu^2 = 0.031$), and prepulse-toxoplasmosis-sex ($p = 0.014$, $\mu^2 = 0.026$) interactions.

One of the most prominent questions of latent toxoplasmosis studies is whether the found effects could be but the consequences of an acute infection or rather a symptom of the latent phase. The anti-*Toxoplasma* IgG antibodies titers stay positive for years after the acute phase, they do, however, decrease with time from infection – statistically; in some cases, they may stay stable or fluctuate. We can, therefore, test the subset of *Toxoplasma*-positive experimental subjects for a statistical association between their anti-*Toxoplasma* antibodies and strength of the effects. In this study, we used CFT titers of 44 *Toxoplasma*-infected subjects and difference between mean

reaction time to the signals with and without the prepulse to discover a negative correlation ($p = 0.0025$, $Tau = -0.201$) between the two variables in a one-sided test. Negative correlation, the higher the CFT titer (thus, the shorter time from the infection), the quicker the reaction, suggest amplified effect of an old latent infection rather than an artifact of a worsened health shortly after an acute phase.

4.5.5 Limitations

The whole experiment, however promising, probably presents more challenges than definitive answers. We would need to test many more experimental subjects, preferably men, since the results were stronger in male subjects than in women, with the population sample divided into subgroups based on their Rhesus factor phenotype (or better genotype), as our previous studies suggested strong interaction of the RhD blood-group with latent toxoplasmosis (Jaroslav Flegr, Novotná, Fialová, Kolbeková, & Gašová, 2010).

The methods of the experiment should also be changed to reveal the mechanisms behind the found effects. We have theorized two probable causes of the observed prepulse facilitation of conscious reaction. First explanation works with the prepulse stimuli decreasing the startle response, thus decreasing the reaction time. A similar effect was demonstrated in a two decades earlier experiment by a group of American researchers; a weak auditory stimulus (prepulse) preceding an unexpected intense noise burst (startle stimuli) by 100 ms reduced errors caused by the noise while firing a rifle (Foss, Ison, Torre Jr, & Wansack, 1989). The second explanation would be that the prepulse stimuli bring the attention of the experimental subjects to the following signal. “Blind” prepulse stimuli without following signals were presented throughout the whole experiment, and there were no reactions on the standalone prepulses by any proband during the whole experiment. Therefore, the shortened reaction times thus could not be caused by experimental subjects learning to react on the prepulse signal before the appearance of the main stimulus.

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Contrasting Effect of Prepulse Signals on Performance of *Toxoplasma*-Infected and *Toxoplasma*-Free Subjects in an Acoustic Reaction Times Test

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Abstract

Background: About 30% of people on Earth have latent toxoplasmosis. Infected subjects do not express any clinical symptoms, however, they carry dormant stages of parasite *Toxoplasma* for the rest of their life. This form of toxoplasmosis is mostly considered harmless, however, recent studies showed its specific effects on physiology, behaviour and its associations with various diseases, including psychiatric disorders such as schizophrenia. Individuals who suffer from schizophrenia have about 2.7 times higher prevalence of *Toxoplasma*-seropositivity than controls, which suggests that some traits characteristic of schizophrenic patients, including the sex difference in schizophrenia onset, decrease of grey matter density in specific brain areas and modification of prepulse inhibition of startle reaction could in fact be caused by toxoplasmosis for those patients who are *Toxoplasma*-seropositive.

Methodology/Principal Findings: We measured the effect of prepulse inhibition/facilitation of the startle reaction on reaction times. The students, 170 women and 66 men, were asked to react as quickly as possible to a startling acoustic signal by pressing a computer mouse button. Some of the startling signals were without the prepulse, some were 20 msec. preceded by a short (20 msec.) prepulse signal of lower intensity. *Toxoplasma*-seropositive subjects had longer reaction times than the controls. Acoustic prepulse shorted the reaction times in all subjects. This effect of prepulse on reaction times was stronger in male subjects and increased with the duration of infection, suggesting that it represented a cumulative effect of latent toxoplasmosis, rather than a fading out after effect of past acute toxoplasmosis.

Conclusions: Different sensitivity of *Toxoplasma*-seropositive and *Toxoplasma*-seronegative subjects on effect of prepulses on reaction times (the toxoplasmosis-prepulse interaction) suggested, but of course did not prove, that the alternations of prepulse inhibition of startle reaction observed in schizophrenia patients probably joined the list of schizophrenia symptoms that are in fact caused by latent toxoplasmosis.

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Introduction

A protozoan parasite *Toxoplasma gondii* infects about 30% of inhabitants of both developed and developing countries [1]. Since 1994, more and more studies have shown that in the latent phase of *Toxoplasma* infection, which was previously considered asymptomatic from a medical point of view, specific changes are induced in behaviour and the physiology of infected humans and of artificially infected animals [2,3]. While some of the observed changes are considered the results of manipulative activity of the parasite aimed to increase the chance of transmission of parasites from intermediate hosts (any warm-blooded animal) to the definitive host of *Toxoplasma*, the cat [2–4], others are probably just non-specific side effects of *Toxoplasma* infection on the host physiology, e.g. of mild but long term impairment of health or of a mild chronic stress [5].

Studies of *Toxoplasma*-induced changes performed in the previous decades showed personality shifts [6,7], changes in simple reaction times and intelligence [8,9], and also suggested possible association between toxoplasmosis and several psychiatric diseases, especially schizophrenia [10–13]. Results of recent metastudies analysing, around 50 studies, showed schizophrenia sufferers to have a, approximately, 2.7 times higher probability of being *Toxoplasma* infected than healthy controls [14–16]. Recent studies showed that at least some known effects of schizophrenia occur only in schizophrenia patients infected with *Toxoplasma,* with a frequently reported difference in an onset of schizophrenia in men and women [17] and a change in grey matter density observed in schizophrenia patients [18] being the most prominent examples. These observations as well as, for example, more serious positive clinical symptoms of schizophrenia suggest that there are at least two types of schizophrenia prevalent in the population with the most serious one associated with toxoplasmosis [12,17,19] as well as that some of the reported differences between schizophrenics and normal controls can be in fact caused by

toxoplasmosis itself rather than by schizophrenia. This can be true for the observed sex differences in the onset of schizophrenia, but not the decrease in density of grey matter as it was observed only in *Toxoplasma*-infected patients but not in *Toxoplasma*-infected controls [20]. Other traits that are considered to be characteristic of schizophrenia patients but could be potentially characteristic for *Toxoplasma* infected subjects are olfactory changes and differences in prepulse inhibition of startle reaction (PPI). Specific changes in olfactory functions and preferences were observed both in schizophrenia patients [21,22] and in *Toxoplasma* infected healthy humans [23] and animals [13,24–27]. Similarly, the effect of schizophrenia on startle reaction in prepulse inhibition test is widely known and is even suggested to be a sensitive tool for research of various types of mental disorders and neurobiological problems [28] including schizophrenia [29,30]. Male schizophrenic patients with early onset of the disease were reported to have profound deficits in PPI of startle response [31]. Modulation of startle response was also found in people with schizotypal personality disorder and even in relatives of schizophrenic patients [32], suggesting possibility to find PPI deficits also in healthy subjects with predispositions to schizophrenia. A recent study showed, that not only schizophrenia patients [33], but also *Toxoplasma*-infected controls have increased latency of startle reflex and decreased effect of prepulse on the latency than *Toxoplasma*-free subjects in acoustic startle reflex inhibition test [34].

While the studies suggesting an association between the latent toxoplasmosis and schizophrenia seem convincing, little is known about the mechanism underlying the connection, with the main suspect being the neurotransmitter dopamine, which plays an important role in aetiology and clinical picture of schizophrenia [35,36]. Research from three main areas back up our understanding that dopamine is also responsible for at least some of the changes associated with latent toxoplasmosis: (1) Genetic research showed *T. gondii* to have two genes encoding thyroxine hydroxylase, a rate-limiting enzyme in dopamine synthesis [37]. (2) Animal research showed changes in dopamine levels in infected mice and in brain tissues [38,39], as well as dopamine role in observed behavioural changes [40,41] and in production of *T. gondii* tachyzoites in infected tissues [42]. (3) In humans, behavioural changes associated with latent toxoplasmosis suggest differences in dopamine levels in seropositive subjects [43,44], and research of human neurotransmitter and neuropeptide systems showed (among other alterations) significant changes in protein levels of DRD1 in cells infected with type I strain of *T. gondii* [45]; DRD1 is involved in negative feedback regulation of dopamine release in the brain [46].

Following our previous studies of simple reaction times [8], we decided to further our understanding of *Toxoplasma*-induced changes in reaction times and sensorimotor gating using an experiment that combines simple reaction times tests with tests of PPI. In experiments conducted with healthy individuals it was shown that a startle signal preceding a go signal decreases reaction time [47]. It would be extremely adaptive for any predation-transmitted parasite to decrease or even switch off this startle facilitated reaction of an infected host, so called startReact [47,48], by, for example, decreasing the intensity of its startle reaction. It is known that PPI plays a significant role in modifying the startle while not affecting the decrease in reaction times [49]. However, in the original study the go signals and startle signals were different and of a different modality, namely using optical go signals and acoustic startle signals. Under natural condition, when a feline predator attracts its prey, the same signal usually plays a role of both go and startle signal. Therefore, we modified the usual setup of startReact experiments by using the startle acoustic signal as the go signal. Using this more ecologically relevant setup we tested the hypothesis that modulation of startle reaction by prepulses could influence reaction times of *Toxoplasma*-infected and *Toxoplasma*-free subjects differently.

Materials and Methods

Ethics statement

The study was approved by IRB of Faculty of Science (Etická komise pro práci s lidmi a lidským materiálem Přírodovědecké Fakulty Univerzity Karlovy) and all experimental subjects signed the informed consent before the start of the study.

Experimental subjects

236 biology students of Charles University in Prague (170 women and 66 men, the ratio corresponding to the sex ratio of Faculty of Science students' population) agreed with participating in the double-blind study. All subjects were Caucasians of a Czech or Slovak (<10%) nationality. A socioeconomic stratification of population of past Czechoslovakia is very low and this is especially true for population of students of Charles University, the most prestigious Czech university. No student reported any hearing problem and visual inspection of data suggested that no subject with such problems was among participants of reaction times test. All students underwent blood sampling for serological analysis, battery of psychological tests (results not used in the present study) and PC test of acoustic prepulse inhibition of simple reaction times. During the experiment, neither the subjects nor the laboratory assistant were aware of results of serological tests for toxoplasmosis. The students were paid 400 CZK (equivalent of $ 20) for their participation in the study.

Serological analysis

Blood samples (1 ml of frozen sera) obtained from participants were sent to National Laboratory for Toxoplasmosis, National Institute of Public Health, Prague and screened for specific anti-*Toxoplasma* IgG antibodies using ELISA (SEVAC, Prague, Czech Republic) and complement fixation test (CFT, SEVAC, Prague, Czech Republic). Samples with high titres of IgG antibodies were tested for IgM (TestLine, Brno, Czech Republic) to exclude possible cases of acute toxoplasmosis. CFT titres from 1:8 to 1:128 are assumed to signify latent *T. gondii* infection [50]. CFT results rather than results established by ELISA were used as CFT is more reliable in case of longer duration *Toxoplasma* infection [50]. Subjects with CFT titres 1:8 and higher and in the same time positive in the ELISA test (positivity index higher than 1.0) were considered *Toxoplasma*-infected, the four subjects with opposite results of CFT and ELISA tests as well as one subject positive in IgM tests were excluded from the study.

Psychomotor test

A PC test of acoustic prepulse inhibition of simple reaction times was developed on our workplace based on previously used test of simple reaction times [51]. The Windows XP, 7, 8 compatible program is available at http://web.natur.cuni.cz/flegr/programy.php.

During the test, a plain grey screen with one button in the middle was presented and the subject was asked to click the left mouse button each time the sound stimuli was played. Bursts of white noise in intensity and duration used in standard tests of PPI were used in our experiment (duration of prepulse signal 20 ms, duration of startle stimuli 40 ms, background white noise present during the whole experiment), the time interval between prepulse

and main stimuli was 20 ms. To avoid reactions to the prepulse instead of the main stimuli, pseudorandom prepulse signals without following stimulus were presented through the test. Stimuli were presented through headphones (Philips SHP1900) with volume set on maximum. All the subjects were tested using identical sets hardware. Three trial signals were played for the experimental subject to get accustomed to the experimental setting before the measured part of the experiment started. 32 prepulse preceded stimuli and 28 plain stimuli were presented in pseudorandom order ensuring similar representation of plain and prepulse preceded stimuli in the first, middle and last part of the program run. The same pseudorandom sequence of signals was used for all subjects. Program ended after the experimental subject responded to the last stimulus. Time from the beginning of the experiment to the last presented stimulus was 4 minutes 17 seconds. Based on a preexperimental testing, the intensity of background noise, prepulses, stimuli and intervals between prepulse and pulse were set to 57 dB, 67 dB, 107 dB and 20 msec., respectively. Under these conditions, different reactions to stimuli with and without prepulses were observed and no reactions of subjects to sole prepulses were observed. The intensity of acoustic signals were measured with calibrated digital storage oscilloscope Agilent DSO 5054A and the acoustic files were edited using Audacity 1.3 software.

Data analysis

Data were manually filtered to exclude all items longer than 1000 ms or shorter than 100 ms (<10% of data). The mean reaction times for signals with (S_p) and without (S_n) prepulse and normalized differences of signals with and without prepulse were computed as $Dn = (S_n - S_p)/S_n$. The program package Statistica v. 9.0 was used for logistic regression, repeated measure GLM analysis, descriptive statistics and for testing the parametric statistical tests presumptions. For performing the nonparametric partial Kendall regression tests [52] we used Kendall Taus computed with the Statistica and an Excel sheet [53] available at http://web.natur.cuni.cz/flegr/programy.php. Data file for possible reanalysis is available at http://web.natur.cuni.cz/flegr/data/audioPrepulse.txt.

Results

The final set contained 236 subjects, 170 women (age 21.8, range 19–30, S.D. 2.12) and 66 men (age 22.2, range 19–31, S.D. 2.74). Forty four (44) *Toxoplasma* infected subjects were older than 192 *Toxoplasma*-free subjects (22.7 vs 21.7, $t_{234} = 2.60$, $p = 0.01$). Logistic regression with sex and age as independent factors showed no effect of sex (O.R. 0.86, $C.I._{95} = 0.42–1.79$, $p = 0.69$) and positive effect of age ($O.R._{95}$ 0.142 (range), $C.I._{95} = 0.03–0.68$, $p = 0.015$) on probability of being *Toxoplasma*-infected.

Students missed less than 2% of stimuli and less than 10% of reaction times were too short (less than 100 msec. or too long (more than 1000 msec.). These suspect data were eliminated manually before the mean reaction times were calculated. No reactions on standalone prepulse signals were registered.

Repeated measure GLM with two dependent variables, namely mean reaction time with and without prepulse and independent variables toxoplasmosis (meaning seropositive subjects with latent infection), sex and age showed no main effect of age ($p = 0.77$, $\mu^2 < 0.001$), toxoplasmosis ($p = 0.128$, $\mu^2 = 0.010$), sex ($p = 0.308$, $\mu^2 = 0.004$) and prepulse ($p = 0.262$, $\mu^2 = 0.005$) but significant effect of prepulse-sex ($p = 0.035$, $\mu^2 = 0.020$) and prepulse-toxoplasmosis-sex ($p = 0.020$, $\mu^2 = 0.023$) interactions, see Fig. 1. The presence of prepulse was associated with shorter reaction times and this effect (prepulse facilitation) was especially strong in *Toxoplasma*-infected men. Since our previous results suggested that toxoplasmosis negatively influences the ability of long-term concentration, rather than reaction times under ideal conditions of maximum concentration at the start of reaction time-tests [8,54], we repeated the GLM analysis with six dependent variables – the mean reaction time with and without prepulse for the 1st, 2nd, and 3rd third of the test, and independent variables toxoplasmosis, sex and age. The results of the test suggested the effects of time-toxoplasmosis-prepulse interaction ($p = 0.010$, $\mu^2 = 0.020$), time-toxoplasmosis-sex ($p = 0.013$, $\mu^2 = 0.019$), time-prepulse-sex ($p = 0.026$, $\mu^2 = 0.016$) but not time-toxoplasmosis-prepulse-sex ($p = 0.095$, $\mu^2 = 0.010$). The separate GLM analyses for three parts of the tests showed that the effects of toxoplasmosis ($p = 0.026$, $\mu^2 = 0.022$), toxoplasmosis-sex interaction ($p = 0.028$, $\mu^2 = 0.021$), prepulse-toxoplasmosis interaction ($p = 0.009$, $\mu^2 = 0.029$), prepulse-sex interaction ($p = 0.006$, $\mu^2 = 0.031$) and prepulse-toxoplasmosis-sex interaction ($p = 0.014$, $\mu^2 = 0.026$), were significant only for the final part of the test (Fig. 2).

To reveal whether observed statistical association represents the cumulative effect of latent toxoplasmosis or rather only a vanishing after-effect of past acute toxoplasmosis, we searched for a possible association between the concentration of specific anti-*Toxoplasma* antibodies (namely CFT titre) and the normalised difference between mean reaction time with and without prepulse. The age of subjects was controlled by using partial Kendall correlation test with ordinal variable CFT titre as a factor and continuous variable age as a covariate. In 44 *Toxoplasma*-positive subjects, the difference between reaction time with and without prepulse correlated negatively with concentration of anti-*Toxoplasma* antibodies ($p = 0.025$, $Tau = -0.201$, one-sided test), Fig. 3. Statistically, concentration of specific anti-*Toxoplasma* antibodies decreases with duration of latent toxoplasmosis. Therefore, existence of a negative correlation between difference in reaction times with and without prepulse suggested that the observed toxoplasmosis-prepulse interaction increased with time from the moment of *Toxoplasma* infection.

Discussion

Results of our study performed on 236 students showed that the *Toxoplasma* infected subjects, especially men, had significantly prolonged reaction times to simple acoustical signals. All subjects reacted more quickly on acoustical signals that were preceded with a weak prepulse and this positive effect of the prepulse was strongest for *Toxoplasma*-infected men. The observed effects of prepulse-toxoplasmosis interaction increased with duration of *Toxoplasma*-seropositivity estimated on the basis of anti-*Toxoplasma* antibodies concentration, suggesting that the effect is most probably the results of slow and cumulative changes induced by latent toxoplasmosis, rather than of the transient after-effect of past acute toxoplasmosis.

Effect of latent toxoplasmosis on simple acoustical-signal reaction times was stronger in the third part of the test. This agrees with already published studies showing that the difference in reaction times between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative subjects was absent in the first minute of the 3-minutes test, increased to its maximum in the second minute of the test when the performance of *Toxoplasma*-seropositive subjects declined, and decreased again in the third minute of the test when the psychomotor performance of *Toxoplasma*-seronegative subjects declined too [8,51,54]. All previous studies showing the effect of latent toxoplasmosis on reaction times used visual rather than acoustical signals without any prepulse. Therefore, the present

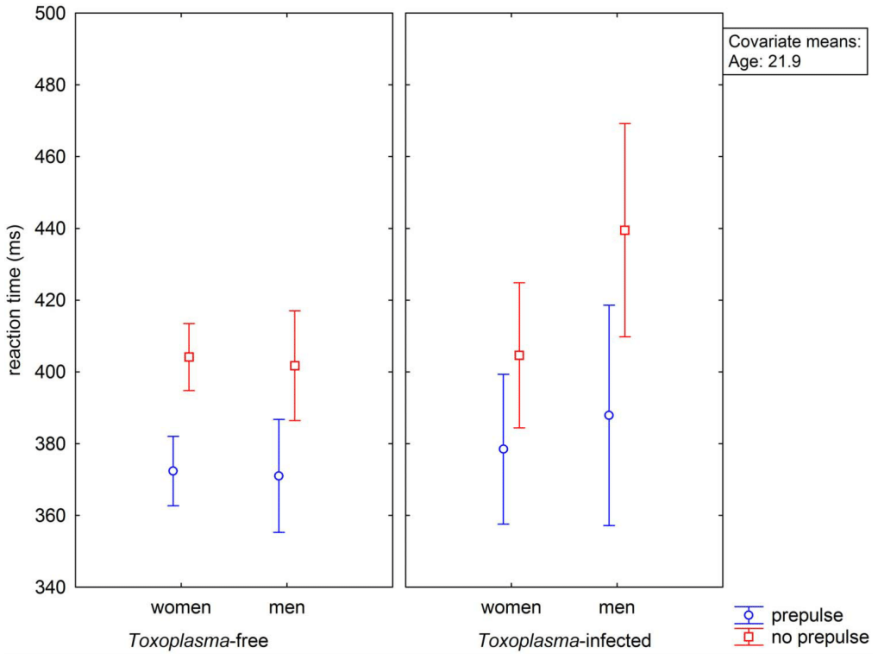

Figure 1. Influence of toxoplasmosis, prepulse and sex on reaction time. Vertical bars denote 0.95 confidence intervals.
doi:10.1371/journal.pone.0112771.g001

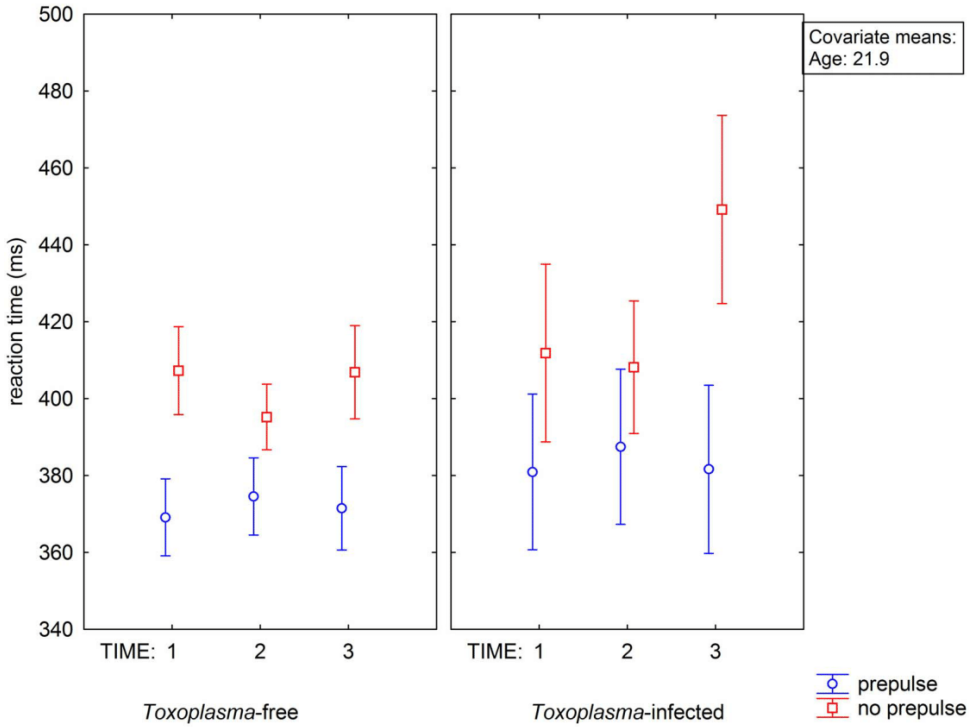

Figure 2. Influence of toxoplasmosis and prepulse on reaction time in different phase of experiment. Vertical bars denote 0.95 confidence intervals.
doi:10.1371/journal.pone.0112771.g002

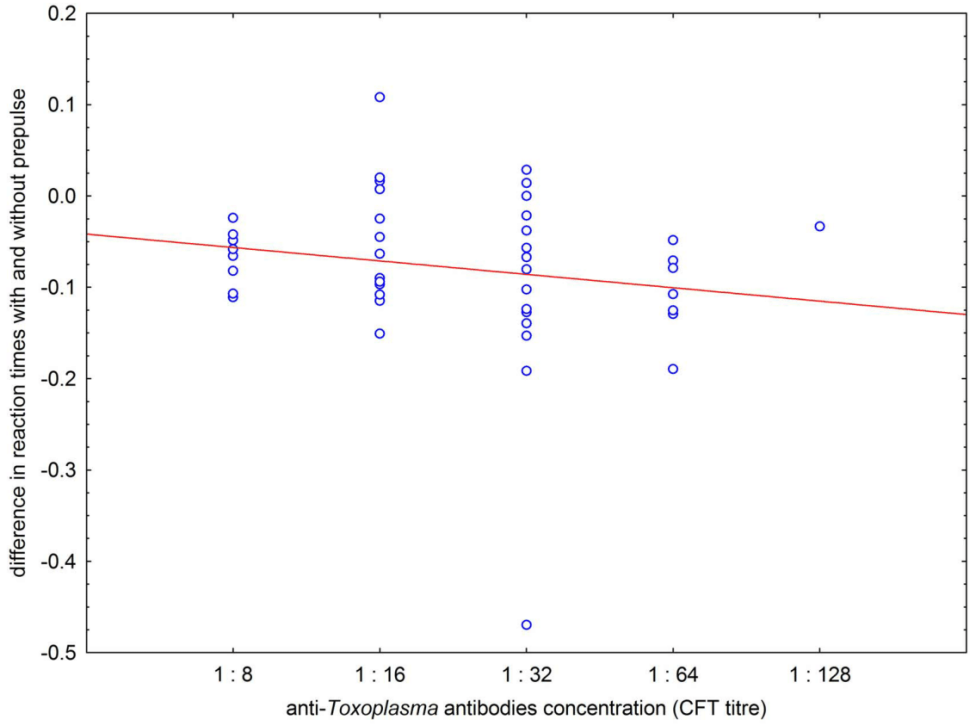

Figure 3. Correlation of anti-*Toxoplasma* antibodies titres with effect of prepulse on reaction times in *Toxoplasma*-infected subjects. Effects of prepulse on reaction time of each rater was calculated as the difference of his mean reaction times with and without prepulse/his mean reaction times without prepulse.
doi:10.1371/journal.pone.0112771.g003

results, namely the decrease of the effect of latent *Toxoplasma* infection on reaction time on prepulse preceded-signals should be confirmed in future studies using visual signal reaction time tests.

Our study revealed very strong facilitating effects of prepulses on the reaction time in all categories of subjects. The difference was about 30 ms for *Toxoplasma*-free and about 70 ms for *Toxoplasma*-infected men. This prepulse-associated decrease of reaction time cannot be explained by students' reacting on prepulse rather than on signals as about same number of prepulses was presented alone, without following signal and the students never responded to the standalone prepulse signals. Moreover, the decrease of latency by 70 ms in *Toxoplasma* infected subjects was larger than the interval between pulse and prepulse. Similar but weaker effect of acoustical prepulses on startle reflex latency (not on the simple reaction time) was already reported in [34]. The most probable explanation of the effect observed in our study is that the prepulse either decreases possible startle response of probands triggered by signals that could otherwise prolong reaction times of all subjects [55], or conveys attention of probands to the following signal.

Our *a priory* hypothesis was based on the former hypothesis, however, the obtained data are in better agreement with the latter one. In the questionnaire studies, the *Toxoplasma*-infected subjects answer that they have no or weak and slow startle reaction [7]. We expected that due to their weaker startle reaction, reaction times of *Toxoplasma*-infected subjects will be less affected by prepulse than the *Toxoplasma*-free controls. However, our results suggest the opposite – the positive effect of prepulse was stronger in the *Toxoplasma*-infected subjects than in the *Toxoplasma*-free subjects. It must be acknowledged, however, that the effects of prepulse on startle reaction, both on its intensity and latency, are highly context dependent and, for example, for many combinations of intensity and signal-prepulse interval the inhibition can turn into the enhancement [49,56,57]. The most parsimonious, but still speculative, explanation of the present phenomenon is that the *Toxoplasma*-infected subjects, especially those that are infected for very long time, lose their concentration during the third part of the reaction time test and the prepulse conveys their "attention" to immediately succeeding signal. It must, however, be emphasized that the experimental subjects did not recognized the existence of two types of signals, the signals with and without the prepulse. Their reactions to the prepulses were therefore purely subconsciously driven by some extra cortical circuit.

The results of present studies supported, but of course not proved, our notion suggesting that many reported effects of schizophrenia, including the aberrant effects of prepulses in the startle inhibition tests, are in fact effects of *Toxoplasma* infection due to increased prevalence of subjects with latent toxoplasmosis in schizophrenia patients [14–16]. However, the study of Pearce et al. [34] showed that schizophrenia patients had larger startle reflex latency than the controls regardless of their toxoplasmosis-status, i.e. no significant schizophrenia-toxoplasmosis interaction was observed in their study. Similarly, they found no effect of schizophrenia-toxoplasmosis-prepulse or toxoplasmosis-prepulse interactions. This seems to contradict our results showing toxoplasmosis-prepulse interaction and also to our hypothesis according to which *Toxoplasma*-free schizophrenia patients are expected to have similar effect of prepulses on startle reflexes as

Toxoplasma-free controls. It must be reminded, however, that startle reaction, which has the latency about 50 ms after the "go" signal is very different process than oriented reaction in simple reaction time tests. Startle reaction is reliably modified by cognitive and emotional processes [56] but the same is even more true for far more complex oriented reaction. Both studies included similar number of *Toxoplasma*-infected subjects, however, by analysing differences of mean with- and without-prepulse reaction time calculated separately for each subject we used more sensitive within-subject technique in searching for toxoplasmosis-prepulse interaction. It must be also reminded that that the commercial diagnostic kits are optimized for diagnosis of acute and postacute toxoplasmosis and have relatively high frequency of false negative results in subjects with old parasite infection and therefore with low concentration of antibodies [58]. Moreover, recent results show that the diagnostic kits have too high specificity, they probably detect just the most common strains of *Toxoplasma gondii* [59]. This can be solved either by using several different tests based on different antigens, which is the only possibility in the clinical practice, or by analyzing the data with special permutation tests for false negative results contaminated-data, rather than with standard statistical test [60], which is the solution applicable in the research project only. While most of latent toxoplasmosis researchers including Pearce et al. [34] use just one diagnostic test for the diagnosis of the *Toxoplasma* infection, we always use two tests and exclude about 10% of subjects with discordant results of tests. The possible presence of a larger subpopulation of false negative subjects with oldest infections and therefore also probably with largest cumulative effect of latent toxoplasmosis in subpopulation of *Toxoplasma*-negative patients than in *Toxoplasma*-negative controls could explain observed effects of prepulses on startle reaction in *Toxoplasma*-negative schizophrenia patients.

Limitations of present study

The effect of toxoplasmosis-prepulse interaction was stronger in male students. In the same time, the number of men in the Faculty of Science biology students and therefore also in our experimental set was rather low – only 14 male students were *Toxoplasma*-infected. It will be therefore necessary to repeat a similar study with a larger sample. Such repetition is very urgent because the effect of *Toxoplasma*-seropositivity is known to depend on RhD phenotype and genotype of subjects [61,62]. The negative effects of *Toxoplasma*-seropositivity are usually much stronger in RhD negative subjects and are sometimes even absent in the most numerous RhD positive heterozygotes [61]. However, the number of RhD negative subjects is about 16% in the Czech population and therefore much larger experimental sample will be necessary to collect for controlling this important confounding factor. It must be noted, however, that the RhD negative subjects were equally represented in *Toxoplasma*-infected and *Toxoplasma*-free men (they were no significantly more frequent in *Toxoplasma*-infected than *Toxoplasma*-free women) and that an uncontrolled but balanced confounding factor increases the risk of false negative but not the false-positive results [2]. *Toxoplasma* is known to influence not only the reaction times but also a level of testosterone and therefore also competitiveness (and motivation) of subjects [63]. Therefore, the effect of *Toxoplasma* infection on performance in particular tests can be even opposite (positive) in different populations [2]. Therefore, our university students-study should be repeated also on different populations such as soldiers or blood donors or on the general population sample. Test subjects in the presented study weren't tested for hearing inaccuracy; although the tests subjects were mostly healthy young individuals, it is possible several of them might have a minor hearing problem that could affect the presented results.

On the basis of the negative correlation between the effect of prepulse stimuli and concentration of anti-*Toxoplasma* antibodies we suggested that the observed effect of latent toxoplasmosis increases with time passed from the moment of infection. It must be mentioned, however, that the concentration of antibodies only indirectly reflects the time passed from the moment of infection and can also be influenced by other factors, e.g. the intensity of the infection and the immunocompetence of a subject.

We confirmed that latent toxoplasmosis influenced the performance of subjects in psychomotor performance tests. We also confirmed earlier observations indicating that the ability of long-term concentration rather than the best reaction times are influenced by the infection. For the first time we demonstrated different effects of prepulse on reaction times of *Toxoplasma*-infected and *Toxoplasma*-free subjects, especially males. Existence of this effect independently suggests that the ability of concentration most probably plays the role in the latent toxoplasmosis-associated impairment of performance in reaction time tests. In the present paper we studied the effect of prepulse on the reaction times and not on the startle reaction, which latter is the subject of most of prepulse studies, or on StartReact [47,48], but on latency facilitation of oriented reactions. Our results support the hypothesis that the increased prevalence of toxoplasmosis in schizophrenia patients in connection with atypical responses of *Toxoplasma*-infected subjects to prepulses could be responsible for the well-known differences in reactions of schizophrenia patients in prepulse inhibition of startle reaction tests.

Author Contributions

Conceived and designed the experiments: JF LP. Performed the experiments: LP BŠ. Analyzed the data: JF. Contributed reagents/materials/analysis tools: JF. Wrote the paper: JF LP.

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4.6 Lower performance of *Toxoplasma*-infected, Rh-negative subjects in the weight holding and hand-grip tests

4.6.1 Reasons for Inclusion

The data for this study were collected during the same experimental sessions as for the previous one and were meant to be analyzed together within the framework of two doctoral projects, mine and that of Blanka Šebánková. Both doctoral projects study the effects of toxoplasmosis on human performance and behavior; hers is more focused on aspects of physical anthropology, mine more on brain function and psychology.

The study might, on the first glance, seem to be concentrating on muscle strength and physical fitness, but as I will explain below, the rationale for conducting the study lies more in the realm of brain functions and is closely related to the reaction times experiments and thus with the thesis' topic.

4.6.2 Introduction

Previous research on simple reaction times in *Toxoplasma*-infected individuals shown a decrease in psychomotor performance with time from the start of the experiment (Havlícek et al., 2001), which led us to plan an experiment working with other types of performance – will the *Toxoplasma*-infected probands tire earlier than the uninfected ones? There was, however, one other thing to test. *Toxoplasma*-infected individuals responded in questionnaires as less willing to fight or rather stop fighting before the fight is over (J Flegr, 2010). The fight here means not just a physical encounter, but also the pursuit of something important.

Indirect evidence for this early surrender could also be seen in one much graver statistics, a percentage of *Toxoplasma*-infected individuals who, so to say, "gave up on life" – that is in suicide statistics. And the evidence there is unpleasantly plentiful. Latent toxoplasmosis was found to be positively correlated with suicide rates in postmenopausal women (Ling, Lester, Mortensen, Langenberg, & Postolache, 2011). Those, who attempted suicide, were found to have higher titers of antibodies against *T. gondii* in contrast with patients without the history of suicidal attempts in a population of patients with mood disorders (although this particular study found no significant association between *Toxoplasma*-seropositivity and number of suicidal

attempts) (Arling et al., 2009). A study comparing 200 healthy volunteers and 200 people following a suicide attempt found anti-*Toxoplasma* IgG seropositivity levels to be significantly lower in healthy volunteers (Yagmur, Yazar, Temel, & Cavusoglu, 2010). Both *T. gondii* seropositivity and antibody titers were positively correlated with suicide attempts in younger schizophrenia patients; the correlation was not significant in older patients (Okusaga et al., 2011). Another study on the schizophrenic population found an association between *T. gondii* seropositivity, together with high blood levels of kynurenine with non-fatal suicidal self-directed violence (Okusaga et al., 2016). A meta-analysis conducted on 24 studies with a total of 2259 cases and 9400 controls suggested a role of latent toxoplasmosis in suicide attempts (as well as in traffic accidents; 4229 cases and 12 234 controls were analyzed in this part) (Arjen L. Sutterland et al., 2019). And the list could go on.

In the study, we wanted to test the "giving-up phenomenon" experimentally using easily available test of physical performance, a Collin dynamometer (primarily measuring isotonic strength) and 5kg of hinged weight (primarily measuring isometric strength).

4.6.3 Materials and Methods[¨]

Our volunteers, mostly students of the Faculty of Sciences, were recruited for the experiment. The experimental session started at 9:00 a.m. with the handgrip test. After careful instructions and demonstration of a proper grasp of the dynamometer by the experimenter, the probands were asked to hold a Collin dynamometer in their dominant hand kept freely along their trunk, and squeeze it as strongly as possible, without moving their body. They were instructed to repeat it two more times with the same hand and then three times with their non-dominant hand.

After the hand-grip test, experimental subjects completed a battery of computer-administered and paper questionnaires and experiments with one short break in the middle. Then, after the battery of tests was over, a short break followed where they were offered snacks to replenish energy (muesli bars, pastries, fruit, and fruit juices). The weight-holding experiment took place at the end of the experimental session. Each proband was instructed to

take the hinged weight (actually a plastic bag with two packs of paper) into both hands and level their forward-stretched arms with shoulders. The experimenter observed the posture and ended the timing when the proband was no longer able to keep their hands leveled or when they gave up themselves.

The data were analyzed using ANCOVA and repeated measures ANCOVA for weight-holding tests and handgrip tests, respectively. *Toxoplasma*-seropositivity, sex, and Rh factor were used as independent variables.

4.6.4 Results and Discussion

289 women (59 *Toxoplasma*-seropositive) and 176 men (29 *Toxoplasma*-seropositive) completed the weight-holding experiment. As anticipated, age (eta^2 = 0.023, p = 0.0004) and sex (eta^2 = 0.20, p < 0.0005) had significant impact on the performance; we have also found a significant toxo-Rh interaction (eta^2 = 0.011, p = 0.026) with lower performance in Rh-negative/*Toxoplasma*-positive probands and higher performance of Rh-positive/*Toxoplasma*-positive in comparison with *Toxoplasma*-negative subjects. The effects weren't significant when analyzed separately for men and women.

The hand-grip test completed 343 women (66 *Toxoplasma*-positive) and 207 men (35 *Toxoplasma*-positive). We have found a significant negative effect of toxoplasmosis on the performance of Rh-negative subjects in men (but not for women). Complete results in a synoptic table can be found in the enclosed publication.

Both experiments showed significant effects of Rh-*Toxoplasma* interactions with Rh-negative subjects performing worse than the Rh-positive ones (with Rh-positive/*Toxoplasma*-positive men performing unexpectedly even better than uninfected male subjects), which is consistent with our findings from other studies (Jaroslav Flegr et al., 2010; Jaroslav Flegr, Novotná, et al., 2008; Novotná et al., 2008).

4.6.5 Limitations

The study mentions a limitation in taking RhD phenotype rather than genotype. We believe the heterozygote advantage to be responsible for

maintaining both Rh-positive and Rh-negative phenotypes in the population (Jaroslav Flegr, 2016; Novotná et al., 2008). To thoroughly test this hypothesis, we would need to distinguish between Rh-positive homozygotes and heterozygotes.

The main problem of this type of study is also that we are not able to tell the causation of the observed phenomena. Latent toxoplasmosis could be responsible for changes in probands' performance, and indeed there is indirect evidence for this causal relationship: As we (and other research groups) have demonstrated in other studies, there often is a negative association between the levels of anti-*Toxoplasma* antibody titers, indicating deepening of the effects with time from infection.

PLOS ONE

RESEARCH ARTICLE

Lower performance of *Toxoplasma*-infected, Rh-negative subjects in the weight holding and hand-grip tests

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Abstract

Background

Toxoplasma, a protozoan parasite of cats, infects many species of intermediate and paratenic hosts, including about one-third of humans worldwide. After a short phase of acute infection, the tissue cysts containing slowly dividing bradyzoites are formed in various organs and toxoplasmosis proceeds spontaneously in its latent form. In immunocompetent subjects, latent toxoplasmosis was considered asymptomatic. However, dozens of studies performed on animals and humans in the past twenty years have shown that it is accompanied by a broad spectrum of specific behavioural, physiological and even morphological changes. In human hosts, the changes often go in the opposite direction in men and women, and are mostly weaker or non-existent in Rh-positive subjects.

Methods

Here, we searched for the indices of lower endurance of the infected subjects by examining the performance of nearly five hundred university students tested for toxoplasmosis and Rh phenotype in two tests, a weight holding test and a grip test.

Results

The results confirmed the existence of a negative association of latent toxoplasmosis with the performance of students, especially Rh-negative men, in these tests. Surprisingly, but in an accordance with some already published data, *Toxoplasma*-infected, Rh-positive subjects expressed a higher, rather than lower, performance in our endurance tests.

Discussion

Therefore, the results only partly support the hypothesis for the lower endurance of *Toxoplasma* infected subjects as the performance of Rh-positive subjects (representing majority of population) correlated positively with the *Toxoplasma* infection.

Introduction

About one third of the world population are infected with the protozoan parasite *Toxoplasma gondii* [1]. In immunocompetent subjects, after a short phase of acute toxoplasmosis promoted by tachyzoites, the disease spontaneously proceeds into its latent phase, which is characterized by the presence of tissue cysts with a slowly dividing form of the parasite, bradyzoites, in various organs and anamnestic IgG antibodies in blood [2]. Latent toxoplasmosis has been considered asymptomatic for a long time, however, during the past 20 years, about one hundred papers have been published showing that *Toxoplasma* seropositive and seronegative subjects differ in many personality traits [3,4], performance in certain psychomotor tests [5,6], cognitive tests [7–10] and also in the incidence and form of many diseases and disorders [11,12]. In some tests, however, *Toxoplasma*-infected subjects score better than *Toxoplasma*-free individuals [13,14]. Very often, these differences deepen with time since the infection [5,15,16] and the analogical changes were nearly always reported to occur in experimentally infected laboratory animals [17–20]. This suggests that the infection is most probably the cause of the behavioral changes, rather than that the special combination of phenotypic traits is the cause of the infection. It has been suggested that these observed behavioral differences are either the product of the manipulation activity of the parasite aimed to increase the probability of transmission of *Toxoplasma* from an intermediate to a definitive host by predation, or potentially as side-effects of the pathological changes accompanying the infection. Very often, personality differences associated with toxoplasmosis differ in scope or even direction between men and women. The stress-coping hypothesis [21,22] explains the opposite reactions of men and women to *Toxoplasma* infection by the fact that men and women cope in an opposite way to mild chronic stress. In contrast to stressed men, who use more individualistic and antisocial (for example aggressive) forms of coping [23,24], stressed women more often seek and provide social support (Stone and Neale 1984, Rosario et al. 1988, Carver et al. 1989), join with others [24], and verbalize towards others or ones' self [25].

Similarly, *Toxoplasma* infection is associated with different phenotypical outputs in Rh-positive and Rh-negative subjects. The Rh-positive subjects, especially Rh-positive heterozygotes, seem to be fully or partly protected against many of the effects of toxoplasmosis, such as personality changes [26], decreased psychomotor performance [27,28], and impaired physical and mental health [29]. The strongest effect of latent toxoplasmosis reported until now, the excessive weight gain of *Toxoplasma* infected women in the 16th week of their pregnancies (4.12 kg vs 2.44 kg, N = 152, 25 *Toxoplasma*-infected), was observed only in Rh-negative mothers [30].

One of the earliest reported effects of latent toxoplasmosis in humans capable of enhancing the transmission of the parasite by predation in infected animals was the phenomenon of lower endurance of infected individuals [5,27,31]. In three independent questionnaire surveys, infected subjects, especially men, responded affirmatively to six of ten statements of the Toxo-92 questionnaire significantly more often, including the following item: "When I am attacked, physically or otherwise, or when I should fight for something important, I stop fighting at one moment. It is not a result of a rational decision not to fight, as in fact I know that I should continue fighting and I would like to do so, but my own subconsciousness betrays me and I lose the will to fight back"[31]. It was suggested that the reason of this premature surrender could be the lack of endurance in *Toxoplasma*-infected subjects. However, no experimental data or other empirical evidence, possibly except an increased rate of suicides or suicide-attempts in the infected subjects [32–36], have thus far been published.

The aim of present study was to search for empirical evidence for a decreased will to fight in the *Toxoplasma*-infected subjects. In double-blind experiments, we measured the endurance

of *Toxoplasma*-infected and *Toxoplasma*-free university students using two tests–a weight-holding tests and a hand-grip test. As the effects of toxoplasmosis very often differ in men and women and in Rh-positive and Rh-negative subjects, we included the variables sex and Rh phenotype into our statistical models.

Material and methods

Population

The population consisted of 347 women (age: 22.8, Std. Dev. 3.95, weight: 62.6, Std. Dev. 9.65, height: 167.4, Std. Dev. 6.18, BMI: 22.3, Std. Dev. 3.18) and 208 men (age 24.1, Std. Dev. 5.37, weight: 74.7, Std. Dev. 11.15, height: 180.4, Std. Dev. 6.82, BMI: 23.0, Std. Dev. 3.02), mostly students of biology of the Faculty of Science, Charles University. They were recruited during undergraduate and graduate level courses of Evolutionary Biology and Methodology of Science between 2010–2015. Usually, more than 70% of students attending these coursed accepted the invitation to participate in projects studying the manipulation activity of the parasite *Toxoplasma* and all except those with visible physical injuries or disabilities were accepted into the endurance tests. They were offered the opportunity to enrol in such experiments during a course entitled Methods of Evolutionary and Experimental Psychology, and compensated for their participation with one credit toward their assessment. The project, including the method of recruitment of participants and obtaining informed consent, was approved by the Institutional Review Board of the Faculty of Science, Charles University (No. 2014/21).

Registered probands signed an informed consent, completed several questionnaires including an anamnestic questionnaire, and provided 3 ml of blood for testing for the presence of anamnestic anti-*Toxoplasma* antibodies and for testing of Rh phenotype. Experiments were separated to three sessions and took about ten hours altogether. Probands participated in all the sessions within several weeks. Both of the tests of performance were completed in the first session, which took place once a week and always started at 9:00 am. After finishing the whole battery of tests, they were informed about results of their serological tests for toxoplasmosis and Rh factor and rewarded with 400 CZK for their participation in the experiment.

First, probands got an email invitation to attend a session ahead of time and chose a date according to their preferences. They were asked to fulfil some requirements: to sleep sufficiently during the night before testing; to refrain from physical exertion for several days beforehand; to cancel their attendance in case of an illness, physical or mental problems or convalescence; to not drink coffee, tea, alcohol, and other energy drinks the day of the experiment. They were, however, offered a snack to replenish their energy during the long experimental session.

Grip test

Hand grip dynamometry is one of the screening procedures measuring fitness in a normal population. Hand grip strength can predict vitality, the nutritional state of an individual, as well as possible functional bodily failure [37,38]. A Collin dynamometer, a type of closed steel spring dynamometer, was used for testing grip strength. Hand grip strength was measured in kg.

The test was performed first in a battery of experiments. Each proband was requested to stand upright, with arms kept freely along their trunk, and to look straight ahead. They took the gauge by their dominant arm and the experimenter explained how they should assumed a fixed position in their hand. The proband was asked to exert the maximal practicable effort on the grip without moving their trunk and shoulders. After the performance, the experimenter recorded the measurement and asked the proband to repeat the sequence two more times with

the same hand, and then three times with their nondominant hand. After the test was over, the experimenter informed the proband of his results and told him which of his or her hands was stronger. This part of the experiment was executed individually in the same room with other probands, but in seclusion.

Weight-holding test

A weight-holding performance test was prepared as a secondary experiment to be implemented in the same session as the grip test. It was placed at the end of a day-long experimental session, preceded by an intelligence and memory test, and several questionnaires distributed in paper and electronic forms. The probands were accommodated with two breaks and a snack in between the parts of the session. Each proband was requested to stand upright, not to lean over or backward, and to look straight ahead with experimenters outside his or her visual field. The proband was fitted with a hinged weight (5 kg) and asked to grasp it with both hands, to tense their arms in a levelled position with their shoulders, and with the backs of their hands up. The timer (measurement in seconds) was started by an experimenter the moment the appropriate position was engaged. The test was finished by the proband or by the experimenter when the proband was no longer capable of stabilizing their arms in a levelled position with shoulders. The probands were informed of their results after completing the test.

Testing for toxoplasmosis and Rh phenotype

Testing for toxoplasmosis was performed at the National Reference Laboratory for Toxoplasmosis, National Institute of Public Health, Prague. The complement-fixation test (CFT), which determines the overall levels of IgM and IgG antibodies of particular specificity, and Enzyme-Linked Immunosorbent Assays (ELISA) (IgG ELISA: SEVAC, Prague) were used to detect the *T. gondii* infection status of the subjects. ELISA assay cut-point values were established using positive and negative standards according to the manufacturer's instructions. In CFT test, the titre of antibodies against *Toxoplasma* in sera was measured in dilutions between 1:4 and 1:1024. The subjects with CFT titres between 1:8 and 1:128 were considered *Toxoplasma* infected (the subjects with higher titres, who were suspected to be in the acute phase of the infection, were excluded from the study). Only subjects with clearly positive or negative results of CFT and IgG ELISA tests were diagnosed as *Toxoplasma*-infected or *Toxoplasma*-free, whilst subjects with different results on these tests, or ambiguous results, were retested or excluded from the study. A standard agglutination method was used for Rh factor examination. A constant amount of anti-D serum (human monoclonal antiD reagent; SeracloneH, Immucor Gamma Inc.) was added to a drop of blood on a white glass plate. Red cells of Rh-positive subjects were agglutinated within 2–5 min.

Statistics

Statistic analyses were performed with program Statistica v. 10.1. The effect of *Toxoplasma* seropositivity and Rh on participants' performance in the weight-holding test was measured with ANCOVA (modul General Linear Models), with the independent binary variables *Toxoplasma* seropositivity (toxo), Rh phenotype (Rh), and sex of a subject (sex), the covariate age of a subject in time of testing (age), and toxo-Rh, toxo-sex, Rh-sex, and Rh-toxo-sex interactions as the independent variables. The effect of toxoplasmosis and Rh on participants' performance in the hand-grip test was measured with repeated measures ANCOVA, with the performance of particular subjects in six trials (3 dominant hand trials and 3-nondominant hand trials) as the repeated measures; the independent variables were toxo, Rh, sex, and two nested factors: hand (dominant/nondominant) and trial (1^{st}, 2^{nd}, and 3^{rd} trial for each hand). The model also

included all interactions of toxo, Rh, sex, hand, and trial. In the follow-up analyses, the same tests (without the factor sex and without all interactions with sex) were performed separately for men and women.

All raw data are available at Figshare https://figshare.com/s/692c3a7d08c3df860c9e

Terminological notes

For the sake of clarity, we abbreviated "*Toxoplasma* seropositivity" to "toxoplasmosis"in the Discussion and to "toxo" in the description of our statistical models. Also, the statistical relations between (formally) dependent and (formally) independent variables are called "effects," despite the fact that the real causal relation between these variables can be different or even non-existent (as reminded in the Discussion).

Results

Weight-holding test

This test was performed by 289 women, 59 (20.4%) *Toxoplasma*-seropositive and 176 men, 29 (16.5%) *Toxoplasma*-seropositive. The seroprevalence of toxoplasmosis did not differ between women and men ($p = 0.339$, Fisher exact test). Similarly, frequency of Rh-negative subjects, 21.1% (61) in women and 19.3% (34) in men, did not differ between men and women or between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative male or female subjects ($Chi^2 = 1.31$, $df = 4$, $p = 0.860$, all partial p values > 0.30). The age of *Toxoplasma*-seropositive subjects was higher than age of seronegative subjects (women: 24.6, SD 6.5 vs 22.6, SD = 3.30, $t_{287} = -3.39$, $p = 0.001$, men: 26.4, SD 9.00 vs 23.9, SD = 4.56, $t_{174} = -2.19$, $p = 0.029$). The effect of *Toxoplasma*-seropositivity and Rh phenotype in the weight-holding test was measured with General Linear Models (GLM) with time of holding as the dependent variable and toxo, Rh, age, sex, toxo-Rh, toxo-sex, Rh-sex, toxo-Rh-Sex as independent variables. Age and especially sex had very significant effects on participants' performance in the test (age: $eta^2 = 0.023$, $p = 0.0004$; sex: $eta^2 = 0.20$, $p < 0.0005$). The effect of toxo depended on the Rh phenotype of subjects (toxo-Rh interaction: $eta^2 = 0.011$, $p = 0.026$). *Toxoplasma*-seropositive, Rh-negative subjects expressed lower performance and *Toxoplasma*-seropositive Rh-positive subjects expressed higher performance than their *Toxoplasma*-seronegative peers, see the Fig 1. Separate GLM analyses performed for men and women showed that the effect was significant neither for men, nor for women (p values > 0.10).

Hand-grip test

The hand-grip test was performed by 343 women, 66 (19.2%) *Toxoplasma*-seropositive and 207 men, 35 (16.9%) *Toxoplasma*-seropositive. The seroprevalence of toxoplasmosis did not differ between women and men ($p = 0.570$, Fisher exact test). Similarly, the frequency of Rh-negative subjects, 21.9% in women and 18.8% in men, did not differ between men and women or between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative male or female subjects ($Chi^2 = 1.48$, $df = 4$, $p = 0.830$, partial p values > 0.11). The age of *Toxoplasma*-seropositive subjects was higher than age of seronegative subjects (women: 24.2, SD 6.3 vs 22.48, SD = 3.09, $t_{345} = -3.24$, $p = 0.001$, men: 25.8, SD 8.38 vs 23.7, SD = 4.45, $t_{206} = -2.12$, $p = 0.035$). The effects of *Toxoplasma*-seropositivity and Rh phenotype in the hand-grip test were measured with repeated measures GLM with the performance in three dominant hand-trials and in three non-dominant hand-trials as nested repeated measures, and toxo, Rh, sex, hand, trial, age, and all interactions of toxo, Rh, sex, hand, and trial as independent variables. Table 1 shows the results of this analysis and analyses performed separately for men and women. Fig 2 shows

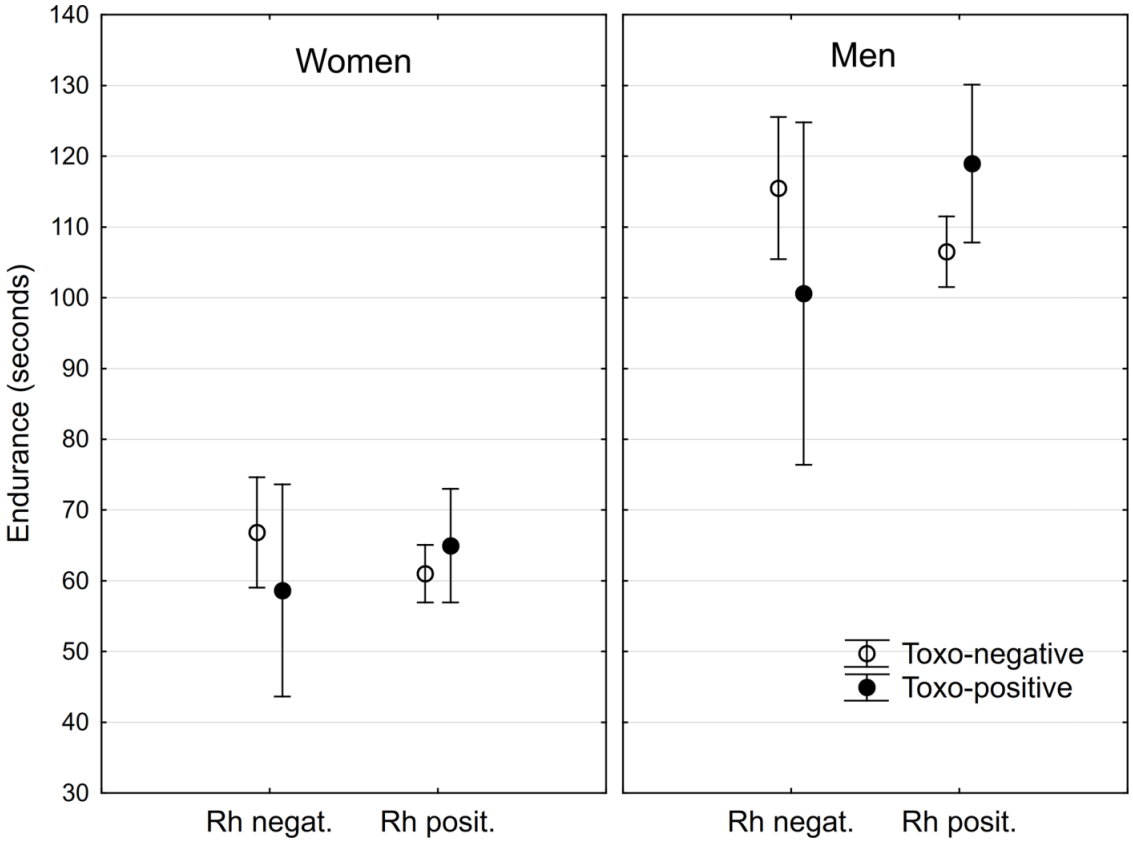

Fig 1. Effects of toxoplasmosis and Rh phenotype on performance in the weight holding test. Points show arithmetic means of endurance computed for covariate at its mean and spreads denote 95% confidence intervals.

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that the effect of trial-Rh-toxo is stronger in men than in women and that the toxoplasmosis had negative effects on the performance of the Rh-negative subjects. Separate analyses performed for men and women showed that the effects were significant for men, but not for women, see Table 1.

Discussion

The results of the present study suggest that *Toxoplasma* seropositivity was related to the performance of infected subjects in two tests that primarily measure the isometric (weight-holding test) and isotonic (hand-grip test) strength of participants. This relation was different in Rh-positive and Rh-negative subjects. *Toxoplasma*-seropositivity was always associated negatively with the performance of Rh-negative subjects, but it had a positive association with endurance in the weight holding test of the Rh-positive subjects, especially the men.

The main reason for doing the present study was to find direct evidence for lower endurance of *Toxoplasma*-infected subjects, which has been suspected to exist on the basis of several questionnaire studies [31]. Our results only partly confirmed this prediction as the lower

Table 1. Effects of toxoplasmosis, Rh phenotype, hand and trial on performance in the hand-grip test.

	All		Women		Men	
	p	η^2	p	η^2	p	η^2
Age	**0.000**	**0.027**	**0.003**	**0.025**	**0.015**	**0.029**
Sex	**0.000**	**0.298**				
Rh	0.721	0.000	0.560	0.001	0.897	0.000
Toxo	0.355	0.002	0.441	0.002	0.562	0.002
Sex-Rh	0.933	0.000				
Sex-Toxo	0.795	0.000				
Rh-Toxo	0.175	0.003	0.364	0.002	0.351	0.004
Sex-Rh-Toxo	0.487	0.001				
Hand	**0.000**	**0.037**	**0.000**	**0.042**	**0.007**	**0.035**
Hand-Age	0.535	0.001	0.158	0.006	0.888	0.000
Hand-Sex	**0.000**	**0.034**				
Hand-Rh	0.064	0.006	0.179	0.005	**0.025**	**0.025**
Hand-Toxo	0.334	0.002	0.461	0.002	0.247	0.007
Hand-Sex-Rh	**0.003**	**0.016**				
Hand-Sex-Toxo	0.093	0.005				
Hand-Rh-Toxo	0.202	0.003	0.266	0.004	0.115	0.012
Hand-Sex-Rh-Toxo	**0.031**	**0.009**				
Trial	**0.000**	**0.015**	**0.008**	**0.014**	**0.028**	**0.018**
Trial-Age	0.429	0.002	0.428	0.003	0.782	0.001
Trial-Sex	**0.032**	**0.006**				
Trial-Rh	**0.001**	**0.012**	0.109	0.007	**0.027**	**0.018**
Trial-Toxo	**0.029**	**0.007**	0.783	0.001	**0.024**	**0.018**
Trial-Sex-Rh	0.157	0.003				
Trial-Sex-Toxo	**0.012**	**0.008**				
Trial-Rh-Toxo	**0.001**	**0.014**	0.199	0.005	**0.012**	**0.022**
Trial-Sex-Rh-Toxo	0.088	0.004				
Hand-Trial	0.092	0.004	0.276	0.004	0.314	0.006
Hand-Trial-Age	0.194	0.003	0.246	0.004	0.623	0.002
Hand-Trial-Sex	0.074	0.005				
Hand-Trial-Rh	0.051	0.005	0.850	0.000	0.060	0.014
Hand-Trial-Toxo	0.502	0.001	0.282	0.004	0.178	0.009
Hand-Trial-Sex-Rh	0.075	0.005				
Hand-Trial-Sex-Toxo	**0.034**	**0.006**				
Hand-Trial-Rh-Toxo	0.062	0.005	0.583	0.002	**0.024**	**0.018**
Hand-Trial-Sex-Rh-Toxo	**0.009**	**0.009**				

Table shows the results, namely p values and partial η^2 for all main effects and all interactions (rows) of repeated measures analyses of variances performed for all subjects (columns 2, 3) and also separately for women (columns 4, 5) and men (columns 6, 7). The models contained the performance in three dominant hand-trials and in three non-dominant hand-trials as nested repeated measures, and toxo, Rh, sex, hand, trial, age, and all interactions of toxo, Rh, sex, hand, and trial as independent variables. Significant effects on the performance are printed in bold; p values < 0.00005 are coded as 0.000.

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endurance in both tests was observed only in the Rh-negative subjects. Moreover, endurance measured with the weight holding test in the Rh-positive subjects (i.e. in majority of the population) was higher in the infected than in the non-infected subjects. Our experimental setup cannot discriminate between whether the lower performance of the seropositive, Rh-negative subjects in the tests was caused by their lower endurance or by their lower physical strength.

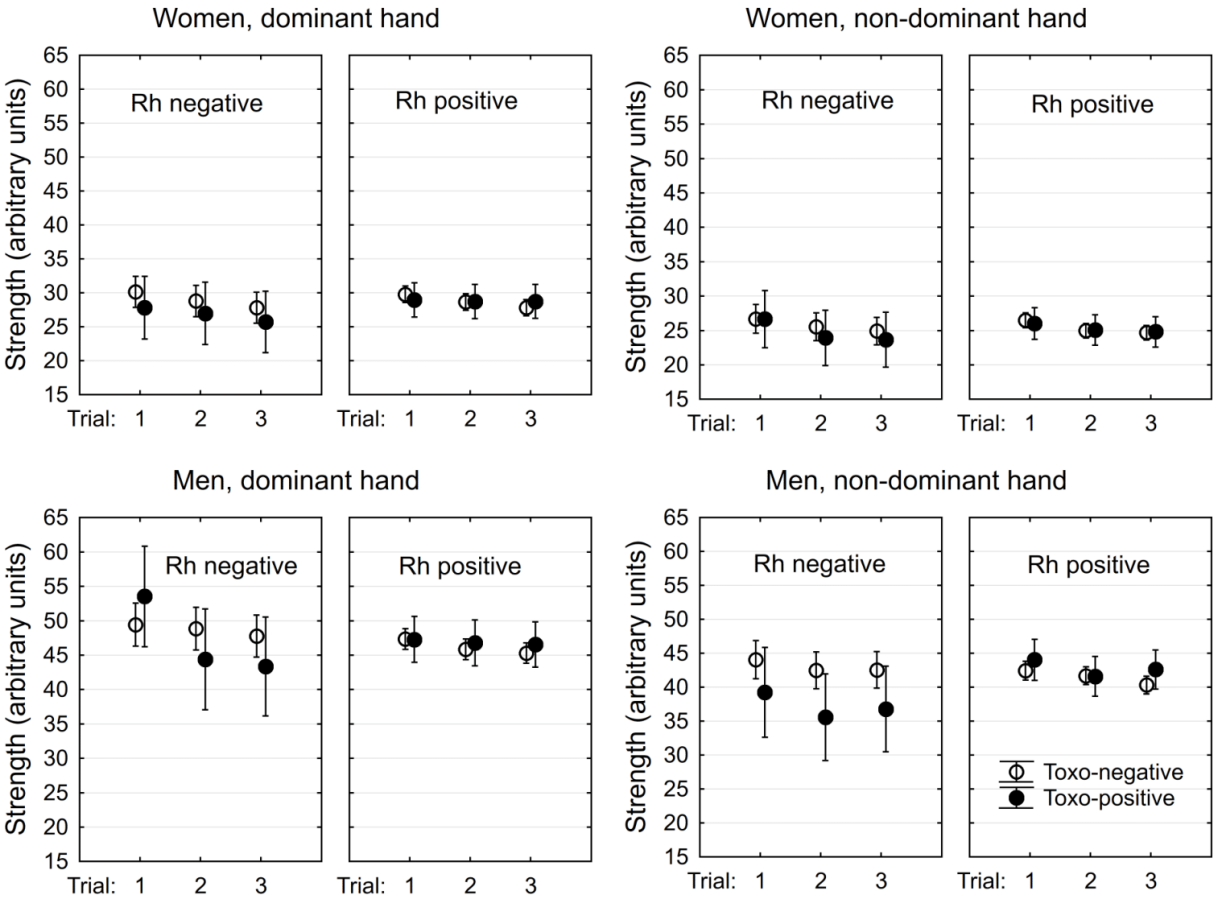

Fig 2. Effects of toxoplasmosis and Rh phenotype on performance in the hand-grip test. Points show arithmetic means of endurance computed for covariate at its mean and spreads denote 95% confidence intervals.

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However, the fact that better rather than worse performance in the hand-grip test was observed in the first dominant-hand trial in men (see the Fig 2) suggests that lower endurance rather than lower physical strength is the more probable explanation of the result.

The existence of significant Rh-Toxo (or Trial-Rh-Toxo) interactions agreed with the results of certain performance tests [27,28] and of certain health status studies [13,29] published earlier. We confirmed that *Toxoplasma*-infected, Rh-negative subjects expressed worse results in performance tests, while this was not always true for Rh-positive subjects. In two previous studies performed on blood donors [27] and students [28], the effects of Rh-toxoplasmosis interaction on performance in simple reaction tests were studied. These studies showed that the *Toxoplasma* free, Rh-negative subjects had shorter reaction times than *Toxoplasma*-free Rh-positive subjects while *Toxoplasma*-infected, Rh-negative subjects had much worse, i.e. longer, reaction times than the *Toxoplasma*-infected, Rh-positive subjects. In the 2008 studies, the authors also showed, but did not discuss, that among the Rh-positive subjects, the

Toxoplasma-infected ones, especially the Rh-positive heterozygotes, had better performance than the *Toxoplasma*-free subjects. A similar paradoxical phenomenon, namely the positive effect of *Toxoplasma* infection on mental health of the Rh-positive subjects, was described in a case-controls study performed on 79 women who had been tested for toxoplasmosis and Rh phenotype. The *Toxoplasma*-infected, Rh-negative women expressed higher neuroticism measured with the N-70 neuroticism inventory and reported more health problems than the *Toxoplasma*-free, Rh-negative women; the opposite was true for Rh-positive women [29]. It can be speculated that the better performance of the *Toxoplasma*-seropositive, Rh-positive subjects in performance tests is a result of an increased level of testosterone, which can be associated with higher levels of competitiveness [39]. However, while the increased level of testosterone was observed only in men [40,41], male mice [42] and non-castrated male rats [43], the better performance of the infected individuals when compared to non-infected individuals in Rh-positive subjects was observed in both men and women. Moreover, the higher competitiveness of *Toxoplasma*-infected subjects can hardly explain the better physical health of infected, Rh-positive subjects [29]. In our evolutionary past in Africa, and probably also in our recent past throughout the world, nearly all people were probably infected with *Toxoplasma*. In Africa 95% of humans are Rh-positive and in Asia the frequency of Rh-positive phenotypes is even higher, about 99%. A higher frequency of Rh-negative subjects, about 16%, is only among Europeans. This higher frequency of Rh-negative subjects was explained by the low abundance of felids and therefore probably the low prevalence of toxoplasmosis in Europe before the recent advent of domestic cat [28]. Among the Toxoplasma-free subjects, the Rh-negative individuals and even more the Rh-positive heterozygotes outperform the Rh-positive homozygotes in physical health and in many psychomotor and cognitive performance-related parameters [27,44–46]. Therefore, the allele for Rh-negativity can spread in low toxoplasmosis prevalence areas, e.g. in ancient Europe. In contrast, in many parts of Africa, Asia and both Americas the abundance of various feline species is much higher and therefore the prevalence of toxoplasmosis in human population is (and always was) also much higher. It is possible that natural status of humans is to be infected with toxoplasma and therefore that the human body is adapted to this infection. Consequently, the performance and health of infected Rh-positive subjects is better than the performance of non-infected Rh-positive subjects.

The results of the reaction time and the endurance tests, however, contrast strongly with the results of intelligence tests performed on a cohort of 502 male soldiers [13]. This study also showed a significant Rh-toxoplasmosis interaction, however, the infected Rh-negative soldiers scored higher and infected Rh-positive subjects scored lower in two different intelligence tests than corresponding *Toxoplasma*-free subjects. We have no explanation for the results of this study, however, the better performance of the infected Rh-negative subjects in the IQ tests suggest that the effects of Rh-toxoplasmosis interaction on performance are context-dependent and rather specific.

Limitation

The main limitation of the present study is that the participants have been screened for the Rh phenotype, not Rh genotype. All available data, however, suggest that the Rh-positive heterozygotes, not homozygotes, are resistant to physiological effects of toxoplasmosis. More informative data will probably be obtained when the Rh-positive participants are tested for their Rh genotype. The number of Rh-negative, *Toxoplasma* seropositive male subjects, the rarest subpopulation in general population, is relatively low. This is probably the reason for the absence of a significant effect of the Rh-*Toxoplasma* interaction in males, despite a relatively large effect size. The general design of a study, the cross-sectional cohort study, can prove the existence of

statistical association between two or more variables–here *Toxoplasma* seropositivity, Rh phenotype and endurance–but cannot solve the question of causality. Of course, neither *Toxoplasma* seropositivity nor Rh phenotype could be affected by the endurance of a human. However, for example, both the probability of the infection and the endurance can be influenced by some unknown third variable, such as the health status of subjects.

Conclusions

Our results partly support the hypotheses that latent toxoplasmosis has negative effects on the endurance of infected subjects, and Rh positivity protects people against some negative effects of toxoplasmosis. It must, however, be stressed that all evidence is only indirect and should be supported by the results of manipulative studies performed on laboratory-infected animals.

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4.7 Footnotes

[1] Most interestingly, a punishment (or a reward) has to be costly to the punishing individual in order to achieve desired effects on free riders. Free punishments seem to be rather ineffective (Balliet, Mulder, & Van Lange, 2011).

[2] First edition of Roethlisberger's and Dickson's Management and the Worker (1939) is now available on https://archive.org/details/managementworker00roet

5 Summary and Foreseeable Future Development

Besides answers to research questions which will be summarized in Conclusion, what is the take-home message of this project? Perhaps that with studies of parasitic manipulation of human hosts and the deteriorating effects this manipulation can have on mental health, we are approaching an extremely wide multidisciplinary field, on which we have to cover everything from parasitology to psychiatry and everything in between, including human behavior and personality traits as well as specific methodology employed in testing. Everything can have an effect: personality traits, sex hormones, lipid metabolism, genetics, prenatal infections, postnatal infections, etc. All of these and many more can affect the final measures of changes in prepulse inhibition of startle reaction. Which is why studies of PPI must be completed by studies of these phenomena on similar populations and effort to understand real connections and real causes of observed effects.

Since the research in effects of latent toxoplasmosis (with and without interaction with the diagnosis of schizophrenia) on startle reactions, prepulse inhibition thereof, as well as on startle-modified reaction times, is at its very beginning, we had to cover also many by-standing topics during my doctoral project, and did not get to study the phenomenon as directly as we would like to. However, we have conducted several experiments which brought us important starting points for the next research development, direct results on difference between latent toxoplasmosis and reaction times and their modifications, lot of yet unpublished data both directly an indirectly related to the thesis' topic, and new ideas of what to focus on next, how, and why. So where to go now?

First, we have a lot of unpublished data in various stage of processing. Computer-administered tests for acoustic startle with and without prepulse modified reaction times as well as visual reaction times modified with distracting weaker stimuli were distributed to adepts of military service. We have collected usable data from about 3,000 individual tests. These data are now preprocessed, connected with serological assays results, and we are

discussing the best approach to analysis. Data from individually administered tests of prepulse inhibition of acoustic startle reaction were collected on low hundreds of volunteers recruited mostly from our university students. We are now developing a biosignals-decoding script that will enable us to analyze the results. Multiple sets of data collected as side projects during these experiments are being worked on right now, with the potential of several interesting publications.

Second, developing our own variants of experimental software to measure StartReact experiments, visual reaction times modified by weak visual stimuli, a computer-administered variant of Stroop test, as well as starting the community of volunteers under the label "Pokusní králíci" helps us develop further experiments.

Third, understanding the limitations of the published studies enable us to design new experiments using different populations (to overcome selection bias), different methods (RhD genotyping instead of testing of phenotype), or different approaches perhaps capable of indicating (or dismissing) causation where it was only suspected.

Our next step is, of course, the analysis and publication of collected data. However, we believe that more experiments should be done regarding possible infectious (parasitic) causes of changes in PPI in healthy individuals, healthy individuals in risk for development of schizophrenia, and schizophrenic patients, since the theory (E. Fuller Torrey & Yolken, 2019), previous research (Pearce et al., 2013), as well as results of our own (Jaroslav Flegr et al., 2018; Příplatová et al., 2014) indicate there really a lot more to be learned. I hope both our research group as well as other laboratories will take up this challenge and perhaps help future generations to deal with some of the mental health issues.

6 Conclusion

The study of changes in reaction times, startle reactions, and prepulse modifications of startle reaction affected by latent infection with a coccidian parasite *Toxoplasma gondii* as well as of the connections between latent

toxoplasmosis and schizophrenia is a work in progress; and, as explained in the previous chapter, probably stays so in future decades. That being said, I think that my doctoral project brought new knowledge into the subject, successfully answered the answerable ones of my initial questions, and can be therefore satisfactorily concluded. What are the most important outputs of the project in the context of the initial research questions?

0. The questions not asked – findings discovered during the latent toxoplasmosis studies, but without direct connections with the thesis' topic – brought two interesting results, the first one being that society's free-riders might be especially interested in punishment of the most altruistic individuals, thus lowering the overall gain of the society. The second finding showed multiple correlations between psychological profiles of men and women and their cortisol and sex hormone levels.
1. In addition to previous studies of our laboratory and others, we have shown new effects of toxoplasmosis on human psychological profile and performance: (a) The grip test and weight-holding test shown negative effects of latent toxoplasmosis with positive RhD phenotype playing a protective role against the effect. (b) We have also found significantly higher levels of extraversion and lower level of conscientiousness in *Toxoplasma*-infected men and women when measured with the NEO-PI-R questionnaire. Moreover, the conscientiousness factor correlated negatively with the length of infection estimated by the decrease in antibody levels, which suggests (albeit not prove) the infection to be the cause of the personality changes and not vice versa.
2. The computer-administered tests of prepulse modification of startle reaction have not shown any significant effect of latent toxoplasmosis on startle itself.
3. It has, however, shown different sensitivity of *Toxoplasma*-infected and *Toxoplasma*-free individuals to the prepulse modifications of reaction times.

4. The experiment has shown that latent toxoplasmosis might be indeed responsible for changes in sensorimotor gating in the processing of startle signals in schizophrenia patients infected with *T. gondii*.

5. As of now, our understanding – based on interpretation of available data, NOT by any means on experimentally or clinically captured effects; as mentioned in the Introduction, this question is yet beyond our experimental reach, and is mentioned and discussed as a distant research goal, with which on mind we are doing most of the current research – is that an infection with *Toxoplasma gondii* can, in predisposed individuals, contribute to manifestation of schizophrenia later in life and worsen some of the symptoms, or perhaps contribute to manifestation of a separate, schizophrenia-like mental illness. We believe that some of the symptoms traditionally attributed to schizophrenia are at least partially caused by the presence of the studied parasite in human tissues and can manifest – expectedly less pronounced – similar symptoms in healthy (i.e., non-schizophrenic) population. Each experiment focused on testing the presence of schizophrenia-like symptoms in non-schizophrenic population with serologically determined latent toxoplasmosis helps establish the bigger picture of differences in quality (as opposed to quantity) of changes in schizophrenics and *Toxoplasma*-infected non-schizophrenic individuals. This might, subsequently, help to focus our attention toward the specific regions of human neurobiology, where the parasitic manipulation contributes to the development of mental illness. And there, perhaps, we might find inspiration for how to fight it. It is in this manner, that the response to the previous question helps, at least a little bit.

And with this slightly optimistic and eager-for-future tone, let's end.

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