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Doctoral Dissertation

A Comprehensive Understanding of Aggressive Behavior at Molecular Level Using Fish *Betta splendens*

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Publications

Trieu-Duc Vu, Yuki Iwasaki, Shuji Shigenobu, Akiko Maruko, Kenshiro Oshima, Erica Iioka, Chao-Li Huang, Takashi Abe, Satoshi Tamaki, Yi-Wen Lin, Chih-Kuan Chen, Mei-Yeh Lu, Masaru Hojo, Hao-Ven Wang, Shun-Fen Tzeng, Hao-Jen Huang, Akio Kanai, Takashi Gojobori, Tzen-Yuh Chiang, H. Sunny Sun, Wen-Hsiung Li, Norihiro Okada. Behavioral and brain-transcriptomic synchronization between the two opponents of a fighting pair of the fish *Betta splendens*. PLoS genetics. 2020 Jun 17;16(6): e1008831.

Trieu-Duc Vu, Kenshiro Oshima, Kenya Matsumura, Yuki Iwasaki, Ming-Tzu Chiu, Masato Nikaido, Norihiro Okada. Alternative splicing plays key roles in response to stress across different stages of fighting in the fish *Betta splendens*. BMC Supplements. (In press, with minor revision process)

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Conference presentation

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Trieu-Duc Vu, Yuki Iwasaki, Norihiro Okada. Neurotranscriptome profiling towards understanding the neurogenomic state of aggression in the fish *Betta splendens.* The 42th Annual Meeting of Molecular Biology Society of Japan, December, 2019, Tokyo.

Trieu-Duc Vu, Kenshiro Oshima, Yuki Iwasaki, Ming-Tzu Chiu, Masato Nikaido, Norihiro Okada. Alternative splicing plays key roles in response to stress across different stages of fighting in the fish *Betta splendens*. The 19th Asia Pacific Bioinformatics Conference, Tainan, Taiwan. February, 2021.

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Abstract

Aggressive behavior is an evolutionary conserved phenomenon and wide-spread among animal kingdom. Using aggression, animals of many species establish dominance hierarchy to regulate social interactions. Previous studies on dominance using fish mainly centered on the differences in brain gene expression between dominants and subordinates after their fighting interactions have ended. Thus, information on how brain gene expression changes according to each stage of aggression generation across different stages of fighting remains to be understood. This study aims to fill the gap by evaluating brain gene expression changes across different fighting stages in males of the fish *Betta splendens* using RNA-seq approach. I discover that brain gene expression between two opponents within a fighting pair changed in a synchrony fashion during fighting, reflecting mutual assessment between them at molecular level. The genes mediated this process are associated with ion transport, signal transduction, and long-term memory. Additionally, the transcriptomic synchronization pattern during fighting was gradually lost after fighting, and brain gene activity reduced to minimum leading to the emergence of a unique neurogenomic state, which is considered to be an energy-saving strategy to adapt to energy depletion among all individuals after fighting. Furthermore, a higher impact of losing in comparison with winning in the nerogenomic state of the individuals was found as indicated by a greater number of long-term memory and autism-risk genes in loser relative to winner. Interestingly, alternative splicing was found to play key roles in response to oxygen stress across the fighting stages among all individuals. Altogether, the findings from this thesis sufficiently elucidate the genes and molecular events underlying aggression generation across different stages of fighting in *B. splendens*, and provide comprehensive information for future research on aggression in vertebrates at molecular levels

List of abbreviations

Abbreviation	Full form
B	**B**efore fighting
D20	**D**uring fighting for **20** minutes
D60	**D**uring fighting for **60** minutes
A0	Just **a**fter end of fighting (once winner chasing loser)
W0	**W**inner collected at just after end of fighting
L0	**L**oser collected at just after end of fighting
A30	**30**-min after end of fighting (after A0)
W30	**W**inner collected **30-min** after just end of fighting
L30	**L**oser collected **30-min** after just end of fighting
TMM	The normalized expression levels of genes
DEG	**D**ifferentially **E**xpressed **G**enes
FDR	**F**alse **D**iscovery **R**ate
Log FC	Log **F**old **C**hange
GoM	**G**rade **o**f **M**embership
LDA	**L**inear **D**iscriminant **A**nalysis
Cv	**C**ovariance of gene expression **v**alue
AS	**A**lternative **S**plicing
DAS	**D**ifferential **A**lternative **S**plicing
ES	**E**xon **S**kipping
IR	**I**ntron **R**etention
A3SS	**A**lternative to **3′*S***plice **S**ite
A5SS	**A**lternative to **5′ S**plice Site
MES	**M**utually **E**xon **S**kipping
IJC	**I**nclusion **J**unction **C**ounts
SJC	**S**kipping **J**unction **C**ounts
IEG	**I**mmediate **E**arly-expressed **G**ene

CHAPTER 1: GENERAL INTRODUCTION

1.1. Background and motivation

Animal behavior displays observed in many organisms generally result from the integration of both internal and external cues (1). Scientific efforts to understand animal behavior had earned Karl von Frisch, Konrad Lorenz, and Nokolaas Tinbergen a shared Nobel Prize in 1973 for elucidating the communication mechanisms amongst animals about a food source. Especially, for their discovery of the information about the location of resources referred to as “dance language” in honey bee (Apis sp.) (2). Since then, understanding what molecular processes are responsible for behavior have fascinated researchers for a long period of time, and it still remains to be understood**.**

The most lasting contribution that inspires researchers of animal behavior is the framework proposed by Nokolaas Tinbergen as known as Tinbergen's four questions, which aimed to answer the question: Why animals behave in the way they do (3). He argued that only by understanding the contributions of both proximate mechanism (causation & development) and ultimate mechanism (evolution & adaptation) to an animal’s behavior, can we begin to get a complete understanding of why and how an animal behaves in the way it does (4). Much of the previous knowledge based on this approach came from the studies of honey bee.

It has been reported that throughout honeybee’s life, individuals of different social status i.e., soldiers, foragers, and workers commonly express different sets of behaviors (as known as behavioral states) that match their competitive ability (5). Thus, individual bees must be able to switch their social status, and this should be mediated by changes of gene expression in the brain that led to distinct transcriptome profiles across the social behavior network (neurogenomic states) (6). Gene. E. Robinson’s lab was the first to show that social information causes mass changes in brain gene expression in honeybee, and provided a frame work to map neurogenomic state onto behavioral state (7). Following this, several studies have conducted to establish these maps in various animals such as songbirds, fish, and mice (8).

Generally speaking, two influence vectors have been widely used to describe the relationship between neurogenomic state and behavioral state, including (i) social information alters brain gene expression to influence behavior and (ii) genetic variations lead to changes in behavior (9). The first demonstrations of the first influence vector focused on immediate early genes (IEGs), and one of these that has proven especially useful is *egr1* in songbird (*Taeniopygia guttata*). It has been shown that the singing of another male bird induces e*gr1* expression in a specific subregion of the auditory forebrain devoted to hearing (10). The well-known demonstration of the second influence vector has been reported in a study that compared two Drosophila species (*D. melanogaster* vs. *D. simulans*), which led to the identification of a specific difference in the *period* gene correlated with temporal differences in song structure. Transferring a small piece of the *period* gene from *D. melanogaster* to *D. simulans* caused the *D. melanogaster* males to produce the *D. simulans* call, rather than the *D. melanogaster* call (11).

In recent years, aggressive behavior has been drawn massive attention among researchers. It is important for regulating social interaction and ensuring the survival of population across animal kingdom (12). Several attempts have been made to quantify the dynamic nature of brain gene expression in relation to aggressive behavior in various animals such as song-bird (13), honeybee (14), mice (15), and other animals. Recently, fish have become one of the most popular vertebrate models for dominance research because they possess easily observed dominance behaviors (16). Hans A. Hofmann’s lab was the first to develop many of the genomic resources for understanding neurogenomic state and behavior state using cichlid fish (17). Then, several related studies using different fish have emerged. For instance, studies examined the activity of genes in established dominant and subordinate males as well as females in cichlid fish (18), zebrafish (19), stickleback fish (20), and other fish.

However, the above fish fighting systems e.g., cichlid fish, zebrafish, or stickleback fish appear to be suitable for understanding the differences in brain gene expression between ultimate winners/losers or dominants/subordinates because their fighting durations are relatively short i.e., 15 to 30 minutes (21-23). Further, most of these studies using microarray technique which are limited by the need for prior knowledge of sequence information and poor detection of low abundant transcripts, allelic variation, and splice variants (21, 24). Thus, the understanding about genes and molecular events underlying the full range of volitional behavior expressed by one opponent toward the other across a longer period of time remains needed. Noticeably, Siamese fighting fish, *Betta splendens*, are notorious for their aggressiveness. Males of this species have stereotypical social displays that are well documented (25), and individual fights can last for more than an hour. Therefore, this fish provides an excellent platform for investigating the relationship between brain gene expression and aggressive behavior given the remarkable behavioral repertoire that they expressed.

We have now detailed knowledge of the two physical substrates responsible for behaviors i.e., the brain and the genome. We also have strong advances in genomic approaches such as RNA-seq, which holds great promise for elucidating the molecular basis of aggressive behavior (26). Together, these have made it possible for us to aim for a comprehensive understanding of aggressive behavior at the gene expression level using *B. splendens*. Therefore, in this PhD project, I provide a linked series of studies that examined changes in *B. splendens* brain transcriptomes associated with experimentally induced changes in behavioral state across different stages of fighting, with the respect to differentially gene expression level, using whole-brain.

Several pairs of male's fish were allowed to fight under different durations namely non-fighting (B), during fighting (D20 & D60), and after fighting (A0 & A30). Two main analyses have been performed, (i) for behavioral measurements, three behaviors including bite/strike, surface-breathing, and mouth-locking were examined in detail in terms of durations and frequencies; and (ii) for brain transcriptome analyses, the brains of fighting opponents in each fighting group were isolated and subsequently used for RNA sequencing, then differentially expressed genes, alternative splicing events, and gene enrichment analyses were carried out.

The findings from this PhD thesis demonstrate that brain gene expression is closely linked with behavior responses, in which changes in brain gene expression mediate those in behavior over multiple timescales. Together, this study will provide comprehensive understanding and a framework to study aggressive behavior at gene expression level in vertebrates.

1.2. Study objective

(1) What is the relationship between brain-transcriptomic activity in two individuals that are engaged in fighting interactions? And how does this relationship change across different timescales to facilitate fighting interactions?

(2) How does energy depletion affect the changes that occur between transcriptomes of fighting opponents after their conflicts have resolved?

(3) Whether are alternative splicing events involved in response to stress across different stages of fighting interaction in this fish, especially with the respect to winners and losers after fighting?

1.3. Thesis outline

Chapter 1: General introduction

Chapter 2: Behavioral and brain- transcriptomic synchronization between the two opponents of a fighting pair of the fish *Betta splendens*.

Chapter 3: A unique neurogenomic state emerges after aggressive confrontations in males of the fish *Betta splendens*.

Chapter 4: Alternative splicing plays key roles in response to stress across different stages of fighting in the fish *Betta splendens*.

Chapter 5: General discussion