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Examiner's Report on habilitation thesis:

RNDr. Michal Vinkler, PhD. **Evolution of Diversity in Avian Innate Immunity**

One of the most famous quotes of Theodosius Dobzansky, a key author of the Synthetic Theory of *Evolution,* who made the first significant synthesis of Charles Darwin's theory of evolution with Gregor Mendel's theory of genetics claimed, "...nothing in biology makes sense except in the light of evolution...". It seems that the scientific path of Dr. Vinkler represents a logical continuation of this thought with the benefit of knowledge of molecular composition in our genetic material and computer analysis that is capable of correlating changes in DNA with evolutionary selection strategies such as the survival from microbial infections. More specifically, the habilitation thesis of Dr. Vinkler represents approximately a 10 year long scientific investigation and his personal journey dedicated to the deeper understanding of evolutionary processes which guide the diversity of Innate immune receptors, the relationship between the health status of birds and its phenotypic manifestation in terms of ornamentation, as well as the correlative nature between the blood cellular composition, inflammatory response and such ornamentation.

It is necessary to emphasize that the ideological premise of such complex investigation is not only based on a relatively new experimental platform which was established by Dr. Vinkler using his favorite model bird species, but it also stems from a recently established paradigm concerning the indispensible role of innate immune receptors in regulation of immune responses in all higher organisms. Specifically, the existence of such receptors, predicted in 1989 by Charles Janeway, was experimentally shown in 1997, when the first human Toll-like receptor (TLR4) was cloned and functionally characterized. From that time, hundreds of different TLRs from a plethora of species were sequenced. In addition, several other types of innate immune receptors were distinguished and their function scrutinized in detail. It is now well established that TLRs, being positioned at the interface between the surrounding environment and our own immune "self", are the main innate immune receptors to sense microbial constituents and the first ones which inform our immune system about the presence of infection. As they often directly interact with and bind the microbe, these receptors are under a strong selective pressure to provide efficient protection of the host.

In my view, the idea of Dr. Vinkler to utilize TLRs for the assessment of their diversity in evolutionary processes which are functionally linked to the resistance of infection and survival, is indeed a genuine one. There are several other considerations which make Vinkler's research very attractive, respectful, and of high scientific and intellectual quality:

a./ Dr. Vinkler and his team were among the first to clone TLRs from selected avian species and gain an initial insight into their diversity. In this endevour, they went a step further using "in silico" analysis, computer simulation, and 3D-structure analysis. Utilizing these tools they attempted deeper analysis regarding how distinct mutations in selected TLRs correlate potential binding affinities to their cognate ligands. It is important to accentuate this methodological achievement, since such correlative and structure-function analyses begin to provide insight into the causative relationship between intra- and interspecific variation in avian TLRs and infection.

b./ It is necessary to emphasize that papers published from Dr. Vinkler's studies are at the forefront of the field of evolution and diversity. Notably, inspite of the fact that the field of evolution is not currently considered to be mainstream, several papers have been published in international and impacted journals, such as Molecular Biology and Evolution (IF=14.797), Frontiers in Immunology (IF=4,716) and Scientific Reports (IF=4,122), to mention just a few. In this regard, this body of work represents an indispensable addition to scientific literature publically available on this topic worldwide.

c./ Dr. Vinkler takes advantage of various avain model organisms which have not been previously recognized and used in rigorous scientific research. The analysis of their immune systems requires the preparation of many different reagents and analytes which are needed for cytometric analysis, gene expression, and computer analysis. These specific requirements, together in combination with extensive field work make such research more difficult and tedious but very unique and of high importance.

Since I feel that the conclusions of presented studies are very important and strong, I would like to use this opportunity to ask following evolution-related questions:

-there is a general consensus that LPS does not bind TLR4 directly but rather to MD2 which then makes contact to TLR4 and allows its dimerization and signaling. If so, and as the result of such binding of LPS, would not genetic variability be manifested preferentially in MD2 than in TLR4? And if so, what does the change in surface charges among various avian and mammalian TLR4 genes mean, i.e. if it it not selected for a direct binding of LPS, why does it change so dramatically? What does it tell us about the evolution of receptor structures and their function? A similar type of question concerns the comment in your article (paper 5, p85) suggesting that avian TLR4, in respect to arginine 393, is identical to equine but distinct from human TLR4. In this respect, what is the type of thinking of evolutionists to such interspecies variation or similarity? Does it really tell us something important? How does MD2 fit in the paradigm concerning the relationship between the variability and functionality of TLRs and their ligands?

-If microbes indeed enforce the variability of TLRs, which ones are driving this process? Infectious exogenous ones or those forming our microbiota? The exogenous microbes do infect us, the latter protects us. Which of these two alternatives would take precedence for driving this evolutionary process of diversification for better function of TLRs?

In summary, Dr. Vinkler has chosen his own path of scientific persuit, one which is very exciting, but also less "travelled" and in some aspects, with an uncertain ending. He examines TLRs from a completely different angle, one which matters the most as it elucidates and defines how TLR evolutionary diversity relates to their immunological performance. It is also obvious that Dr. Vinkler put together a very proficient and competitive research team, established a collaborative network, and dedicated a lot of time to educate his students. Undoubtly, his work in this field has pushed experimental boundaries of evolutionary ecology a step further. Given the quality of Dr. Vinkler's work, I fully recommend this habilitation thesis to be accepted by the relevant committees and counsels as the fulfilment of the requirement for awarding the relevant degree.

Dominik Filipp