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Molecular Biomarkers of Welfare in Poultry

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Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

— Marie Curie

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Abbreviations

3'UTR	3' Untranslated region
ACTH	Adrenocorticotropic hormone
AGP	α1-acid glycoprotein
ANS	Autonomic nervous system
APP	Acute phase protein
APR	Acute phase response
AVP	Arginine vasopressin
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
DOA	Death on arrival
eIF2α	Eukaryotic translation initiation factor 2α
FB	Fibrinogen
GI	Gastrointestinal
HE	Haemorrhagic enteritis
HEV	Haemorrhagic enteritis virus
Hp	Haptoglobin
HPA	Hypothalamic-pituitary-adrenal
IBV	Infectious bronchitis virus
IFN	Type I interferon
IL-1	Interleukin-1
IL-6	Interleukin-6
LPS	Lipopolysaccharides
MAP	Multiple Antigen Presentation
miRISC	miRNA-induced silencing complex
miRNA	Micro-RNA
mRNA	Messenger RNA
MW	Molecular weight
PABP	Poly(A) binding protein
Pig-MAP	Pig major acute phase protein
RNAPII	RNA polymerase II

ROS	Reactive oxygen species
RT	Room temperature
SAA	Serum amyloid A
SG	Stress granule
SNS	Sympathetic nervous system
SRCR	Scavenger receptor cystein-rich
TNF-α	Tumor necrosis factor-alpha
α-MSH	α-melanocyte stimulating hormone

Abstract

The thesis aims to explore the relationship between stress and immune response in poultry, focusing on acute phase proteins (APPs) and microRNAs (miRNAs) as welfare biomarkers for transport- and disease-related stress. We worked on two poultry species, namely turkey and chicken. Turkey (*Meleagris gallopavo*) was studied in order to provide new information in this species, which is poorly characterised for what concerns immunity and welfare. Existing studies related with avian species are mostly carried out on chicken (*Gallus gallus*), but there is still lacking information that needs to be addressed. In fact, we have complemented information, already available in chicken, describing the extrahepatic expression of APPs.

The effects of transport-related stress on liver and adipose tissue gene expression of four APPs, namely α1-acid glycoprotein (AGP), Serum Amyloid A (SAA), PIT54 (Haptoglobin-like) and C-Reactive Protein (CRP) was investigated. The expression of AGP and CRP mRNA was found to be increased in liver and adipose tissue from birds after road transport. The presence of AGP protein was also confirmed. In addition, the expression of the same APPs was studied under a disease-related stress, caused by a heamorrhagic enteritis virus (HEV), which is a stressful disease for turkeys. As preliminary results, the expression of AGP, PIT54 and SAA mRNA was found to be increased in liver and adipose tissue from HEV-affected birds, while CRP expression was decreased in jejunum from HEV-infected birds. These results showed that road transport and HEV disease induce the mRNA expression of AGP, PIT54, SAA and CRP, suggesting their use as welfare biomarkers in turkey species. Although APPs are produced mainly by liver, extrahepatic production has been described in many species but not in poultry. The expression and location of five APPs, namely AGP, SAA, PIT54, CRP and OVT, was studied in 20 different tissues from healthy chickens. SAA, OVT and CRP mRNA expression was particularly higher in gastrointestinal tract, respiratory system and lymphatic system. AGP and OVT proteins were detected in different organs. Moreover, the expression of five miRNAs, namely miR-22, miR-155-5p, miR-181a-3p, miR-204 and miR-365-3p, was analyzed under transport-related stress in turkeys. The expression levels of miR-22, miR-155 and miR-365 were increased, suggesting they can be used to discriminate stressed- and unstressed animals. In order to better characterize the disease and to investigate a possible source of biomarkers, the intestinal proteome of turkeys was also determined to study the effects of HEV disease on intestinal mucosa. A total of 523 proteins were identified by SWATH-MS strategy between healthy and

HEV-affected birds. A number of 64 of them were found to be differentially expressed. The effective presence of fifteen proteins related with immune system was also validated by mRNA expression. These findings can help us to better understand the effect of HEV in turkeys.

Riassunto

La tesi si propone di esplorare il rapporto tra stress e risposta immunitaria nel pollame, concentrandosi su proteine di fase acuta (APPs) e microRNA (miRNAs) come biomarcatori per il transporto e le malattie correlate a stress. Abbiamo lavorato su due specie di pollame, tacchino e pollo. Il tacchino (*Meleagris gallopavo*) è stato studiato in modo da fornire nuove informazioni in merito a questa specie, che è scarsamente caratterizza per quanto riguarda l'immunità e il benessere. Gli studi esistenti relativi alle specie aviarie sono stati effettuati sul pollo (*Gallus gallus*) e per completare le informazioni disponibili in letteratura, abbiamo descritto l'espressione extraepatica delle APPs in questa specie.

Sono stati indagati gli effetti dello stress correlati al trasporto, studiando l'espressione genica di quattro APPs nel fegato e nel tessuto adiposo: α1-acid glycoprotein (AGP), Serum Amyloid A (SAA), PIT54 (Haptoglobin-like) and C-Reactive Protein (CRP). L'espressione di mRNA di AGP e CRP è risultata essere aumentata nel fegato e nel tessuto adiposo degli uccelli dopo il trasporto su strada. È stata confermata la presenza della proteina AGP. Inoltre, l'espressione delle stesse APPs è stata studiata anche nella patologia causata dal virus della enterite emorragica (HEV) e associata allo stress. Come risultati preliminari, l'espressione di mRNA di AGP, PIT54 e SAA era aumentata nel fegato e nel tessuto adiposo di uccelli HEV positivi; al contrario, l'espressione di CRP era ridotta nel digiuno di uccelli HEV-positivi. Questi risultati hanno dimostrato che il trasporto su strada e la malattia HEV inducono l'espressione di mRNA di AGP, PIT54, SAA e CRP, suggerendo il loro uso come biomarcatori del welfare nel tacchino. Sebbene le APPs siano prodotte principalmente dal fegato, la produzione extraepatica è stata descritta in molte specie, ma non nel pollame. L'espressione e la localizzazione delle cinque APPs (AGP, SAA, PIT54, CRP e OVT) è stata studiata in 20 diversi tessuti di polli sani. L'espressione di mRNA di SAA, OVT e CRP era particolarmente elevata nel tratto gastrointestinale, nelle vie respiratorie e nel sistema linfatico. Le proteine AGP e OVT sono state trovate in diversi organi. Inoltre, l'espressione di cinque miRNAs (miR-22, miR-155-5p, miR-181a-3p, miR-204 e miR-365-3p) è stata studiata nei tacchini in condizioni di stress legate al trasporto. Il livelli di espressione di miR-22, miR-155 e miR-365 erano aumentati, suggerendo il loro utilizzo per discriminare animali stressati e non stressati. Per caratterizzare meglio la malattia e individuare una possibile fonte di biomarcatori, è stato determinato anche il proteoma intestinale dei tacchini per studiare gli effetti della malattia HEV sulla mucosa intestinale. Sono state identificate 523

proteine utilizzando la strategia di SWATH-MS confrontando tacchini sani e malati e 64 proteine sono risultate essere differenzialmente espresse. È stata quindi validata l'espressione genica di quindici proteine correlate con il sistema immunitario. Questi risultati possono aiutare a comprendere meglio l'effetto di HEV nei tacchini.

PhD thesis rationale

The main aim of this thesis is to identify biomarkers to evaluate animal welfare and food quality in poultry species. A fundamental issue of farm animal welfare is to keep animals clinically healthy, without diseases or stress, in order to produce safe and high quality food. This topic is highly relevant for both Governments and food industries worldwide, being directly linked to public health and animal well-being.

It is known that domestic farm animals, in modern production, have to deal with several stressors during their life such as weighting, vaccination and a lot of environmental and management procedures such as road transport. These factors can affect the behavioural, physiological and performance responses of animals. Road transport has been identified as one of the most stressful events for animals, especially for poultry. The task of monitoring animals' welfare is challenging. Reliable biomarkers, with few exceptions, are virtually absent, and most of the measurements to assess animal welfare still rely on animal behaviours features. The potential use of molecular biomarkers seems to be a good way to control if the animals are healthy and ensure their welfare.

The expression of acute phase proteins (APPs) has been shown to depend on inflammatory status but also on different diets and stress levels, in different domestic species. APPs belong to a large family of structurally unrelated proteins that are produced as part of the systemic inflammatory response and play a role in modulating innate immunity and scavenging inflammatory by-products. Although APPs are produced mainly in liver, they can be synthesized also in other tissues. APP are commonly used to monitor welfare in animals.

MicroRNAs (miRNAs) have also been identified in humans as biomarkers for a high number of diseases and biological processes. Research in animal science is lagging behind. The modulation of miRNAs expression is also an early event in stressful conditions. MiRNAs are small regulatory non-coding RNAs that modulate gene expression and play key roles in immune defenses and inflammation. Moreover, miRNAs are non-invasive biomarkers and can be easily extracted from extracellular body fluids.

In the last years, many knowledge has emerged in farm animals, but not in all the species (e.g. turkey). Although turkey (*Meleagris gallopavo*) farming is economically relevant, no

information regarding APPs is available, as compared with other poultry species, and there is very limited information on the welfare of turkeys during transport. From a clinical perspective, the use of APPs as biomarkers for stress in poultry has not been adequately addressed so far. The identification of suitable biomarkers of stress and disease is of paramount importance, with the final aim to reduce as much as possible the stress level in animals. Moreover, the APP expression in poultry is usually linked to a pathological status and a widespread expression study has never been done so far.

In this study, we focused on two models of stress: the stress related to management (e.g. stress due to transport) and the stress related to disease (e.g. the stress due to an extremely stressful disease in turkeys, namely viral heamorrhagic enteritis).

We also integrated the state of art of the knowledge about APPs in poultry with new information, derived from APP expression in a model of stress due to transport and characterization of local APP production in healthy chicken tissues, to assess where APP are produced and their tissue expression amount. The major APPs in turkey during transport were identified and their potential use as indicators of transport-related stress was investigated. As a preliminary study, the mRNA expression of APPs in a model of disease due to heamorrhagic enteritis virus infection was also investigated in turkeys. The potential use of microRNA as welfare biomarkers in a similar transport model was assessed. Some miRNAs related with stressful events and immune response were evaluated as non-invasive markers of transport stress.

In the first part of the thesis, extrahepatic expression of five APPs – AGP, SAA, PIT54, CRP and OVT, was studied in a large panel of 20 chicken tissues. Avian acute phase response and APP studies only focus on inflammation and liver is considered the major source of APP, although extrahepatic expression has been described before for other animal species. We tried to understand how much the other tissues could contribute for the innate and adaptive immunity by producing APPs in healthy conditions. Immunolocalization was also identified using available antibodies. Results of this study are reported in Chapter 5.1.

In the second part of the thesis, four APPs, previously found to be overexpressed in chicken – AGP, SAA, PIT54 and CRP, were investigated under a model of transport-related stress in turkeys, in order to determine whether mRNA expression in liver and adipose tissue was increased after a stressful condition. Immunolocalization was also identified using available

antibodies. In the case of absence of antibodies, we tried to produce them. In particular, the expression of the already known biomarkers, such as APPs, is presented in Chaper 5.2.

In the third part of the thesis, innovative biomarkers (miRNAs) were studied under a model of transport-related stress in turkeys, in order to determine if they can be considered as welfare biomarkers. The serum levels of five miRNAs – miR-22, miR-155, miR-181a, miR-204 and miR-365, were quantified in order to investigate whether transport-related stress modifies the expression of circulating miRNAs and if there is a potential scope in their use as welfare biomarkers. The expression of the novel biomarkers (miRNAs) is presented in Chaper 5.3.

In the fourth part of the thesis, the intestinal proteome of turkey was characterized. Gastrointestinal (GI) tract is regarded as one essential organ for nutrient absorption, pathogen prevention and immune response in poultry. Virus infections of the GI tract occur regularly in poultry and impacts negatively the bird welfare. However, no information about turkey intestine proteome is available. The proteome from healthy and haemorrhagic enteritis (HE) virus-infected animals was then determined, focusing again on the local expression of APPs rather then the systemic one. Haemorrhagic enteritis was identified as a model for disease because it affects particularly turkeys, and it is geographically widespread and endemic in many areas. Turkey intestine proteome is described in Chapter 5.4.

In the fifth part of the thesis, four APPs – AGP, SAA, PIT54 and CRP, were investigated under a model of HE virus infection in turkeys, in order to determine whether mRNA expression in liver, adipose tissue and small intestine (jejunum) was increased in sick birds. Preliminary results are presented in Chaper 5.5.

Chapter 1. Welfare, stress and road transport

1.1. Animal welfare

Welfare is not only the absence of cruelty and suffering. It is a multidimensional concept that embraces three animals' states: physical, mental and natural state (Figure 1), as well as high levels of biological perfomance and the potential for animals to have positive experience (Fraser et al., 1997).

Physical state

McGlone (1993) stated that welfare is poor when physiological systems are disturbed to the point that survival and/or reproduction is impaired. High mortality rates are indicative of poor welfare. Instead, Broom (1986) have referred to how an animal can cope with its environment. Coping is an essentially reflection of the physical condition, although nowadays mental states are known to contribute to this circumstance. Physical state envisages that the animals are healthy, have high levels of biological performance, successful reproduction rates and can cope with the environment.

Mental state

Mental states is one of the central points in welfare. They are more difficult to understand and are becoming more investigated and explored, especially by scientists. Welfare is strongly related to what the animal feels and fears, even more than health, lack of stress or fitness (Duncan, 1993). Well-being of the animals also involves their capability to have positive experiences and various forms of pleasure (Broom, 1998).

Natural state

Natural state refers to the ability of the animal to fulfil its natural needs and desires. Not being able to cope with its natural needs, the animal feels frustrated and this harms its welfare. Welfare means not only control of pain and suffering, but also considers the fulfilment of the animals' nature (Rollin, 1993).

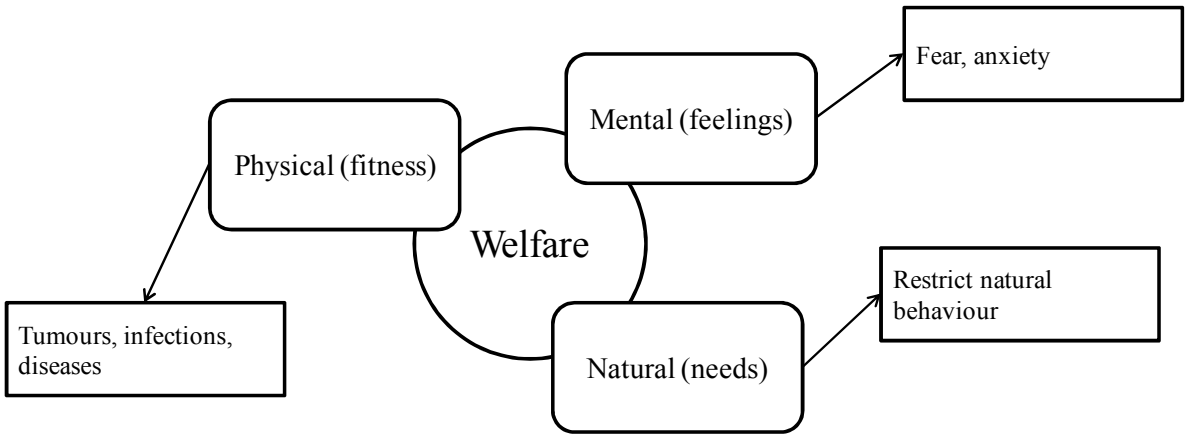

Figure 1. Welfare framework. Three animals' states (physical, mental and natural state) are referred together to describe the welfare concept.

Given this background, the definition of animal welfare is often debated. Welfare concerns the quality of an animals' life and not only how long the life lasts (quality versus quantity). The knowledge about the needs of animals has proposed to give animals' defined "freedoms". The "Five Freedoms", which were originally developed by the UK Farm Animal Welfare Council (FAWC, 1993), are often used as a framework to assess welfare, providing valuable guidance on animal well-being.

The "Five Freedoms" are:

- Freedom from hunger and thirst – access to water and a correct diet to maintain health and vigour;
- Freedom from discomfort – appropriate environment, including shelter and a comfortable resting area;
- Freedom from pain, injury and disease – prevention for diseases or rapid diagnosis and treatment;
- Freedom to express normal behaviour – sufficient space, proper facilities and company of their own species;
- Freedom from fear and stress – proper handling conditions and treatment to avoid mental stress.

The current importance of animal welfare is not only focused on ethics and the belief that animals can suffer, but it has also received a major consideration in meat quality and quantity

(Gispert et al., 2000; Schwartzkopf-Genswein et al., 2012; Terlouw et al., 2008). Healthy and happy animals will provide better animal derived food for humans.

1.2. What is Stress?

Stress occurs when the organism perceives a threat of disruption of homeostasis, although it is an abstract internal state without any clear definition. Stress is a part of life and all organisms have evolved mechanisms to cope with it. When applied to animal production, stress usually indicates a real or perceived perturbation that adversely affects homeostasis or welfare. Two types of stress shall be differentiated. The animal can develop a non-threatening stress response (often referred to as "good stress") or the stress response truly threatens the biological state and has a negative effect on the animals' welfare (referred to as "bad stress" or distress) (NRC, 2008). For this thesis, the term stress will be used in general to refer to a negative experience for the animals' homeostasis.

Stressors are events or threads that can activate any mechanisms or adaptive responses, including behavioural reactions, activation of the hypothalamic-pituitary-adrenal (HPA) axis and adrenal gland, activation of the sympathetic branch of the autonomic nervous system (ANS), secretion of stress hormones (e.g. glucocorticoids), and/or mobilization of the immune system (Minton, 1994). Although a stress response is involved in, at least, one of the above systems, none of them are exclusive to identify a stressful state (Moberg, 2000; Schneiderman et al., 2005). Physiological and behavioural reactions to stress are dependent from the original stressor and it is well established that different animal species and even animals of the same species but different genetic background responds differently to the same stressor to restore its own homeostasis (Hall et al., 1998).

The following list includes some considered stressors:

- Viral or bacterial infections;
- Threat of physical harm;
- Drugs;
- Noises;
- Dark places;
- Climatic changes (intensive cold or heat);
- Restraint;
- Food and water deprivation;
- Pre-mixing and no-familiar social interactions;
- Road transport.

In a response to stress, the body activates behavioural or physiological mechanisms to counter this perturbation and return to its homeostasis. The animal may not always manifest stress with recognizable evident behaviours but, instead, with subclinical pathological changes, sometimes imperceptible.

Moberg (1985) has divided response of animals' stress into three general stages: the recognition of a stressor, the biological defense against the stressor and the consequences of the stress response. Fear and pain are very strong and common causes of stress in livestock and, when suffering from severe stress, animals can develop diseases, fail to reproduce and/or stop to grow up properly. Thus, the condition of a stressed animal is considered atypical and regarded as undesirable (Stott, 1981).

1.3. Biological basis of stress

A stress response begins with the central nervous system (CNS) perceiving a stressor that can be positive or negative (Moberg, 2000). The CNS controls the action of endocrine glands through the release of neurotransmitters (e.g. catecholamines and indoleamines). Specific brain areas are recognized to be involved in the stress response organization. Amygdala, hippocampus and prefrontal cortex are connected with hypothalamus, which modulates the final stress response through activation of HPA and the sympathetic nervous system (SNS) (Mora et al., 2012). Once the CNS perceives a threat, it develops a biological response that consists of combination of the four general biological defense responses (Figure 2):

- behavioural response;
- autonomic nervous system response;
- neuroendocrine response;
- immune response.

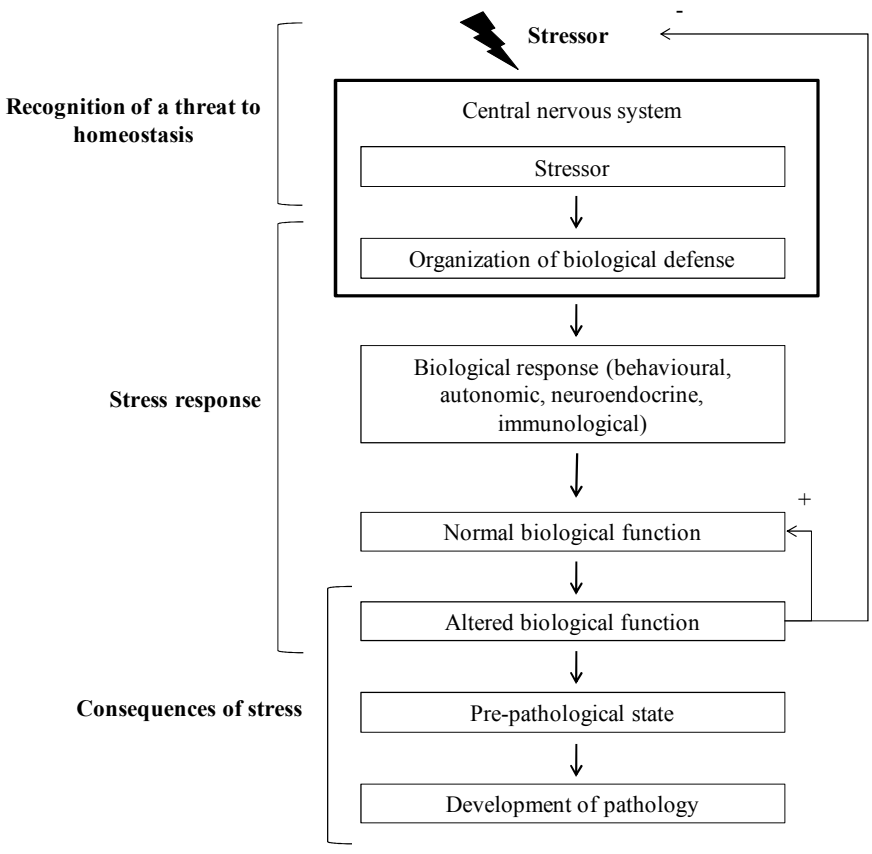

Figure 2. A model of the biological response of animal to stress. Adapted from Moberg (2000).

The HPA axis, often referred to as the "stress response system", is one of the primary endocrine system that reacts to stressors (Chrousos, 2000; Moberg, 2000). In response to a stressful situation, the hypothalamus synthesizes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), which travel to the anterior pituitary and cause the release of adrenocorticotropic hormone (ACTH) into the circulatory system. AVP is considered the second most important modulator of pituitary ACTH secretion (Antoni, 1993). ACTH acts selectively on specific receptors in the adrenal cortex, resulting in the release of steroids by promoting the uptake of cholesterol and its enzymatic conversion to glucocorticoids (cortisol and corticosterone) (Figure 3). In turn, corticosteroids mobilize energy stores in response to the perceived stress and affects a diverse number of biological systems, including the cardiovascular system, gastrointestinal system, exocrine glands and adrenal medulla (Uchoa et al., 2014). Cortisol is the primary glucocorticoid in humans and most mammals, whereas corticosterone is the primary glucocorticoid in the rodents. Glucocorticoids play an important role in gluconeogenesis by stimulating the conversion of glycogen into glucose for energy, and by promoting lipolysis to break fats into usable sources of energy (Brindley and Rollan, 1989). Glucocorticoids also support the stress response by potentiating the synthesis and action of catecholamines released by the adrenal medulla.

When the stressor is removed, glucocorticoids bound to receptors in the hypothalamus and anterior pituitary initiate negative feedback that causes a decrease in the production and release of CRH and ACTH, thus terminating the hormonal response and completing the negative feedback loop (Keller-Wood, 2015).

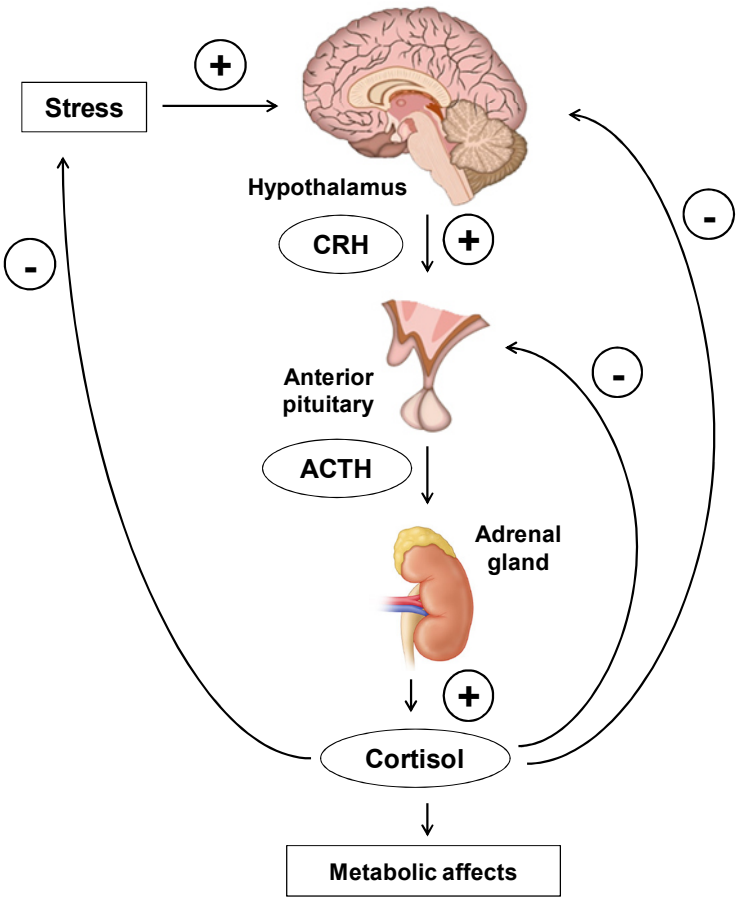

Figure 3. Schematic diagram of the major components of the hypothalamic–pituitary–adrenal (HPA) axis. Adapted from BGD Lecture-Endocrine Histology available at https://embryology.med.unsw.edu.au.

Stressors can also activate the sympathetic division of the ANS (Chrousos, 2000; Moberg, 2000). This system is the basis of Cannon's proposed "flight-or-fight" response during stress (Cannon, 1929). The "flight-or-fight" response (also called acute stress response) is a physiological reaction that occurs in response to a perceived harmful event. Animals react to threats with a general discharge of the SNS, preparing for fighting or fleeing (Figure 4).

Adrenal medulla is part of the sympathetic division of the ANS, working as an endocrine organ and playing a role in the overall regulation of the HPA axis at several levels, including the brain and the pituitary gland. Activation of the sympathetic pathway initiates release of a hormonal cascade from the adrenal medulla that results in the secretion of catecholamines (e.g. epinephrine or adrenaline and norepinephrine or noradrenaline), which act on various target organs and tissues, and stimulate CRH secretion. While both adrenaline and noradrenaline increase alertness

and awareness, adrenaline and the indolamine serotonin seems to be associated more with anxiety and fear (Abelson et al., 1996; Arroyo et al., 2016; Fell et al., 1985).

This activation leads to increased heart rate, blood pressure, breathing, increased mobilization of nutrients, and redistribution of blood flow (Blanc et al., 1991). Some stressors may also increase the activity of the parasympathetic division, affecting both core body temperature and the gastrointestinal system (Goldstein, 2013). However, because the autonomic responses affect very specific biological systems and its effects are of relatively short duration, ANS does not have a significant impact on an animals' long-term welfare.

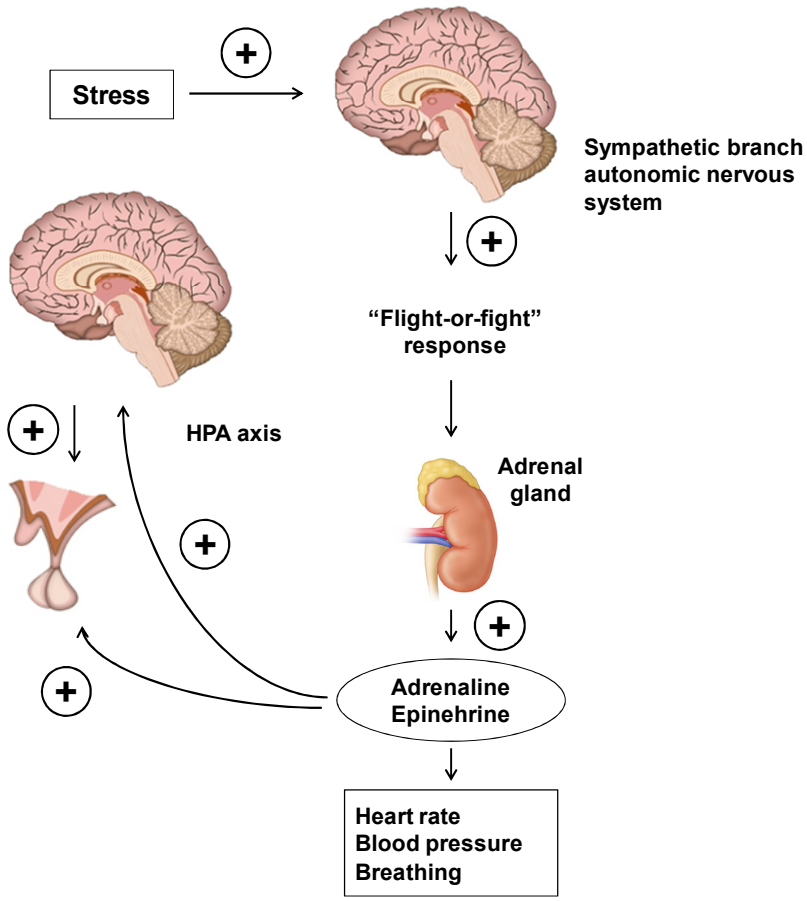

Figure 4. Schematic diagram of the major components of the sympathetic division of the autonomic nervous system (ANS).

Signalling pathways link the brain with the immune system, combining the HPA axis and the immune system activation (Dhabhar, 2009; Nance and Sanders, 2007). During stress, lymphocytes and macrophages are activated and express receptors for a variety of hormones and neurotransmitters, while the spleen and thymus are innervated by the ANS (O'Connor et al., 2003; ThyagaRajan and Priyanka, 2012). Cytokines and other humoral mediators of inflammation have also been demonstrated to act as potent activators of the central stress response. Three main inflammatory cytokines – tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) – are able to enhance the synthesis and secretion of CRH and AVP at the level of the hypothalamus (Bernardini et al., 1990; Naitoh et al., 1988), whereas ACTH secretion have direct effects on glucocorticoids secretion (Figure 5) (Fukata et al., 1989; Salas et al., 1990).

Adaptive immune responses, that involve antigen recognition by T cells, have been shown to be invariably affected during acute stress (Fleshner et al., 1995).

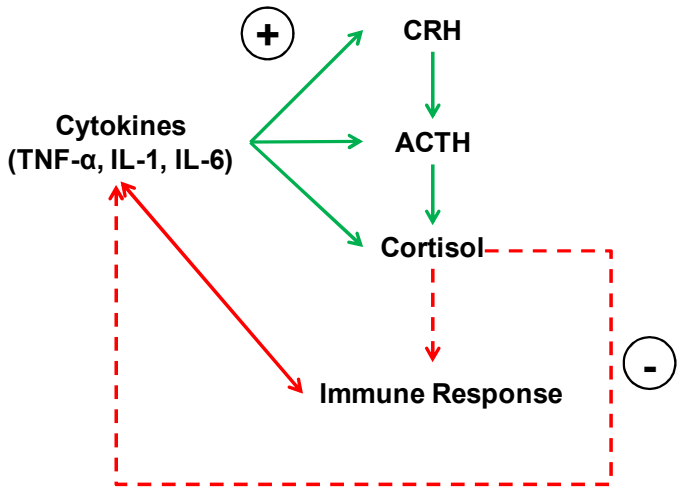

Figure 5. A simplified schematic representation of the interactions between the HPA axis and the immune system. Solid lines represent stimulatory effects and dashed lines represent inhibitory effects.

The activation of the HPA axis has inhibitory effects on the inflammatory immune response. Glucocorticoids act as potent anti-inflammatory and immunosuppressive factors by inhibiting vital functions of the immune cells. They decrease the production of cytokines and other mediators of inflammation (e.g. platelet-activating factor, nitric oxide and prostanoids), induce

cytokine resistance and inhibit the expression of receptors on the surface of immune cells (Chrousos, 1995). Glucocorticoids and catecholamines secreted during stress exert an immunomodulative effect by suppressing the T-helper response, thus protecting the tissues from the potentially destructive actions of immune system (Elenkov et al., 1999).

1.4. Welfare and road transport

Road transport is a necessary practice that livestock encounters during their lifetime. Animals need to be transported for various reasons – for feeding and growth, harvest and breeding purposes, re-stocking or before slaughtering process (Broom, 2005; Schwartzkopf-Genswein et al., 2012).

Road transport is a complex procedure including:

- assembly and loading of animals at their place of origin;
- confinement on a moving or stationary vehicle, sometimes without food, water and rest;
- unloading and lairage at their final destination.

The protection of animals at the time of transport is covered in European Union (EU) by the Council Regulation (EC) No 1/2005 of 22 December 2004. The document information can be found at (http://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:32005R0001). It relates to protecting the animals' welfare during transportation to prevent injuries or unnecessary suffering, and applies to the transport of live vertebrate animals carried out within the EU. When animals are subjected to unusual conditions or circumstances, the welfare of these animals should be taken in account for them to avoid suffering unnecessary discomfort, stress or injuries.

Among the general conditions of transportation, it is recomended that:

- The animals should be adequately prepared for the transport;
- The handling of animals should be carried out by trained and competent people;
- Any methods such as violence, fear, injuries or suffering should be avoid;
- The animals should have access to water, feed and appropriate rest at suitable intervals;
- Transportation to the destination must involve regular checks on the welfare.

The regulation also gives stricter rules for longer journeys and efficient checks at the point of departure and on a random basis thereafter.

If a good welfare associated with the transport of animals is guaranteed, the mortality rates and carcass downgrading are reduced and the financial income increases. In addition, if the general public starts to be aware of animals' welfare concerns during transport, they will take it in account when deciding whether or not to purchase animal products.

The properties of meat that are of major interest to the consumer (e.g. tenderness, juiciness and flavour) are strongly affected by pre-treatments before slaughter. Animals often experience physical and physiological insults when they are transported and handled, decreasing welfare and meanwhile reducing carcass yield and meat quality. Weschenfelder et al. (2012) has published a study evaluating the effects of road transportat practices in pig on animal behaviour, physiological parameters as welfare markers, carcass and meat quality.

In cattle, "bad welfare" lowers the glycogen meat content and consequently elevates pH (Lahucky et al., 1998). Glycogen is required as energy for muscle activity in the animals. In a healthy and non-stressed animal, the glycogen content of the muscle is high. After the animal has been slaughtered, the glycogen in the muscle is converted into lactic acid and the carcass becomes firm. If the animal is stressed before or during slaughter procedures, the glycogen is depleted due to the increase need of energy and the lactic acid level that develops in the meat decreases (Lambert et al., 1998). This will have serious adverse effects on meat quality, since the lactic acid has the effect of retarding the growth of bacteria and contributes to meat properties, such as color, taste and tenderness.

1.4.1. Road transport as stress model

Road transportation for short or long periods can impose stress to animals. Stress responses have been demonstrated in different animal species by, for example, increases in heart rate (Kent and Ewbank, 1986), increased adrenal cortical activity (Ruiz-de-la-Torre et al., 2001), decreased humoral immunity (Machenzie et al., 1997) and increased morbidity and mortality due to infectious diseases in the few weeks following transportation (Chirase et al., 2004; Hartung, 2003).

Road transportation had been demonstrated to be a stressful event prior to slaughter for:

- cattle (Grigor et al., 2004; Schwartzkopf-Genswein et al., 2012);
- bulls (Earley et al., 2013);
- pigs (Piñeiro et al., 2007; Salamano et al., 2008; Schwartzkopf-Genswein et al., 2012);
- poultry (Schwartzkopf-Genswein et al., 2012).

This kind of stress situation may impose to animals both physical and emotional effects in varying degree. An animal may experience emotional stress during transport followed by physical stress (Tarrant, 1990; Tarrant et al., 1992).

Turkey species makes no exception and road transportation is also regarded as one of the most stressful event in the turkeys' lifetime as well (Marchewka et al., 2013), reflecting direct effects on economical losses due to injuries and/or the increased death on arrival (DOA) rate (Petracci et al., 2006). In fact, most of the studies on the impact of road transport on poultry addressed its effect on the carcass quality, conversion of muscle, meat color, texture, and related negative effects on processed products (Schwartzkopf-Genswein et al., 2012).

Intensive rearing of the birds at geographically dispersed sites requires transport by road to centralized processing facilities to slaughter. The birds may be exposed to a variety of potential stressors during transit, including the thermal demands of the transport microenvironment, acceleration, vibration, motion, impacts, fasting, social disruption and noise (Mitchell and Kettlewell, 1998). The adverse effects may range from mild discomfort and aversion to death. Responses of birds to transportation involve many factors such as health status before transportation, how the birds are handled, loaded and unloaded, truck design, loading density, environmental condition, transit duration, lairage duration, and length of feed withdrawal (Schwartzkopf-Genswein et al., 2012). Studies carried by Bayliss and Hinton (1990) described that 40% of DOA during pre-slaughter are due to the poultry transport to the abattoir. Beside this economical perspective, there is also an ethical aspect that has to be addressed. The identification of suitable biomarkers of stress is of paramount importance in order to develop handling and transport procedures that minimize the stress level in animals.

1.5. Welfare and diseases

Diseases can spread rapidly among poultry because they are usually kept together in a cage or in overcrowded areas. They also share the same food and water bowls. In this farming status, diseases provide to animals heavy disturbance of welfare.

There are four main types of diseases affecting poultry, which are briefly listed hereby:

- metabolic and nutritional diseases;
- behavioural diseases;
- parasitic diseases;
- infectious diseases.

Metabolic and nutritional diseases

In poultry, these are conditions associated with a disturbance of normal metabolic functions either through a genetic defect, inadequate or inappropriate nutrition, such as increased metabolism, rapid growth rate or high egg production. These include Fatty Liver, Kidney Syndrome, Perosis, Rickets, Layer Fatigue and various cardiovascular diseases and metabolic disorders associated with egg production (Julian, 2005).

Behavioural diseases

Abnormal behavioural patterns can lead to injury or even ill health of the birds. These include fightings, aggressive pecking, or even cannibalism (Dennis et al., 2004; Lay et al., 2011).

Parasitic diseases

Parasitic diseases are often contracted through contact with an intermediate vector, but may occur as the result of direct exposure. The prevalence of most parasitic diseases in poultry contribute significantly to the low productivity (Permin and Hanson, 1998). The most frequent parasitic diseases include Coccidiosis, Cryptosporidiosis, Histomoniasis, Parasitic Worms (or Helminths), Toxoplasmosis and Trichomoniasis.

Infectious diseases

Infectious diseases can be spread directly or indirectly from one bird to another. These include Marek's disease, Infectious bronchitis, Infectious bursal disease, Newcastle disease, Fowlpox,

Avian encephalomyelitis, Chicken infectious anemia, Salmonellosis, Colibacillosis, Mycoplasmosis, Avian influenza, Avian tuberculosis, Chlamydiosis, Fowl cholera, Pasteurelloses, Infectious laryngotracheitis and Haemorrhagic enteritis (Brioudes et al., 2014; Miller et al., 2013; Saif, 1998).

1.5.1. Haemorrhagic enteritis virus as disease model to measure welfare

Virus infection of the gastrointestinal tract regularly occur in poultry. Many pathogens establish contact at intestinal mucosa surface. In field, these infections are always complicated by other infectious agents and, due to mucosa damage, leads to malnutrition, weight loss and death of the birds. Enteritis in poultry provide a very good model for health-related welfare disturbance.

Haemorrhagic enteritis (HE) is an acute gastrointestinal (GI) disorder affecting young turkeys at 4–5 weeks old, and can compromise the commercial production of livestock. The etiologic agent is the haemorrhagic enteritis virus (HEV), a member of the family *Adenoviridae*, type II, a non-enveloped, icosahedral DNA virus and 70–90 nm in diameter (Guy, 1998). Although HEV has been recognised since 1937 (Pomeroy and Fenstermacher, 1937), the mechanisms of viral immunopathogenesis and immunosuppression are still under study. Chickens are also susceptible to HEV but the greater severity of this disease occurs in turkeys (Rautenschlein et al., 1998).

HEV is geographically widespread and considered endemic in areas where turkeys are raised commercially. The usual route of infection is oral, and virus is often introduced into previously uninfected birds by equipment contaminated with infectious feces. The virus can survive in feces for months and in litter for weeks.

HE disease is characterized by acute onset of depression, pallor of mucosa, splenomegaly, diarrhea, intestinal haemorrhage and sudden death. The virulence of the virus varies but morbidity may approach 100%. Mortality can also be observed but it is rare because of extensive use of vaccines available. However, birds that survive experience a transient immunodepression and immunosuppression related to the lymphotrophic and lymphocytopathic nature of the virus. This often manifests itself in the form of HEV-related secondary infections with opportunistic pathogens agents such as the avian pathogenic *Escherichia coli*, bacterial respiratory diseases or septicemia (Koncicki et al., 2012). Prior exposition to Necrotic enteritis virus, Coccidiosis, Newcastle disease virus or *Mycoplasma gallisepticum* can exacerbate the problem, resulting in

mortality rates from 10–60% (Rautenschlein and Sharma, 2000). Usually, animals with less than 4 weeks old are resistant to infection because of the presence of maternal antibody (Fadly and Nazerian, 1989).

Following oral inoculation, HEV is detected in the intestine, bursa of Fabricius and caecal tonsils on day 1 day after infection. HEV replicates in these organs before entering the systemic circulation and migrating to the spleen. Inside the spleen, the virus reaches its peak levels of replication on day 4. The spleen is usually enlarged, friable and mottled white. Viral replication in B cells and macrophages induces necrosis and apoptosis of these cells, which leads to the depletion of IgM+ B cells and of functional macrophages. However, the spleen architecture returns to normal status on day 10 (Rautenschlein and Sharma, 2000; Saunders et al., 1993; Soback et al., 1985; Suresh and Sharma, 1995).

Infected lymphocytes circulate through other organs but the organs remain anatomically intact. At day 5, haemorrhagic lesions and bleeding in the intestine start to occur. Very low levels of virus are detected in the intestinal tissue, even in the presence of intestinal lesions (Suresh and Sharma, 1996; Rautenschlein et al., 1998). The virus is detected in the lamina propria of the intestine but not in mucosal epithelial cells (Hussain et al., 1993). However, birds reveal congestion and occasional intraluminal haemorrhage in the small intestine.

The role of T cells in HEV infection has not been elucidated in details. T cells are also attracted to the spleen and the relative percentage of CD4+ cells increase between days 4 and 6, while CD8+ levels are observed only at day 16 after infection (Suresh and Sharma, 1995). T cells also induce the production of various inflammatory cytokines (eg. TNF-α, IL-1β and IL-6). The production of these cytokines is massive, unregulated and leads to a systemic shock. Thus, it has been suggested that the HEV lesions and the degeneration of the intestinal epithelial cells is not due to the effects of the virus but, instead, a virally induced cytokine-mediated reaction, which leads to a haemorrhagic shock in the birds (Figure 6) (Suresh and Sharma, 1996).

In endemic areas, turkey flocks are vaccinated by drinking water, using avirulent HEV strains, or marble spleen disease virus (MSDV), a closely related virus that is non-pathogenic for turkeys. Despite vaccination, outbreaks occurs in the field. Diagnosis of HEV is based on clinical signs and lesions, and PCR techniques to detect hemorrhagic enteritis viral DNA (Hess et al., 1999).

We identified HEV as a model for stress during disease because the enteritis caused by the virus can have severe effects on the GI tract condition and negatively affect health and performance of

birds in commercial turkey flocks. Haemorrhagic enteric diseases are a major issue to the turkey industry, affecting loss of productivity, increasing mortality, and they are regarded as major treat over animal welfare.

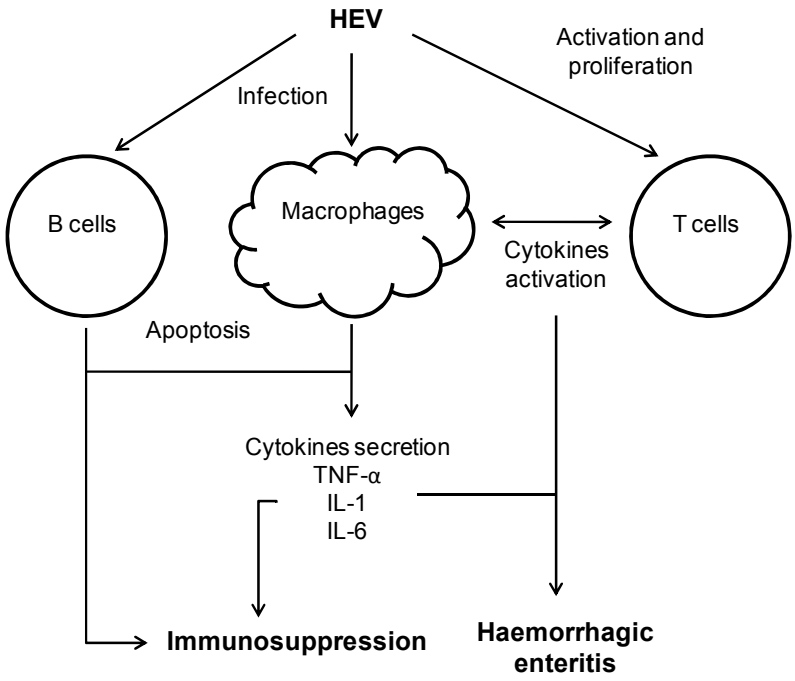

Figure 6. Model of haemorrhagic enteritis virus (HEV) immunopathogenesis. HEV replicates in B cells and macrophages. HEV induces apoptosis of B cell and macrophages, leading to immunosuppression. HEV induce T cells to produce cytokines, which activate macrophages. Activated macrophages secrete inflammatory cytokines that cause intestinal haemorrhage. Adapted from Rautenschlein et al. (2000).

1.6. Methods to assess stress

Assessment of stress is different from species to species and husbandry conditions, as well as in each individual animal. To effectively evaluate stress, the collection of multiple behavioural and physiological parameters can be employed by various teams, including researchers, veterinarians, technicians and animals'caretakers.

Behavioural parameters are often the most obvious indicator that an animal is having a difficult time to cope with a particular situation. The animal develops behaviours to avoid an unpleasant state. In addition, internal physiological indicators, such as changes in heart and respiratory rates, adrenal response and neurotransmitters will also increase or decrease. Physiological parameters are mediated through the endocrine, neural and immune system and can be quantified by the changes of stress hormone levels, including glucocorticoids, catecholamines, indolamines, growth hormone and prolactin.

Methods to assess animals' stress include:

Behavioural observation:

- feeding behaviour;
- sexual behaviour;
- maternal behaviour;
- attention to threat;
- unusual scratching.

Physiological measures:

- temperature change;
- respiratory rate;
- heart rate;
- blood pressure;
- body weight;
- physiological parameters (e.g. glucocorticoids, catecholamines and indolamines).

These methods are often used to assess animal welfare (Hill and Broom, 2009; Marchewka et al., 2013; Piñeiro et al., 2007; Tuchscherer et al., 2014).

Given the direct association between stress and HPA axis, glucocorticoid levels can be used as indicators for the strength of a stressor and cortisol is the most frequently employed biomarker to assess the effect of several stressors in animals (Hart, 2012). Cortisol is typically measured in blood serum or plasma but can also be quantified in saliva, urine, feces and hair (Abelson et al., 2005; Escribano et al., 2015; Kersey and Dehnhard, 2014; Meyer and Novak, 2012).

Beyond the major hormones of the HPA axis, stressors stimulate the secretion of other endocrine factors (e.g. prolactin, growth hormone, luteinizing hormone, α-melanocyte stimulating hormone (α-MSH), and oxytocin). Serum levels of these hormones can be effectively used to monitor the temporal dynamics of stress responses (Ranabir and Reetu, 2011). Cholesterol and low density lipoprotein-associated cholesterol (LDL-cholesterol) were also identified as a stress marker in pigs housed at different stocking densities (Marco-Ramell et al., 2011), as well as serotonin and dopamine were involved in the pig's response to handling at the slaughterhouse and to fear by tonic immobility (Arroyo et al., 2016). Serotonin has related to aggressiveness in chickens (Dennis et al., 2013). Blood metabolites were also assessed as oxidative stress markers in broilers exposed to higher temperature (Lin et al., 2006). In addition, various other biochemical parameters such as non-esterified fatty acids, triglycerides, cholesterol, urea, creatinine and fecal corticosterone were validated as indicators of stress in cows and pigs under housing and feed stress (Marco-Ramell et al., 2016; Saco et al., 2008). Because these compounds are also released in response to other stimuli, it is necessary to take into consideration and control normal patterns of secretion in order to accurately interpret their concentration levels.

Although methods to assess stress are focused on identifying hormones and compounds levels outside of a "normal range", there is no scientific standardized ranges for basal stress markers concentration. On the other hand, both positive and negative stimuli can activate the HPA axis, and hormonal changes are not necessarily present under all clearly stressful conditions (Bomholt et al. 2005; Ulrich-Lai et al. 2006). Most of these stress markers identified so far show a rapid response and their values quickly return to basal levels once the stressor disappears (Soler et al., 2013), and their meaningful interpretation is subjected to limitations.

In conclusion, the measurement of welfare is difficult and subjective. Although solid and standard welfare protocols exist for poultry, the Welfare Quality® (2009), Assessment Protocol for Poultry, reports that these protocols cannot be applied successfully to turkey species (http://www.welfarequalitynetwork.net/network/45848/7/0/40).

The knowledge of how welfare management tools, previously applied to other species, can be applied also to turkeys is unclear, and this prevents a correct quantification of the effects of management practices on turkey productivity and welfare. The standard protocols to assess animal welfare are often incomplete or unsuitable, since they differ in the thresholds set to differentiate high versus poor welfare, and/or in the way the information is integrated to form an overall evaluation judgment (Botreau et al., 2007).

Chapter 2. Acute phase proteins as welfare biomarkers

2.1. Acute phase proteins (APPs)

APPs are plasma proteins, mainly synthesized by hepatocytes, as part of the acute phase response (APR), and mediated by the major pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), which are released during inflammation at the site of injury (Gabay and Kushner, 1999; Moshage, 1997; Schrödl et al., 2016). The APR is a core part of the innate immune response, which carry out an important role in the early defense mechanism and serves to initiate the acquired immune response to prevent infection, eliminate potential pathogens and initiate the inflammatory process (Figure 7).

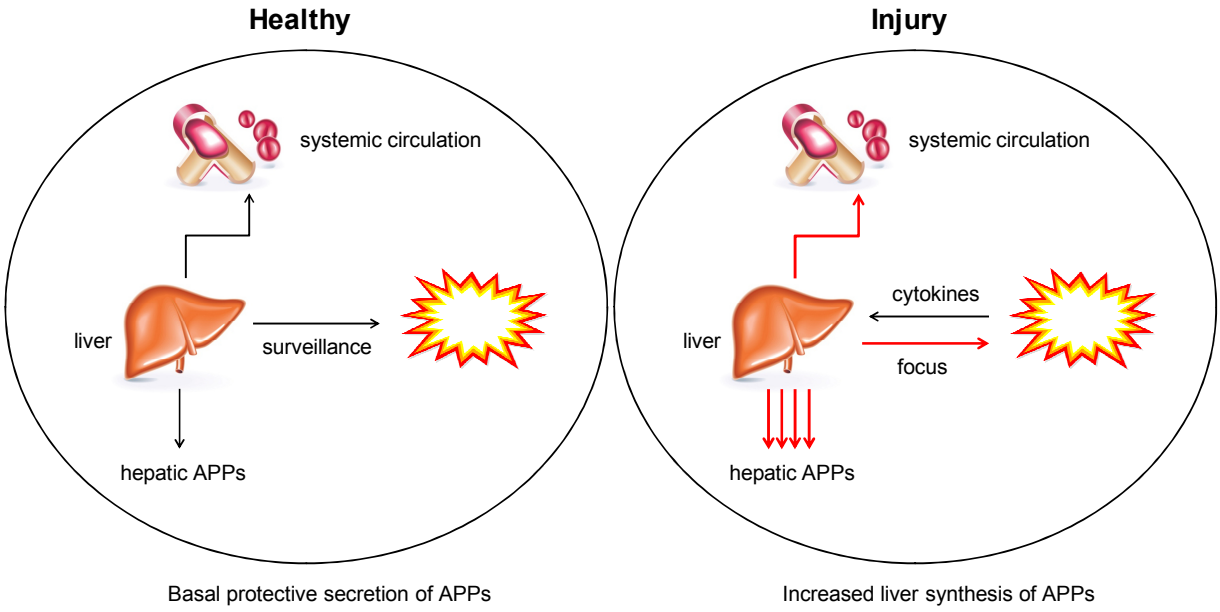

Figure 7. APPs secretion by the liver during healthy and injury condition. Liver is the main organ to contribute for circulating APP, protecting healthy state and preventing injury state.

APP concentration can change significantly as a consequence of many health problems, which can be an inflammation caused by physical trauma, microbial infections, immunological disorders, neoplasia, or non-inflammatory stress (Gabay and Kushner, 1999; Murata, 2007). APP

demonstrate “major”, “moderate” or “minor” responses depending on the magnitude of increase during the APR (Cray et al., 2009; Eckersall and Bell, 2010):

- **Major APP**

Major APP has a low serum concentration (< 1 g/L) in healthy animals, which rises dramatically by 10– to 1000–fold after stimulation, peaking at 24–48 h, and then declining rapidly during the recovery phase.

- **Moderate APP**

Moderate APP increases around 2– to 10–fold after activation, peaking after 2–3 days, and then decreasing more slowly than a major APP.

- **Minor APP**

Minor APP shows only a slight increase of slight 1– to 2–fold after activation. These APP are not considered as a marker during APR.

- **Negative APP**

Negative APP falls in concentration during the inflammatory response, such as albumin and transferrin.

From a clinical perspective, APPs also provide useful biomarkers to detect the early onset of diseases or to assess welfare. APPs can be used in diagnosis, prognosis and in general health screening. As biomarkers of disease, they are highly sensitive indicators of inflammation, although lacking specificity. APP response also differs among species (Table 1).

Table 1. List of major and moderate APPs that respond to inflammatory stimuli in common farm animals.

Species	Major APPs	Moderate APPs
Pig	MAP, SAA, CRP	Hp
Cow	Hp, SAA	AGP
Goat	Hp, SAA	FB
Sheep	Hp, SAA	AGP, CRP
Rabbit	Hp, SAA	AGP, CRP, FB
Chicken	N/A	AGP, SAA, PIT54, OVT

Hp, haptoglobin; SAA, serum amyloid A; AGP, alpha 1-acid glycoprotein; CRP, C-reactive protein; MAP, major acute phase protein; PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like); OVT, ovotransferrin; FB, fibrinogen.

In swine, pig major acute phase protein (pig-MAP), serum amyloid A (SAA) and C-reactive protein (CRP) are major APPs, whereas haptoglobin (Hp) is a moderate APP (Piñeiro et al., 2013; Pomorska-Mól et al., 2013; Quereda et al., 2012). However, in cattle, Hp is considered a major APP together with SAA and α1-acid glycoprotein (AGP) is a moderate APP (Ceciliani et al., 2012; Gånheim et al., 2007). A similar response to cattle occurs on goat, sheep and rabbit. In these three species, Hp and SAA are considered as major APPs (Eckersall et al., 2007; Gonzalez et al., 2008). In sheep, AGP and CRP show a moderate response after inflammation (Chemonges et al., 2014). Fibrinogen (FB) was also being described as a moderate APP in goat and rabbit (Cray et al., 2009).

2.2. APPs as stress biomarkers

APPs had also been described as useful biomarkers in the assessment of animal welfare (Murata et al., 2004; Schrödl et al., 2016).

Many researchers have investigated the potential use of APPs as indicators of stress. Although the mechanism of APP induction in response to stress is yet to be elucidated, activation of the HPA axis and the central and peripheral components of the ANS are responsible for crucial functions of the stress system (Kyrou et al., 2006). Inadequate, excessive or prolonged reactions to a stress condition may lead to a disease state and activate pathways involved in metabolism and immunity (Kyrou and Tsigos, 2009; McEwen, 1998). Glucocorticoids also lead to the production and release of APPs from liver into bloodstream (Murata, 2007; Tsigos and Chrousos, 2002).

An increase in several APPs levels has been assessed in various species during road transport, indicating their potential use as an objective parameter to evaluate stress. A list of works that relate APPs as stress biomarkers is presented below:

In cattle

- Conner et al. (1988) have described the first increment of APPs in response to stress stimuli. Later, Alsemgeest et al. (1995) have reported that cattle stressed by housing on a slippery floor had effects on the concentrations of SAA. Saco et al. (2008) have also found SAA as an indicator of stress under different systems of housing and feeding.
- Murata and Miyamoto (1993) found serum Hp concentrations increased after 1400 km over 2 days–transport, whereas Lomborg et al. (2008) have showed that the serum concentrations of Hp and SAA were already significantly increased after 4 to 6– hours of transport.
- The effects of 5–hours of transport together with lairage for 48 h were showed to increase Hp and SAA concentrations immediately 24 hours after transport (Giannetto et al., 2011).

In pig

- Piñeiro et al. (2007) have evaluated the pig APP response after 24 and 48 hours–transport. Pig-MAP, Hp, SAA and CRP were significantly increased. Salamano et al. (2008) that investigated the effects of a housing period following long road transport

observed the same. They found Hp, CRP and pig-MAP more increased after arrival than 28 days after housing.

- Soler et al. (2013) have proposed the use of SAA and cortisol, collected from saliva, as stress marker in pigs after a 45 min–short transport. SAA have shown a more prolonged response than cortisol, which has decreased immediately after arrival.
- Hp were also identified as a marker for housing stress in pigs (Marco-Ramell et al., 2016).

Monitoring APP could be considered as useful marker of welfare during transport and routine management in animals.

2.3. APPs in poultry

Major APPs described in poultry include α1-acid glycoprotein (AGP), C-reactive protein (CRP), Serum amyloid A (SAA), Haptoglobin-like protein (PIT54) and Ovotransferrin (OVT) (O'Reilly and Eckersall, 2014).

2.3.1. α1-acid glycoprotein (AGP)

In chickens, AGP has clinical significance (O'Reilly and Eckersall, 2014). AGP is one of the most important binding proteins in plasma, carrying out important anti-inflammatory and immunomodulatory roles to control inflammation (Luo et al., 2015). Furthermore, AGP can act as a non-specific antimicrobial agent, binding to LPS and inhibiting its activity and numerous drugs, sustaining their transport levels in the cells (Koppenol et al., 2015; Murata et al., 2004). Elevated serum AGP levels have been observed in chickens infected with various bacterial or viral pathogens (Inoue et al., 1997; Nakamura et al., 1996; Packialakshmi et al., 2016; Takahashi et al., 1994; Takahashi et al., 1998).

2.3.2. Serum amyloid A (SAA)

SAA is a major APP in vertebrates and can easily increase their concentration up to 1000–fold as response to a stimuli. SAA belongs to a family of apolipoproteins which is incorporated into high-density lipoprotein (HDL) and then it is released into the circulation (Benditt and Eriksen, 1977). The major SAA function is to modulate HDL transport and metabolism during APR. In mammals, multiple SAA genes have been described (Uhlar and Whitehead, 1999; Upragarin et al., 2005) but in chicken just one SAA gene has been identified (Ovelgönne et al., 2001). Persistently elevated serum SAA levels in chicken may result in the formation of AA amyloid protein, a SAA precursor. The accumulation of this precursor is able to cause joint amyloidosis, showing signs similar with chronic arthritis (Landman, 1999; Woldemeskel, 2012). Chamanza et al. (1999) found SAA overexpressed in broilers after turpentine injection and *Staphylococcus aureus* infection. Asasi et al. (2013) detected significant increases in SAA in chickens infected with Infectious Bronchitis Virus (IBV). SAA was also identified as a useful APP to monitoring the progression of disease in falcons, reflecting the severity of inflammation (Caliendo et al., 2013).

2.3.3. Haptoglobin-like protein (PIT54)

Hp is the major hemoglobin-binding protein found in mammals. Under certain pathological conditions, hemoglobin may leak into the bloodstream in high concentrations that can be toxic for the surrounding tissues. This hemoglobin is then sequester by Hp, inhibiting its oxidative activity (Nielsen and Moestrup, 2009). In birds' genome there is no gene coding a similar protein. Instead, the protein referred as PIT54 was identified, which seems to have the same role as Hp (Wicher and Fries, 2006). PIT54 belongs to the Scavenger receptor cysteine-rich (SRCR) superfamily and is composed by four repetitive SRCR domains. Until now, PIT54 is found only in avian species, having been identified in geese, ostriches, emus, chicken and *Galapagos finches* (Wicher and Fries, 2006; Wicher and Fries, 2010; Zylberberg et al., 2013). PIT54 (mammalian-like Haptoglobin) has been associated with *Staphylococcus gallinarium* and *S. aureus* infections (Garcia et al., 2009; Millet et al., 2007), *E. coli* and *Eimeria tenella* infections (Georgieva et al., 2010) and IBV infections (Asasi et al., 2013).

2.3.4. C-reactive protein (CRP)

CRP was the first APP been described and is one of the major APP in humans and dogs (Eckersall and Bell, 2010). CRP has the ability to binds directly to degenerating cells, residues and polysaccharides on bacteria, fungi, and parasites, acting as an opsonin. It can activate the complement system when bound to one of its ligands and can bind to phagocytic cells, interacting together with humoral and cellular systems of inflammation (Cray et al., 2009; Gruys et al., 2005; Marnell et al., 2005). CRP has also been identified in birds with *E. coli*, *Pasturella multocida*, *S. aureus* infection (Patterson and Mora, 1964), IBV (Seifi et al., 2014), birds infected with *Salmonella typhimurium* (Rauber et al., 2014) and in birds exposed to heat stress (Sohail et al., 2010).

2.3.5. Ovotransferrin (OVT)

The transferrins are a group of iron-binding glycoproteins present in vertebrates and invertebrates. In poultry, transferrin protein is an APP, which occurs as two forms with the same gene sequence expressed in liver and oviduct, called ovotransferrin (OVT). Both forms have the same amino acid sequence and protein structure, differing only in the extent of glycosylation and

composition of attached carbohydrate side groups (Thibodeau et al., 1978; Williams, 1968; Xie et al., 2002). In chicken, OVT is a positive APP, increasing its circulating concentration in response to a stimuli and remaining elevated as long as inflammation persists (Cray et al., 2009). OVT has the ability to sequester iron with high affinity, an essential nutrient for bacterial, limiting bacterial growth. Chickens infected with *Staphylococcus* spp., *E. coli* or parasites as *E. maxima* and *E. tenella* showed a significant increase in the levels of serum OVT (Chamanza et al., 1999; Rath et al., 2009). OVT concentrations were also higher when chickens were challenged with lipopolysaccharides (LPS) (Horrocks et al., 2011; Koppenol et al., 2015).

There is no information available regarding APPs as road transport biomarkers in poultry; however they have been related with stress in birds.

- Salamano et al. (2010) have found higher AGP concentration in laying hens reared in different housing system and subject to stressful events.
- Stress by high temperature and overcrowding have increased serum AGP and OVT concentration in broilers laired at different stocking densities (Najafi et al., 2015).
- Feed deprivation in poultry farming imposes stress to the birds and adversely affects their welfare. Serum levels of OVT and AGP were increased following 30 hours after feed deprivation (Najafi et al., 2016).

2.4. APPs extrahepatic expression

Liver mostly produces APPs, but extrahepatic expression has been widely demonstrated. Meek and Benditt (1986) reported for the first time the extrahepatic expression of SAA in different mouse tissues: adrenal gland, small intestine, large intestine, lung, kidney, brain, heart, spleen, testis, skeletal muscle, stomach and pituitary. Kalmovarin et al. (1991) have also described APPs mRNA expression in a variety of mouse tissues. Since then, data on expression of APPs in extrahepatic tissues have arise constantly.

- SAA family is known to be expressed in various normal extrahepatic tissues in humans (Urieli-Shoval et al., 1998), cattle (Berg et al., 2011; Lecchi et al., 2012), horses (Berg et al., 2011), rabbit, mink and mouse (Marhaug et al., 1997; Ramadori et al., 1985), even without any association with specific diseases;
- Hp is a major APP in cattle and several investigations have described its extrahepatic expression (Cooray et al., 2007; Dilda et al., 2012; Lecchi et al., 2012);
- AGP is also known to be expressed in many other tissues beside liver (Lecchi et al., 2009; Liu et al., 2014; Rahman et al., 2015);
- CRP is also synthesized by a variety of tissues (Agassandian et al., 2014; Calabro et al., 2005; Jabs et al., 2003).

The list where APPs were found so far includes tongue, trachea, esophagus, stomach, abomasum, forestomach, small and large intestine, tonsil, pancreas, spleen, lung, prostate, skin, kidney, pituitary, placenta, bladder, testis, ovary, uterus, mammary gland, bone marrow, lymph nodes, thymus, thyroid, monocytes, leukocytes, heart, brain, cerebellum, eye, adipose tissue, skeletal muscle, synovial membrane and adrenal gland. No information about the extrahepatic expression in poultry is presently available.

In general, the extrahepatic expression rate is lower in healthy status in comparison to that of the liver, but it might increase considerably under local homeostasis disturbance (Figure 8).

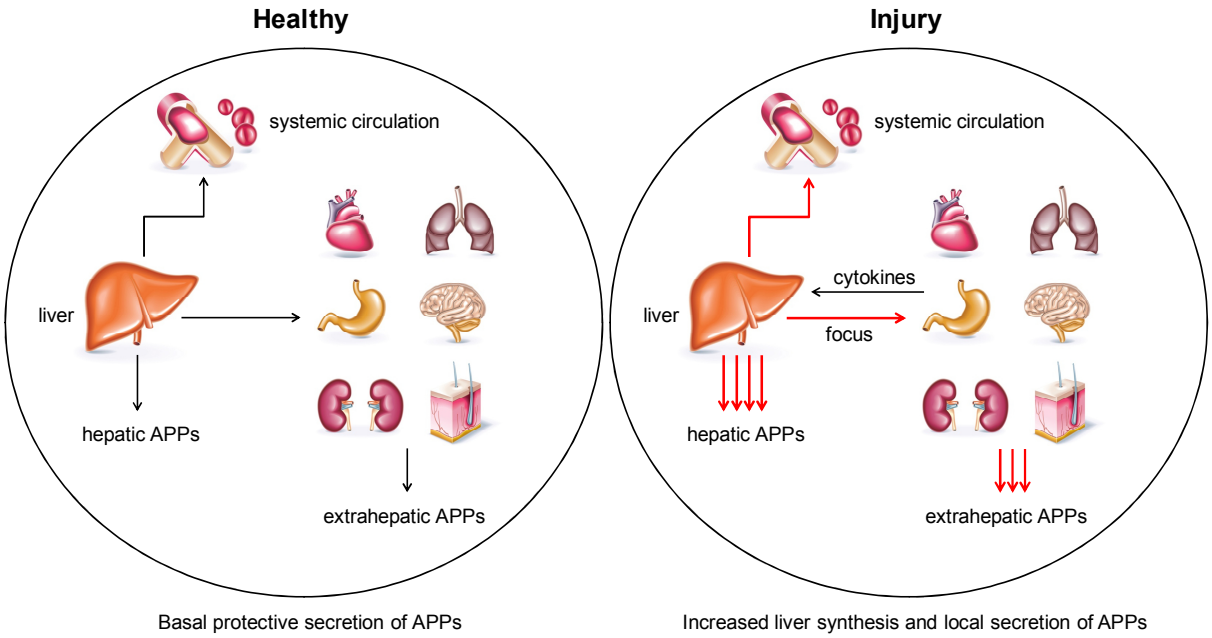

Figure 8. APPs secretion by liver and extrahepatic tissues during healthy and injury condition. Extrahepatic APPs play several tissue-specific roles in a healthy state, provide local protection, and repair in the event of a local injury.

Chapter 3. MicroRNAs as welfare biomarkers

3.1. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) comprise a large family of short nucleotides (19–24 nucleotides in length) that have emerged as key post-transcriptional regulators, and control many developmental and cellular processes in eukaryotic organisms. MiRNAs are non-coding RNAs that regulate messenger RNA (mRNA) or protein levels either by promoting mRNA degradation or by attenuating protein translation.

In 1993, the first miRNAs were identified in *Caenorhabditis elegans*, a nematode (Lee et al., 1993). Since then, miRNAs have been reported in a wide variety of organisms ranging from single-cell algae to humans, suggesting that miRNA-mediated biological function is an ancient and critical cellular regulatory element (Bartel and Chen, 2004; Zhao et al., 2007). The number of miRNAs in the genome appears to be correlated with the complexity of the developmental program, with mammals having the largest number of miRNAs. It has been estimated that more than 60% of mammalian mRNA is targeted by at least one miRNA (Friedman et al., 2009).

3.2. miRNAs biogenesis and regulation

MiRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed by RNA polymerase II (RNAPII)-specific transcripts of independent genes or from introns of protein-coding genes (Krol et al., 2010). The pri-miRNAs fold into hairpins, acting as substrates for two members of the RNase III family of enzymes – Drosha and Dicer, operating in complexes with dsRNA-binding proteins (dsRBPs), for example DGCR8, transactivation-responsive (TAR) RNA-binding protein (TRBP) and protein activator of PKR (PACT). MiRNAs biogenesis is illustrated in Figure 9.

In the first step, the Drosha processes pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), characterized by a stem-loop structure, inside the nucleus. Some pre-miRNAs are also produced from short introns, called mirtrons, because of splicing and disbranching, avoiding the Drosha step. Then, pre-miRNAs are exported to the cytoplasm, via Exportin-5. Dicer, assisted by TRBP, processes pre-miRNA to a ~20-nucleotide miRNA/miRNA* duplex. One strand of this duplex – miRNA/miRNA*, representing a mature miRNA, is then incorporated into the miRNA-induced silencing complex (miRISC) and the other strand (miRNA*) is degraded. As part of miRISC, miRNAs targets mRNAs and induce their translational repression or deadenylation and degradation. Deadenylation of mRNAs is mediated by glycine-tryptophan protein of 182 kDa (GW182), which interacts with Argonaute (AGO) proteins and act downstream of them. AGO are core components of the miRISC and different species express multiple AGO homologues (eg. AGO1–AGO4 in mammals). While the amino-terminal part of GW182 interacts with AGO, the carboxy-terminal part interacts with the poly(A) binding protein (PABP) and recruits the deadenylases CAF1 and CCR4.

Argonaute RISC catalytic component 2 (AGO2) has dual functions in the processing of miRNA precursors and in target silencing. First, AGO2 functions as a Dicer co-factor in pre-miRNA processing, as part of the RISC-loading complex. AGO2, which has a robust RNaseH-like endonuclease activity, supports Dicer processing by cleaving the 3′ arm of some pre-miRNAs, thus forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA) (Diederichs and Haber, 2007). Second, AGO2 is loaded with a guide miRNA strand, making an active miRISC (Chendrimada et al., 2004).

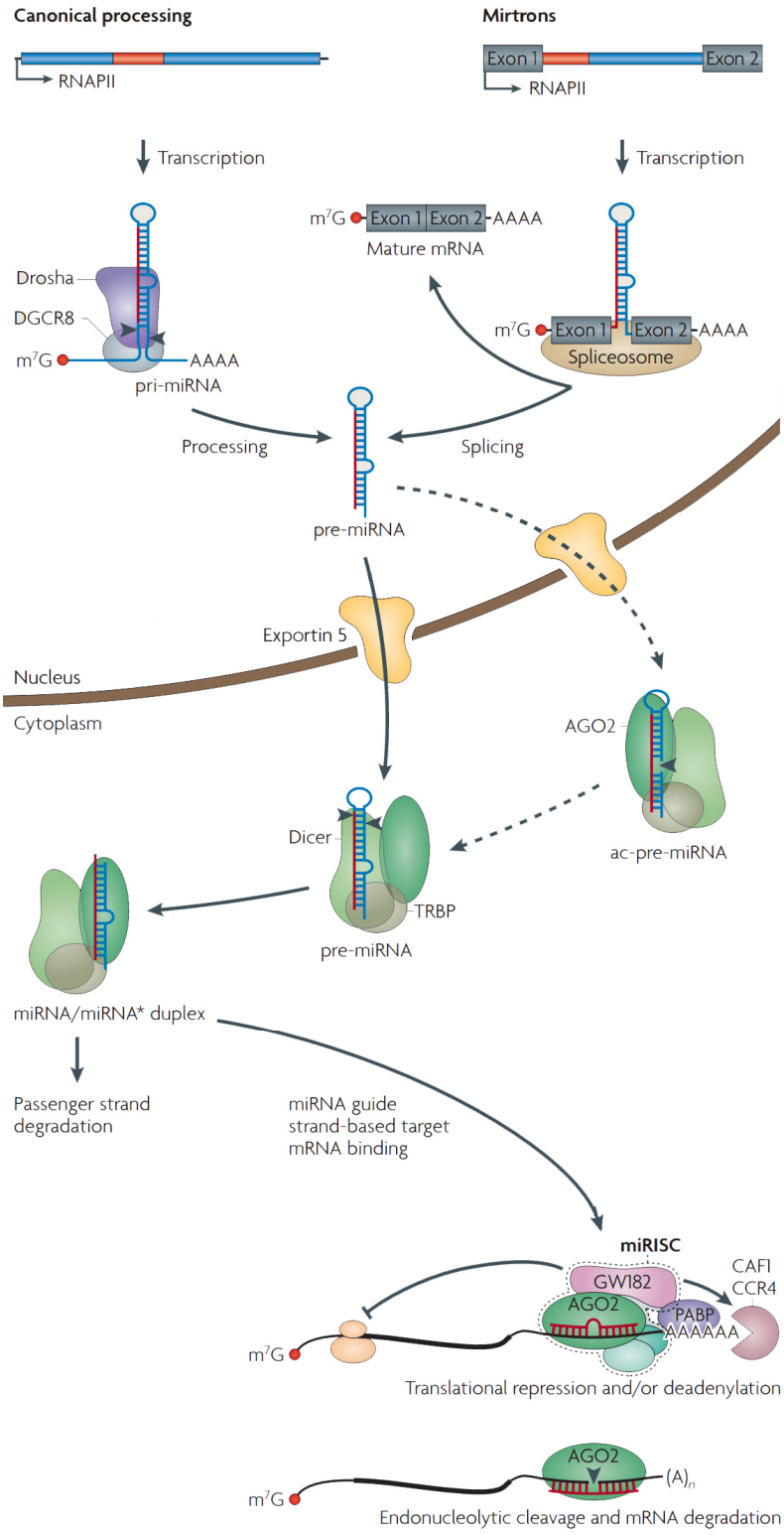

Figure 9. MiRNA biogenesis. Following transcription of the pri-miRNA, the pre-miRNA is excised by Drosha and transported by Exportin-5 into the cytoplasm. Splicing of the pre-miRNA by Dicer generates the miRNA duplex that contains the mature miRNA strand, which together with several accessory proteins assembles into the RISC complex to enable post-transcriptional regulation of target mRNA translation. Adapted from Krol et al. (2010).

A miRNA regulates the expression of its target mRNA in three manners, depending on the degree of complementarity between them (Figure 10):

- Translational repression;
- Cleavage of the target mRNA;
- mRNA deadenylation.

The small size of miRNAs provides a limited amount of sequence information for specificity. A partial pairing between a miRNA and a target is often sufficient. In animals, the vast majority of miRNAs only forms partial complementation with their targets, and the most mRNAs targets studied to date are regulated through 3'untranslated region (3'UTR) interactions. Nucleotides 2 to 8 at the 5' end of the miRNA, also called "seed" sequence, must be perfectly complementary to the target (Bartel, 2004; Pasquinelli, 2012).

An imperfect complementarity leads to translational repression whereas a perfect or nearly perfect complementarity leads to cleavage of the target mRNA by endonucleotic cleavage activity of AGO (Wang et al., 2013). Some animal miRNAs can cause the cleavage of their target mRNAs despite imperfect complementarity between them (Yekta et al., 2004). Binding of the miRISC, which includes GW182 protein to 3'UTR target sequences can result in the recruitment of deadenylation factors that remove the poly(A) tail and make the mRNA susceptible to degradation (Wu et al., 2006).

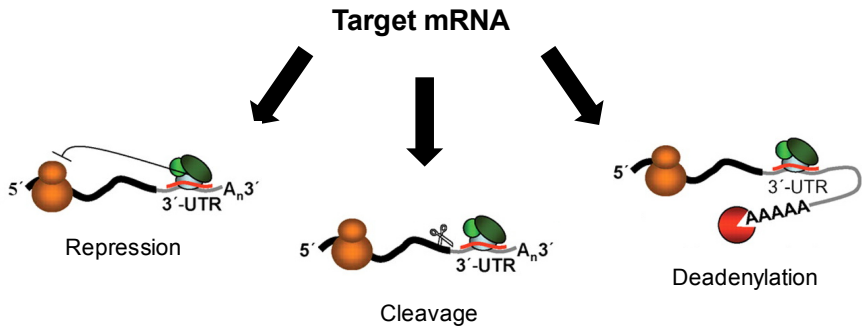

Figure 10. MiRNAs regulate gene expression through multiple pathways. Translational repression can be induced by blocking translation initiation. Perfect pairing between miRNA and its target induces endonucleolytic cleavage by AGO, leading to rapid degradation of the mRNA. Partial pairing of the miRNA complex and its target to 3'UTR site can result in deadenylation of the mRNA. Adapted from Fazi and Nervi (2008).

Transcription of miRNA genes is regulated in a similar manner to that of protein-coding genes. A common regulatory mechanism for miRNA gene expression is by regulatory feedback loops. MiRNAs can participate in feedback circuits and repress mRNA that encode factors involved in the biogenesis or function of the same miRNA (Ha and Kim, 2014; Krol et al., 2010).

3.3. miRNAs involved in cellular stress pathways

MiRNAs have been identified in animal species as markers for various biological processes. They play key roles in immune system, act as specific targets for inflammation and contribute for the diagnosis of a variety of diseases and stress disorders (Andersen et al., 2014; Rebane and Akdis, 2013; Wang et al., 2013).

There is a link between the Dicer complex and the cellular stress response (Figure 11). When the cell senses a stress signal, Dicer modifications can activate or inhibit miRNAs. Stressors, such as reactive oxygen species (ROS), UV radiation or type I interferons (IFNs), cause a reduction in the expression of Dicer (Wiesen and Tomasi, 2009). Loss of Dicer function reduces stress tolerance, whereas Dicer overexpression confers stress resistance. In addition, Dicer co-factors – AGO, PACT and TRBP, which participates in the connection between Dicer and miRISC complex, can be influenced by stress. Stress signalling can interfere with AGO2 function by post-translational modifications.

Under stress, three modifications can alter AGO2, which loses its capacity to bind Dicer complex and, consequently, inhibits pre-miRNA processing. P38/MAPK phosphorylates AGO2 at Serine 387, epidermal growth factor receptor (EGFR) modifies tyrosine 393, and/or proline 700 can be hydroxylated (Emde and Hornstein, 2014). These post-translational modifications contribute to AGO2 translocation into stress granules (SGs). SGs are known to be sites where miRNAs accumulate when the cell is under stress, and contain enzymes responsible for miRNA decapping, deadenylation and degradation (Anderson and Kedersha, 2006).

PACT and TRBP participate in the cellular stress response via interaction with PKR. PKR is an interferon-induced dsRNA-regulated protein kinase, which binds to dsRNA and is involved in the host inflammatory response. PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α), activating SGs and, thereby, causing global reduction of protein synthesis (Taylor et al., 2005). AGO2 and PACT are recruited into stress granules, together with other proteins involved in miRNA regulation pathways (Emde and Hornstein, 2014). Stress response is also extended to the regulation of miRISC activity, leading to miRNA degradation.

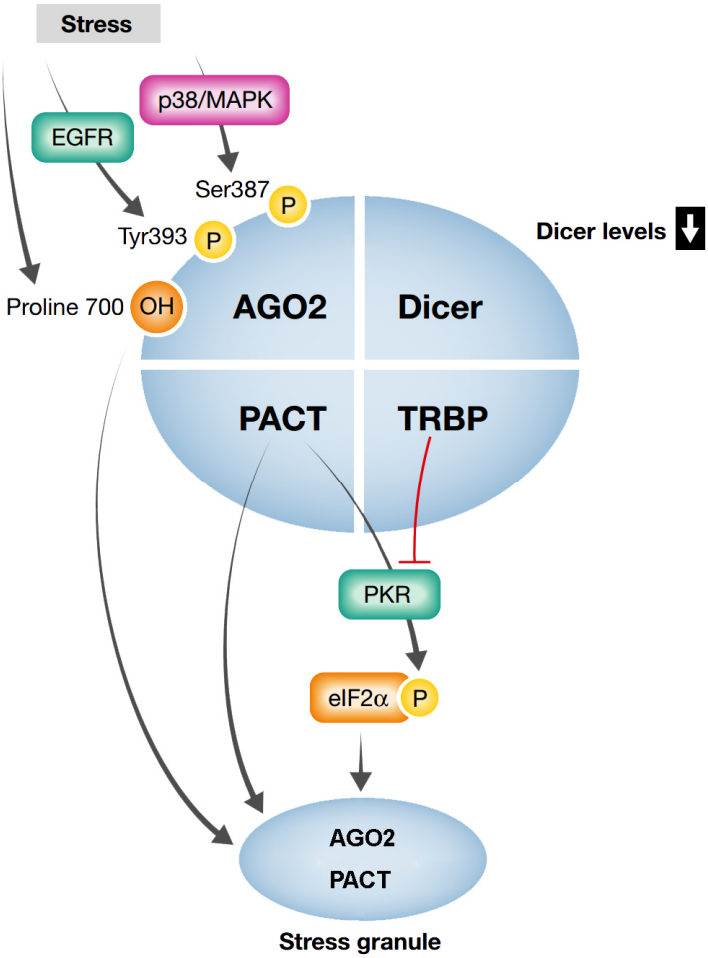

Figure 11. Connection between the miRNA biosynthesis, stress and stress granules (SGs). Stress is suggested to modify AGO2. When SGs are formed, both PACT and AGO2 are recruited into them. The interactions between cellular stress and the Dicer/miRISC complex affects Dicer activity and inhibit miRNA processing. Adapted from Emde and Hornstein (2014).

Chapter 4. Adipose tissue

Adipose tissue (AT) is a loose connective tissue, distributed as fat depots throughout the whole body and being a necessary survival characteristic of the organism during periods of famine. In humans, AT has an important role in the obesity and overweight. However, in farm animals, obesity is not an issue, due to the controlled environment in which they live. Actually, it has been demonstrated that AT influences animal health and meat quality, and has an economic value by affecting parameters such as tenderness, juiciness and taste (Wood et al., 2008).

Fat depots are mainly classified as subcutaneous or visceral adipose tissue. Subcutaneous AT includes depots located beneath the skin, whereas visceral AT is located in the intra-abdominal cavities, surrounding specific organs, such as kidney and heart, or distributed among peritoneum layers, such as mesenteric and omental fat. Subcutaneous and visceral AT have unique adipokines expression profiles, and different metabolic characteristics (Ibrahim et al., 2010; Peinado et al., 2010). Figure 12 presents the major location of visceral and subcutaneous AT depots in chicken.

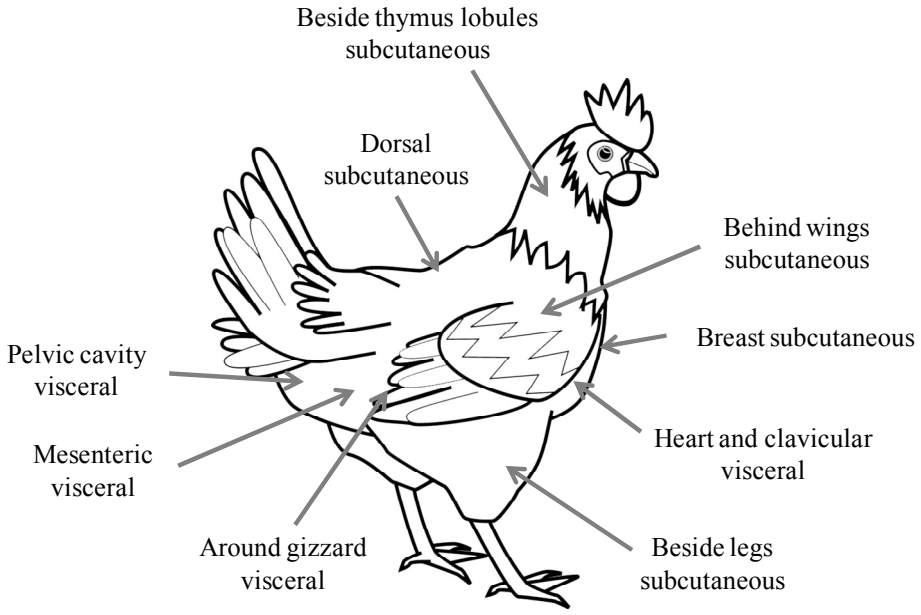

Figure 12. The distribution of subcutaneous and visceral adipose tissue in chicken. Adapted from Sauerwein et al. (2014).

For a long time, AT has been considered as a passive organ of energy storage. In 1994, AT was identified as the source of the hormone leptin, transforming the tissue in an active participant in hormonal regulation of homeostasis (Zhang et al., 1994). Since then, AT was proved to be involved at countless pathways such as homeostasis and metabolic regulation, reproduction, as well as innate and adaptive immune response, through production and secretion of messenger molecules which are called as "adipokines" (Cunningham et al., 1999; Kershaw and Flier, 2004; Lord, 2002; Rajala and Scherer, 2003).

Adipokines include a wide range of hormones and chemokines, produced exclusively by AT (e.g. leptin, adiponectin and visfatin), and molecules involved in the immune system (e.g. interleukins, cytokines and APPs) (Figure 13) (Deng and Scherer, 2010; Sauerwein et al., 2014).

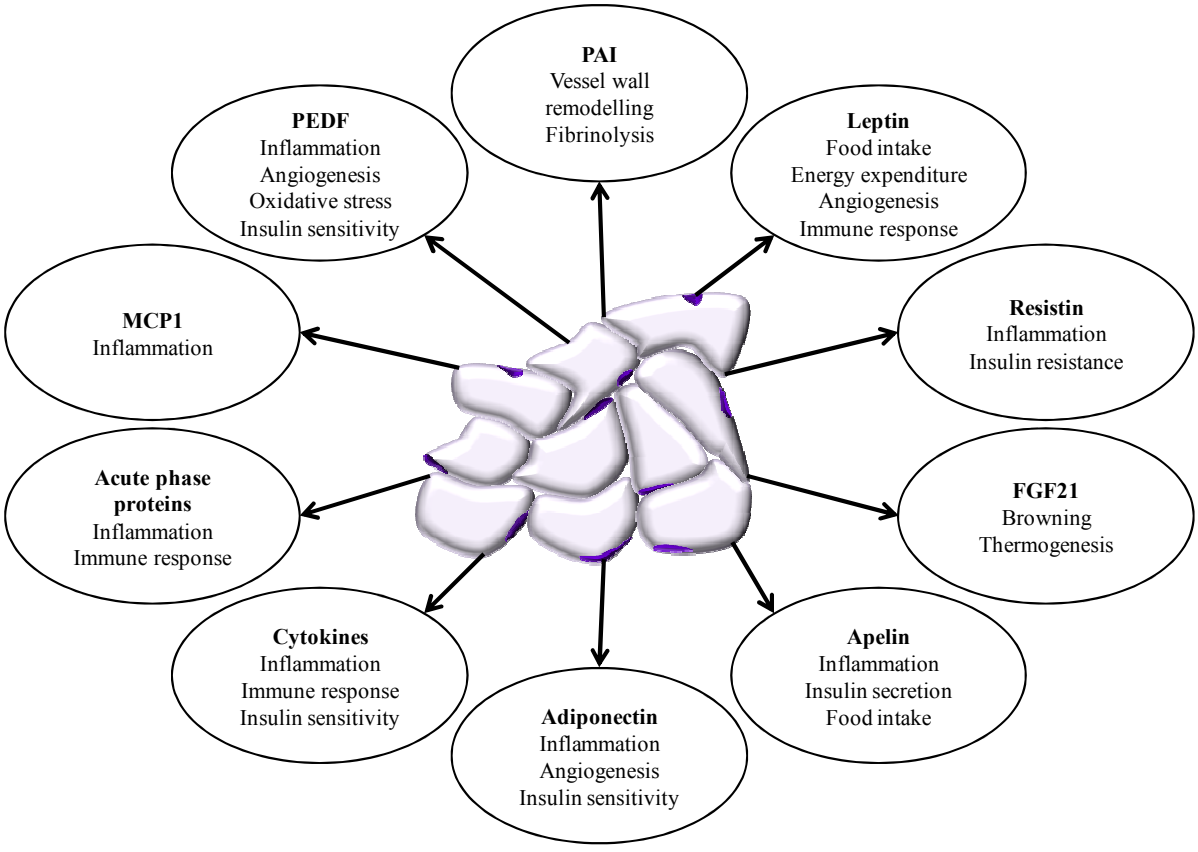

Figure 13. Example of adipokines synthesized and secreted from adipose tissue and their main functions.

The role of the adipocytes as a hormone and cytokine secreting cells has already been clarified (Wisse, 2004). Adiposity has been correlated with the higher number of macrophages in obese animals, which tends to aggregate and form cells characteristic of chronic inflammation (Weisberg et al., 2003). Pre-adipocytes are also potent phagocytes similar to macrophages in both morphology and pattern of gene expression (Cousin et al., 1999). The AT production of leptin increases during acute infection and fat contributes to elevated circulating TNF-α, TGF-β1, IL-1β, IL-6, IL-8, IL-10, as well as other factors that participate directly in immune response (e.g. APP) (Trayhurn and Wood, 2004; Vettor et al., 2005). Moreover, the fact that a list of adipokines are involved in inflammation has lead to the view that the obesity is state of chronic low-grade inflammation, relating acute phase response with metabolic syndrome. Visceral depots are known by having a higher lipolytic activity and expression of pro-inflammatory cytokines than subcutaneous depots (Wronska and Kmiec, 2012). Although the AT contribution to the secretory inflammatory cytokines has been addressed in animals, the role of AT in the immunologic pathways is unknown in poultry.

Adipose tissue seems to be very reactive to a stress condition and there is an intricate relationship between the systems that are activated in response to stress and the metabolism. Glucocorticoids are the main stress effectors produced after the activation of HPA axis and they leads to a redistribution of energy sources (e.g. increased glucose production, adipocyte differentiation and increasing the visceral adiposity) (Chrousos, 2000; Pasquali et al., 2006). Glucocorticoids mediate the conection between adipocytes and the HPA axis, linking obesity and metabolic diseases with stress-related pathologies (Kloet et al., 2015; Peckett et al., 2011).

Chapter 5. Experimental designs and results

Some aspects are of particular relevance when we planned our experimental design related with poultry species:

- Information about turkey (*Meleagris gallopavo*) are scarce in literature. There is no available information regarding how to evaluate welfare in this species, especially for what concerns APPs and miRNAs as welfare biomarkers. On the contrary, this species is very important from an economic perspective.

- Chicken (*Gallus gallus*) is the closest species and we resorted to this to complete the missing information in turkey.

In this study, APP and miRNAs were characterized for the first time as molecular welfare biomarkers and as markers for stress-related transport in turkey species. Intestinal mucosa proteome was also characterized in healthy animals and in the presence of a gastrointestinal disease. The distribution of APP extrahepatic expression was studied in poultry, in this case in chicken species.

The results of this investigation are presented in this thesis in two published papers, in two papers ready for publication and in a project still going on:

Paper 1 (pp 64-84): Widespread extrahepatic expression of acute phase proteins in healthy chicken (*Gallus gallus*) tissues – Andreia Tomás Marques, Laura Nordio, Cristina Lecchi, Guido Grilli, Chiara Giudice, Fabrizio Ceciliani. Submitted to *Veterinary Immunology and Immunopathology*.

Paper 2 (pp 85-106): The effect of transport stress on turkey (*Meleagris gallopavo*) liver acute phase proteins gene expression – Andreia Tomás Marques, Cristina Lecchi, Guido Grilli, Chiara Giudice, Sara Rota Nodari, Leonardo James Vinco, Fabrizio Ceciliani. *Research in Veterinary Science*. 2016 Feb;104:92-5. doi: 10.1016/j.rvsc.2015.11.014.

Paper 3 (pp 107-119): Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkeys – Cristina Lecchi, Andreia Tomás Marques, Miriam Redegalli, Sarah Meani, Leonardo James Vinco, Valerio Bronzo, Fabrizio Ceciliani. *Animal.* 2016 Jul;10(7):1213-7. doi: 10.1017/S1751731115003043.

Paper 4 (pp 120-165): Proteome analysis of intestinal mucosa in turkey (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus – Andreia Tomás Marques, Sandra Anjo, Mangesh Bhide, Ana Varela Coelho, Cristina Lecchi, Guido Grilli, Bruno Manadas, Fabrizio Ceciliani. To be submitted to *Journal of Proteomics*.

The experimental design planned for this thesis is presented in Figure 14.

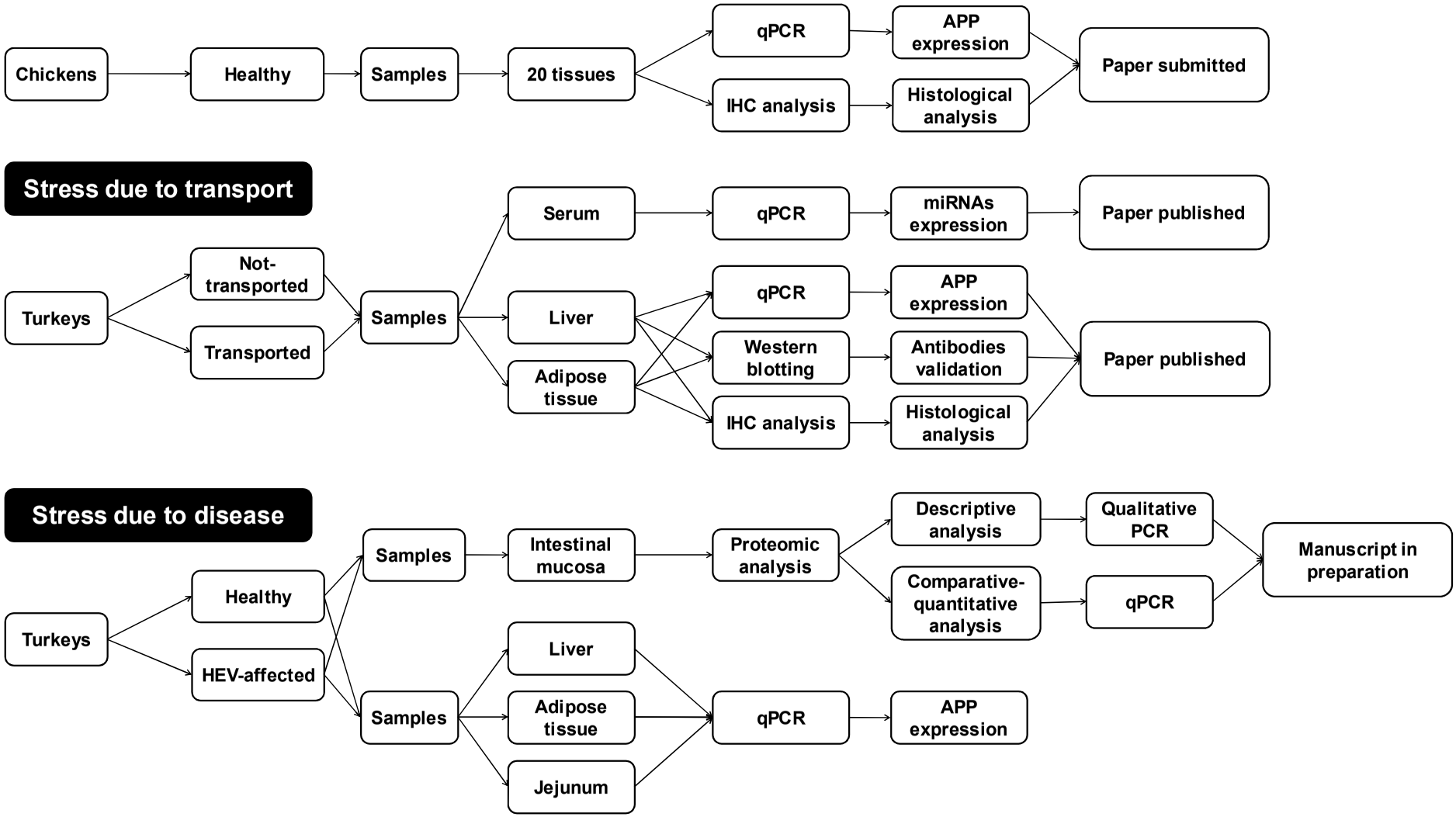

Figure 14. Thesis experimental design's scheme.

The main aims for each study are summarized below:

Chapter 5.1. Widespread extrahepatic expression of acute phase proteins in healthy chicken (*Gallus gallus*) tissues

The aim of the present study was to investigate the distribution pattern of the five major APPs in twenty tissues collected from six healthy chicken.

The study was carried out by measuring APP mRNA with quantitative Real Time PCR and by immunohistochemistry to detect AGP and OVT localization, using available antibodies, in order to understand their potential source, role in local immunity, and their impact in chicken welfare.

Chapter 5.2. The effect of transport stress on turkey (*Meleagris gallopavo*) liver and adipose tissue acute phase proteins gene expression

The aim of this study was to identify the major APP in turkey species and evaluate their potential use as indicators of transport stress.

- In a first instance, we focused our attention on the liver gene expression and, where antibodies were available, at protein level.
- In the second part of the study, the gene expression modification of APP mRNA and protein localization was extended to visceral adipose tissue.
- In the last part of the study, we tried to produce antibodies against CRP and PIT54, since there is already an antibody against AGP and a comercial assay for SAA available in poultry.

The study was carried out by measuring APP mRNA abundance by quantitative Real Time PCR and immunohistochemistry to detect AGP localization, after validating an anti-AGP antibody by Western blotting. Antibodies against CRP and PIT54 proteins were also raised and validated in turkey species.

Chapter 5.3. Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkey (*Meleagris gallopavo*)

The aim of the present study was to determine whether transport-related stress modulates the expression of circulating miRNA and investigate the potential use of differentially expressed miRNA as biomarkers to measure transport-related stress. Five miRNAs, previously demonstrated to be related to stressful events in chicken and immune defenses, were selected for our study and road-transportation was identified as stressful model.

The study was carried out by measuring those miRNAs by quantitative Real Time PCR using TaqMan probes.

Chapter 5.4. Proteome analysis of intestinal mucosa in turkey (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus

The aim of this work was to undertake in-depth and comparative studies of the protein expression pattern of turkey intestinal mucosa. Currently, proteomic studies in poultry are limited to chicken. To the best of our knowledge, no proteomic data about intestine is available in turkey.

A SWATH-MS strategy was used to characterize and compare the proteome composition from healthy and HEV-affected turkeys.

Chapter 5.5. Acute phase proteins mRNA changes in the liver, adipose tissue and jejunum of turkeys (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus

The aim of this study was to evaluate the APP potential use as indicators of a heamorrhagic enteritis disease in turkeys. The APP gene expression was quantified in liver, adipose tissue and jejunum, the tissue were the lesions were found, in both healthy and sick turkeys.

The study was carried out by measuring APP mRNA abundance by quantitative Real Time PCR.

5.1. Widespread extrahepatic expression of acute phase proteins in healthy chicken (*Gallus gallus*) tissues

Manuscript of this work is submitted to *Veterinary Immunology and Immunopathology*:

Widespread extrahepatic expression of acute phase proteins in healthy chicken (*Gallus gallus*) tissues.

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Keywords: acute phase proteins, animal welfare, chicken, extrahepatic expression, *Gallus gallus*.

Abstract

Acute phase proteins (APPs) are plasma proteins that can modify their expression in response to inflammation caused by tissue injury, infections, immunological disorders, or stress. Although APP are produced mainly in liver, extrahepatic production has also been described. The aim of this work was to study the extrahepatic expression of five APP, namely AGP, SAA, PIT54, CRP and OVT in twenty tissues collected from healthy chicken (*Gallus gallus*) by quantitative Real Time PCR and immunohistochemistry. As expected, APP gene expression was higher in liver compared with other tissues. The mRNA coding for CRP, OVT and SAA was detected in all analyzed tissues with a higher expression in gastrointestinal tract, respiratory and lymphatic samples. SAA expression was particularly high in cecal tonsil, lung, spleen and Meckel's diverticulum, whereas OVT in lung, bursa of Fabricius and pancreas. AGP and PIT54 mRNA expression were detected in all tissues but at very low levels. Immunohistochemical expression of AGP and OVT was variably detected in different organs, being identified in endothelium of every tissue. Positive cells were present in the epithelium of the mucosal layer of gastrointestinal tract and kidney. Lung and central nervous system stained for both proteins. No positive staining was detected in lymphoid tissues and muscle. These results suggest that each tissue is able to express different amount of APP even in healthy conditions and mount a local acute phase reaction.

1. Material and methods

1.1. Samples collection and preservation

The samples were collected during routinely slaughtering procedures from six healthy female chicken, 55 days old (Table 1). The clinical status of the animals was assessed by ante- and post-mortem inspection. Figure 1 presents the chicken anatomy.

Portions of each tissue were removed immediately after slaughtering, preserved in RNAlater (Sigma–Aldrich) or frozen into liquid nitrogen and afterwards stored at −80ºC before RNA extraction. Adipose tissue was stored without previous immersion in RNAlater. Samples for immunohistochemistry analysis were fixed in 10% buffered formalin.

Table 1. Samples list.

Adipose tissue	Subcutaneous adipose tissue Visceral adipose tissue Pericardial adipose tissue	
Muscular system	Pectoral muscle	
Lymphatic system	Thymus Spleen Cecal tonsil Bursa of Fabricius	
Central nervous system	Brain Cerebellum	
Urogenital system	Kidney	
Respiratory system	Lung	
Gastrointestinal tract	Crop	
	Stomach	Mucosa of proventriculus Mucosa of gizzard
	Pancreas	
	Intestine	Duodenum Caecum
	Meckel's diverticulum	
	Liver	

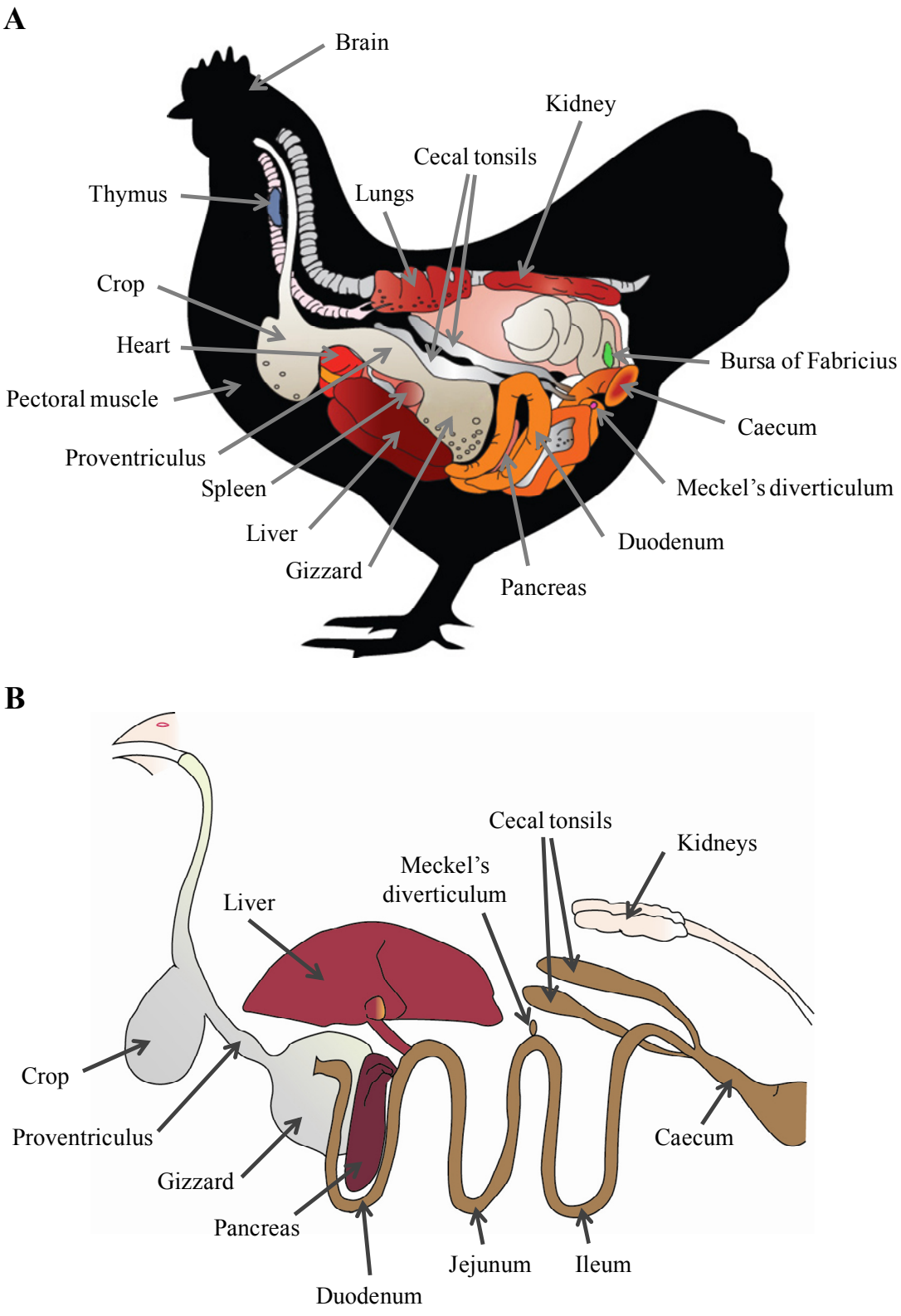

Figure 1. Chicken anatomy. A, general anatomy; B, gastrointestinal tract anatomy in detail. Images available in Pinterest (https://www.pinterest.com) and modified.

1.2. Qualitative and quantitative mRNA expression

Total RNA was extracted using QIAzol lysis reagent according to the manufacturer's protocol (Qiagen) and treated with DNase I (Thermo Fisher Scientific, Fermentas). Total RNA was quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. Reverse transcription was carried out with 1 µg RNA using the iScript cDNA Synthesis Kit (BioRad). The cDNA was used as template for PCRs.

Qualitative PCR was performed in 10 µL final volume containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (LeGene Biosciences). PCR conditions were 35 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 45 sec (Eppendorf Mastercycler). PCR products were visualized on 1.6% agarose gel stained with ethidium bromide. The same primers were used in qualitative and quantitative PCR (Table 2).

Table 2. List of the genes under study and their respective primers sequences, accession numbers and fragment lengths.

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length (bp)
AGP	NM_204541.2	GGTGTACATCATGGGTGCCT	CGCATGTTTCATTCAGCCTCA	143
SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145
OVT	NM_205304.1	AGCCATTGCGAATAATGAGG	ATGGGCTTCAGCTTGTATGG	90
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136
YWHAZ	XM_003205203.1	TTCCCTTGCAAAAACGGCTT	TTCAGCTTCGTCTCCTTGGG	148

AGP, alpha 1-acid glycoprotein; SAA, serum amyloid A; PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like); CRP, C-reactive protein; OVT, ovotransferrin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL4, ribosomal protein L4; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta. SAA, PIT54, CRP, GAPDH, RPL4 and YWHAZ were from Marques et al. (2016). AGP and OVT primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0).

Quantitative real time PCR (qPCR) was performed using 12 µL Eva Green Supermix (BioRad), 250 nM AGP and PIT54, 300 nM SAA, CRP and OVT and 400 nM GAPDH, YWHAZ and RPL4 primers, using Eco Real-Time PCR System (Illumina). GAPDH, RPL4 and YWHAZ were selected as reference genes based on previous studies and literature (Yang et al., 2013; Yue et al., 2010).

In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing fourfold serial dilution starting from the pooled sample composed by a liver cDNA mix from six animals. Each sample was tested in duplicate. Non-reverse transcribed controls were performed by omitting reverse transcription and no template controls were conducted by adding nuclease free water. The thermal profile used (95°C for 10 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec; for melting curve construction, 55°C for 15 sec and 80 cycles starting to 55°C and increasing 0.5°C each 10 sec) was the same for each target gene. The MIQE guidelines were followed (Bustin et al., 2009). For all genes studied, the standard curves derived from serial dilution of pooled sample gave correlation coefficients (R^2) greater than 0.990 and efficiencies greater than 94%. Results were compared using the comparative $^{\Delta\text{-}\Delta}$Cq method.

1.3. Western blotting analysis

Western blotting analysis was carried out on chicken serum using an anti-bovine AGP antibody. Proteins were separated on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membrane. Before gel separation, 1 µl ß-mercaptoethanol (Sigma Aldrich) was added to each sample. Immunolabeling was performed using a rabbit polyclonal anti-bovine AGP (Ceciliani et al., 2007) as primary antibody (1:4000 dilution for 1 h min at RT), while an anti-rabbit IgG labelled with peroxidase (Vector Laboratories) was used as secondary antibody (1:4000 dilution for 45 min at RT). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

To further assess the specificity of the antibody for chicken protein, Western blotting experiment was repeated after overnight incubation of the anti-boAGP antibody with purified bovine AGP using a 1:10 molar ratio, in order to block every anti-AGP specific reactive sites and to detect

any possible non-specific reactions. The solution was then utilized as primary antibody in immunolabeling detection procedure (1:4000 dilution for 1 h at RT).

1.4. Immunohistochemistry studies

The immunolocalization was carried out as previously described (Rahman et al., 2015). Formalin-fixed tissue specimens were routinely processed for histology and paraffin embedded. Sections of 5 μm thick were obtained from paraffin blocks and mounted on poly-lysine-coated slides.

The sections were then deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was performed by heating the slides in citrate buffer solution (pH=6.5) in a water bath at 95°C for 30 min. The sections were cooled for 40 min at RT and then rinsed in Tris buffered saline (TBS). The slides were therefore incubated for 20 min at RT with normal goat serum (1:70) to block any nonspecific protein binding. Sections were incubated at 4°C overnight in a humidified chamber with primary antibodies: polyclonal rabbit anti-AGP, 1:200 (Ceciliani et al., 2007); polyclonal rabbit anti-OVT, 1:800 (MyBioSource).

The sections were then rinsed in TBS for 3 times of 3 min each and then incubated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences) for 20 min at RT. After three washes in TBS, the chromogen 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) was applied for 20 min and, after rinsing in tap water, slides were counterstained with Mayer's haematoxylin (Diapath srl) for 2 min. Slides were therefore rinsed in tap water for 5 min, dried in a stove at 45°C for 12 h and therefore mounted in aqueous mounting agent (Aquatex, Merck). Immunohistochemical staining was evaluated and semi-quantitatively scored as mild, moderate or intense. Histological sections of liver served as positive controls. Additionally, paraffin embedded samples of oviduct from normal hens were retrieved from the departmental archives and served as positive controls for OVT. Negative controls were prepared by replacing the respective primary antibody with normal rabbit IgG (Santa Cruz Biotechnology).

1.5. Statistical analysis

For all statistical procedures, mean and standard error of the mean (SEM) values were calculated. Student t-test, with a two-tailed distribution and an homoscedastic variance was used for all statistical analyses. Significance accepted for p-values ≤ 0.05.

2. Results

2.1. Detection and measurement of acute phase protein mRNA abundance in chicken tissues

In a preliminary set of experiments, the primers quality was assessed by qualitative PCR. Gene specific amplification was confirmed by a single band with the expected size in agarose gel electrophoresis as showed in Figure 2, and confirmed by a single peak in melt-curve analysis by qPCR.

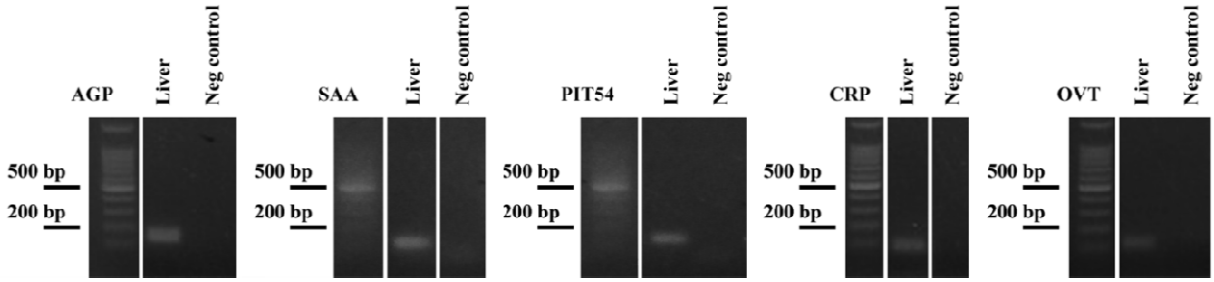

Figure 2. PCR amplification products of AGP, SAA, PIT54, CRP and OVT in liver mRNA separated by agarose gel electrophoresis (1.6%) stained with ethidium bromide. Non-template reaction was used as negative control.

qPCR was used to quantify extrahepatic expression of AGP, SAA, PIT54, CRP and OVT in the following tissues: subcutaneous adipose tissue, visceral adipose tissue, pericardial adipose tissue, pectoral muscle, thymus, spleen, brain, cerebellum, kidney, lung, crop, mucosa of proventriculus, mucosa of gizzard, pancreas, duodenum, caecum, Meckel's diverticulum, cecal tonsil and bursa of Fabricius. Liver was used as reference tissue (mRNA expression=1) (Table 3).

Table 3. Extrahepatic expression of APP ± standard error of the mean (SEM) compared to liver expression values (set to 1). Data shows the mean value of 6 animals.

Tissues	Genes				
	AGP	SAA	PIT54	CRP	OVT
Subcutaneous adipose tissue	5.30E-05 ± 0.0000	7.26E-02 ± 0.0495	9.49E-05 ± 0.0001	6.26E-03 ± 0.0030	6.67E-02 ± 0.0130
Visceral adipose tissue	3.05E-05 ± 0.0000	1.33E-02 ± 0.0033	2.10E-05 ± 0.0000	3.17E-02 ± 0.0197	9.93E-02 ± 0.0503
Pericardial adipose tissue	5.27E-04 ± 0.0005	1.38E-01 ± 0.0310	3.65E-04 ± 0.0003	9.38E-03 ± 0.0030	1.89E-01 ± 0.0330
Pectoral muscle	9.70E-06 ± 0.0000	2.38E-03 ± 0.0010	8.24E-06 ± 0.0000	2.65E-04 ± 0.0002	3.41E-03 ± 0.0009
Thymus	2.05E-05 ± 0.0000	7.09E-02 ± 0.0201	3.27E-04 ± 0.0001	1.91E-03 ± 0.0009	8.55E-03 ± 0.0019
Spleen	8.70E-04 ± 0.0003	2.57E-01 ± 0.0631	9.99E-04 ± 0.0003	1.08E-03 ± 0.0004	1.91E-02 ± 0.0028
Brain	1.23E-04 ± 0.0000	9.32E-03 ± 0.0037	1.45E-04 ± 0.0000	5.90E-02 ± 0.0576	1.08E-01 ± 0.0273
Cerebellum	7.58E-05 ± 0.0000	1.68E-02 ± 0.0070	1.02E-04 ± 0.0000	3.53E-04 ± 0.0001	1.07E-01 ± 0.0330
Kidney	9.67E-04 ± 0.0004	6.56E-02 ± 0.0274	5.59E-04 ± 0.0002	3.69E-03 ± 0.0013	3.44E-02 ± 0.0097
Lung	5.33E-04 ± 0.0003	6.94E-01 ± 0.3031	2.91E-04 ± 0.0002	8.14E-03 ± 0.0033	2.59E-01 ± 0.0676
Crop	3.48E-05 ± 0.0000	3.79E-02 ± 0.0276	4.76E-05 ± 0.0000	2.99E-04 ± 0.0002	1.40E-02 ± 0.0073
Mucosa of proventriculus	3.40E-04 ± 0.0001	1.36E-02 ± 0.0036	2.26E-04 ± 0.0001	7.35E-03 ± 0.0056	2.78E-02 ± 0.0044
Mucosa of gizzard	1.55E-04 ± 0.0001	1.16E-01 ± 0.0507	2.06E-04 ± 0.0002	5.22E-03 ± 0.0020	3.51E-02 ± 0.0103
Pancreas	1.45E-04 ± 0.0001	3.30E-02 ± 0.0117	2.67E-04 ± 0.0001	2.21E-03 ± 0.0010	1.12E-01 ± 0.0253
Duodenum	1.61E-05 ± 0.0000	1.83E-02 ± 0.0068	1.26E-04 ± 0.0001	6.41E-04 ± 0.0003	1.78E-02 ± 0.0135
Meckel's diverticulum	6.61E-06 ± 0.0000	2.06E-01 ± 0.1290	1.34E-05 ± 0.0000	5.44E-03 ± 0.0047	3.13E-02 ± 0.0227
Caecum	1.77E-05 ± 0.0000	2.41E-02 ± 0.0059	7.65E-06 ± 0.0000	5.71E-04 ± 0.0001	8.39E-03 ± 0.0012
Cecal tonsil	5.08E-05 ± 0.0000	7.22E-01 ± 0.2917	1.93E-05 ± 0.0000	8.26E-04 ± 0.0002	4.11E-02 ± 0.0113
Bursa of Fabricius	5.23E-05 ± 0.0000	3.00E-02 ± 0.0134	3.39E-05 ± 0.0000	2.68E-03 ± 0.0013	1.26E-01 ± 0.0280

mRNA coding for SAA, CRP and OVT was detected in all tissues analysed, although its concentration appeared to vary considerably between different tissues (Figure 3). Its amounts ranged from 0.002 to 0.72, 0.001 to 0.06 and 0.003 to 0.26, respectively, as compared to liver, with cecal tonsil and lung having the highest concentration. SAA mRNA expression was higher in cecal tonsil, lung, spleen, Meckel's diverticulum, pericardial adipose tissue and mucosa of gizzard (0.72, 0.69, 0.26, 0.21, 0.13 and 0.12, respectively). CRP mRNA expression was high just in brain and visceral adipose tissue (0.06 and 0.03, respectively). OVT was more expressed in lung, followed by pericardial adipose tissue and bursa of Fabricius (0.26, 0.19 and 0.13, respectively), whereas brain, cerebellum, pancreas, visceral adipose tissue and subcutaneous adipose tissue showed around 0.10 mRNA expression.

Expression of AGP and PIT54 was lower as compared to liver (from 0.0001 to 0.001). Nonetheless, both APP were detected in all the tissues (Figure 4).

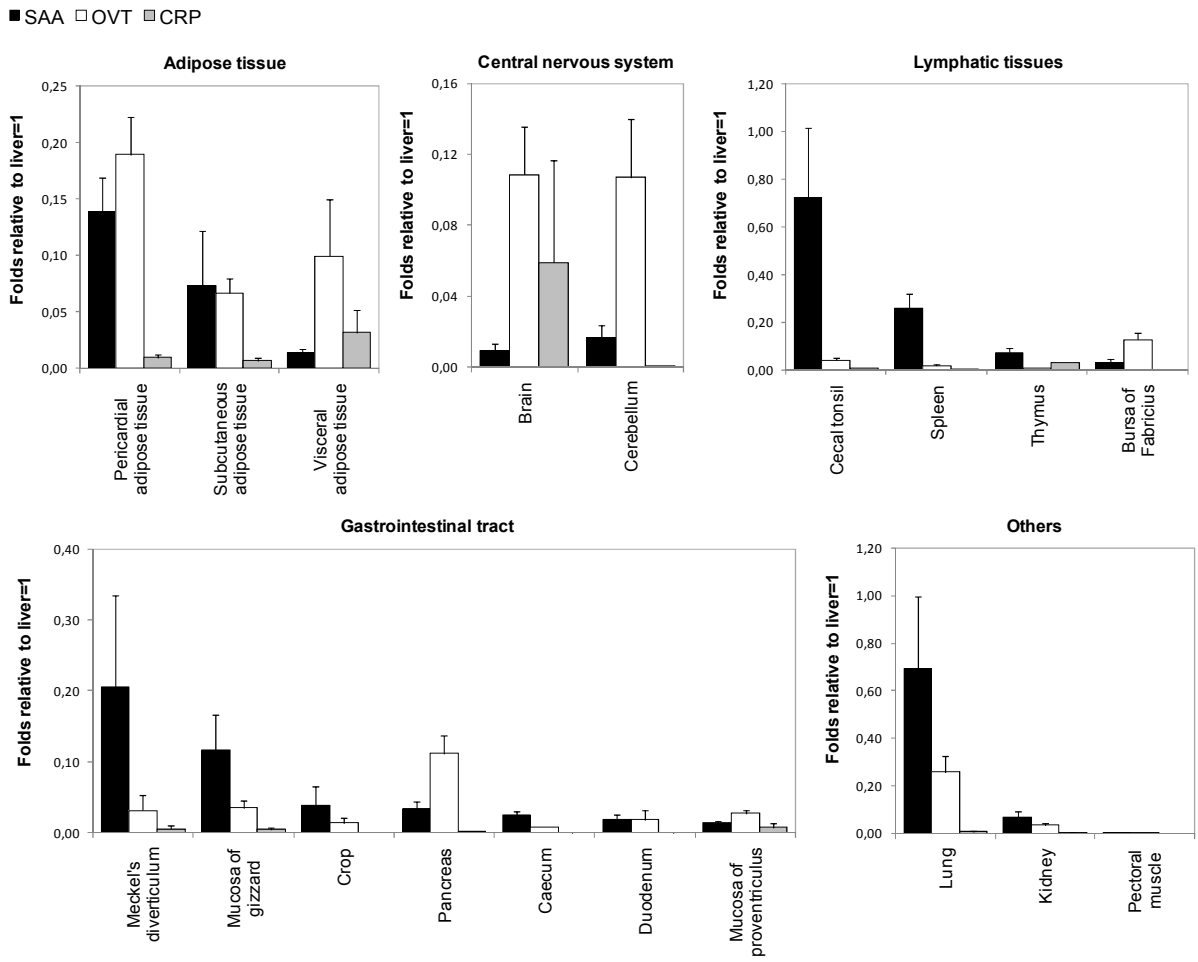

Figure 3. Relative extrahepatic expression of SAA, OVT and CRP in chicken healthy tissues studied by qPCR. Liver was used as reference tissue and data are means ± SEM of six animals.

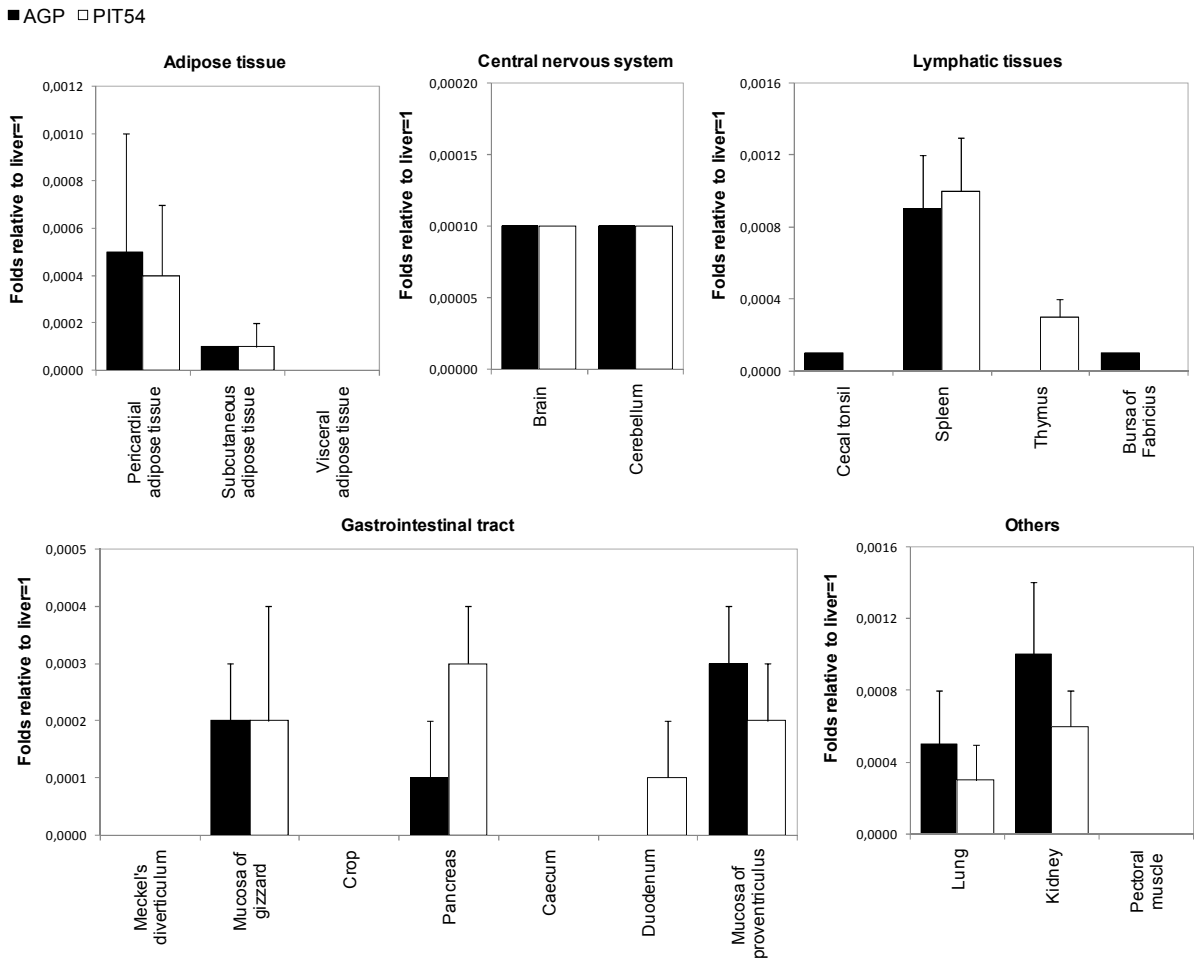

Figure 4. Relative extrahepatic expression of AGP and PIT54 in chicken healthy tissues studied by qPCR. Liver was used as reference tissue and data are means ± SEM of six animals.

2.2. Validation of anti-bovine AGP antibody by Western blotting

To the best of our knowledge, no antibody specific for chicken CRP, PIT54 and SAA has also been reported in literature so far, but there is a commercial antibody available for OVT. Then, attention was focused on validating an antibody against AGP, already validated for turkey species (Marques et al., 2016), but not in chicken. The presence of AGP was confirmed by Western blotting using an anti-bovine AGP antibody (Ceciliani et al., 2007) that was shown to cross-react with a most prominent band with a molecular weight (MW) of 55–65 kDa in serum (Figure 5).

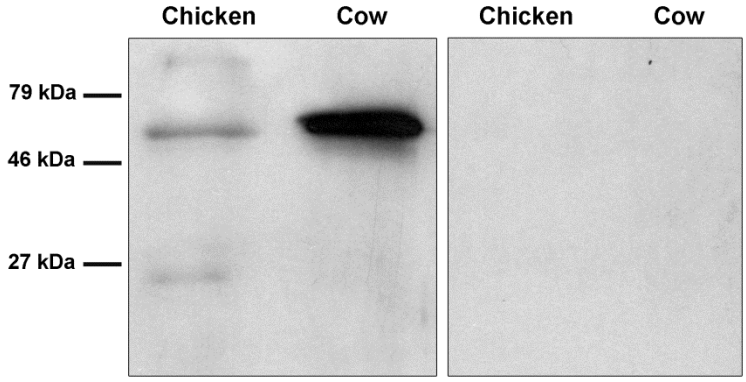

Figure 5. Detection of chicken AGP by Western blotting in serum. Left panel: anti-boAGP primary antibody. Right panel: anti-boAGP primary antibody after blocking specific sites with purified bovine AGP.

2.3. Localization of APPs in chicken tissues by immunohistochemistry

No antibodies specific for chicken CRP, PIT54 and SAA are available. Immunohistochemistry studies were then performed using only AGP and OVT antibodies. A polyclonal anti-chicken OVT was purchased (MyBioSource) and a polyclonal anti-bovine AGP antibody (Ceciliani et al., 2007) was validated in chicken.

The presence of AGP and OVT proteins was clearly identified in endothelium of every tissue, confirming they are circulating proteins that can easily reach any tissue by bloodstream. At immunohistochemical examination, liver and oviduct were used as positive controls and stained for AGP and both AGP and OVT, respectively. Immunohistochemical expression of AGP and

OVT was variably detected in tested tissues, with similar pattern of expression for both markers (Figure 6). Detailed results are given in Table 4.

Briefly, multifocal positivity was detected in adipose and connective tissue for both AGP and OVT. Scattered positive cells, for both markers, were variably present in the epithelium of the mucosal layer of organs of the gastrointestinal tract (crop, mucosa of proventriculus, duodenum, caecum and meckel's diverticulum). In the liver, hepatocytes were mildly and diffusely positive, whereas ductal epithelium exhibited intense multifocal granular intracytoplasmic positivity, as also seen in kidney tubular epithelium. Pancreatic islets were intensively positive but acini always negative. Lung was diffusely positive in its different anatomic regions. Central nervous tissue was multifocally positive according to different areas. No positive staining was detected in lymphoid tissues (thymus, intestinal Peyer's patches, bursa of Fabricius, spleen) and muscle.

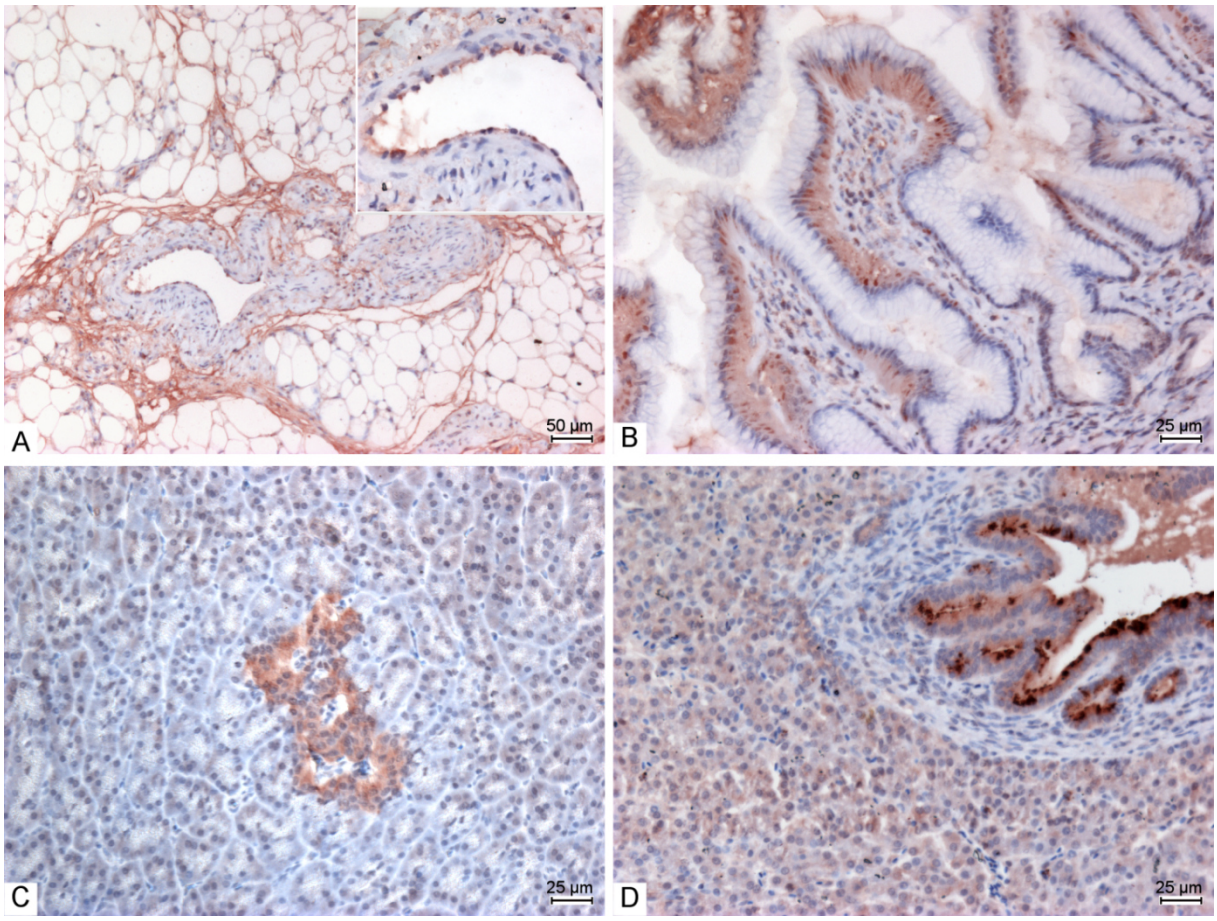

Figure 6. Immunolocalization of AGP and OVT in chicken. Immunohistochemical staining (standard ABC method, AEC red chromogen). A, subcutaneous adipose tissue, anti-AGP, 10X: multifocal positive adipose tissue surrounding a central arterial vessel with negative tunica media and positive endothelium (inset). B, mucosa of proventriculus, anti-OVT, 20X: diffuse positivity in the basal portion of mucosal columnar epithelium. C, pancreas, anti-OVT, 20X: positive islet and negative acini. D, liver, anti-OVT, 20X: multifocal intense positivity in ductal epithelium and mild diffuse positivity in hepatocytes section of liver. Bar, 25 and 50 micrometers.

Table 4. Results of the immunohistochemical examination.

Tissue		AGP	OVT
Subcutaneous adipose tissue	**Adipocytes**	+ (intense, with scattered less positive)	+ (multifocal to diffuse intense)
	Connective tissue	+ (multifocal)	+ (multifocal)
Visceral adipose tissue	**Adipocytes**	+ (intense multifocal to diffuse)	+ (multifocal)
	Connective tissue	+ (multifocal)	+ (multifocal)
Pericardial adipose tissue	**Adipocytes**	+ (diffuse)	+ (multifocal)
Pectoral muscle	**Muscular fibers**	Negative	Negative
Thymus	**Lymphocytes**	Negative	Negative
Spleen	**Lymphocytes**	Negative	Negative
Brain	**Neurons**	+ (mild diffuse)	+ (mild diffuse)
	Axons	Negative	Negative
	Choroid plexus	+ (diffuse)	+ (diffuse)
Cerebellum	**White matter**	Negative	Negative
	Granule cell layer, Purkinje cell layer	Negative	Negative
	Molecular layer	+ (mild)	+ (mild)
Kidney	**Tubular epithelium**	+ (multifocal cytoplasmic)	+ (multifocal granular cytoplasmic)
	Glomeruli	Negative	Negative
Lung	**Parabronchi, atria, air capillaries, bronchi**	+	+
Crop	**Epithelium**	stratum corneum: +, stratum lucidum and stratum granulosum: -, stratum spinosum and stratum basale: +	stratum corneum: +, stratum lucidum and stratum granulosum: -, stratum spinosum and stratum basale: +
	Muscle	Negative	Negative
Mucosa of proventriculus	**Mucosal columnar epithelium**	+ (in the basilar zone of the cytoplasm)	+ (in the basilar zone of the cytoplasm)
	Ductal epithelium	+ (faint diffuse)	+ (moderate multifocal)
	Oxynticopeptic cells	+ (faint patchy)	+ (faint patchy)
Mucosa of gizzard	**Epithelium, koilin, muscle**	Negative	Negative
Pancreas	**Islets**	+ (intense)	+ (intense)
	Acini	Negative	Negative
Intestine	**Duodenum, ceacum epithelium, Meckel's diverticulum epithelium**	+ (mild scattered single cells)	+ (mild scattered single cells)
	Lymphoid follicles (GALT, cecal tonsil)	Negative	Negative
Bursa of Fabricius	**Lymphocytes**	Negative	Negative
	Epithelium	+ (mild multifocal)	+ (mild multifocal)
Liver	**Hepatocytes**	+ (faint diffuse)	+ (faint multifocal)
	Ductal epithelium	+ (mild multifocal)	+ (intense multifocal)

+/- = mild, + = moderate, ++ = intense

3. Discussion

The present study reports for the first time the extrahepatic expression of five APP, namely AGP, SAA, PIT54, CRP and OVT, in twenty tissues collected from healthy chicken. Where antibodies were commercially available, protein localization was also determined by immunohistochemical studies. Acute phase proteins mRNA was detected in all tissues included in this experiment, and liver was confirmed to be the main source of APP. Extrahepatic production of APP mRNA was found to be particularly abundant in the gastrointestinal tract, lymphatic system and lung.

Among the five APP, SAA mRNA was found to be the most abundantly expressed. SAA is a major APP in vertebrates and can easily increase its concentration up to 1000-fold as response to a stimuli (Gabay and Kushner, 1999). SAA belongs to a family of apolipoproteins that are incorporated into high-density lipoprotein to be afterward released into the circulation (Benditt and Eriksen, 1977). In mammals, multiple SAA genes have been described (Uhlar and Whitehead, 1999; Upragarin et al., 2005). In chickens, on the other hand, only one SAA gene has been identified so far (Ovelgönne et al., 2001). SAA exhibits significant immunological activity being chemotactic for neutrophils and mast cells, and takes part to the so called cytokine-serum amyloid A-chemokine network (De Buck et al., 2016). In humans, SAA can act as pathogenic pattern recognition protein by opsonizing Gram-negative bacteria, increasing meanwhile macrophages and neutrophil phagocytosis (Shah et al., 2006). Furthermore, SAA can activate the inflammasome cascade (Eklund et al., 2012). Given the background of its immune-modulatory activity, it is not surprising that the extrahepatic tissues where SAA was found to be mostly expressed were those related to immune defenses, such as for example cecal tonsils, in which SAA mRNA abundance almost equals the amount produced by liver (0.72). This finding supports the hypothesis that cecal tonsil, which is the major lymphoid district within the gut-associated lymphoid tissue (GALT) (Yun et al., 2000), is probably a second main source of SAA in chicken. Spleen was the other tissue where SAA was found to be abundantly expressed (0.26 as compared with liver), in agreement with previous findings reporting splenic expression of SAA in chickens orally infected with *S. enteritidis* (Matulova et al., 2012) and *Ascaridia galli* (Dalgaard et al., 2015). The mRNA abundance of SAA in the cecal tonsils and spleen tissues may confirm in chicken that, as has been demonstrated in humans and laboratory rodents, SAA is also involved in the development of adaptive immunity, as shown by its capability to induce the expression of IL-17A and IL-17F from CD4+ T cells (Ather et al., 2011; 2013). This

hypothesis is also supported by the finding of SAA mRNA in Meckel's diverticulum, which is regarded as a fully mature lymphoid tissue in chicken (Casteleyn et al., 2010). SAA mRNA expression was also detected in pancreas, in a similar way already reported by Lecchi et al. (2012) and, for the first time, in gizzard's mucosa, which is a part of the digestive tract of birds. The potential presence of SAA in this district is interesting, since provides the evidence that also locally produced innate-immunity related proteins can protect the mucosa of the mechanical stomach of poultry. The last tissue where SAA was found to be highly expressed was lung (0.60 as compared with liver). Respiratory system provides the second main entrance for many pathogens in mammals as well as in avian species (Smiałek et al., 2011). The finding of SAA expression in lung is consistent with what has been recently reported about lung and spleen SAA overexpression in chickens experimentally challenged with H5N1 influenza virus (Burggraaf et al., 2014).

Beside SAA, also OVT was found to be heavily expressed in lungs (0.26 as compared to liver). The product of expression, namely the OVT protein, was also detected at the protein level by immunohistochemistry, identifying lungs as another important source of APP in chicken (Geus and Vervelde, 2013). Ovotransferrin provides an useful biomarker of inflammatory diseases in chicken (Rath et al., 2009), but no information about its extrahepatic expression was available before the present study. The high level of expression of OVT in lungs may also explain the early appearance of this protein in serum after experimental challenging with avian influenza virus (Sylte and Suarez, 2012). Ovotransferrin is a multi-functional protein with a major role in avian natural immunity (Giansanti et al., 2012). It was shown that OVT could permeate *E.coli* outer membrane, accessing to their inner membrane and causing ion leakage inside bacteria, eventually driving to the uncoupling the respiratory-dependent energy production (Aguilera et al., 2003). Consistently with its immune-related function, OVT mRNA was found expressed also in bursa of Fabricius (0.13 as compared with liver), which is a central lymphoid organ for B cells production and lymphocyte maturation (Ratcliffe, 2002). On the background of its distribution along the mucosa and the immune districts associated to gastroenteric system, we may therefore speculate that OVT may contribute to the first line of defense against invading pathogens. High expression of OVT was also detected in pancreas (0.11), pericardial adipose tissue (0.19) and brain (0.11). Its identification in brain suggests that OVT may fulfil its activity of iron-binding uptake (Taylor and Morgan, 1991) also in chicken central nervous system. We may also not rule out the possibility that one of OVT function in brain includes the scavenging of iron radical in

order to protect neural cells from reactive oxygen species damages. The physiological role of OVT in pancreas remains elusive.

CRP mRNA was found to be moderately expressed in brain and visceral adipose tissue (0.06 and 0.03 as compared to liver, respectively). CRP has the ability to bind directly to necrotic cells, cellular debris and polysaccharides on bacteria, fungi, and parasites, thus acting as an opsonin. In as such, CRP activates the complement system when bound to one of its ligands and binds to phagocytic cells, modulating both humoral and cellular components of inflammation (Petersen et al., 2004). In humans, CRP over-expression in brain and adipose tissue is associated to brain diseases, or metabolic disorders related to inflamed adipose tissue, respectively (Brooks et al., 2010; Islam, 2016). The biological significance of CRP expression in these two tissues in chickens has yet to be elucidated.

Although detected in all tissues included in this studies, the extrahepatic mRNA expression of AGP and PIT54 was found to be negligible as compared to liver (<0.01). For what concerns AGP, these findings look somehow contradictory if compared with immunohistochemistry result, since the presence of the protein was demonstrated in many tissues. This result can be partially explained by the long half-life of AGP after its synthesis (Ceciliani and Pocacqua, 2007). It cannot also be ruled out the possibility that the protein found in the tissues also derive, at least partially, from the liver and carried out to tissues via blood circulation.

4. Conclusions

In conclusion, the data presented here demonstrated that the five acute phase proteins AGP, SAA, PIT54, CRP and OVT, are constitutively expressed in all chicken tissues even in absence of a systemic acute phase response. The APP mRNA abundance of SAA and OVT was found to be higher in three systems, namely respiratory, gastrointestinal and lymphoid system. We confirmed the presence of AGP and OVT corresponding proteins directly in almost all tissues.

The results presented in this study suggest the hypothesis that extrahepatic APP, in particular those produced at mucosal levels, might play important roles in the innate and adaptive immunity by providing the first line of defense against pathogens or locally modulating the inflammation. Probably, the locally APP production can immediately support and control the local defense and initiate repairs, while liver supplies vast amounts of these reactants to all body parts.

Further research is needed to investigate the relationship between local and systemic reactions and to determine the significance of its local production in avian species. Understanding where APP are produced and secreted is the first step for their proper utilization as biomarkers during diseases.

5.2. The effect of transport stress on turkey (*Meleagris gallopavo*) liver and adipose tissue acute phase proteins gene expression

Part of this work is already published as:

The effect of transport stress on turkey (*Meleagris gallopavo*) liver acute phase proteins gene expression.

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Keywords: acute phase proteins, animal welfare, *Meleagris gallopavo*, road transportation stress, turkey.

Abstract

The aim of this study was to investigate the effects of transport-related stress on liver and adipose tissue gene expression of four acute phase proteins (APP), namely α1-acid glycoprotein (AGP), Serum Amyloid A (SAA), PIT54 and C-Reactive Protein (CRP) in turkey (*Meleagris gallopavo*). A group of seven BUT BIG 6 commercial hens was subjected to a 2-hour long road transportation and the quantitative gene expression of APP in the liver and adipose tissue was compared to that of a not transported control group. The expression of AGP and CRP mRNA was found to be increased in animals slaughtered after road transport. AGP mRNA expression was increased in both liver and adipose tissue, whereas CRP mRNA expression was increased in liver alone. The presence of AGP protein was also confirmed by immunohistochemistry and Western blotting. Two antibodies against CRP and PIT54 proteins were raised but their validation was not succeeded. The results of this study showed that road-transport might induce the mRNA expression of immune related proteins. The finding that AGP and CRP can be upregulated during transport could suggest their use as for the assessment of turkey welfare during transport.

1. Material and methods

1.1. Samples collection and preservation

Liver and visceral adipose tissue samples were collected during routinely slaughtering procedures from fourteen 92 day-old clinically healthy BUT BIG 6 breeding line female turkeys. Clinical status of the animals was verified following a clinical examination of the animals and the absence of evident pathologic lesions in the carcasses.

The first group of seven animals was slaughtered and sampled in an abattoir located on site (control group – not transported animals), the second group of seven animals received the same treatment after a two hours transport (transport–stressed animals). The transport of turkeys was carried out on a truck that was authorized by Italian Health Institutions following the Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97). Animal were transported in plastic coop 1 m width × 60 cm length × 39"L × 40 cm high. Temperature ranged from 20 to 22°C. Both groups underwent the same handling procedure (namely catching and caging and the same slaughtering procedure), with the exception of road-transport.

Portions of liver and visceral adipose tissue were removed immediately after slaughtering, snap frozen into liquid nitrogen and afterwards stored at −80ºC (for gene expression analyses). Samples for immunohistochemistry analysis were fixed in 10% buffered formalin.

1.2. Primers design

The nucleotide sequences of four APP, namely AGP, SAA, PIT54 and CRP, were identified among the sequences available in NCBI. Primers were designed on turkey sequences available in NCBI by using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0), preventing possible secondary structures with the mfold Web Server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and ensuring the specificity of the sequence by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). Primer sequences and fragment sizes are described in Table 1.

Table 1. List of the genes under study and their respective primers sequences, accession numbers and fragment lengths.

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length (bp)
AGP	XM_003211214	TCCCTGCCGAAATAGACAAC	TCCTTCATCTCAGCCATGTG	103
SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136
YWHAZ	XM_003205203.1	TTCCCTTGCAAAAACGGCTT	TTCAGCTTCGTCTCCTTGGG	148

AGP, alpha 1-acid glycoprotein; SAA, serum amyloid A; PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like); CRP, C-reactive protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL4, ribosomal protein L4; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

1.3. RNA extraction and cDNA synthesis

Total RNA was extracted using TriZol standard protocol (Invitrogen). RNA concentration and quality were validated as ratio A260/A280 by NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc.). RNA was treated with DNase (Fermentas) and the first-strand cDNA synthesis was carried out with 1 μg RNA using iScript cDNA synthesis kit (BioRad).

1.4. Qualitative mRNA expression

The cDNA was used as template for PCRs, which were performed in 10 μL final volume containing 1x buffer (LeGene Biosciences), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 μM each primer (Table 1) and 0.025 U Taq polymerase (LeGene Biosciences). PCR conditions were 35 cycles of 94ºC for 30sec, 60ºC for 30sec and 72ºC for 45sec (Eppendorf Mastercycler). PCR products were visualized on 1.6% agarose gel stained with ethidium bromide.

1.5. Quantitative mRNA expression

Quantitative Real Time PCR (qPCR) was performed using 12 µL Eva Green Supermix (BioRad), 250 nM AGP and PIT54, 300 nM SAA and CRP and 400 nM GAPDH, YWHAZ and RPL4 primers, using Eco Real Time PCR detection System (Illumina).

In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing four-fold serial dilution starting from the pooled sample composed by liver cDNA of five not transported animals. Each sample was tested in duplicate. No-RT controls were performed by omitting reverse transcription and no template controls were conducted by adding nuclease free water. The thermal profile used was the same for each target gene: 95°C for 10 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec; for melting curve construction, 55°C for 15 sec and 80 cycles starting to 55°C and increasing 0.5°C each 10 sec. The MIQE guidelines were followed (Bustin et al., 2009). For all genes studied, the standard curves derived from serial dilution of pooled sample gave correlation coefficients (R^2) greater than 0.990 and efficiencies greater than 94%. Results were compared using the comparative $^{\Delta\text{-}\Delta}$Cq method (Giulietti et al., 2001).

Three housekeeping genes were selected (GAPDH, RPL4 and YWHAZ) based on previous studies and literature (Lecchi et al., 2012; Yang et al., 2013; Yue et al., 2010) and geometric mean was used for normalization purposes.

1.6. Western blotting analysis

In order to assess the cross-reactivity of turkey AGP with available anti-AGP antibodies, Western blotting analysis was carried out on turkey liver and adipose tissue using an anti-bovine AGP antibody. No antibody specific for turkey CRP, PIT54 and SAA has been reported so far in literature.

Aliquots of 50 mg of liver and 100 mg of adipose tissue were mechanically homogenized in 6 volumes (w/v) of lysis buffer (50 mM Tris-HCl pH=7.6, 150 mM NaCl, 5 mM EDTA, 4% IGEPAL (NP-40), 1% Triton X-100, 1% Zwitterion) added with a protease inhibitors cocktail (1:100) (Sigma Aldrich) and incubated for 1 h on ice. After incubation, aliquots were centrifuged at 14000 x g for 15 min at RT. For adipose tissue, an extra centrifugation was done at 14000 x g

for 10 min at RT. The protein content of the supernatant was then quantified at 280 nm by JASCO Model V-530 UV-vis Spectrophotometer (JASCO Inc.).

A total of 100 µg (liver) and 150 µg (adipose tissue) total protein were separated on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membrane. Before gel separation, 1 µl ß-mercaptoethanol (Sigma Aldrich) was added to each sample. Immunolabeling was performed using a rabbit polyclonal anti-bovine AGP (Ceciliani et al., 2007) as primary antibody (1:4000 dilution for 1 h min at RT), while an anti-rabbit IgG labelled with peroxidase (Vector Laboratories) was used as secondary antibody (1:4000 dilution for 45 min at RT). A purified AGP from bovine (1 µg/µL) was used as positive control. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescent HRP Substrate (Millipore). A purified AGP from bovine serum (1 µg/µL) was used as positive control (Ceciliani et al., 2005).

In order to further assess the specificity of the primary antibody, the Western blotting experiment was repeated after overnight incubation of bovine anti-AGP antibody (Ceciliani et al., 2007) coupled with bovine AGP (Ceciliani et al., 2005) using a 1:10 molar ratio, in order to block every anti-AGP specific reactive sites and to detect any possible non-specific reactions. The solution was then utilized as primary antibody in immunolabeling detection procedure (1:4000 dilution for 1 h at RT).

1.7. Immunohistochemistry studies

Immunolocalization in liver was carried out as previously described (Rahman et al., 2015). Briefly, immunohistochemistry was performed on fixed in 10% buffered formalin and embedded in paraffin tissue sections to identify AGP in the different cell types present in liver and adipose tissue. Sections of 5 µm thick were obtained and mounted on poly-lysine-coated slides.

The sections were deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was then performed by heating the slides in a pressure cooker for 10 min at 95ºC in citrate buffer solution (pH=6.5). The sections were cooled for 40 min at RT and then rinsed in Tris buffered saline (TBS). The slides were then incubated for 20 min at RT with normal goat serum (1:70) to block any nonspecific protein binding. After

incubation with the same primary antibody used for Western blotting at a dilution of 1:200 for anti-bovine AGP (Ceciliani et al., 2007), the slides were incubated at 4°C overnight in a humidified chamber.

The sections were rinsed in TBS for 3 times of 5 min each and treated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences) for 20 min at RT. After three washes in TBS, the chromogen, 3-amino-9-ethylcarbazole (Vector Laboratories), was applied for 20 min. The slides were then rinsed in tap water, counterstained with Mayer hematoxylin (Diapath srl) for 1 min and mounted with glycerine jelly (Kaiser's glycerol gelatine, Merck).

Negative controls were prepared by replacing the respective primary antibody with normal rabbit serum (non-immune rabbit serum, Dakocymation). In addition, to rule out unspecific cross-reactivity of the antibodies included in this study the anti-AGP specific sites were blocked by pre-incubation with bovine purified AGP, prior to IHC as previously described (Ceciliani et al., 2005; Rahman et al., 2010).

1.8. Anti-CRP and anti-PIT54 antibodies production and validation

CRP and PIT54 protein sequence from turkey and chicken were aligned against CRP and Hp rabbit sequences. Two small peptides composed by 15 amino acids 100% conserved between turkey and chicken species, but not between poultry and rabbit, were selected (Table 2). The peptides were synthesize as Multiple Antigen Presentation (MAP) technique. Using a BlastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the new designed peptides were confirmed not have homology with rabbit.

Four rabbits were inoculated with new produced peptides. Two rabbits were inoculated with CRP peptide and two rabbits were inoculated with PIT54 peptide. Blood was collected from the animals after a two months period and antibodies were purified by affinity chromatography using the respective peptide as column ligands. This part of the project was carried out in cooperation with the group of Biochemistry of the department, with the support of Dr. Vitaliano Borromeo and Dr. Anna Berrini.

Table 2. CRP and PIT54 peptide sequence from turkey and chicken.

Protein name	Peptide sequence
CRP	YVTFRVPENRGEWE
PIT54	ALGGARYGRGPDVIW

CRP, C-reactive protein; PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like).

Reactivity between new produced antibodies against CRP and PIT54 turkey and chicken proteins was validated by Western blotting analysis. Serum from turkey and chicken were separated on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane. Immunolabeling was performed using the new produced rabbit polyclonal anti-CRP and anti-PIT54 antibodies as primary antibody (1:2000 and 1:1000 dilutions, respectively, for 2 h at RT), while a goat anti-rabbit IgG with peroxidase (Vector Laboratories) was used as secondary antibody (1:10000 dilution for 45 min at RT). CRP and PIT54 peptides (0.05 μg/μL) were used as positive control.

The specificity of the primary antibodies was confirmed after overnight incubation of anti-CRP and anti-PIT54 antibodies coupled with CRP and PIT54 peptides, respectively, using a 1:10 molar ratio, in order to block every anti-CRP and anti-PIT54 specific reactive sites. The solution was then utilized as primary antibody in immunolabeling detection procedure (1:2000 dilution for 2 h at RT).

1.9. Statistical analysis

Statistical analysis was performed using a General Linear Model procedure of SAS (SAS version 9.2 Inst. Inc., Cary, NC). Least significant difference was used to compare mean values. Statistical significance was accepted at p-value ≤ 0.05.

2. Results

2.1. Expression of APP mRNA in turkey liver and adipose tissue

In order to make a first screening of APP in turkey, the expression of AGP, SAA, PIT54 and CRP was investigated by qualitative PCR in liver and adipose tissue. Gene specific amplification was confirmed by a single band with the expected size in agarose gel electrophoresis (Figure 1). Results from qualitative PCR revealed the presence of AGP, SAA, PIT54 and CRP in liver and adipose tissue mRNA.

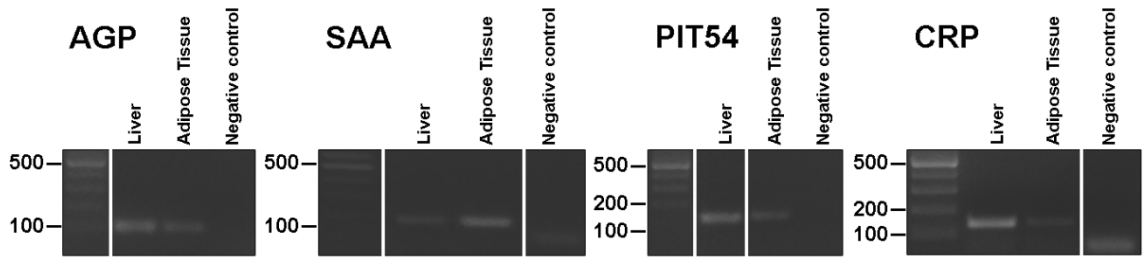

Figure 1. PCR amplification products of AGP, SAA, PIT54 and CRP in liver and adipose tissue mRNA separated by agarose gel electrophoresis (1.6%) stained with ethidium bromide. Non-template reaction was used as negative control.

2.2. Transport stress influences mRNA abundance of AGP and CRP in liver

APP mRNA abundance in liver from not transported and transport–stressed animals was determined by qPCR after normalization against reference genes. Results are presented in Figure 2. Liver AGP and CRP mRNA abundance was found to be statistically significantly increased three-folds in animals slaughtered after 2 h transport, as compared with not transported animals (p-value = 0.0205 and 0.0109, respectively). The mRNA abundance of PIT54 and SAA did not show any statistical significant difference after road transportation.

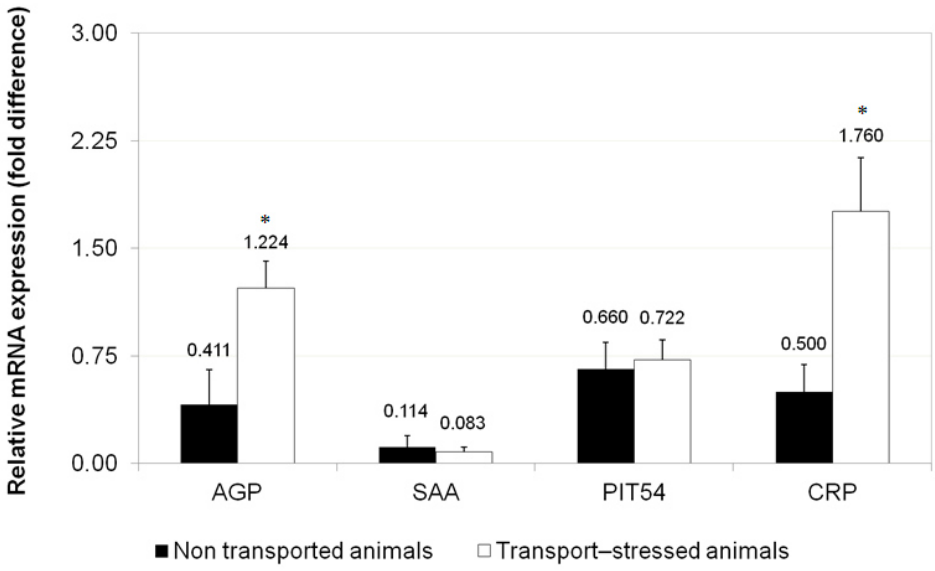

Figure 2. Relative expression of AGP, SAA, PIT54 and CRP in liver of not transported and transport–stressed animals studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Data are means ± SEM of seven animals. Folds relative to liver=1.

2.3. Transport stress influences the mRNA abundance of AGP in adipose tissue

APP mRNA abundance in adipose tissue from not transported and transport–stressed animals was measured by qPCR after normalization against reference genes. Results are presented in Figure 3. The mRNA abundance of all the four APP was lower as compared to that found in liver. In fact, it was not possible to differentially quantify mRNA coding for AGP and PIT54 in the adipose tissue of not transported animals. The mRNA abundance of AGP in adipose tissue amounted, in average, to only 0.01 – 0.05% as compared to that found in liver, but it increases in a statistically significant way (p-value = 0.0301) in transport–stressed animals. Unlike liver, adipose tissue PIT54 seems to be more expressed after the road transportation (126-folds as compared to its expression in not transported animals), but the results are not statistically significant even if there is a positive trend (p-value = 0.0806). CRP have shown a slight increase (2.7-folds) in transport–stressed animals when compared with not transported animals but the results are not significant due to high individual variability. For SAA, a slight increase (3-folds) was also observed, but its expression remains at a basal level and it is not statistically significant.

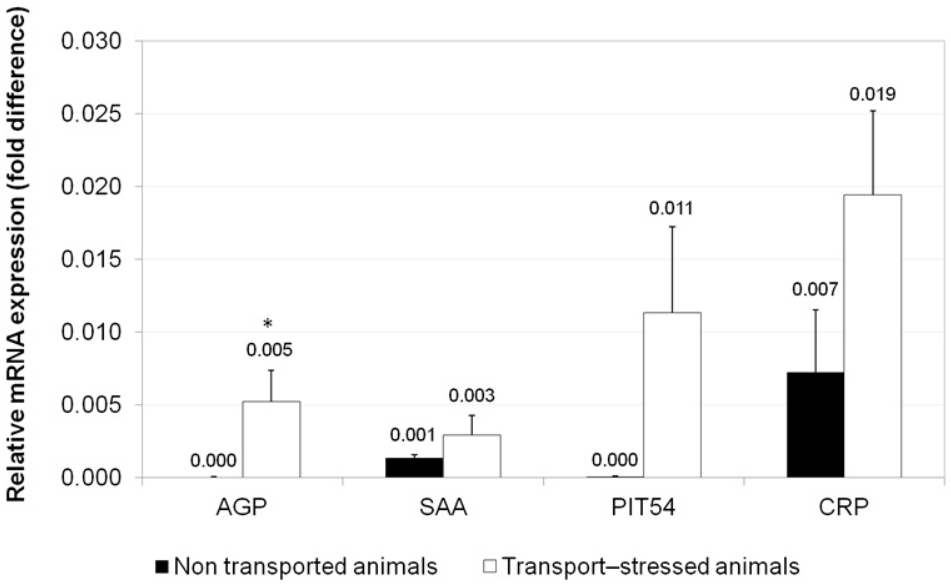

Figure 3. Relative expression of AGP, SAA, PIT54 and CRP in adipose tissue of not transported and transport–stressed animals studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Folds relative to liver. Data are means ± SEM of seven animals. Folds relative to liver=1.

2.4. Validation of anti-bovine AGP antibody by Western blotting

To the best of our knowledge, no antibody specific for turkey CRP, PIT54 and SAA has been reported in literature at least so far. Attention was thus focused on AGP only, given that its expression was found to be statistically significantly overexpressed in liver and adipose tissue.

The presence of AGP was confirmed by Western blotting using an anti-bovine AGP antibody (Ceciliani et al., 2007) that was shown to cross-react with a most prominent band with a molecular weight (MW) of 55–65 kDa in liver (Figure 4A) and adipose tissue (Figure 5A). The electrophoretical pattern (MW and presence of high molecular weight glycoforms) is consistent between turkey and bovine. A low molecular weight band (MW – 35 kDa), corresponding to deglycosylated AGP, is present in turkey liver but not evident in bovine liver.

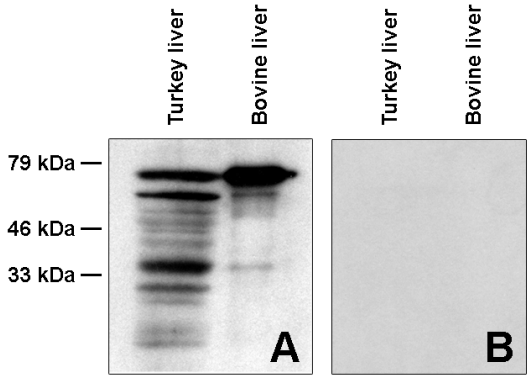

Figure 4. Detection of turkey AGP by Western blotting in liver tissue. A, anti-bovine AGP primary antibody. B, anti-bovine AGP primary antibody after blocking specific sites with purified bovine AGP.

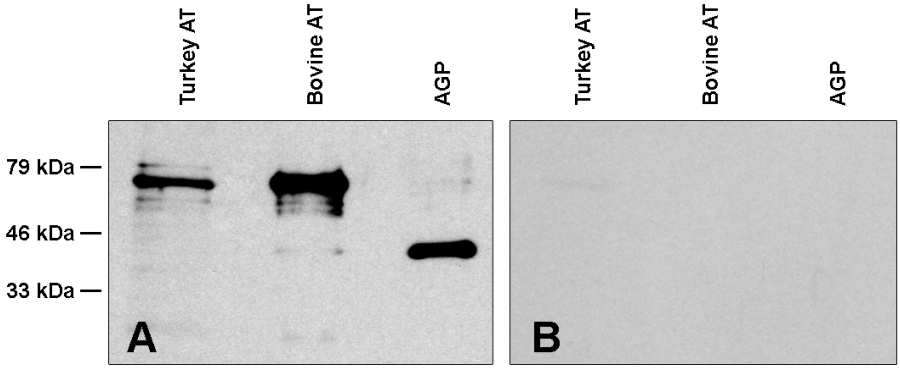

Figure 5. Detection of turkey AGP by Western blotting in adipose tissue (AT). A, anti-bovine AGP primary antibody. B, anti-bovine AGP primary antibody after blocking specific sites with purified bovine AGP.

In order to rule out any unspecific cross-reactivity, blocking of the anti-bovine AGP antibody specific sites was carried out by incubating an aliquot of antibody with a purified bovine AGP (Rahman et al., 2015). The mixture was then utilized as primary antibody on Western blotting membrane. Neither turkey nor bovine reactive bands' or tissue immunodetection was detected, thus confirming that all bands visualized were derived from AGP (Figure 4B and 5B).

2.5. The localization of AGP in turkey liver and adipose tissue

An anti-AGP positive immunostaining was detected in liver and adipose tissue. Results are presented in Figure 6.

In hepatocytes (Figure 6A), anti-AGP positive intracytoplasmic finely granular immunostaining (red staining) was multifocally detected. Positive signal was not diffuse and small groups of positive hepatocytes were scattered throughout the liver parenchyma, without any evident pattern.

In adipose tissue section (Figure 6B), anti-AGP stained an elevated number of adipocytes. Specifically, anti-AGP immunostaining was localized at the periphery of adipocyte cytoplasm, lining cell borders. Immunostaining was not detected in adipose tissue fibrous septa nor in vessels.

As already demonstrated during Western blotting experiments, the positive immunolabeling of control and fat tissues completely disappeared when tissue sections were stained with the pre-blocked antibodies, confirming the specificity of anti-AGP antibody for its antigen on tissues as well. No immunostaining was observed in negative control samples.

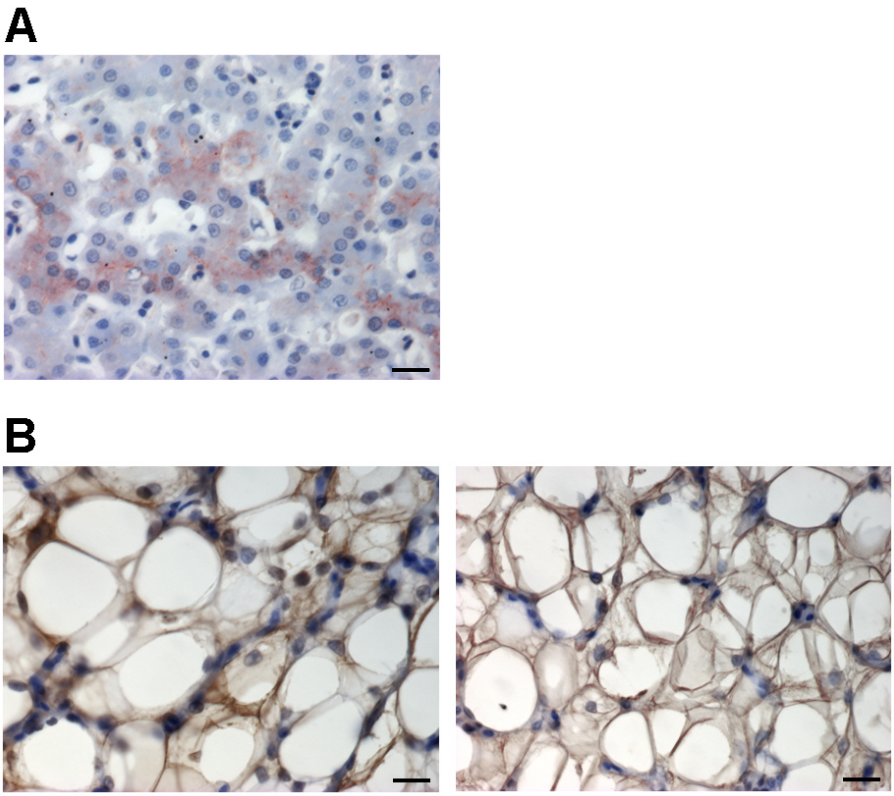

Figure 6. Immunolocalization of AGP in turkey. Immunohistochemical staining anti-AGP (standard ABC method, AEC chromogen). A, section of liver. A positive, intracytoplasmic immunostaining (red/brown staining) is visible in a moderate number of hepatocytes. B, section of adipose tissue. Immunostaining was localized at the periphery of adipocyte cytoplasm and borders. Bar, 20 micrometers.

2.6. CRP and PIT54 antibodies production

This part of the work was aimed to raise antibodies against CRP and PIT54. The peptides were synthesize as Multiple Antigen Presentation (MAP) technique, which allows the multiple presentation of the same peptide via binding four chains with three residues of lysine. The MAP approach is an effective method to chemically synthesize and deliver epitopes as the constituents of a single immunogen. It multiplies the number of identical immunogens, meanwhile increasing the size of the peptide, which become more manageable for ELISA assays, for example, or as positive standard for Western blotting. The following Figure 7 presents the MAP concept:

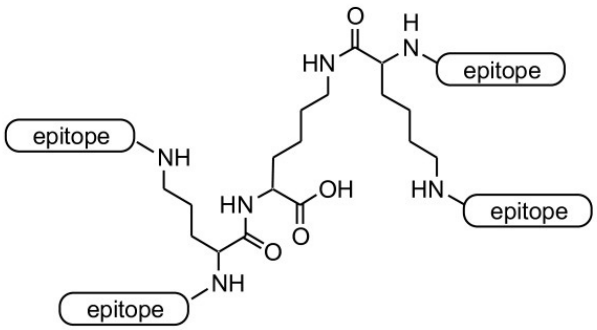

Figure 7. Example of Lys-based Multiple Antigen Presentation (MAP) vaccines (Fujita and Taguchi, 2011).

After purification, 1.5 ml of anti-CRP antibodies with a concentration of 1.14 mg/ml and 0.858 mg/ml, and 2 ml of anti-PIT54 antibodies with a concentration of 0.629 mg/ml and 0.387 mg/ml were obtained. The validation of newly produced antibodies was checked by Western blotting that was shown a very similar pattern for both anti-CRP and anti-PIT54 antibodies, reacting with a band with a MW of 26 kDa and a block of bands with a MW range of 50–75 kDa (Figure 8). CRP individual subunits have approximately 24 kDa, whereas the MW of the entire molecule is 120 kDa (Thiele et al., 2015). Members of the PIT54 family were previously identified as a single peptide, with a molecular size of 54 or 66 kDa in the absence or presence of 2-mercaptoethanol (Iwasaki et al., 2001).

As negative control, anti-CRP and anti-PIT54 antibodies specific sites were blocked with CRP and PIT54 peptides before using them as primary antibody. A very strong attenuation of the signal for both anti-CRP and anti-PIT54 antibodies was obtained, thus confirming that antibodies are binding specifically (Figure 9).

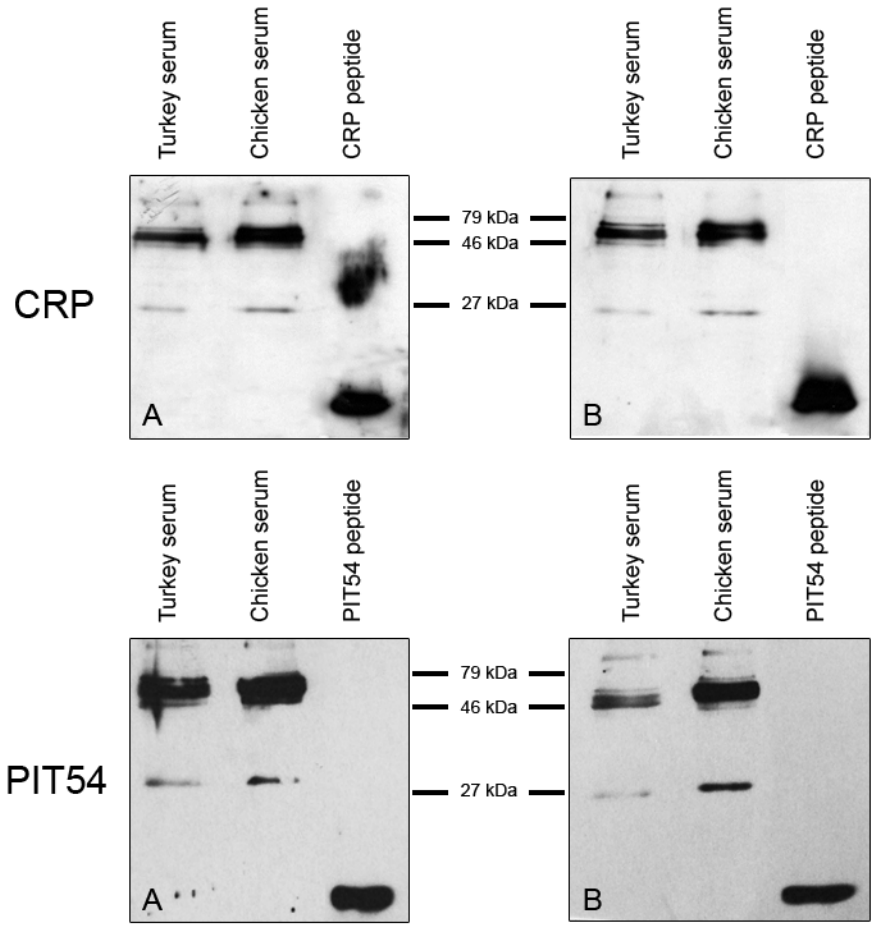

Figure 8. Detection of CRP and PIT54 by Western blotting in turkey and chicken serum. A, anti-CRP 1 and anti-PIT54 1 primary antibodies, respectively. B, anti-CRP 2 and anti-PIT54 2 primary antibodies, respectively.

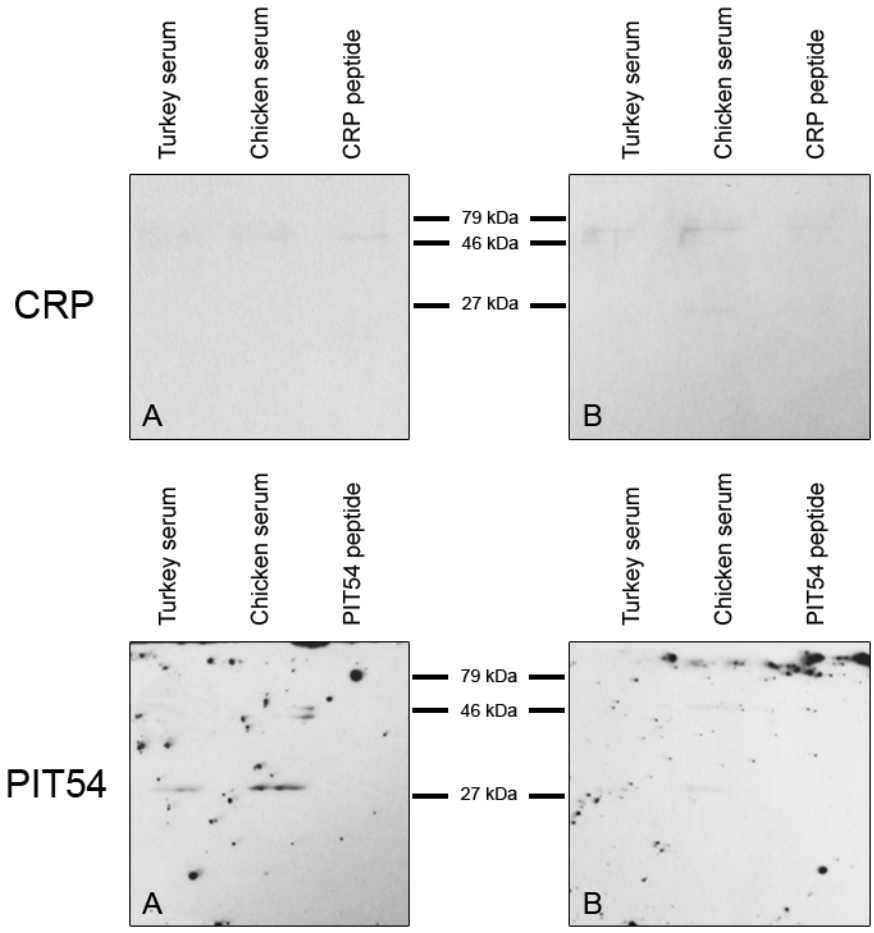

Figure 9. Detection of CRP and PIT54 by Western blotting in turkey and chicken serum after blocking. A, anti-CRP 1 and anti-PIT54 1 primary antibodies after blocking specific sites with peptide CRP and PIT54, respectively. B, anti-CRP 2 and anti-PIT54 2 primary antibodies after blocking specific sites with peptide CRP and PIT54, respectively.

Since anti-CRP and anti-PIT54 antibodies detected bands with same MW, a hypothesis made was that antibodies were reacting against chicken immunoglobulins and, probably, these bands were masking the region of our proteins. IgY is the main serum immunoglobulin in chicken and the functional equivalent of mammalian IgG. IgYconsists of two light chains with a MW of 22 kDa and two heavy chains with a MW of 66 kDa (Santos et al., 2014). In fact, when anti-CRP and anti-PIT54 antibodies were tested against other species (cattle and pig), bands with the same MW were obtained, showing that our antibodies are reacting against immunoglobulin heavy and light chains in all species analized (Figure 10).

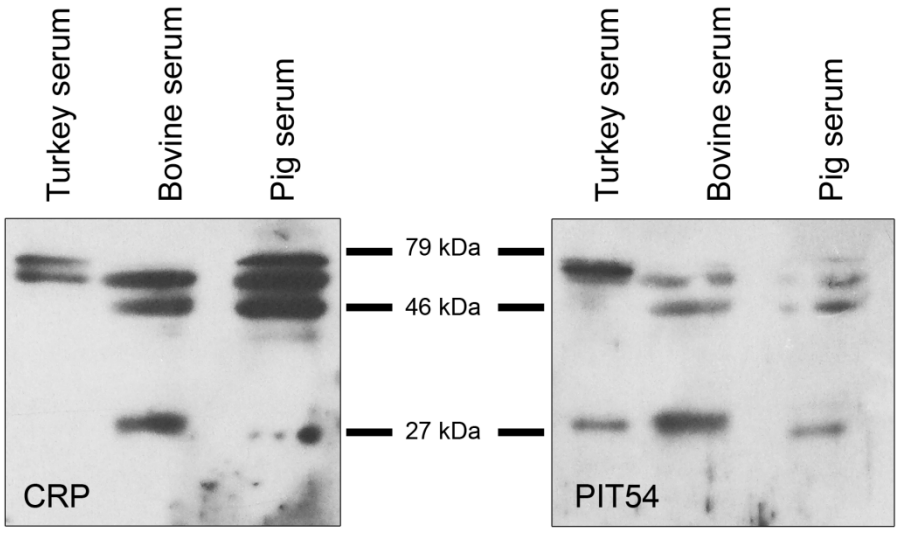

Figure 10. Detection of CRP and PIT54 by Western blotting in turkey, cow and pig. Proteins were identified after immunostaining recently produced anti-CRP and anti-PIT54 antibodies. Anti-CRP and anti-PIT54 antibodies cross-reacted against other species.

Dr. Anna Berrini from Biochemistry group carried out an ELISA analysis using the antibodies against respective peptides with a very strong and specific signal. Furthermore, both antibodies showed no reaction against a purified bovine immunoglobulin (data not shown). The Biochemistry group also performed another Western blotting to check possible mistakes or contaminations but the same results were obtained and the anti-CRP and anti-PIT54 antibodies were dropped.

3. Discussion

The present study provides, for the first time, information about acute phase protein in turkey. We have demonstrated that 2h-long road transportation is capable to modify AGP and CRP mRNA liver expression. Remarkably, adipose tissue was also found to be reactive to road transportation, since AGP mRNA abundance has also increased in transport–stressed animals.

The effective presence of the AGP protein was confirmed by Western blotting and immunohistochemistry. Western blotting electropherogram clearly identified in liver and adipose tissue a 55–66 kDa band, with the same molecular weight of the corresponding bovine AGP (Rahman et al., 2015).

AGP is one of the most widely measured APP in chicken (O'Reilly and Eckersall, 2014) and its expression has been found to be related with depressive disorders and stress in humans (Healy et al., 1991). AGP belongs to the family of lipocalins, e.g. binding proteins which also fulfil immune-modulatory function, including inhibition of phagocytosis (Lecchi et al., 2013), neutrophil degranulation, chemotaxis and respiratory burst (Lecchi et al., 2008; Miranda-Ribera et al., 2010; Rinaldi et al., 2008) and monocyte apoptosis (Ceciliani et al., 2007). The immunomodulatory activity of AGP is focused on dampening the side effects of inflammation.

Given that AGP mRNA was found to be statistically significantly overexpressed in both liver and adipose tissue, AGP was identified as one of the major acute phase proteins in turkey, at least for what concerns stress measurement after road transport. This conclusion is supported by previous studies in chicken, which demonstrated that measurement of AGP titration could provide useful information on both health and welfare of laying hens reared in different housing systems, which were subject to stressful events (Salamano et al., 2010). The serum concentration of AGP was also significantly increased in organically produced broilers as compared to conventionally produced ones (Tuyttens et al., 2008).

Liver function is strongly influenced by circulating glucocorticoid levels, and gene array studies have shown that hepatic response follows acute stress in chicken (Désert et al., 2008). A recent study (Sherlock et al., 2012) determined the differential hepatic gene expression in broilers in response to combined stressors (feed withdrawal, catching and transport), identifying 733 genes which were differently expressed but the use of APP as biomarkers for transport stress in poultry has not been adequately addressed so far. On the background that corticosteroids can upregulate

AGP gene expression (Ceciliani and Pocacqua, 2007), it can be therefore speculate that the overexpression of AGP in liver is induced by the increase of cortisol concentration induced by transport-related procedures.

Among the other APP included in the present study, only liver CRP mRNA was shown to be upregulated (3.5-folds higher) in transport–stressed animals as compared with control. CRP is a major APP in humans and dogs and frequently used in veterinary field (Eckersall and Bell, 2010), but little evidence is available in poultry (O'Reilly and Eckersall, 2014) and none in turkey. Although Sohail et al. (2010) found that CRP serum concentrations increased in chicken exposed to a heat stress conditions, and a very recent study correlated CRP concentration with the intensity of inflammatory reaction after *Salmonella typhimurium* LPS treatment (Rauber et al., 2014), the use of CRP as an APP in chicken is yet to be established. The present results are encouraging, and provide for the first time clues that CRP is related to stress induced by road transport in turkey.

Neither PIT54 nor SAA liver mRNA abundance was modified after road transport, confirming in turkey what has already been demonstrated in chicken (Garcia et al., 2009; Georgieva et al., 2010), that the use of PIT54 and SAA as biomarkers for diseases or welfare deserves further investigations, since they probably need a more intense challenge than that provided in this study.

In the second part of this study, the measurement of mRNA abundance of the four candidate APP was extended to the visceral adipose tissue. Adipose tissue is a loose connective tissue that, beside its role in regulating metabolism and innate immunity, is also linked to hypothalamic-pituitary-adrenal axis and stress (Bose et al., 2009; Chrousos, 2000). Adipose tissue affects a wide variety of body functions by actively secreting messenger molecules, called as “adipokines”, which include hormones, chemokines, cytokines and also acute phase proteins in both humans and farm animals (Sauerwein et al., 2014). Adipose tissue is also extremely reactive and sensitive to stress conditions (Charmandari et al., 2005). In previous works, AGP and SAA expression has been reported in different adipose tissue depots in cattle (Ceciliani et al., 2015; Rahman et al., 2015). The findings of this work presented the evidence that turkey visceral adipose tissue can produce mRNA of all four APP included in this experiment, albeit at lower levels as compared to liver (0.01 – 2%), and that road transport induces an overexpression of AGP in a statistically significant way. Moreover, the increase of mRNA abundance of PIT54 is

very close to the statistical significance (p-value = 0.0806), a value that probably can be improved in further studies increasing the number of animals.

PIT54 is homologous to the scavenger receptor cysteine-rich family of proteins and has been identified as the major hemoglobin-binding protein – haptoglobin in mammals – in chicken and goose plasma (Quaye, 2008; Wicher and Fries, 2006). Remarkably, PIT54 mRNA abundance was found to be increased only in adipose tissue and not in liver. The mammalian equivalent protein, haptoglobin, ranks high in the list of possible stress biomarkers in pigs (Piñeiro et al., 2007; Soler et al., 2013), but the present findings can confirm in turkey that only adipose tissue PIT54 mRNA is overexpressed after transport. The impact of PIT54 and AGP production from adipose tissue on the overall amount of circulating protein is unknown and deserves further investigation.

Previous studies on cattle, pigs and rats suggest that SAA may be used as marker of stress (Deak et al., 1997; Hicks et al., 1998; Lomborg et al., 2008; Soler et al., 2013) but in the current study no statistical increase of SAA mRNA abundance was observed, neither in liver nor in adipose tissue.

4. Conclusions

In conclusion, the present study provides information on acute phase proteins in turkey, not available until now. On the background of mRNA abundance data, the concentration of AGP and CRP might be modified in turkey after road transport and may provide useful clinical indicators of stress in poultry. Although encouraging, these results must be considered as preliminary, and need to be paralleled by the measurement in serum of the respective transcript product, in order to confirm that AGP and CRP protein are overexpressed after road transportation and that its serum concentration can provide a suitable measure of turkey welfare.

We have also demonstrated that adipose tissue is capable to mount a local acute phase response during a stress condition such as road transportation, through the increase expression of AGP and PIT54 mRNA.

We tried to develop antibodies against poultry CRP and PIT54; however antibodies produced were not specific.

Further research is needed to refine the use of these measures in the assessment of turkey welfare. However, the present results will pave the way for the development of turkey specific assays, where AGP, CRP and PIT54 might be explore as suitable biomarkers of transport stress.

5.3. Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkey (*Meleagris gallopavo*)

This work is already published as:

Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkeys.

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Keywords: biomarkers, circulating miRNA, stress, turkey, welfare.

Abstract

MicroRNA (miRNA) have been identified in circulating blood and might have the potential to be used as biomarkers for several pathophysiological conditions. To identify miRNA that are altered following stress events, turkeys (*Meleagris gallopavo*) were subjected to 2h of road transportation. The expression levels of five circulating miRNA, namely miR-22, miR-155-5p, miR-181a-3p, miR-204 and miR-365-3p, were detected and assessed by quantitative polymerase chain reaction using TaqMan® probes, as potential biomarkers of stress. The areas under the receiver operating characteristic curves were then used to evaluate the diagnostic performance of miRNA. A panel of three stress-responsive miRNA, miR-22, miR-155 and miR-365 were identified; their expression levels were significantly higher after road transportation and the area under the curve (AUC) were 0.763, 0.71 and 0.704, respectively. Combining the three miRNA, a specificity similar to the one found for the three miRNA separately was found. The AUC of the weighted average of the three miRNA was 0.763. This preliminary study suggests that the expression levels of circulating miR-22, miR-155 and miR-365 are increased during transport-related stress and that they may have diagnostic value to discriminate between stressed- and unstressed animals.

1. Material and methods

1.1. Samples collection and preservation

Blood was collected from sixteen clinically healthy 105-day-old turkeys BUT BIG 6 by branchial vein venipuncture using serum collection tubes during routine disease testing. After a 2h road transportation, further blood samples were collected during routine slaughtering process from the neck vessels cut by the automatic processing killer. Road-transport was carried out according to EU procedures for animal transport (Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97). Serum was stored at −80°C until RNA extraction.

1.2. miRNA extraction and reverse transcription

Total RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen). Serum was thawed on ice and centrifuged at 3000 × g for 5 min at 4°C. An aliquot of 150 µl per sample was transferred to a new tube and 1 ml of Qiazol was added. The *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen) was used as synthetic spike-in control due to lack of sequence homology to avian miRNA. After an incubation at RT for 5 min, 3.75 µl (25 fmol final concentration) of spike-in control was added and the samples vortexed to ensure complete mixing. The RNA extraction was then carried out according to manufacturer's instruction.

Total RNA concentration and quality were validated as ratio A260/A280 by NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc.). The reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using miRNA-specific stem-loop RT primers as according to manufacturer's instructions. Reverse transcription reactions were performed in 15 µl volume reactions containing 1.5 µl 10× miRNA RT buffer, 1 µl MultiScribe reverse transcriptase (50 U/µl), 0.30 µl 100 mM dNTP mix, 0.19 µl RNase Inhibitor (20 U/µl), 6 µl of custom RT primer pool and 3.01 µl of nuclease-free water. The custom RT primer pool was prepared combining 10 µl of each individual 5× RT primer in a final volume of 1000 µl; the final concentration of each primer in the RT primer pool was 0.05×

each. Serum RNA of 3 µl were added to each RT reaction. RT reaction mixture were incubated on ice for 5 min, 16°C for 30 min, 42°C for 30 min and then 85°C for 5 min.

1.3. Quantitative Real Time PCR

The qPCR experiments were designed following MIQE guidelines (Bustin et al., 2009). Small RNA TaqMan assays were performed according to manufacturer's instruction. The selected primer/probe assays (Life Technologies) included cel-miR-39-3p (assay ID000200), hsa-miR-22 (assay ID398), hsa-miR-155-5p (assay ID479), hsa-miR-181a-3p (assay ID516), hsa-miR-204 (assay ID508), hsa-miR-365-3p (assay ID1020) (Table 1). Quantitative reactions were performed in duplicate in scaled-down (12 µl) reaction volumes using 6 µl TaqMAN 2X Universal Master Mix II (Applied Biosystems), 0.6 µl miRNA specific TaqMan Assay 20× and 1 µl of the RT product per reaction on Eco Real Time PCR detection System (Illumina). The standard cycling program was 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Data were normalized relative to the expression of cel-miR-39. miRNA expression levels are presented in terms of fold change normalized to cel-miR-39 expression using the formula $2^{-\Delta\Delta Cq}$ (Giulietti et al., 2001).

Table 1. List of the probe assays and their respective primers sequences and references.

Assay Name	Mature microRNA Sequence	References
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG	Roberts et al., 2014
hsa-miR-22	AAGCUGCCAGUUGAAGAACUGU	Wang et al., 2014
hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGG	Yao et al., 2013
hsa-miR-181a	ACCAUCGACCGUUGAUUGUACC	Wang et al., 2014
hsa-miR-204	UUCCCUUUGUCAUCCUAUGCCU	Ahanda et al., 2014; Wang et al., 2014
hsa-miR-365	UAAUGCCCCUAAAAAUCCUUAU	Ahanda et al., 2014

cel-miR-39-3p, assay ID000200; hsa-miR-22, assay ID398; hsa-miR-155-5p, assay ID479; hsa-miR-181a-3p, assay ID516; hsa-miR-204, assay ID508; hsa-miR-365-3p, assay ID1020.

1.4. Statistical analysis

Normality of the distribution of each of the miRNA variables was assessed using Shapiro–Wilk test. Since data were not normally distributed a non-parametric method, the Wilcoxon test, was used in the analysis of differences in miRNA expression. A receiver operating characteristic (ROC) curve was used to determine the sensitivity and specificity of the assay in discriminating between pre- and post-transported animals. The area under the curve (AUC) for the ROC curves was calculated. Statistical analysis was performed using XLSTAT for Windows (Addinsoft) and MedCalc 14.0 (MedCalc Software bvba).

2. Results

2.1. miR-22, miR-155 and miR-365 levels are elevated in the blood serum of stressed turkeys

The expression of miR-22, miR-155, miR-181a, miR-204 and miR-365 was quantified by qPCR after normalization against miRNA cel-miR-39 (Qiagen). The comparative analysis demonstrated that three circulating miRNA, namely miR-22, miR-155 and miR-365, were differentially expressed in serum samples collected after road transportation as compared with those collected before transportation on the same animals.

In detail, the levels of miR-22, miR-155 and miR-365 were significantly higher after road transportation (all p-value < 0.05) (Figure 1). The median expression levels of miR-22, miR-155 and miR-365 were 0.90 (range, 0.22 to 7.90), 0.97 (range, 0.43 to 2.23) and 0.66 (range, 0.27 to 1.00) before transportation, and 1.81 (range, 0.19 to 30.13), 1.61 (range, 0.57 to 4.07) and 1.59 (range, 0.23 to 5.76) after transportation, respectively.

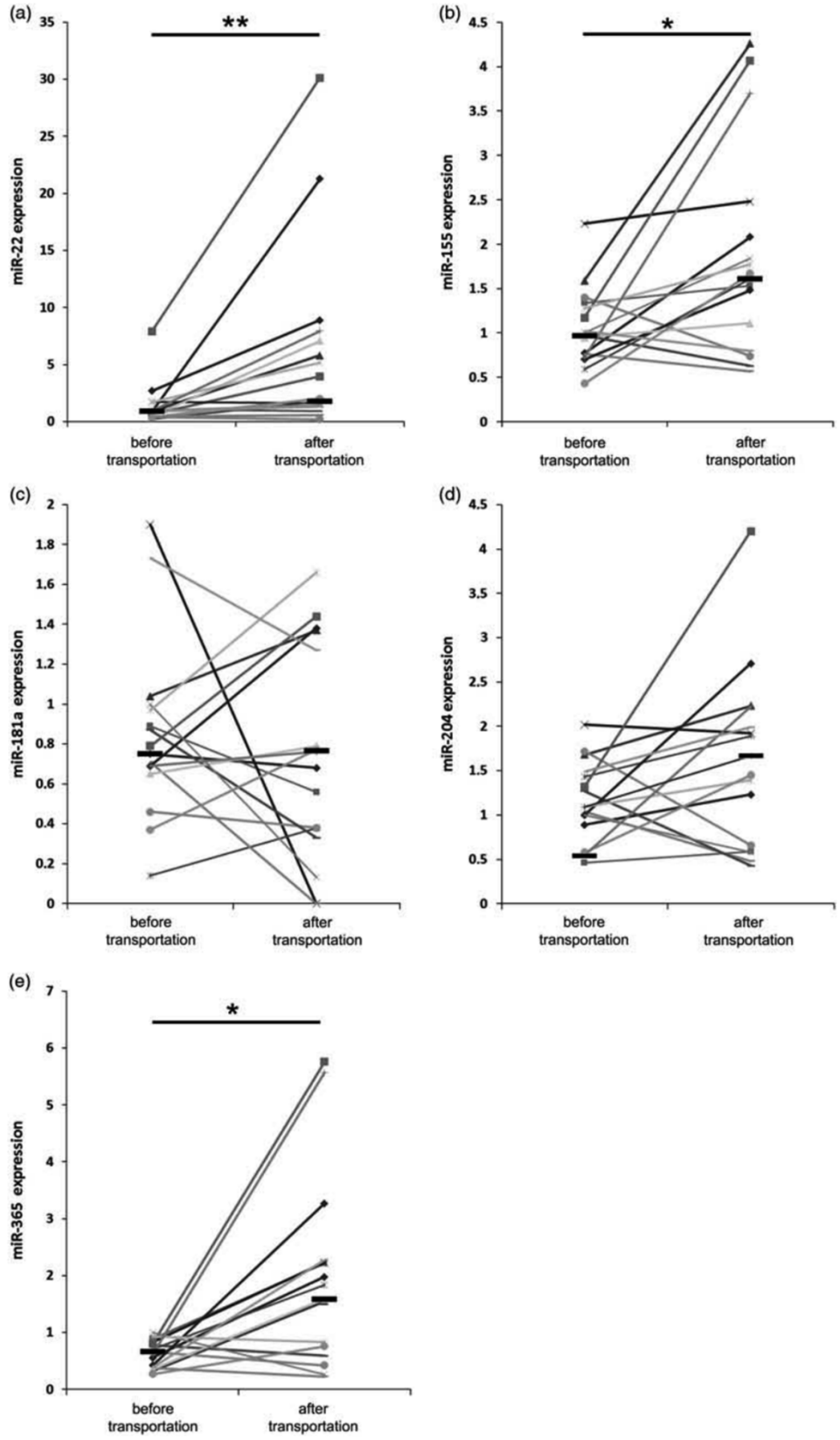

Figure 1. Circulating stress-related miRNA levels of turkeys before and after road transportation. Levels of (a) miR-22, (b) miR-155, (c) miR-181a, (d) miR-204 and (e) miR-365. The black lines mark the median expression levels. **p*-value < 0.05; ***p*-value < 0.01.

2.2. Diagnostic performance of miR-22, miR-155 and miR-365

The potential use of the three miRNA, which were found to be differentially regulated as biomarker predictors of transport-related stress, was explored. Receiver-Operating Characteristic (ROC) analysis was used to estimate the diagnostic value of miR-22, miR-155 and miR-365, as well as the combination of the three miRNA.

In statistics, a ROC curve is a graphical plot that illustrates the performance of a test. It creates a curve by plotting the true positive rate, called sensitivity, against the false positive rate which is calculated through the expression 1 - specificity. The sensibility is the probability of our test give us a positive result and the animal is stressed, whereas the specificity is the probability of our test give us a negative result and the animal is not stressed. A test with perfect discrimination has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity) with no overlap in the two distributions.

The area under the curve, also referred as AUC, can quantify the ability of the test to discriminate between stressed and not-stressed animals. A perfect test has an area about 1, and a bad test has an area about 0.5. Above 0.7, we consider that a test has the ability to differentiate both groups. It is difficult to have a sensibility and specificity both at 100%, so we selected a cut-off with a good balance between them.

The ROC analysis was carried out by plotting the true positive (sensitivity) versus false positive (1-specificity). Cut-off points were set in order to maximize the sum of sensitivity and specificity. The cut-off points for miR-22, miR-155 and miR-365 were 1.31, 1.48 and 1.59, respectively (Table 2). The diagnostic accuracy of miR-22, miR-155 and miR-365, as measured by the area under the curve (AUC) and it was 0.763 (95% CI 0.586 to 0.941), 0.710 (95% CI 0.512 to 0.908) and 0.704 (95% CI 0.497 to 0.912), respectively (Figure 2).

Table 2. Cut-off, sensitivity, specificity and AUC for each candidate miRNA and for their combination.

Stress marker	Cut-off	Sensitivity (%)	Specificity (%)	AUC (95% CI)
miR-22	1.31	76.9	76.9	0.763 (0.586 – 0.941)
miR-155	1.48	61.5	92.3	0.710 (0.512 – 0.908)
miR-365	1.59	53.8	100	0.704 (0.497 – 0.912)
miR-22, miR-155, miR-365	2.131	69.2	84.6	0.763 (0.557 – 0.906)

hsa-miR-22, assay ID398; hsa-miR-155-5p, assay ID479;hsa-miR-365-3p, assay ID1020.

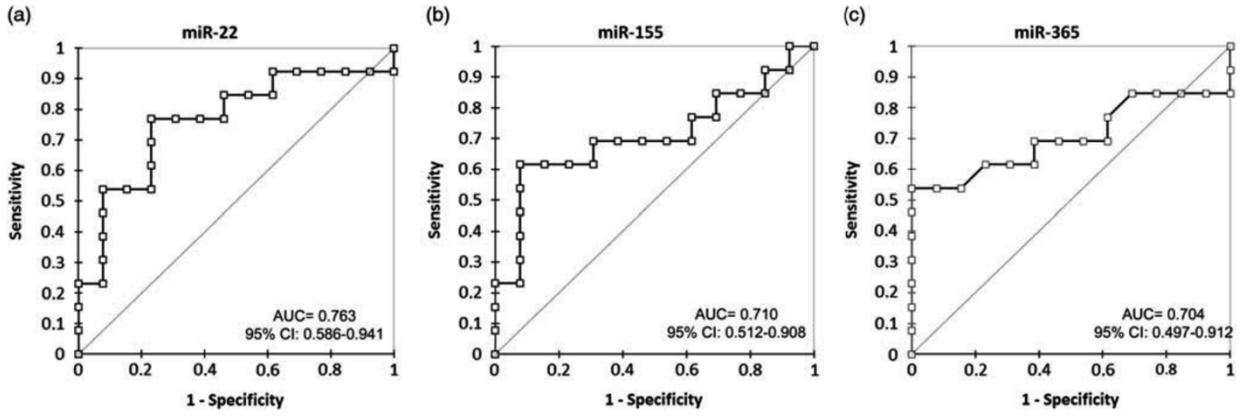

Figure 2. Receiver-operator characteristics (ROC) curve analysis of candidate stress-related miRNA. ROC plots for (a) miR-22, (b) miR-155 and (c) miR-365 were used to differentiate stressed from not-stressed animals. AUC, area under the curve; CI, confidence interval.

Further statistical analysis was performed considering the weighted average relative quantification values of the three stressed-related miRNA. The median expression levels were 1.02 (range, 0.62 to 1.38) and 1.97 (range, 1.73 to 6.26) before and after transportation, respectively (Figure 3a).

The logit model based on the three miRNA [logit= (0.065 x expression level of miR-22) + (0.562 x expression level of miR-155) + (1.395 x expression level of miR-365)] was used to assess the predicted probability of being discriminated as stressed-animals and to construct a ROC curve (Figure 3b). The AUC for the combined miRNA was 0.763 (95% CI 0.557 to 0.906) (Table 2)

and suggests that serum levels of miR-22, miR-155 and miR-365 can discriminate transported from not-transported animals alone or in combination.

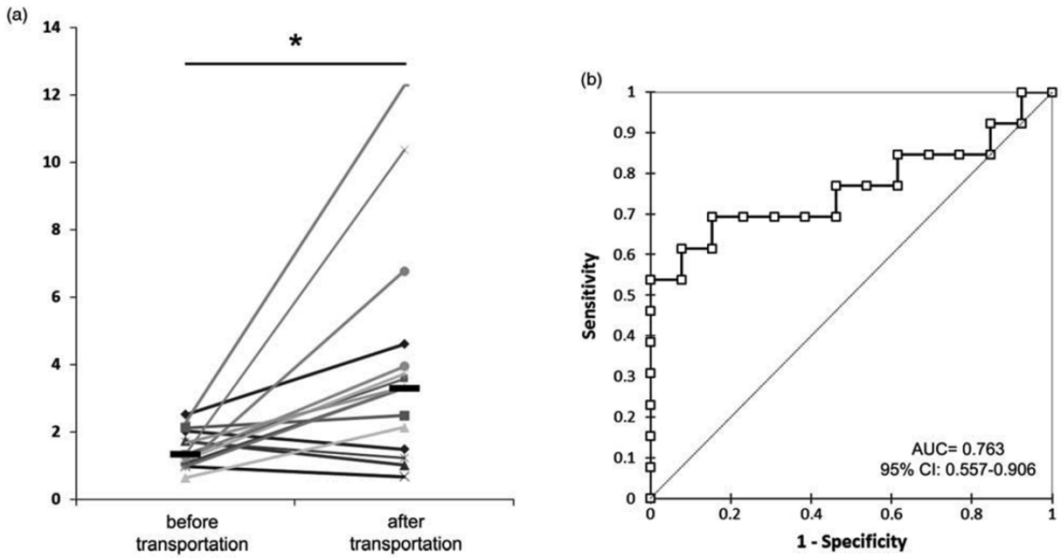

Figure 3. The average expression of the three candidate stress-related miRNA. (a) The weighted average relative quantification levels of miR-22, miR-155 and miR-365 before and after road transportation. The black lines mark the median expression levels. * *p*-value< 0.05. (b) Receiver operating characteristics (ROC) curve analysis of miR-22, miR-155 and miR-365, constructed using the logit model. AUC, area under the curve; CI, confidence interval.

3. Discussion

Five miRNAs, previously demonstrated to be related to stressful events in chicken and immune defenses, were selected for our study – miR-22, miR-155, miR-181a, miR-204 and miR-365. MiR-22 is involved in hypothalamic fear and stress response in chicken after tonic immobility (Wang et al., 2014). MiR-155 is conserved between many species and it was found to play critical roles in various physiological and pathological processes such as immunity in chicken (Mashima, 2015; Yao et al., 2013). MiR-181a is studied in farm animals and seems to be related with immunity and stress in chicken (Wang et al., 2013; Wang et al., 2014). MiR-204 and miR-365 are related with feed deprivation stress in chicken (Ahanda et al., 2014).

The present work provided the evidence that three miRNA, namely miR-22, miR-155 and miR-365, initially chosen as regulation members of innate immunity, are upregulated in a statistically significant way during road transport. Moreover, ROC analysis demonstrated that the combination of the three miRNA which were found upregulated may be regarded as a potential diagnostic biomarker panel to determine transport-related welfare in turkey species.

Accurate determination of stress in poultry, and farm animals in general, is critical, since it is often difficult to measure quantitatively stress-related parameters. Beside the use of behavioural scoring system, biochemical parameters have been proposed to provide a broad assessment of animal welfare including corticosteroids and acute phase proteins (Marchewka et al., 2013; Piñeiro et al., 2007). MiRNA have been identified in animal species as biomarkers (Wang et al., 2013) and they can be easily extracted from extracellular body fluids (Gilad et al., 2008; Roberts et al., 2014). The modulation of miRNAs expression is also an early event in stressful conditions (Biggar and Storey, 2015; Emde and Hornstein, 2014; Leung and Sharp, 2010).

The hypothesis of this study was that circulating miRNA could provide a useful source of biomarkers for objective measurements of animal welfare. This hypothesis was validated by demonstrating that three miRNA (from five selected miRNA) were significantly upregulated during road transport in turkeys. Given their involvement in stressful events and modulation of immune response (Ahanda et al., 2014; Mashima, 2015; Wang et al., 2014), the present results suggest that transport-related procedures may interfere with the immune status of the animals by modifying the gene expression level of immune-related miRNA.

MiRNA act as regulators of gene expression during many different pathophysiological pathways, including those involved in neuropsychiatric disorders and stress (Kocerha et al., 2015), moreover a recent study reported that miRNome is capable of quickly reacting to feed deprivation stress in chicken (Ahanda et al., 2014).

In particular, miR-155 is physiologically expressed at low levels in B and T cells, macrophages, dendritic cells, and progenitor/stem cell populations, and is upregulated after their activation by immune stimuli, evading to modulation of humoral and innate cell-mediated immune responses (Elton et al., 2013).

MiR-22 is one of the very few ubiquitously expressed miRNA, and is likely to be involved in buffering cellular activities that are common to the vast majority cells. Among others activities, it is involved in the hematopoiesis process, in the down regulation of IL6 and in the differentiation of Th17 cells (Liang et al., 2015).

The role of miR-365 is still not well understood. MiR-365 expression has been so far associated to cancer development and its progression (Zhou et al., 2013). The finding that miR-365 may be related to transport-stress confirms what has been recently reported by Ahanda et al. (2014), who demonstrated that miR-365 family members are present in plasma and red blood, but not white blood cells, and their expression is modulated by food deprivation stress.

4. Conclusions

In conclusion, this preliminary study highlighted that transport-related procedures are capable of modifying expression of immune-related miRNA, providing for the first time a molecular link between stress and immune defenses in turkey species.

In the second part of this investigation, we demonstrated by ROC analysis that miR-22, miR-155 and miR-365, individually or in combination, may be useful to discriminate between stressed- and not-stressed animals.

In order to confirm the diagnostic value of these candidate miRNA, and develop a minimally invasive screening tool for assessing turkey welfare, further studies on a higher number of samples and different transport conditions are required to validate the candidate miRNAs in the field.

5.4. Proteome analysis of intestinal mucosa in turkey (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus

Manuscript of this work is already in preparation to be submitted to *Journal of Proteomics*:

Proteome analysis of intestinal mucosa in turkey (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus (HEV).

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Keywords: haemorrhagic enteritis virus, intestine, LC-MS/MS, *Meleagris gallopavo*, proteome.

Abstract

Haemorrhagic enteritis (HE) is a viral disease, which negatively affects intestinal integrity and barrier function in turkey (*Meleagris gallopavo*). Birds exhibit HEV-induced immunosuppression condition and can easily be infected with secondary infections agents, resulting in significant economic loss. To our best knowledge, intestinal proteome studies is turkey have not been presented yet. Therefore, this study integrate a proteomic approach to investigate the effects of HE disease on intestinal mucosa, focusing on proteins involved in immunity and inflammatory pathways. A total of 523 unique proteins were identified by SWATH-MS strategy between healthy and HEV-affected intestine. Sixty-four intestinal mucosa proteins were found to be differentially abundant. Twenty-two of them are related to energy metabolism, 16 with protein metabolism, 7 with cytoskeleton, and their changes in abundance could comprise intestinal integrity and function. The up-regulation of proteins involved in carbohydrate metabolism, fatty acids metabolism and oxidative phosphorylation suggested that HE disease disturbs energy metabolism and thus induce oxidative stress. We also found seven proteins related with immune system, all of them up-regulated in HEV-affected intestine. A qualitative PCR further confirmed the individual mRNA presence for six of these proteins. These findings have important implications for understanding the effect of HE in turkeys.

1. Material and methods

1.1. Samples collection and preservation

Samples were obtained during routinely slaughtering procedures from 92-day-old females BUT BIG 6 breeding line female turkeys. A group of four turkeys were clinical healthy and PCR HEV negative (control group). A second group of four turkeys were PCR HEV positive (HEV-infected animals). Clinical status of the animals was also assessed by ante- and post-mortem inspection.

Portions of intestine were removed immediately after slaughtering, mucosa was scratched, snap frozen into liquid nitrogen and afterwards stored at −80ºC.

1.2. Protein extraction

Protein extraction of intestinal mucosa was carried out on ice, following a general protocol for lysis of cells with 2X lysis buffer. In short, 400 mg of each tissue was washed with cold PBS, traces of blood were removed and tissue was homogenized in 1 ml lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% IGEPAL (NP-40), 0.1% SDS) with 10 µl Protease Inhibitor Cocktail (Sigma-Aldrich). Tissue disruption was performed using a tissue homogenizer (Precellys24, Bertin Technologies) (4 cycles, 5500 rpm, 20 sec) and glass beads (0.1 mm, Bertin Technologies) and sonicated on ice (10 cycles, 10 sec). Protein extracts were centrifuged for 10 min, 10000 x g, 4°C. Supernatant containing protein extract was stored at –80°C until prior use.

Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as a protein standard. BSA solution was prepared in PBS 1x with a concentration of 2 mg/ml. A series of protein solutions were prepared by serial dilution. The absorbance was measured at 610 nm using Odyssey® CLx Infrared Imaging System (LI-COR, Biosciences). The concentration of protein was plotted against the corresponding absorbance resulting in a standard curve, which was used to determine the protein content (R^2=0.9964).

1.3. Protein digestion

Protein extracts were hydrolyzed in-solution, essentially as described in Llombart et al. (2015). Samples were adjusted to a concentration of 2 µg/µL with 6 M Urea and 50 mM NH_4HCO_3 pH=8.5. Two µg of green fluorescence protein (GFP) was used as internal standard for each sample. Reduction was performed by addition of dithiothreitol (DTT) at a concentration of 10 mM and incubated for 1 h at RT. As alkylating agent, iodoacetamide (IAA) was used at a concentration of 30 mM and incubated for 30 min at RT in the dark. IAA was quenched by adding N-Acetyl-L-cysteine (NAC) at a concentration of 37.5 mM and incubated for 15 min at RT. Urea concentration was lowered to 1 M by adding 50 mM NH_4HCO_3 pH=8.5. Finally, samples were digested with trypsin (Promega) (trypsin:protein=1:20, w/w) for 18 h at 37 °C and, afterwards, the reaction was stopped by addition of 0.5% formic acid (FA).

1.4. Sample preparation for LC-MS/MS

The digested peptides were de-salted and concentrated using C18 Bond Elut OMIX solid phase extraction pipette tips (Agilent Technologies) as described in Silva et al. (2016). Each mixture was completely dried and then re-suspended in 100 µl of 2% acetonitrile (ACN) with 1% FA and sonicated in the ultrasound bath for 5 min with low amplitude. The tip column was hydrated with 100 µL of 50% ACN and equilibrated with 100 µL of 2% ACN with 1% FA. The peptides were loaded into the column five times followed by a wash step with 100 µL of 2% ACN with 1% FA. Peptides were then eluted with 100 µL of 70 % ACN with 0.1 % FA and after evaporation the pellets were stored at –20ºC until LC-MS/MS analysis.

The washed pellets were resuspended in 50 µL of a solution of 2% ACN and 0.1% FA, and 10 µL of each replicate (in a total of 4 replicates per condition) were used to create a pooled sample for protein identification. In order to remove insoluble material, the peptide mixture were then centrifuged for 5 min at 14000 x g and collected into the proper vial for LC-MS injection.

1.5. LC-MS/MS analysis

Samples were analyzed on a Triple TOF^{TM} 5600 System (ABSciex®) in two phases: information-dependent acquisition (IDA) of the pooled samples and, SWATH (Sequential

Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition of each individual sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXPTM C18CL (300 µm ID × 15 cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5 µL/min with a multistep gradient: 0-2 min linear gradient from 2 to 5%, 2-45 min linear gradient from 5 % to 30 % and, 45-46 min to 35 % of ACN in 0.1 % FA and 5% DMSO. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSprayTM Source, ABSciex®) with a 50 µm internal diameter (ID) stainless steel emitter (NewObjective).

The information dependent acquisition (IDA) experiments were performed for each pooled samples in two acquisitions per pool. The mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 100 MS/MS scans (100–1500 m/z from a dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 25 seconds (mass spectrometer operated by Analyst® TF 1.7, ABSciex®). Rolling collision was used with a collision energy spread of 5.

For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode (Gillet et al., 2012) and the same chromatographic conditions used as in the IDA run described above. A set of 60 windows (Table 1) of variable (width containing 1 m/z for the window overlap) was constructed covering the precursor mass range of 350-1250 m/z. A 250 ms survey scan (350-1500 m/z) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from 100–1500 m/z for 50 ms resulting in a cycle time of 3.25 s from the precursors ranging from 350 to 1250 m/z. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with variable collision energy spread (CES) according with the window.

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments, and used for subsequent SWATH processing.

Table 1. SWATH-MS method.

	m/z range	Width (Da)	CES
Window 1	349.5-360.9	11.4	5
Window 2	359.9-375.2	15.3	5
Window 3	374.2-389.2	15	5
Window 4	388.2-402.2	14	5
Window 5	401.2-415.3	14.1	5
Window 6	414.3-427.4	13.1	5
Window 7	426.4-439.1	12.7	5
Window 8	438.1-449.9	11.8	5
Window 9	448.9-460.7	11.8	5
Window 10	459.7-471.1	11.4	5
Window 11	470.1-480.5	10.4	5
Window 12	479.5-490	10.5	5
Window 13	489-499	10	5
Window 14	498-508	10	5
Window 15	507-516.5	9.5	5
Window 16	515.5-525.1	9.6	5
Window 17	524.1-533.2	9.1	5
Window 18	532.2-540.8	8.6	5
Window 19	539.8-548.5	8.7	5
Window 20	547.5-555.7	8.2	5
Window 21	554.7-563.4	8.7	5
Window 22	562.4-570.6	8.2	5
Window 23	569.6-577.8	8.2	5
Window 24	576.8-585.4	8.6	5
Window 25	584.4-592.6	8.2	5
Window 26	591.6-600.3	8.7	5
Window 27	599.3-607.9	8.6	5
Window 28	606.9-615.6	8.7	5
Window 29	614.6-623.2	8.6	5
Window 30	622.2-630.9	8.7	5
Window 31	629.9-638.5	8.6	5
Window 32	637.5-646.2	8.7	5
Window 33	645.2-653.8	8.6	5
Window 34	652.8-661.5	8.7	5
Window 35	660.5-669.1	8.6	5
Window 36	668.1-677.2	9.1	5
Window 37	676.2-685.3	9.1	5
Window 38	684.3-693.9	9.6	5
Window 39	692.9-702.9	10	5
Window 40	701.9-711.9	10	5
Window 41	710.9-721.3	10.4	5
Window 42	720.3-731.2	10.9	5
Window 43	730.2-741.6	11.4	5
Window 44	740.6-752.4	11.8	5
Window 45	751.4-763.6	12.2	5
Window 46	762.6-775.8	13.2	5
Window 47	774.8-787.9	13.1	5
Window 48	786.9-800.5	13.6	5
Window 49	799.5-814.5	15	8
Window 50	813.5-829.3	15.8	8
Window 51	828.3-845.5	17.2	8
Window 52	844.5-865.3	20.8	8
Window 53	864.3-886.5	22.2	8
Window 54	885.5-911.2	25.7	8
Window 55	910.2-939.1	28.9	8
Window 56	938.1-972	33.9	8
Window 57	971-1008.4	37.4	10
Window 58	1007.4-1053.4	46	10
Window 59	1052.4-1120	67.6	10
Window 60	1119-1249.6	130.6	10

1.6. Database searches and quantification

Libraries were obtained using Protein Pilot™ software (v5.1, ABSciex®), using the following parameters: i) search against a database composed by all the entries for the species *Meleagris gallopavo* (https://www.ncbi.nlm.nih.gov/protein?LinkName=genome_protein&from_uid=112) which comprised 24245 non-redundant sequences and *Gallus gallus* (https://www.ncbi.nlm.nih.gov/protein?LinkName=genome_protein&from_uid=111) that comprised 41611 non-redundant sequences from NCBI (release at July 2016), and malE-GFP (IS); ii) iodoacetamide alkylated cysteines as fixed modification; iii) trypsin as digestion type. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR (Sennels et al., 2009; Tang et al., 2008).

Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®) (Lambert et al., 2013). Briefly, peptides were selected automatically from the library using the following criteria: (i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and (ii) peptides that contained biological modifications and/or were shared between different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH™ quantitation was attempted for all proteins in library file that were identified below 5% local FDR from ProteinPilot™ searches. In SWATH™ Acquisition data, peptides are confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide.

Target fragment ions, up to 5, were automatically selected and peak groups were scored following the criteria described in Lambert et al. (2013). Peak group confidence threshold was determined based on a FDR analysis using the target-decoy approach and 1% extraction FDR threshold was used for all the analyses. Peptide that met the 1% FDR threshold in at least three of the four biological replicates were retained and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 4 minutes with 100 ppm XIC width.

The levels of the proteins were estimated by summing all the transitions from all the peptides for a given protein that met the criteria described above (an adaptation of Collins et al., 2013) and

normalized to the total intensity of each sample. The internal standard (malE-GFP) was used to performed retention time alignment.

1.7. Functional annotation and grouping

The retrieved protein FASTA sequences were loaded on the open source online tool Blast2GO 4.0.2 software (http://www.blast2go.com) which is a comprehensive high-throughput tool for gene ontology (GO) online tool. The default parameters were used and, for the basic local alignment search tool (BLAST), protein sequences were mapped against the NCBI database. The functional analysis was further narrowed by PANTHER classification (http://www.pantherdb.org). WEGO (http://wego.genomics.org.cn) has used to build graphics. Kyoto encyclopedia of genes and genomes (KEGG) database (released October 2016) was used to classify identified proteins into specific functional terms and metabolic pathways.

1.8. Validation of proteomic results by qualitative PCR

Qualitative PCR of selected genes, involved in immunity, inflammatory pathways and response to stimulus, was used to confirm protein expression data. Total RNA was extracted from the same tissue samples as those used for proteome analysis.

RNA extraction was made using TriZol standard protocol (Invitrogen) and DNase treatment was carried out with DNase (Fermentas). RNA concentration and quality were validated as ratio A260/A280 by NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc.). 1 µg RNA was retrotranscribed using the iScript cDNA synthesis kit (Biorad).

The resulting cDNA was amplified by means of a qualitative PCR, performed in 10 µL final volume containing 1x buffer (LeGene Biosciences), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (LeGene Biosciences). PCR conditions were 35 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 45 sec (Eppendorf Mastercycler). PCR products were visualized on 1.6% agarose gel stained with ethidium bromide.

Primers were designed on turkey sequences available in NCBI by using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0), preventing possible secondary structures with the mfold Web

Server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and ensuring the specificity of the sequence by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). Primer sequences and fragment sizes are described in Table 2.

Table 2. List of the genes under study and their respective primers sequences, accession numbers and fragment lengths.

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length (bp)
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146
SELENBP1	XM_010724013.1	AACCTGAAAGACGAGCTCCA	AGATCAGGCATGGGAGAATG	103
HPX	XM_010706282.1	CTTCCAGGGTGAGAAGGTCTG	CTCCTCAGGGTGACACTCCA	130
ES1	XM_003208390.2	ACGTGAAAGGACAGCCAACT	TCCAAAGCCACCTGGTATGA	137
CORO2A	XM_010726179.1	GTCCCTCGATGGTCAGAGTC	GTCCAGAGCCTGGTCTCAAG	139
FGB	XM_003205349.2	TCAGGCAGAGAATGTGAGGA	GGCTTGGTAAAAGGATCTGGC	86
MPST	XM_010711524.1	CAAGCCAGATGTTGCTGTGT	TCCCAAATCCAGTTTCAAGG	156
PGRMC1	NM_001271939.1	GAGGTCTTGCCACTTTCTGC	TTCAGCAGCTTACCAACGTG	148
CANX	XM_003210435.2	ATGGAAACCCAGGAAGATCC	CACAGCACTGAAGGGAGTCA	79
IGLL1	XM_010720331.1	ACACCAACAGACCCTCGAAC	CAGTGCTGCTGTCGTAGCTC	135
PPIA	NM_001166326.1	GTGCCCATAACAGCAGAGAAC	ACCACCCTGACACATGAAGC	111
GPA33	XM_010721705.1	ATGACAGTCAGCGTTGTCCC	TTCTCTTGCCGTCTCCTCGT	150
CALM	NM_205005.1	ACTTGGTCAGAACCCCACAG	TCAATTGTGCCATTGCCATCAG	78
SDSL	XM_010720582.1	GCCAAGACTGTGTCAGAACG	TCATCCAGGAACCTTTCCAC	110
BDH2	XM_003205765.2	AATCTAACCCAGACCAGGCAC	CCACTTCTTCAGCGGTAGCC	78

PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like); SELENBP1, selenium-binding protein 1-A; HPX, hemopexin; ES1, ES1 protein homolog; CORO2A, coronin-2A; FGB, fibrinogen beta chain; MPST, 3-mercaptopyruvate sulfurtransferase; PGRMC1, membrane-associated progesterone receptor component; CANX, calnexin; IGLL1, immunoglobulin lambda-like polypeptide 1; PPIA, peptidyl-prolyl cis-trans isomerase A; GPA33, cell surface A33 antigen; CALM, calmodulin; SDSL, serine dehydratase; BDH2, 3-hydroxybutyrate dehydrogenase type 2.

1.9. Statistical analysis

Data analysis was conducted with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) using SIMCA P software package (Umetrics) in order to observe intrinsic clusters and obvious outliers (based on the principles of Hotelling T^2) within the data set. Student t-test, with a two-tailed distribution and an homoscedastic variance was used for all statistical analyses. Significance accepted for p-values ≤ 0.05.

2. Results and discussion

In order to study protein expression characteristics of turkey intestine, we carried out a proteome mapping of intestinal mucosa collected from healthy and HEV-infected animals. Protein target database resources of turkey are still incomplete and poorly annotated. To compensate, we have combined two databases, turkey and chicken, composed by nonredundant proteins from NCBI database (version of July 2016) to boost the number of identified proteins.

Using SWATH-MS strategy, a library was created with 7013 identified peptides, corresponding to 929 different proteins. After filtering for proteins which were quantified in at least three of four biological replicates and with at least one peptide (FDR = 1% and a confidence of 99%), this library allowed the quantification of 523 proteins among eight samples, which were included in this comparative and descriptive analysis (Supplementary Table 1).

2.1. Functional grouping of intestinal mucosa proteome

From our dataset composed by 523 proteins, only 185 protein number identifiers were successfully mapped on UniProtKB website (http://www.uniprot.org) and 45 were mapped on PANTHER website (http://www.pantherdb.org). Since more than 60% of the UniProtKB proteins were lacking and/or were classified as “Uncharacterized protein”, we ran BLAST using Blast2GO software (https://www.blast2go.com) to annotate our protein dataset.

Gene Ontology (GO) analysis was performed using the three main ontologies (Cellular Component, Molecular Function and Biological Process). Blast2GO retrieved 841 unique Biological Process GO terms, 541 unique Molecular Function GO terms and 800 unique Cellular Component GO terms associated with our dataset. From 523 total proteins, 18 sequences were not annotated. For a clearer representation, graphics were designed using WEGO website (http://wego.genomics.org.cn) (Figure 1).

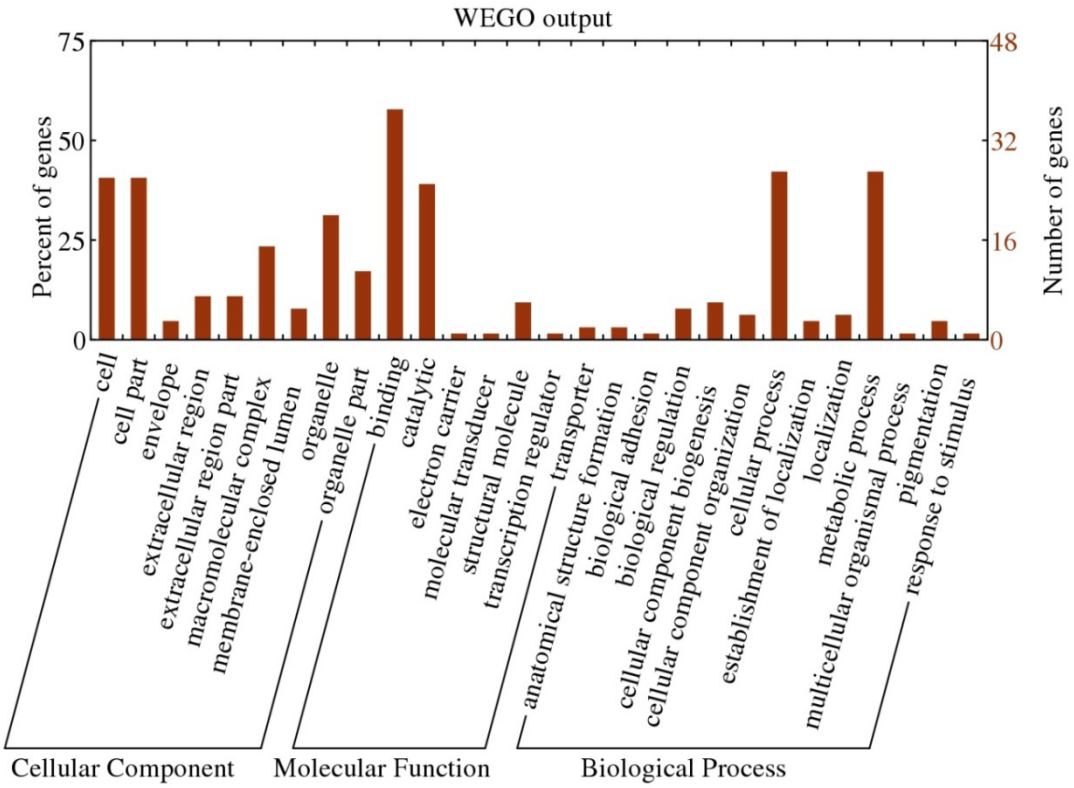

Figure 1. Proteome characteristics of mucosa intestine. GO classification using Blast2GO shows level 2 categories for Cellular Components, Molecular Function and Biological Processes.

523 proteins were grouped into 28 GO terms divide into three ontologies:

Cellular component: 40.6% cell, 40.6% cell part, 31.3% organelle, 23.4% macromolecular complex, 17.2% organelle part, 10.9% extracellular region, 10.9% extracellular region part, 7.8% membrane-enclosed lumen and 4.7% envelope.

Molecular function: 57.8% binding, 39.1% catalytic activity, 9.4% structural molecule activity, 3.1% transporter activity, 1.6% electron carrier activity, 1.6% molecular transducer activity and 1.6% transcription regulator activity.

Biological process: 42.2% metabolic process, 42.2% cellular process, 9.4% cellular component biogenesis, 7.8% biological regulation, 6.3% cellular component organization, 6.3% localization, 4.7% pigmentation, 3.1% anatomical structure formation, 1.6% biological adhesion, 1.6% biological process, 1.6% multicellular organismal process and 1.6% response to stimulus.

2.2. Data Analysis

Due to the large amount of data generated in proteomics studies and its complexity, multivariate methods could be applied, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), allowing identification of proteins that can explain the differences we observed.

PCA is a powerful statistical tool/analysis that finds the most important trends in a dataset. Each sample is represented by a point in a score-plot, being classified as a member of one class if they fall within its boundaries. Samples which lie close to each other in the score-plots have similar profiles, whereas samples which lie far apart are very different and are classified as a member of a different class. In the other side, PLS-DA is a more powerful technique for discriminating between classes previously established, in our case, healthy and sick animals. Similar to PCA, it works by calculating a multivariate projection of the data and maximises the separation of the classes.

PCA and PLS-DA were applied to the 523 proteins quantified as multivariate statistical method. Based on the principles of Hotelling T^2 (95% confidence limit), four healthy animals and four disease-affected animals were separated into two groups (Figure 2). In PCA score-plot, we can see a disperse pattern for all samples (Figure 2A). However, HEV-affected samples tends to stay in the right panel whereas healthy samples aggregates itself in the middle. Since we already know the samples that compose our two groups and, after perform a PLS-DA, a more powerful separation was obtained and both groups were separated completely (Figure 2B). Healthy samples are on the left side, whereas HEV-affected samples stayed on the right side of score-plot, perfectly divided in two groups. This suggest that from our list of proteins, some of them, when combined together, allow us to distinguish clearly healthy animals from HEV-affected animals.

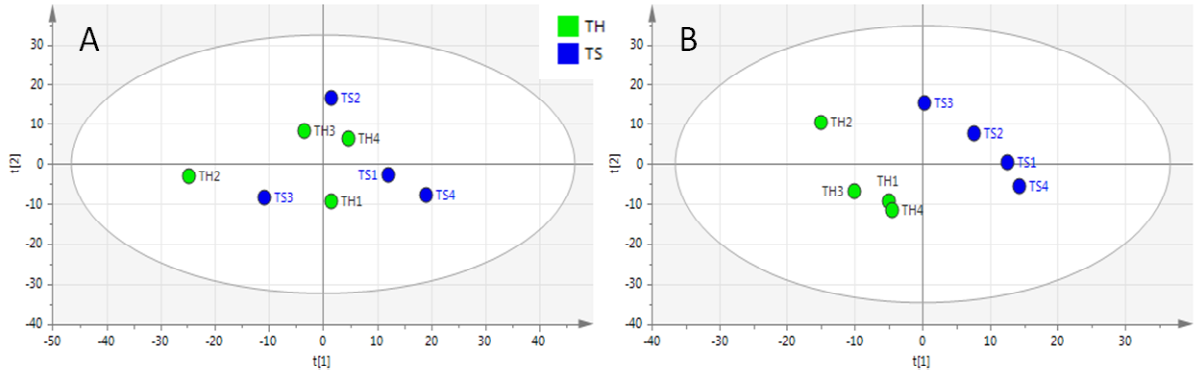

Figure 2. PCA and PLS-DA score-plots from healthy and HEV-affected groups. A, Score-plot obtained from PCA modelling, 2 components extracted. Healthy group, green. HEV-infected group, blue. The first component explains 35% ($R^2X[1]=0.345$) of the variation and the second component 17% ($R^2X[2]=0.167$). B, Score-plot obtained from PLS-DA modelling, 2 components extracted. Healthy group, green. HEV-infected group, blue. The first component explains 30% ($R^2X[1]=0.269$) of the variation and the second component 20% ($R^2X[2]=0.20$).

2.3. Proteins differentially expressed

A protein was considered differentially expressed when the *p*-value was below 0.05. From 523 proteins, following statistical analysis, 64 proteins were found to be differentially expressed in the intestinal mucosa from healthy and HEV-affected turkeys (Table 3).

Table 3. List of proteins differentially expressed in the intestinal mucosa in healthy and HEV-affected intestine. Protein fold changes in HEV-affected compared to healthy intestine are shown.

Accession	Name	Protein	Fold	*p*-value
gi|733885686	CASP6	PREDICTED: caspase-6 [Meleagris gallopavo]	4.63	0.0385
gi|733883237	DECR1	PREDICTED: 2,4-dienoyl-CoA reductase, mitochondrial [Meleagris gallopavo]	4.41	0.0438
gi|475808004	SDHA	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial [Gallus gallus]	4.40	0.0427
gi|513231603	LOC427292	PREDICTED: S-adenosylmethionine synthase isoform type-2-like isoform X2 [Gallus gallus]	3.79	0.0031
gi|971417755	HSDL2	PREDICTED: heterogeneous nuclear ribonucleoprotein A/B isoform X2 [Gallus gallus]	3.36	0.0429
gi|733926850	HNRPAB	PREDICTED: hydroxysteroid dehydrogenase-like protein 2, partial [Meleagris gallopavo]	3.36	0.0298
gi|733929326	DHDH	PREDICTED: trans-1,2-dihydrobenzene-1,2-diol dehydrogenase [Meleagris gallopavo]	3.30	0.0078
gi|733921537	SELENBP1	PREDICTED: selenium-binding protein 1-A isoform X2 [Meleagris gallopavo]	3.09	0.0017
gi|971370598	HIST1H2B5L	PREDICTED: histone H2B 1/2/3/4/6-like [Gallus gallus]	2.94	0.0290
gi|733919423	PCYOX1	PREDICTED: prenylcysteine oxidase 1 [Meleagris gallopavo]	2.58	0.0216
gi|971378941	HPX	PREDICTED: hemopexin [Gallus gallus]	2.53	0.0114
gi|971384254	RPL7	PREDICTED: 60S ribosomal protein L7 isoform X1 [Gallus gallus]	2.52	0.0408
gi|733893517	RPL14	PREDICTED: 60S ribosomal protein L14 [Meleagris gallopavo]	2.40	0.0283
gi|733910438	ATP5J2	PREDICTED: ATP synthase subunit f, mitochondrial [Meleagris gallopavo]	2.38	0.0040
gi|326924446	ES1	PREDICTED: ES1 protein homolog, mitochondrial-like [Meleagris gallopavo]	2.30	0.0459
gi|733868906	PIT54	PREDICTED: soluble scavenger receptor cysteine-rich domain-containing protein SSC5D-like [Meleagris gallopavo]	2.23	0.0411
gi|71897175	COPA	coatomer subunit alpha [Gallus gallus]	2.19	0.0192
gi|733928504	LOC104915868	PREDICTED: pyruvate carboxylase, mitochondrial-like, partial [Meleagris gallopavo]	2.18	0.0275
gi|971392610	ETFDH	PREDICTED: electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial isoform X2 [Gallus gallus]	2.16	0.0371
gi|733894380	HSPE1	PREDICTED: 10 kDa heat shock protein, mitochondrial [Meleagris gallopavo]	2.13	0.0232
gi|971375846	GK	PREDICTED: glycerol kinase isoform X4 [Gallus gallus]	2.11	0.0023

gi|733918251	RPL22	PREDICTED: 60S ribosomal protein L22, partial [Meleagris gallopavo]	2.06	0.0314
gi|50755667	NDUFB10	PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 [Gallus gallus]	1.99	0.0023
gi|733890971	PSMA3	PREDICTED: proteasome subunit alpha type-3 [Meleagris gallopavo]	1.95	0.0338
gi|733926972	CORO2A	PREDICTED: coronin-2A [Meleagris gallopavo]	1.89	0.0140
gi|487442401	SDHB	succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial isoform 2 precursor [Gallus gallus]	1.78	0.0297
gi|733884061	FGB	PREDICTED: fibrinogen beta chain [Meleagris gallopavo]	1.76	0.0004
gi|166091440	SRSF1	serine/arginine-rich splicing factor 1 [Gallus gallus]	1.73	0.0186
gi|971420700	SEPT2L	PREDICTED: septin-2 isoform X2 [Gallus gallus]	1.70	0.0192
gi|326922507	HSPD1	PREDICTED: 60 kDa heat shock protein, mitochondrial [Meleagris gallopavo]	1.67	0.0251
gi|971440071	HSD17B4	PREDICTED: peroxisomal multifunctional enzyme type 2 isoform X1 [Gallus gallus]	1.62	0.0468
gi|733870043	MPST	PREDICTED: 3-mercaptopyruvate sulfurtransferase isoform X2 [Meleagris gallopavo]	1.60	0.0448
gi|971437427	TLN1	PREDICTED: talin-1 isoform X3 [Gallus gallus]	1.60	0.0181
gi|429903872	PGRMC1	membrane-associated progesterone receptor component 1 [Gallus gallus]	1.57	0.0166
gi|733911356	UBE2L3	PREDICTED: ubiquitin-conjugating enzyme E2 L3 [Meleagris gallopavo]	1.50	0.0343
gi|733909243	CANX	PREDICTED: LOW QUALITY PROTEIN: calnexin [Meleagris gallopavo]	1.47	0.0229
gi|733881362	ECI2	PREDICTED: enoyl-CoA delta isomerase 2, mitochondrial isoform X1 [Meleagris gallopavo]	1.46	0.0205
gi|733925751	RPS6	PREDICTED: 40S ribosomal protein S6 [Meleagris gallopavo]	1.44	0.0388
gi|733912151	IGLL1	PREDICTED: immunoglobulin lambda-like polypeptide 1 isoform X2 [Meleagris gallopavo]	1.41	0.0362
gi|733921095	DLAT	PREDICTED: dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial [Meleagris gallopavo]	1.36	0.0416
gi|261490820	PPIA	peptidyl-prolyl cis-trans isomerase A [Gallus gallus]	1.33	0.0218
gi|326919055	MTTP	PREDICTED: microsomal triglyceride transfer protein large subunit [Meleagris gallopavo]	1.32	0.0385
gi|733888683	CPT1A	PREDICTED: carnitine O-palmitoyltransferase 1, liver isoform [Meleagris gallopavo]	1.30	0.0386
gi|733872460	GPA33	PREDICTED: cell surface A33 antigen [Meleagris gallopavo]	1.29	0.0374
gi|971380110	HNRNPA2B1	PREDICTED: heterogeneous nuclear ribonucleoproteins A2/B1 isoform X5 [Gallus gallus]	1.26	0.0369
gi|45384366	CALM	calmodulin [Gallus gallus]	1.24	0.0432

gi|733897806	GOT1	PREDICTED: aspartate aminotransferase, cytoplasmic, partial [Meleagris gallopavo]	1.22	0.0140
gi|743405597	DDX4	probable ATP-dependent RNA helicase DDX4 [Gallus gallus]	1.22	0.0397
gi|733893848	LOC100547885	PREDICTED: UDP-glucuronosyltransferase 1-1-like isoform X5 [Meleagris gallopavo]	1.12	0.0217
gi|326928043	ACOX2	PREDICTED: peroxisomal acyl-coenzyme A oxidase 2 [Meleagris gallopavo]	-1.19	0.0492
gi|52138693	WDR1	WD repeat-containing protein 1 [Gallus gallus]	-1.23	0.0445
gi|482661640	CORO1C	coronin-1C isoform 1 [Gallus gallus]	-1.26	0.0150
gi|733870073	MYH9	PREDICTED: myosin-9 [Meleagris gallopavo]	-1.33	0.0471
gi|971431933	ATP5L	PREDICTED: ATP synthase subunit g, mitochondrial [Gallus gallus]	-1.36	0.0216
gi|971411979	TPM1	PREDICTED: tropomyosin alpha-1 chain isoform X16 [Gallus gallus]	-1.37	0.0044
gi|50746903	CISD2	PREDICTED: CDGSH iron-sulfur domain-containing protein 2 [Gallus gallus]	-1.41	0.0201
gi|733903686	SI	PREDICTED: sucrase-isomaltase, intestinal [Meleagris gallopavo]	-1.46	0.0418
gi|45382893	CALB1	calbindin [Gallus gallus]	-1.61	0.0344
gi|733873176	DDX3X	PREDICTED: ATP-dependent RNA helicase DDX3X [Meleagris gallopavo]	-1.67	0.0250
gi|733927467	LOC104915494	PREDICTED: fatty acyl-CoA hydrolase precursor, medium chain-like [Meleagris gallopavo]	-1.76	0.0429
gi|733905607	LOC104912905	PREDICTED: creatine kinase U-type, mitochondrial-like [Meleagris gallopavo]	-1.78	0.0065
gi|733922734	ACE	PREDICTED: LOW QUALITY PROTEIN: angiotensin-converting enzyme [Meleagris gallopavo]	-1.79	0.0117
gi|733912766	LOC100539678	PREDICTED: serine dehydratase-like [Meleagris gallopavo]	-2.12	0.0254
gi|326919083	BDH2	PREDICTED: 3-hydroxybutyrate dehydrogenase type 2 [Meleagris gallopavo]	-2.15	0.0409

A volcano plot was built for the 523 proteins to identify interesting proteins in our dataset (Figure 3).

A volcano plot is a type of scatter plot that is used to represent the most-meaningful changes of proteins that are considered both statistically significant (p-value ≤ 0.05) and differentially expressed (increased or decreased folds change). It makes clear the relationship between a statistical test and the magnitude of the difference in expression values of the samples. Therefore, the volcano plot enables a quick visual identification that contributes to identify possible targets in our results.

The up-regulated proteins in HEV-affected animals with corresponding p-value ≤ 0.05 are shown in the right side of Figure 3 (red dots), whereas the down-regulated proteins with p-value ≤ 0.05 are the red dots in the left side. Black dots are the proteins without statistically significant p-value > 0.05.

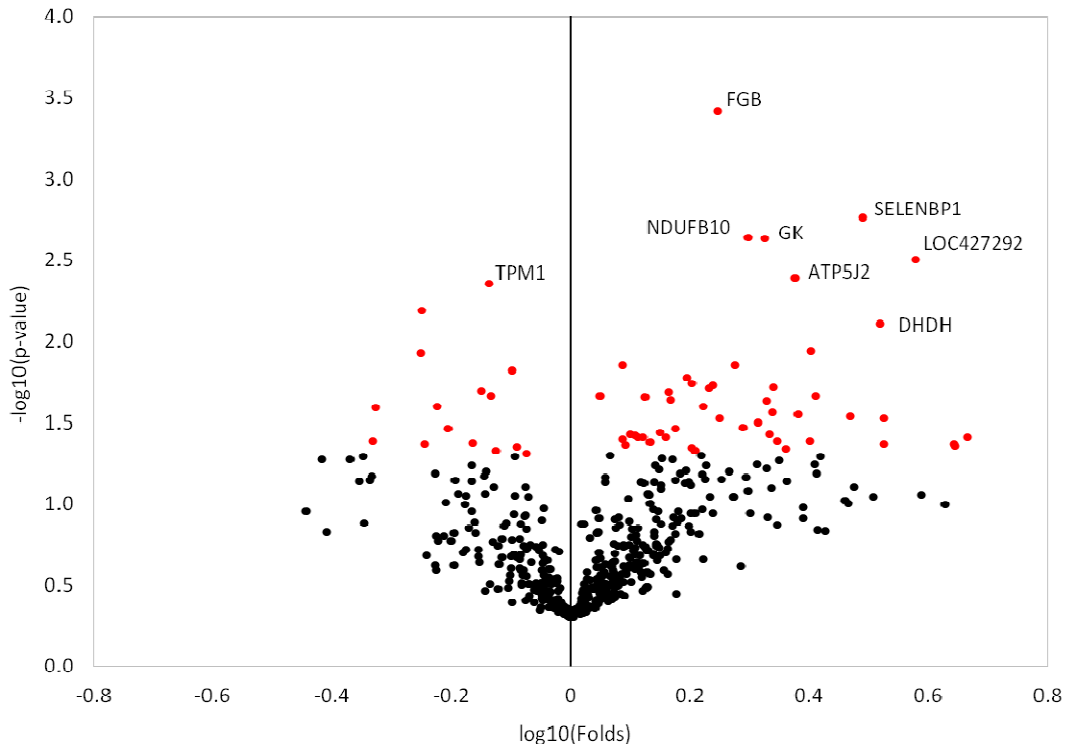

Figure 3. Volcano plot of the 523 protein list. Each point represents a protein. The red dots indicate statistically significant proteins with p-values ≤ 0.05 and the black dots represent statistically non-significant proteins (p-values > 0.05). Negative log10(Folds) values indicate down-regulated proteins and positive values indicate up-regulated proteins in HEV-affected animals.

Among all differentially expressed proteins, S-adenosylmethionine synthase isoform type-2-like isoform X2 (LOC427292), trans-1,2-dihydrobenzene-1,2-diol dehydrogenase (DHDH), selenium-binding protein 1-A isoform X2 (SELENBP1), ATP synthase subunit f mitochondrial (ATP5J2), glycerol kinase isoform X4 (GK), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 (NDUFB10), fibrinogen beta chain (FGB) and tropomyosin alpha-1 chain isoform X16 (TPM1) had the meaningful changes.

Sixty-four proteins of differential abundance were grouped into nine classes based on their putative functions: amino acid and protein metabolism (25%), carbohydrate metabolism (19%), fatty acids metabolism (16%), cytoskeleton (11%), immune system (11%), nucleic acid biogenesis (6%), ribosome (6%), binding and transport (5%) and apoptosis (1%) (Figure 4).

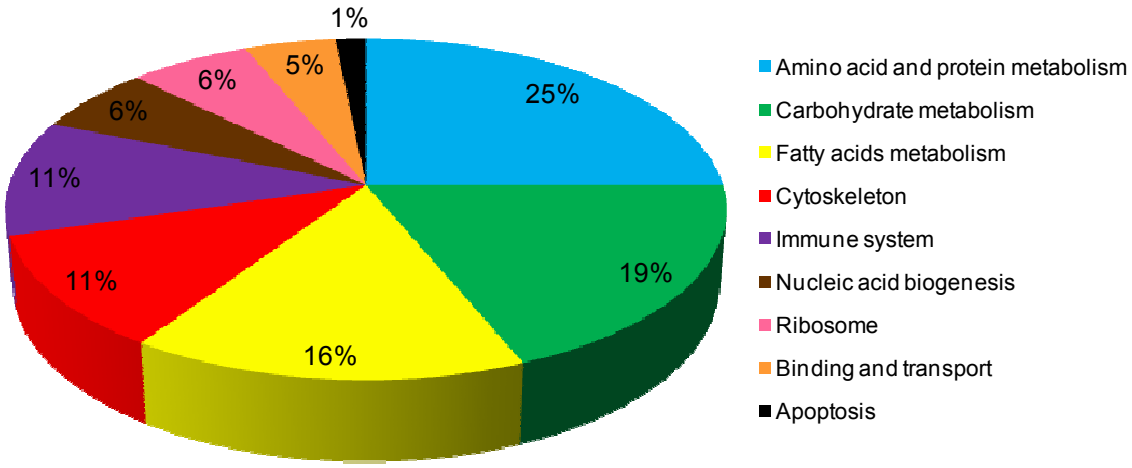

Figure 4. Functional classification of the proteins of differential abundance identified from the intestinal mucosa of turkey.

Those related to amino acid and protein metabolism, carbohydrate metabolism, fatty acids metabolism, cytoskeleton and immune system were predominant and accounted for approximately 80% of the differential proteins.

A comparison of proteins differential abundant between both groups showed that 49 proteins were up-regulated and 15 down-regulated in HEV-affected animals (Figure 5).

The 49 up-regulated proteins were distributed in ten classes: 13 in amino acid and protein metabolism, 9 in carbohydrate metabolism, 7 in fatty acids metabolism, 7 in immune system, 4 in nucleic acid biogenesis, 4 in ribosome, 3 in cytoskeleton, 1 in binding and transport and 1 in apoptosis.

The 15 down-regulated protein were distributed in six classes: 4 in cytoskeleton, 3 in amino acid and protein metabolism, 3 in carbohydrate metabolism, 3 in fatty acids metabolism and 2 in binding and transport.

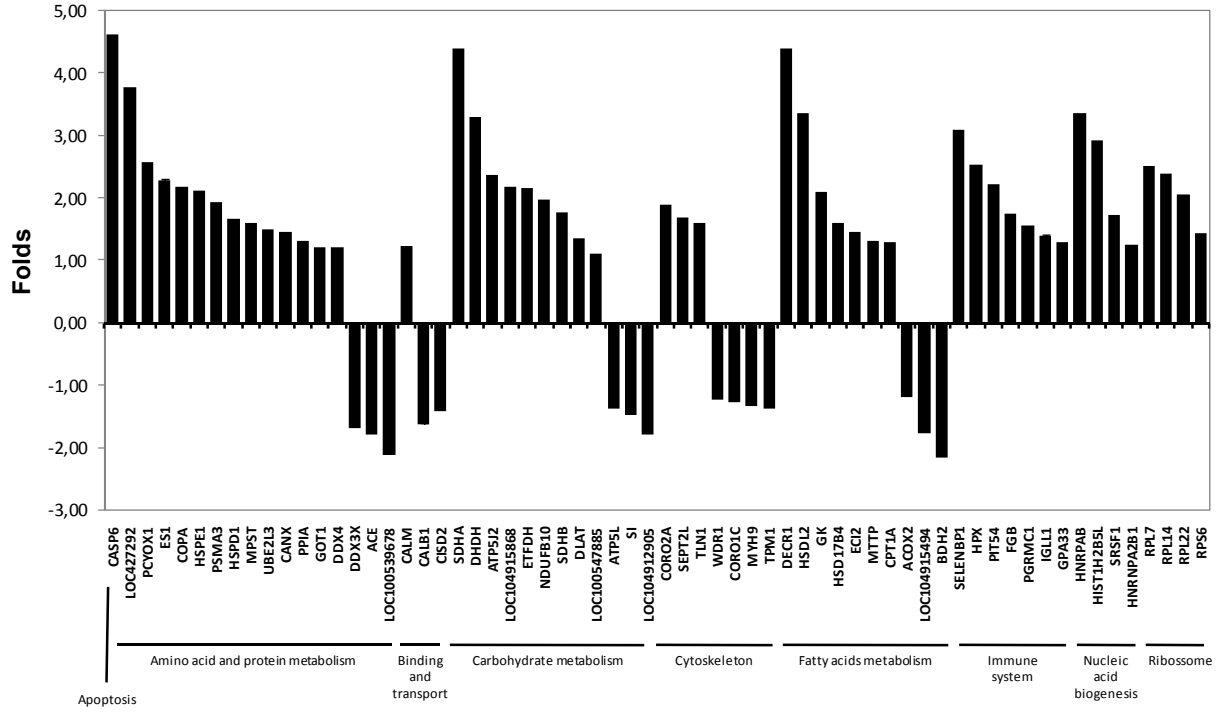

Figure 5. Quantitative analysis of the proteins of differential abundance from HEV-affected intestinal mucosa.

2.4. mRNA expression of selected proteins related with immune system

The intestine is crossed by a wide capillary network. The finding of a protein by proteomic techniques does not confirm its expression by intestine. Several proteins can be expressed by liver or other tissues and then delivered to intestine through blood. Therefore, the effective presence of proteins related with immune response on intestinal mucosa was confirmed by qualitative PCR through detection of their respective mRNAs.

Fifteen proteins (selenium-binding protein 1-A (SELENBP1), hemopexin (HPX), PIT54, fibrinogen beta chain (FGB), membrane-associated progesterone receptor component (PGRMC1), immunoglobulin lambda-like polypeptide 1 (IGLL1) and cell surface A33 antigen (GPA33) grouped into immune system classe; ES1 protein homolog (ES1), coronin-2A (CORO2A), 3-mercaptopyruvate sulfurtransferase (MPST), calnexin (CANX), peptidyl-prolyl cis-trans isomerase A (PPIA) and calmodulin (CALM) related with inflammation, viral and bacterial defenses and response to external stimulus; and serine dehydratase (SDSL) and 3-hydroxybutyrate dehydrogenase type 2 (BDH2) up-regulated more than 2 times in healthy mucosa) were selected for functional validation at RNA level using qualitative PCR. No studies have investigated their mRNA expression in turkey intestine to date. Results are summarized in Table 4 and pictures of the agarose gel electrophoresis can be seen in Figure 6. Liver was also included in this analysis as an internal control.

Thirteen and 11 genes out of 15 were found expressed by HEV-affected and healthy mucosa, respectively. Four genes out of 15 were not found expressed by intestinal mucosa of healthy animals, suggesting that these proteins are delivered to intestine through blood in a normal state. These include selenium-binding protein 1-A (SELENBP1), hemopexin (HPX) and fibrinogen beta chain (FGB). Interesting, mRNA of these genes, except HPX, was detected in intestinal mucosa affected with HEV. HPX mRNA was undetectable in intestinal mucosa of HEV-affected animals, suggesting that intestine cannot produce this protein and it needs to be delivered via blood capillaries. ES1 protein homolog (ES1) was not detected either in liver nor intestine. We cannot rule out the hypothesis that primers are not working since liver was negative and another positive control was not included.

Table 4. mRNA expression of inflammation and innate immunity-related proteins in turkey intestinal mucosa. Qualitative PCR results: P, presence; A, absence.

Gene name	Liver	Healthy intestinal mucosa	HEV-affected intestinal mucosa
SELENBP1, selenium-binding protein 1-A	P	A	P
HPX, hemopexin	P	A	A
PIT54, haptoglobin-like	P	P	P
FGB, fibrinogen beta chain	P	A	P
PGRMC1, membrane-associated progesterone receptor	P	P	P
IGLL1, immunoglobulin lambda-like polypeptide 1	P	P	P
GPA33, cell surface A33 antigen	A	P	P
ES1, ES1 protein homolog	A	A	A
CORO2A, coronin-2A	A	P	P
MPST, 3-mercaptopyruvate sulfurtransferase	P	P	P
CANX, calnexin	P	P	P
PPIA, peptidyl-prolyl cis-trans isomerase A	P	P	P
CALM, calmodulin	P	P	P
SDSL, serine dehydratase	P	P	P
BDH2, 3-hydroxybutyrate dehydrogenase type 2	P	P	P

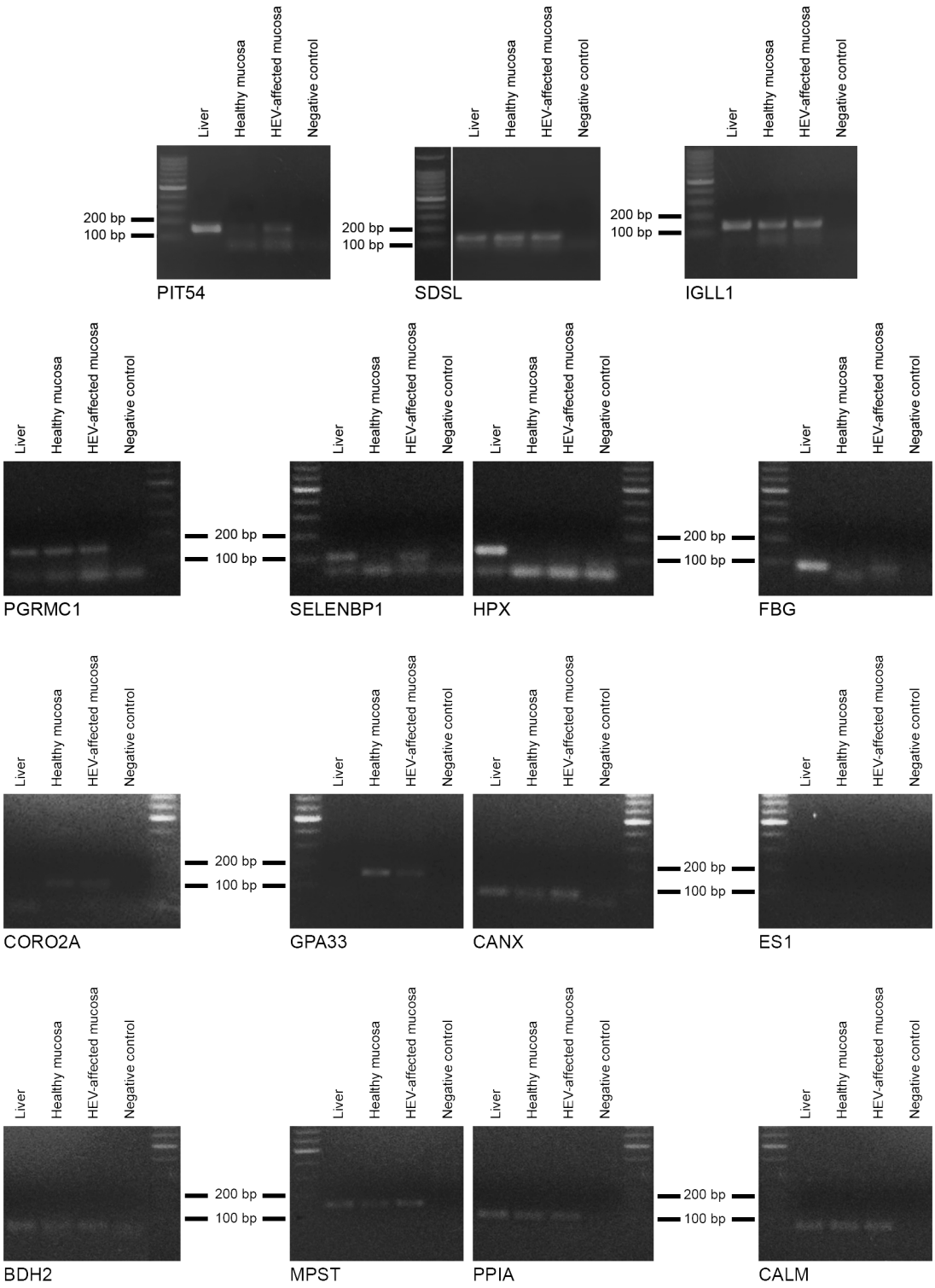

Figure 6. PCR amplification products of PIT54, SDSL, IGLL1, PGRMC1, SELENBP1, HPX, FGB, CORO2A, GPA33, CANX, ES1, BDH2, MPST, PPIA and CALM in liver, healthy and HEV-affected intestinal mucosa mRNA separated by agarose gel electrophoresis (1.6%) stained with ethidium bromide. Non-template reaction was used as negative control.

The presence of FGB in turkey HEV-intestine confirms previous studies in chicken infected with gastrointestinal diseases (Georgieva et al., 2010). Fibrinogen is a glycoprotein synthetized by hepatocytes and considered an APP in chicken. The RNA expression of SELENBP1 by intestine in poultry has never been investigated, but SELENBP1 has been observed in several types of human cancers, including colorectal cancer (Kim et al., 2006). SELENBP1 is a member of selenium-binding protein family, which have been shown to bind selenium covalently (Porat et al., 2000). SELENBP1 role in intestine is not known, however SELENBP1 is a target of hypoxia-inducible factor-1α to respond to reactive oxygen species (ROS) (Huang et al., 2012) and it is associated with alterations of lipid/glucose metabolism-related proteins (Ying et al., 2015). Also mRNA presence of PGRMC1 has never been described in intestine. PGRMC1 is a single transmembrane protein that forms part of a multi-protein complex that binds to progesterone and other steroids, and it is associated with cancer in humans (Cahill, 2007). There is evidence that progesterone modulates immune function in mammals (Dosiou et al., 2008) and promotes T cell differentiation (Piccinni et al., 1995).

Intestine is regarded as one essential organ for nutrient absorption, pathogen prevention and immune response (Furness et al., 1999; Lillehoj and Trout, 1999), established interaction between different microbial species and contributing to the animal welfare. The important role of intestinal mucosa in the immune response is demonstrated by the RNA expression of acute phase proteins and genes related with B-lymphocytes differentiation and epithelial mucosa integrity, namely PIT54, IGLL1 and GPA33. PIT54 is homologous to haptoglobin in mammals and has been identified as the major hemoglobin-binding protein in birds (Quaye, 2008; Wicher and Fries, 2006). As already described before, PIT54 is considered one APP in poultry and its extrahepatic expression is described in this thesis. The IGLL1 gene encodes a component of the pre-B-cell receptor, which is crucial for B cells development (Chen et al., 2016). Zandi et al. (2012) have also found IGLL1 gene expressed in cattle fetal tissues, including intestine, however its expression by avian intestine has never been reported. The malfunction of the epithelial cells caused by HEV can be demonstred by the expression of GPA33, a gene identified in human and mice intestine, and overexpressed in many tumors (Johnstone et al., 2002; Solmi et al., 2004). GPA33 is an intestinal epithelium-specific cell surface marker and a component of the tight junction-associated proteins of the immunoglobulin superfamily (Ackerman et al., 2008; Johnstone et al., 2000) and it is involved in immune signalling (Mallegol et al., 2007; Van Niel et al., 2003). GPA33 is also crucial for the maintenance of intestinal barrier function (Williams et al., 2015). We did not detect GPA33 expression in liver and, in fact, literature refers its

expression only in intestine. Interestingly, HPX mRNA was not found, although its protein has been increased by 2.5-folds in HEV-affected intestine. HPX is considered another APP induced by inflammation in chicken (Adler et al., 2001). Takagi et al. (2012) have also reported the presence of HPX protein in injured intestinal mucosa of rats, but not at mRNA level. HPX has the highest binding affinity to heme group, sequestering heme from pathogens and protecting cells against toxicity (Dooley et al., 2010; Tolosano and Altruda, 2002), suggesting the importance of this protein to be translocated into intestine by blood.

Other proteins that have been identified by proteomics and validated by gene expression studies include CORO2A, MPST, CANX, PPIA, CALM, ES1, SDSL and BDH2. Although these proteins were not classified as immune system-involved, after a bibliographic search, we found them associated with innate immunity, inflammation, viral and bacterial responses and external stimulus. Coronin proteins are known as regulators of actin-based cellular processes, and CORO2A is associated with human colon carcinoma (Rastetter et al., 2005). Huang et al. (2011) have shown evidence that CORO2A controls inflammatory response and is required for de-repression of TLR target genes by macrophages. CORO2A expression by liver has also not been reported yet. CANX is an important chaperone of the endoplasmic reticulum and is involved in secretory pathways. Its involvement in innate immunity and intestinal paneth cells' differentiation have already been reported (Gassler et al., 2002; Huang et al., 2016). PPIA (or better known as Cyclophilin A) is reported in literature in association with viral infections in chicken, such as Influenza Virus and Infectious Bursal Disease Virus (Frausto et al., 2013; Wang et al., 2015; Xu et al., 2010), and heat intestinal stress in pigs (Pearce et al., 2015). Moreover, it has been reported that CypA is involved in T cell activation and in the IFN-I or IL-2 responses during virus infections (Dawar et al., 2016; Rauf et al., 2011).

MPST is another protein, which gene we found in intestine. Although not directly involved with immune system, MPST can help with reactive oxygen species (ROS) higher levels generated during haemorrhagic shock and playing an important role in cyanide detoxification, avoiding in this way the significant damage to cell structures (Iciek et al., 2005). CALM is a multifunctional calcium sensor protein that participates in various immune functions such as programmed cell death, autophagy, inflammation and the immune response (Tidow and Nissen, 2013). We found CALM protein more expressed by HEV-affected intestine and the presence of its mRNA was also validated. Consistent with our findings, Connell et al. (2013) found the same gene associated with avian colonization by *Campylobacter jejuni* in chicken, suggesting the importance of the calcium absorption in the maintenance of intestinal homeostasis.

ES1 gene was also included in this analysis. ES1 protein homolog mRNA was induced in intestine of chickens infected with *Salmonella enterica* (Matulova et al., 2013; Rychlik et al., 2014). However, in our study the mRNA was undetected in any samples included, then we have to consider the possibility that our designed primers did not work.

Finally, SDSL and BDH2 are two proteins involved in protein and fatty acid metabolism, respectively, which folds were 2-times increased in healthy animals. Therefore, its mRNA expression was investigated. Both mRNA were found in healthy and sick intestine. It is notorious that serine dehydratase members have already been reported in the colonization of the avian gut by *C. jejuni* (Velayudhan et al., 2004), whereas we found it more expressed by healthy birds. No previous studies have investigated BDH2 expression in chicken intestinal mucosa and validated by mRNA expression. Inflammation of the gastrointestinal tract generates a stressful condition, which can lead to a intestine shutdown and decreases enzymes and secretions needed for digestion. A normal metabolism is occuring in the healthy birds, while the need for digestion enzymes was stopped in affected animals.

3. Conclusions

It has become increasingly clear that intestine plays important roles in a wide range of biological processes, including metabolic control, oxidative stress, inflammation, as well as for both innate and adaptive immune response in poultry. However, depth knowledge of the biochemistry and molecular characteristics of intestinal mucosa has not yet properly been addressed so far.

To the best of our knowledge, we have here presented the first comparison of healthy and HEV-affected intestinal mucosa proteomes in turkey. The analysis was carried out by SWATH-MS strategy and the global proteome mapping allowed the identification of 523 proteins, being 64 differentially expressed. HEV disease induced changes in a higher number of proteins related with protein, carbohydrate and lipid processes, and immune system. Furthermore, the validation at gene expression level of proteins involved in inflammation, infection and immune response demonstrated that intestine expresses, at least, 13 genes involved in these mechanisms. Little is so far known about the intestinal immunity in turkey species, although it is of key importance for the management of animal health and also for economic gain.

Further research is needed to investigate if the quantitative protein results found by proteomic technique is consistent with quantitative gene expression. Quantitative Real Time PCR will be done to quantify the fifteen genes validated by qualitative PCR.

Supplementary Table 1. List of proteins identified and quantified in healthy and HEV-affected intestine. TH, turkey healthy; TS, turkey sick; SD, standard deviation; %COV, covariance percentage.

N	No. Peptides (max.)	Accession	Name	Mean TH	Mean TS	SD TH	SD TS	%COV TH	%COV TS
1	14	gi|733870073	PREDICTED: myosin-9 [Meleagris gallopavo]	1.06E-02	7.95E-03	1.29E-03	1.08E-03	12.19	13.60
2	13	gi|326919055	PREDICTED: microsomal triglyceride transfer protein large subunit [Meleagris gallopavo]	6.53E-03	8.63E-03	2.62E-03	2.42E-03	40.18	28.00
3	14	gi|45382893	calbindin [Gallus gallus]	7.51E-02	4.67E-02	2.62E-02	9.72E-03	34.91	20.81
4	12	gi|733894925	PREDICTED: villin-1 [Meleagris gallopavo]	7.00E-03	5.64E-03	1.68E-03	1.03E-03	23.98	18.20
5	4	gi|733913411	PREDICTED: spectrin alpha chain, non-erythrocytic 1 isoform X2 [Meleagris gallopavo]	1.08E-03	1.08E-03	4.06E-04	1.74E-04	37.51	16.14
6	10	gi|971424700	PREDICTED: actin, cytoplasmic 2 isoform X1 [Gallus gallus]	1.36E-02	1.50E-02	9.12E-03	5.78E-03	66.90	38.48
7	15	gi|326919555	PREDICTED: keratin, type II cytoskeletal cochleal [Meleagris gallopavo]	2.44E-02	2.08E-02	1.24E-02	4.23E-03	50.59	20.33
8	14	gi|733884696	PREDICTED: serum albumin [Meleagris gallopavo]	3.36E-02	1.99E-02	2.33E-02	1.21E-02	69.52	60.62
9	5	gi|971425641	PREDICTED: clathrin heavy chain 1 isoform X1 [Gallus gallus]	1.49E-03	1.92E-03	2.90E-04	4.51E-04	19.39	23.43
10	5	gi|733869766	PREDICTED: aconitate hydratase, mitochondrial [Meleagris gallopavo]	2.34E-03	3.17E-03	1.15E-03	1.37E-03	49.04	43.05
11	13	gi|733923780	PREDICTED: elongation factor 2 [Meleagris gallopavo]	1.10E-02	9.89E-03	2.16E-03	2.11E-03	19.73	21.37
12	12	gi|733871820	PREDICTED: sodium/potassium-transporting ATPase subunit alpha-1 [Meleagris gallopavo]	5.72E-03	5.98E-03	1.84E-03	1.57E-03	32.18	26.22
13	2	gi|971437052	PREDICTED: tubulin alpha-1B chain [Gallus gallus]	1.30E-03	2.89E-03	9.42E-04	2.11E-03	72.64	72.91
14	15	gi|728798448	protein disulfide-isomerase precursor [Gallus gallus]	2.32E-02	2.09E-02	4.47E-03	4.84E-03	19.28	23.16
15	12	gi|326931252	PREDICTED: malate dehydrogenase, mitochondrial [Meleagris gallopavo]	3.28E-02	3.70E-02	6.68E-03	6.12E-03	20.40	16.54
16	11	gi|733926812	PREDICTED: fructose-bisphosphate aldolase B [Meleagris gallopavo]	1.63E-02	1.63E-02	4.04E-03	6.01E-03	24.72	36.99
17	11	gi|733918334	PREDICTED: alpha-enolase isoform X1 [Meleagris gallopavo]	1.08E-02	9.85E-03	1.22E-03	1.01E-03	11.31	10.25
18	7	gi|733905521	PREDICTED: aminopeptidase N [Meleagris gallopavo]	4.17E-03	3.50E-03	6.00E-04	5.54E-04	14.37	15.82
19	11	gi|326934148	PREDICTED: keratin, type I cytoskeletal 20 [Meleagris gallopavo]	2.30E-02	2.02E-02	1.20E-02	3.86E-03	52.33	19.12
20	10	gi|733904108	PREDICTED: pyruvate kinase PKM isoform X2 [Meleagris gallopavo]	8.26E-03	9.23E-03	7.68E-04	1.42E-03	9.29	15.44
21	13	gi|733914241	PREDICTED: 78 kDa glucose-regulated protein [Meleagris gallopavo]	5.86E-03	6.31E-03	9.41E-04	6.63E-04	16.06	10.51
22	12	gi|733906284	PREDICTED: fatty acyl-CoA hydrolase precursor, medium chain-like [Meleagris gallopavo]	1.56E-02	1.65E-02	2.08E-03	7.37E-03	13.33	44.58
23	11	gi|733890560	PREDICTED: heat shock protein HSP 90-alpha [Meleagris gallopavo]	6.67E-03	9.21E-03	2.73E-03	3.02E-03	40.96	32.78
24	10	gi|54020687	elongation factor 1-alpha 1 [Gallus gallus]	5.52E-03	6.06E-03	3.15E-03	3.52E-03	57.13	57.98

25	15	gi|326933304	PREDICTED: heat shock cognate 71 kDa protein [Meleagris gallopavo]	2.00E-02	2.13E-02	2.97E-03	2.08E-03	14.85	9.75
26	2	gi|733888559	PREDICTED: mucin-2 isoform X2 [Meleagris gallopavo]	9.01E-04	7.46E-04	2.89E-04	2.88E-04	32.12	38.54
27	2	gi|153792017	tubulin beta-3 chain [Gallus gallus]	2.20E-04	4.25E-04	2.13E-04	3.31E-04	96.85	77.87
28	8	gi|326917889	PREDICTED: cadherin-17 [Meleagris gallopavo]	3.59E-03	3.76E-03	1.96E-03	3.69E-04	54.57	9.80
29	1	gi|971398818	PREDICTED: spectrin beta chain, non-erythrocytic 5 [Gallus gallus]	8.55E-05	6.02E-05	7.82E-05	2.30E-05	91.51	38.28
30	4	gi|733872815	PREDICTED: carbonyl reductase [NADPH] 1 [Meleagris gallopavo]	2.99E-03	2.81E-03	8.55E-04	1.27E-03	28.65	45.25
31	7	gi|733903686	PREDICTED: sucrase-isomaltase, intestinal [Meleagris gallopavo]	3.67E-03	2.51E-03	5.42E-04	6.49E-04	14.79	25.81
32	7	gi|733896251	PREDICTED: bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2 isoform X2 [Meleagris gallopavo]	3.88E-03	4.55E-03	1.98E-03	2.46E-03	51.04	54.08
33	6	gi|733907702	PREDICTED: filamin-B isoform X2 [Meleagris gallopavo]	1.63E-03	1.37E-03	2.17E-04	1.52E-04	13.35	11.11
34	9	gi|733890623	PREDICTED: LOW QUALITY PROTEIN: creatine kinase B-type [Meleagris gallopavo]	5.67E-03	6.19E-03	2.11E-03	5.07E-04	37.14	8.19
35	3	gi|448261627	ATP synthase subunit beta, mitochondrial precursor [Gallus gallus]	1.37E-03	1.03E-03	1.00E-03	5.54E-04	73.34	53.74
36	3	gi|326916634	PREDICTED: trifunctional enzyme subunit alpha, mitochondrial [Meleagris gallopavo]	7.82E-04	8.73E-04	6.27E-04	5.79E-04	80.09	66.32
37	13	gi|733877610	PREDICTED: epoxide hydrolase 1-like isoform X1 [Meleagris gallopavo]	6.98E-03	9.89E-03	5.36E-04	2.59E-03	7.68	26.22
38	5	gi|733882112	PREDICTED: cytosolic non-specific dipeptidase [Meleagris gallopavo]	4.60E-03	3.70E-03	8.78E-04	7.65E-04	19.11	20.68
39	7	gi|733885214	PREDICTED: hydroxyacyl-coenzyme A dehydrogenase, mitochondrial [Meleagris gallopavo]	4.97E-03	5.88E-03	2.60E-03	4.16E-04	52.20	7.06
40	7	gi|733905603	PREDICTED: protein disulfide-isomerase A3 [Meleagris gallopavo]	2.13E-03	2.38E-03	8.31E-04	8.59E-04	39.01	36.01
41	2	gi|971421516	PREDICTED: aldehyde dehydrogenase, mitochondrial [Gallus gallus]	4.04E-04	5.34E-04	1.53E-04	1.69E-04	37.83	31.70
42	3	gi|733927011	PREDICTED: cytoplasmic aconitate hydratase [Meleagris gallopavo]	1.34E-03	1.27E-03	5.08E-04	3.56E-04	38.02	28.02
43	9	gi|46048961	glyceraldehyde-3-phosphate dehydrogenase [Gallus gallus]	5.06E-03	7.49E-03	3.69E-03	4.32E-03	72.89	57.70
44	5	gi|326922507	PREDICTED: 60 kDa heat shock protein, mitochondrial [Meleagris gallopavo]	7.55E-04	1.26E-03	5.52E-04	6.56E-04	73.22	52.20
45	3	gi|363738106	PREDICTED: C-factor-like isoform X2 [Gallus gallus]	4.08E-03	3.83E-03	3.15E-03	1.17E-03	77.30	30.53
46	12	gi|57524986	stress-70 protein, mitochondrial precursor [Gallus gallus]	3.28E-03	4.15E-03	6.41E-04	1.17E-03	19.53	28.26
47	7	gi|733929822	PREDICTED: unconventional myosin-Ia [Meleagris gallopavo]	9.75E-04	1.37E-03	3.07E-04	3.51E-04	31.48	25.64
48	2	gi|113206112	transitional endoplasmic reticulum ATPase [Gallus gallus]	6.39E-04	7.31E-04	3.05E-04	1.77E-04	47.80	24.24
49	10	gi|971419880	PREDICTED: 4-aminobutyrate aminotransferase, mitochondrial [Gallus gallus]	1.93E-03	2.61E-03	5.68E-04	5.20E-04	29.40	19.93
50	2	gi|45383752	creatine kinase U-type, mitochondrial precursor [Gallus gallus]	3.48E-03	3.65E-03	4.63E-04	6.84E-04	13.29	18.74
51	11	gi|733912136	PREDICTED: glutathione S-transferase theta-1-like [Meleagris gallopavo]	5.10E-03	7.12E-03	2.67E-03	2.50E-03	52.43	35.08
52	9	gi|733886337	PREDICTED: UDP-glucose 6-dehydrogenase [Meleagris gallopavo]	4.53E-03	5.86E-03	2.27E-03	2.18E-03	49.98	37.12
53	6	gi|733894667	PREDICTED: isocitrate dehydrogenase [NADP] cytoplasmic [Meleagris gallopavo]	3.08E-03	4.04E-03	1.04E-03	2.42E-04	33.75	5.98

54	7	gi|733867992	PREDICTED: rab GDP dissociation inhibitor beta [Meleagris gallopavo]	4.29E-03	3.93E-03	6.18E-04	1.64E-03	14.41	41.63
55	9	gi|971401116	PREDICTED: glutamate dehydrogenase 1, mitochondrial [Gallus gallus]	8.92E-03	8.04E-03	1.02E-03	8.70E-04	11.49	10.81
56	6	gi|733907734	PREDICTED: cytochrome b-c1 complex subunit 1, mitochondrial [Meleagris gallopavo]	1.78E-03	2.03E-03	3.56E-04	3.59E-04	20.00	17.70
57	5	gi|733897658	PREDICTED: glutathione S-transferase omega-1-like [Meleagris gallopavo]	2.35E-03	3.33E-03	7.23E-04	6.68E-04	30.82	20.02
58	4	gi|971398918	PREDICTED: alpha-actinin-1 isoform X3 [Gallus gallus]	1.27E-03	1.14E-03	1.45E-04	1.60E-04	11.41	14.02
59	5	gi|733920829	PREDICTED: apolipoprotein A-IV [Meleagris gallopavo]	1.63E-03	1.08E-03	5.11E-04	2.87E-04	31.33	26.52
60	11	gi|356640688	glutathione S-transferase alpha class A1.1 [Meleagris gallopavo]	2.69E-02	2.14E-02	1.29E-02	8.96E-03	48.13	41.85
61	4	gi|326925288	PREDICTED: alcohol dehydrogenase [NADP(+)] [Meleagris gallopavo]	1.09E-03	1.28E-03	1.73E-04	3.67E-04	15.81	28.62
62	6	gi|733896644	PREDICTED: putative hexokinase HKDC1 [Meleagris gallopavo]	2.27E-03	2.22E-03	1.68E-04	6.44E-04	7.41	28.93
63	9	gi|733871499	PREDICTED: triosephosphate isomerase [Meleagris gallopavo]	4.74E-03	5.01E-03	8.42E-04	1.22E-03	17.76	24.42
64	1	gi|733914806	PREDICTED: peroxisomal acyl-coenzyme A oxidase 1 isoform X1 [Meleagris gallopavo]	6.81E-04	4.21E-04	2.48E-04	2.79E-04	36.42	66.24
65	6	gi|91260417	cytochrome P450 3A37 [Meleagris gallopavo]	3.19E-03	2.94E-03	1.42E-03	1.21E-03	44.44	41.20
66	5	gi|487442792	fructose-1,6-bisphosphatase 1 [Gallus gallus]	2.28E-03	1.87E-03	6.52E-04	4.50E-04	28.68	24.06
67	5	gi|971411927	PREDICTED: annexin A2 isoform X1 [Gallus gallus]	2.07E-03	1.70E-03	1.07E-03	3.72E-04	51.61	21.83
68	6	gi|733885503	PREDICTED: annexin A5 [Meleagris gallopavo]	3.43E-03	2.26E-03	2.06E-03	4.37E-04	59.91	19.34
69	4	gi|971397363	PREDICTED: L-lactate dehydrogenase A chain isoform X1 [Gallus gallus]	2.48E-03	2.06E-03	1.31E-03	5.99E-04	52.84	29.00
70	1	gi|733903496	PREDICTED: 3-ketoacyl-CoA thiolase, mitochondrial [Meleagris gallopavo]	5.23E-04	1.34E-03	3.99E-04	1.02E-03	76.28	76.13
71	5	gi|45383562	endoplasmin precursor [Gallus gallus]	2.11E-03	2.38E-03	8.94E-04	7.11E-04	42.30	29.87
72	4	gi|733917093	PREDICTED: adenosine deaminase, partial [Meleagris gallopavo]	4.19E-03	5.80E-03	1.23E-03	9.09E-04	29.38	15.69
73	10	gi|733931404	PREDICTED: calreticulin [Meleagris gallopavo]	4.77E-03	4.71E-03	1.78E-03	1.57E-03	37.30	33.30
74	4	gi|971409716	PREDICTED: plastin-1 isoform X2 [Gallus gallus]	1.01E-03	9.33E-04	6.97E-04	5.15E-04	68.77	55.23
75	6	gi|733897036	PREDICTED: cytochrome P450 2H2 [Meleagris gallopavo]	3.74E-03	4.76E-03	7.33E-04	1.70E-03	19.60	35.68
76	9	gi|733920834	PREDICTED: apolipoprotein A-I [Meleagris gallopavo]	5.81E-03	5.47E-03	1.77E-03	1.18E-03	30.51	21.66
77	10	gi|734703908	glutathione S-transferase [Meleagris gallopavo]	2.71E-03	3.50E-03	5.92E-04	1.44E-03	21.87	41.05
78	9	gi|733900438	PREDICTED: 4-trimethylaminobutyraldehyde dehydrogenase [Meleagris gallopavo]	3.27E-03	3.59E-03	1.09E-03	4.49E-04	33.20	12.52
79	1	gi|733869642	PREDICTED: histone H2A-IV [Meleagris gallopavo]	1.42E-04	1.42E-04	1.46E-04	1.74E-04	102.91	122.70
80	9	gi|971401669	PREDICTED: voltage-dependent anion-selective channel protein 2 isoform X1 [Gallus gallus]	5.05E-03	4.85E-03	1.14E-03	9.68E-04	22.68	19.97
81	1	gi|733893841	PREDICTED: UDP-glucuronosyltransferase 1-1-like isoform X2 [Meleagris gallopavo]	9.94E-04	8.38E-04	1.86E-04	2.07E-04	18.67	24.67
82	7	gi|733926877	PREDICTED: prostaglandin reductase 1 [Meleagris gallopavo]	3.03E-03	3.62E-03	8.26E-04	1.12E-03	27.28	30.92

83	4	gi|733897873	PREDICTED: long-chain-fatty-acid--CoA ligase 5 [Meleagris gallopavo]	2.94E-03	3.41E-03	5.36E-04	9.53E-04	18.24	27.94
84	9	gi|733901608	PREDICTED: peroxiredoxin-1 [Meleagris gallopavo]	6.93E-03	7.49E-03	8.24E-04	9.22E-04	11.88	12.32
85	2	gi|733880423	PREDICTED: trifunctional enzyme subunit beta, mitochondrial isoform X1 [Meleagris gallopavo]	1.45E-03	1.40E-03	7.02E-04	4.68E-04	48.36	33.44
86	4	gi|971425780	PREDICTED: 14-3-3 protein epsilon isoform X1 [Gallus gallus]	7.47E-04	9.59E-04	2.86E-04	1.65E-04	38.27	17.25
87	9	gi|944095982	malate dehydrogenase, peroxisomal isoform MDH1x [Gallus gallus]	6.92E-03	7.69E-03	1.26E-03	1.57E-03	18.18	20.36
88	5	gi|971436997	PREDICTED: citrate synthase, mitochondrial isoform X3 [Gallus gallus]	2.10E-03	1.79E-03	3.53E-04	4.87E-04	16.80	27.19
89	3	gi|971410607	PREDICTED: eukaryotic initiation factor 4A-II isoform X1 [Gallus gallus]	1.49E-03	1.28E-03	4.30E-05	4.65E-04	2.89	36.40
90	6	gi|971437846	PREDICTED: ATP synthase subunit alpha, mitochondrial [Gallus gallus]	1.54E-03	4.04E-03	7.71E-04	2.50E-03	50.08	61.76
91	3	gi|733883695	PREDICTED: annexin A13 isoform X1 [Meleagris gallopavo]	8.36E-04	1.14E-03	4.96E-04	5.61E-04	59.29	49.37
92	11	gi|733887177	PREDICTED: fatty acid-binding protein, liver [Meleagris gallopavo]	3.23E-02	3.62E-02	1.82E-02	2.18E-02	56.24	60.14
93	8	gi|326924446	PREDICTED: ES1 protein homolog, mitochondrial-like [Meleagris gallopavo]	4.85E-03	1.12E-02	3.49E-03	4.88E-03	71.86	43.68
94	3	gi|733922734	PREDICTED: LOW QUALITY PROTEIN: angiotensin-converting enzyme [Meleagris gallopavo]	1.55E-03	8.67E-04	3.69E-04	1.60E-04	23.79	18.47
95	4	gi|733895562	PREDICTED: glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic-like [Meleagris gallopavo]	1.73E-03	2.55E-03	8.39E-04	1.25E-03	48.35	48.94
96	4	gi|733905525	PREDICTED: isocitrate dehydrogenase [NADP], mitochondrial [Meleagris gallopavo]	1.40E-03	1.46E-03	4.73E-04	9.55E-05	33.87	6.55
97	5	gi|733922929	PREDICTED: prohibitin [Meleagris gallopavo]	1.74E-03	2.24E-03	9.06E-04	5.91E-04	52.13	26.44
98	6	gi|733870808	PREDICTED: aldose reductase-like [Meleagris gallopavo]	1.18E-03	1.23E-03	3.47E-04	6.84E-04	29.37	55.59
99	8	gi|733903200	PREDICTED: intestinal-type alkaline phosphatase-like [Meleagris gallopavo]	4.84E-03	4.55E-03	2.09E-03	1.79E-03	43.21	39.42
100	2	gi|733915767	PREDICTED: NADPH--cytochrome P450 reductase isoform X2 [Meleagris gallopavo]	2.28E-04	2.43E-04	1.08E-04	5.33E-05	47.53	21.97
101	6	gi|733930993	PREDICTED: LOW QUALITY PROTEIN: keratin, type I cytoskeletal 18 [Meleagris gallopavo]	2.35E-03	2.61E-03	8.15E-04	5.85E-04	34.75	22.44
102	6	gi|734703860	malic enzyme 1, NADP(+)-dependent, cytosolic [Meleagris gallopavo]	1.67E-03	1.41E-03	3.51E-04	5.82E-04	20.98	41.28
103	4	gi|971430201	PREDICTED: 2-oxoglutarate dehydrogenase, mitochondrial isoform X3 [Gallus gallus]	1.52E-03	1.70E-03	3.45E-04	1.15E-04	22.65	6.76
104	5	gi|733900955	PREDICTED: epithelial chloride channel protein-like [Meleagris gallopavo]	2.80E-03	2.81E-03	5.94E-04	1.55E-03	21.20	55.23
105	1	gi|733878596	PREDICTED: ezrin [Meleagris gallopavo]	1.20E-03	1.22E-03	1.21E-03	6.95E-04	100.75	57.19
106	1	gi|733880307	PREDICTED: apolipoprotein B-100 [Meleagris gallopavo]	2.30E-04	2.14E-04	1.12E-04	4.46E-05	48.48	20.87
107	3	gi|733890845	PREDICTED: cytoplasmic dynein 1 heavy chain 1 [Meleagris gallopavo]	8.90E-04	8.23E-04	1.74E-04	1.74E-04	19.56	21.15
108	8	gi|733916541	PREDICTED: LOW QUALITY PROTEIN: fatty aldehyde dehydrogenase [Meleagris gallopavo]	3.51E-03	2.73E-03	8.78E-04	4.45E-04	25.04	16.27
109	2	gi|971410902	PREDICTED: uncharacterized protein FCGBP [Gallus gallus]	5.33E-04	4.64E-04	1.72E-04	1.52E-04	32.21	32.72

110	7	gi|261490820	peptidyl-prolyl cis-trans isomerase A [Gallus gallus]	8.05E-03	1.07E-02	2.16E-03	1.54E-03	26.81	14.32
111	3	gi|733918993	PREDICTED: aflatoxin B1 aldehyde reductase member 2-like [Meleagris gallopavo]	1.49E-03	1.80E-03	6.76E-04	1.10E-03	45.37	61.04
112	2	gi|733870229	PREDICTED: thiosulfate sulfurtransferase [Meleagris gallopavo]	7.66E-04	1.12E-03	4.35E-04	2.63E-04	56.70	23.50
113	11	gi|733881646	PREDICTED: carboxymethylenebutenolidase homolog [Meleagris gallopavo]	8.03E-03	6.68E-03	3.19E-03	1.67E-03	39.75	25.04
114	4	gi|733897806	PREDICTED: aspartate aminotransferase, cytoplasmic, partial [Meleagris gallopavo]	9.18E-04	1.12E-03	7.38E-05	5.92E-05	8.03	5.27
115	4	gi|733881362	PREDICTED: enoyl-CoA delta isomerase 2, mitochondrial isoform X1 [Meleagris gallopavo]	8.63E-04	1.26E-03	2.02E-04	1.03E-04	23.43	8.20
116	3	gi|733903182	PREDICTED: LOW QUALITY PROTEIN: nucleolin [Meleagris gallopavo]	5.46E-04	9.15E-04	1.17E-04	4.46E-04	21.45	48.71
117	1	gi|733902954	PREDICTED: peroxisomal bifunctional enzyme, partial [Meleagris gallopavo]	1.62E-04	2.69E-04	9.88E-05	7.24E-05	60.93	26.88
118	8	gi|733902290	PREDICTED: retinol-binding protein 2 [Meleagris gallopavo]	2.78E-02	2.36E-02	4.25E-03	7.27E-03	15.27	30.88
119	5	gi|733919450	PREDICTED: annexin A4 isoform X2 [Meleagris gallopavo]	2.75E-03	2.48E-03	8.84E-04	5.98E-04	32.14	24.14
120	1	gi|326919053	PREDICTED: alcohol dehydrogenase 1 [Meleagris gallopavo]	7.80E-05	1.91E-04	4.59E-05	1.73E-04	58.91	90.68
121	5	gi|733907614	PREDICTED: transketolase [Meleagris gallopavo]	1.41E-03	1.17E-03	3.81E-04	3.52E-04	26.96	30.06
122	1	gi|733925391	PREDICTED: ras GTPase-activating-like protein IQGAP2 isoform X2 [Meleagris gallopavo]	2.06E-04	3.42E-04	6.97E-05	2.37E-04	33.90	69.14
123	2	gi|326920316	PREDICTED: catalase [Meleagris gallopavo]	4.79E-04	4.86E-04	1.03E-04	6.66E-05	21.50	13.70
124	4	gi|733919112	PREDICTED: glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1 isoform X4 [Meleagris gallopavo]	7.51E-04	7.41E-04	1.95E-04	2.19E-05	25.89	2.95
125	2	gi|54020693	ADP/ATP translocase 3 [Gallus gallus]	1.71E-03	3.65E-03	1.14E-03	2.91E-03	66.66	79.61
126	4	gi|733887615	PREDICTED: bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing) [Meleagris gallopavo]	5.59E-04	9.02E-04	3.12E-04	1.84E-04	55.81	20.44
127	5	gi|326913992	PREDICTED: hemoglobin subunit beta [Meleagris gallopavo]	8.52E-03	3.32E-03	1.31E-02	4.91E-03	154.06	148.13
128	5	gi|733905676	PREDICTED: fatty acyl-CoA hydrolase precursor, medium chain-like [Meleagris gallopavo]	8.04E-03	8.87E-03	1.33E-03	5.54E-04	16.56	6.25
129	2	gi|326914482	PREDICTED: ester hydrolase C11orf54 homolog [Meleagris gallopavo]	4.42E-04	5.61E-04	2.43E-04	8.87E-05	54.99	15.82
130	2	gi|733874768	PREDICTED: plastin-2 [Meleagris gallopavo]	6.52E-04	6.23E-04	2.95E-04	1.64E-04	45.25	26.33
131	7	gi|733868906	PREDICTED: soluble scavenger receptor cysteine-rich domain-containing protein SSC5D-like [Meleagris gallopavo]	1.90E-03	4.22E-03	1.29E-03	3.02E-03	68.06	71.47
132	2	gi|733909243	PREDICTED: LOW QUALITY PROTEIN: calnexin [Meleagris gallopavo]	4.59E-04	6.76E-04	3.12E-04	3.14E-04	67.84	46.49
133	1	gi|356640676	glutathione S-transferase alpha class A1.3 [Meleagris gallopavo]	1.90E-04	2.39E-04	1.53E-04	9.71E-05	80.41	40.57
134	4	gi|733905685	PREDICTED: glucose-6-phosphate isomerase, partial [Meleagris gallopavo]	2.24E-03	2.16E-03	3.43E-04	3.17E-04	15.29	14.70
135	5	gi|733880463	PREDICTED: bifunctional epoxide hydrolase 2 [Meleagris gallopavo]	1.45E-03	1.20E-03	5.94E-04	2.74E-04	40.98	22.94
136	5	gi|45382953	aspartate aminotransferase, mitochondrial precursor [Gallus gallus]	1.41E-03	1.66E-03	4.09E-04	7.80E-04	28.92	47.10

137	2	gi|733885911	PREDICTED: uncharacterized protein LOC100549463 [Meleagris gallopavo]	3.20E-04	8.26E-04	1.44E-04	6.23E-04	44.96	75.35
138	3	gi|971437028	PREDICTED: myosin light polypeptide 6 isoform X2 [Gallus gallus]	1.26E-03	1.28E-03	4.32E-04	3.35E-04	34.43	26.15
139	4	gi|733873018	PREDICTED: amine oxidase [flavin-containing] B-like [Meleagris gallopavo]	6.73E-04	1.13E-03	2.33E-04	3.88E-04	34.53	34.24
140	1	gi|57529350	14-3-3 protein beta/alpha [Gallus gallus]	3.80E-05	6.25E-05	1.95E-05	4.41E-05	51.39	70.53
141	1	gi|326913821	PREDICTED: sulfotransferase 1C1-like [Meleagris gallopavo]	1.81E-04	7.04E-04	1.68E-04	5.01E-04	92.54	71.16
142	4	gi|733887752	PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial [Meleagris gallopavo]	1.32E-03	1.41E-03	4.31E-04	6.04E-05	32.66	4.28
143	7	gi|733921406	PREDICTED: transgelin-2 [Meleagris gallopavo]	2.85E-03	2.56E-03	9.41E-04	6.74E-04	32.97	26.32
144	2	gi|733896042	PREDICTED: actin-related protein 3 [Meleagris gallopavo]	7.37E-04	1.03E-03	4.17E-04	1.77E-04	56.62	17.15
145	2	gi|733905496	PREDICTED: 60S ribosomal protein L4 [Meleagris gallopavo]	4.51E-04	5.53E-04	3.76E-04	2.31E-04	83.45	41.77
146	4	gi|733907349	PREDICTED: aminoacylase-1 [Meleagris gallopavo]	1.44E-03	1.57E-03	6.33E-04	1.89E-04	43.91	12.00
147	2	gi|733897690	PREDICTED: xaa-Pro aminopeptidase 1 [Meleagris gallopavo]	3.17E-04	4.36E-04	1.18E-04	2.22E-04	37.14	50.87
148	4	gi|363744876	PREDICTED: hydroxysteroid dehydrogenase-like protein 2 [Gallus gallus]	8.04E-04	1.21E-03	4.18E-04	6.64E-04	51.97	55.06
149	3	gi|733901397	PREDICTED: non-specific lipid-transfer protein [Meleagris gallopavo]	5.81E-04	8.88E-04	1.37E-04	3.62E-04	23.56	40.81
150	7	gi|71895985	phosphoglycerate mutase 1 [Gallus gallus]	2.16E-03	2.05E-03	3.37E-04	4.26E-04	15.60	20.75
151	1	gi|733904379	PREDICTED: sorbitol dehydrogenase [Meleagris gallopavo]	6.61E-05	9.47E-05	6.81E-05	6.19E-05	103.04	65.36
152	2	gi|733894061	PREDICTED: putative methyltransferase DDB_G0268948 [Meleagris gallopavo]	1.08E-03	8.83E-04	4.70E-04	2.73E-04	43.67	30.91
153	3	gi|326933776	PREDICTED: corticosteroid 11-beta-dehydrogenase isozyme 1-like [Meleagris gallopavo]	9.37E-04	1.30E-03	2.99E-04	6.51E-04	31.95	49.95
154	1	gi|733891463	PREDICTED: ADP-ribosylation factor 1 [Meleagris gallopavo]	2.36E-04	2.87E-04	7.64E-05	2.56E-04	32.33	89.26
155	1	gi|971411979	PREDICTED: tropomyosin alpha-1 chain isoform X16 [Gallus gallus]	4.25E-04	3.10E-04	2.67E-05	5.98E-05	6.30	19.33
156	3	gi|734703936	transferrin precursor [Meleagris gallopavo]	1.23E-03	1.41E-03	9.07E-04	1.19E-03	73.66	84.72
157	2	gi|733900704	PREDICTED: ATP-binding cassette sub-family D member 3-like [Meleagris gallopavo]	6.59E-04	4.97E-04	2.02E-04	1.79E-04	30.58	36.08
158	3	gi|733900852	PREDICTED: glutamine synthetase [Meleagris gallopavo]	5.67E-04	6.37E-04	2.17E-04	2.99E-04	38.19	46.92
159	4	gi|971440071	PREDICTED: peroxisomal multifunctional enzyme type 2 isoform X1 [Gallus gallus]	7.68E-04	1.24E-03	2.44E-04	3.13E-04	31.72	25.19
160	4	gi|733906777	PREDICTED: NAD(P)H dehydrogenase [quinone] 1 [Meleagris gallopavo]	1.41E-03	1.68E-03	1.02E-03	5.32E-04	71.76	31.67
161	1	gi|733880917	PREDICTED: protein disulfide-isomerase A4 isoform X2 [Meleagris gallopavo]	1.53E-04	2.28E-04	1.03E-04	5.67E-05	67.22	24.86
162	4	gi|45384366	calmodulin [Gallus gallus]	3.72E-03	4.60E-03	6.03E-04	4.47E-04	16.20	9.72
163	5	gi|326929282	PREDICTED: hemoglobin subunit alpha-A [Meleagris gallopavo]	3.63E-03	3.08E-03	1.81E-03	1.78E-03	49.86	57.60
164	1	gi|71897175	coatomer subunit alpha [Gallus gallus]	1.75E-04	3.83E-04	1.08E-04	1.52E-04	61.72	39.75

165	5	gi|734703894	ras homolog family member A [Meleagris gallopavo]	2.38E-03	2.27E-03	1.22E-03	9.70E-04	51.22	42.83
166	1	gi|733881908	PREDICTED: succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial [Meleagris gallopavo]	1.60E-04	3.01E-04	2.06E-04	4.96E-05	128.75	16.48
167	2	gi|733898401	PREDICTED: plastin-3 [Meleagris gallopavo]	4.26E-04	4.85E-04	2.50E-04	1.41E-04	58.77	29.10
168	1	gi|733926622	PREDICTED: endoplasmic reticulum aminopeptidase 1 [Meleagris gallopavo]	7.11E-05	7.00E-05	6.71E-05	3.66E-05	94.32	52.26
169	3	gi|326922143	PREDICTED: 40S ribosomal protein SA [Meleagris gallopavo]	6.94E-04	6.94E-04	2.33E-04	2.49E-04	33.56	35.86
170	4	gi|733923433	PREDICTED: lamin-B2, partial [Meleagris gallopavo]	9.47E-04	1.07E-03	8.45E-05	2.78E-04	8.92	26.12
171	4	gi|971439426	PREDICTED: heterogeneous nuclear ribonucleoprotein K isoform X3 [Gallus gallus]	5.88E-04	8.06E-04	2.58E-04	3.77E-04	43.90	46.73
172	7	gi|733904262	PREDICTED: cytochrome c oxidase subunit 5A, mitochondrial [Meleagris gallopavo]	3.07E-03	3.65E-03	4.10E-04	6.97E-04	13.33	19.09
173	1	gi|733927467	PREDICTED: fatty acyl-CoA hydrolase precursor, medium chain-like [Meleagris gallopavo]	1.05E-03	5.97E-04	7.30E-04	3.91E-04	69.63	65.53
174	1	gi|733891027	PREDICTED: kinectin isoform X5 [Meleagris gallopavo]	2.65E-04	2.38E-04	9.89E-05	1.24E-04	37.38	52.23
175	2	gi|52138659	receptor of activated protein C kinase 1 [Gallus gallus]	2.56E-04	2.86E-04	6.24E-05	2.24E-04	24.34	78.34
176	2	gi|733911696	PREDICTED: sarcoplasmic/endoplasmic reticulum calcium ATPase 2 [Meleagris gallopavo]	4.90E-04	4.04E-04	8.66E-05	1.14E-04	17.65	28.24
177	10	gi|326911881	PREDICTED: galectin-2 [Meleagris gallopavo]	8.31E-03	7.89E-03	8.39E-04	1.06E-03	10.10	13.50
178	2	gi|733912488	PREDICTED: 60S acidic ribosomal protein P0, partial [Meleagris gallopavo]	3.85E-04	5.43E-04	2.01E-04	3.73E-04	52.25	68.70
179	2	gi|971390215	PREDICTED: 14-3-3 protein theta isoform X1 [Gallus gallus]	2.97E-04	2.98E-04	7.67E-05	8.08E-05	25.85	27.10
180	3	gi|733876597	PREDICTED: calpain-13 isoform X2 [Meleagris gallopavo]	6.82E-04	7.16E-04	2.39E-04	4.20E-05	35.10	5.86
181	2	gi|971370598	PREDICTED: histone H2B 1/2/3/4/6-like [Gallus gallus]	1.03E-03	3.02E-03	5.23E-04	1.48E-03	50.87	48.93
182	3	gi|971428595	PREDICTED: adenosylhomocysteinase [Gallus gallus]	1.18E-03	1.27E-03	9.35E-05	2.77E-04	7.89	21.71
183	3	gi|971383602	PREDICTED: myosin regulatory light chain 2, smooth muscle minor isoform isoform X2 [Gallus gallus]	5.81E-04	6.73E-04	3.66E-04	2.23E-04	62.95	33.06
184	5	gi|733926880	PREDICTED: LOW QUALITY PROTEIN: thioredoxin [Meleagris gallopavo]	2.13E-03	1.98E-03	2.89E-04	4.10E-04	13.57	20.66
185	3	gi|733913470	PREDICTED: carnitine O-acetyltransferase [Meleagris gallopavo]	6.76E-04	5.18E-04	2.87E-04	1.05E-04	42.41	20.30
186	2	gi|733917232	PREDICTED: dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 [Meleagris gallopavo]	6.46E-04	4.40E-04	2.30E-04	1.02E-04	35.59	23.19
187	1	gi|733869112	PREDICTED: T-complex protein 1 subunit beta [Meleagris gallopavo]	2.08E-04	1.93E-04	1.18E-04	1.21E-04	56.71	62.55
188	6	gi|733912151	PREDICTED: immunoglobulin lambda-like polypeptide 1 isoform X2 [Meleagris gallopavo]	5.16E-03	7.29E-03	2.10E-03	2.48E-03	40.68	33.93
189	4	gi|733888470	PREDICTED: cathepsin D, partial [Meleagris gallopavo]	1.35E-03	1.25E-03	1.74E-04	2.00E-04	12.89	16.00
190	3	gi|347360989	adseverin [Gallus gallus]	1.05E-03	8.33E-04	3.17E-04	3.81E-04	30.26	45.72
191	3	gi|326918682	PREDICTED: estradiol 17-beta-dehydrogenase 11-like [Meleagris gallopavo]	1.36E-03	2.26E-03	3.66E-04	7.19E-04	26.89	31.90
192	1	gi|971396970	PREDICTED: coatomer subunit beta isoform X1 [Gallus gallus]	5.14E-04	3.76E-04	2.14E-04	3.74E-04	41.65	99.28

193	3	gi|363743079	PREDICTED: ATP synthase F(0) complex subunit B1, mitochondrial [Gallus gallus]	1.95E-04	2.73E-04	1.45E-04	1.83E-04	74.50	67.01
194	4	gi|733909233	PREDICTED: heterogeneous nuclear ribonucleoprotein H isoform X5 [Meleagris gallopavo]	1.15E-03	1.30E-03	1.99E-04	4.21E-04	17.31	32.42
195	1	gi|733887582	PREDICTED: bile acid-CoA:amino acid N-acyltransferase isoform X2 [Meleagris gallopavo]	1.75E-04	2.11E-04	9.37E-05	1.16E-04	53.55	54.91
196	3	gi|326929269	PREDICTED: hemoglobin subunit alpha-D [Meleagris gallopavo]	8.32E-04	1.21E-03	2.26E-04	1.11E-03	27.22	91.84
197	1	gi|733890024	PREDICTED: dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial, partial [Meleagris gallopavo]	5.13E-04	6.41E-04	1.58E-04	6.22E-05	30.89	9.70
198	4	gi|733909027	PREDICTED: nucleophosmin [Meleagris gallopavo]	2.14E-03	1.90E-03	7.14E-04	3.99E-04	33.40	20.94
199	3	gi|733904664	PREDICTED: electron transfer flavoprotein subunit alpha, mitochondrial [Meleagris gallopavo]	7.26E-04	8.62E-04	8.01E-04	5.97E-04	110.44	69.32
200	1	gi|733930975	PREDICTED: major vault protein [Meleagris gallopavo]	1.43E-04	1.30E-04	3.12E-05	5.86E-05	21.81	45.13
201	3	gi|733918286	PREDICTED: 6-phosphogluconate dehydrogenase, decarboxylating [Meleagris gallopavo]	9.67E-04	9.75E-04	1.01E-04	4.35E-04	10.49	44.59
202	1	gi|733905614	PREDICTED: ras GTPase-activating-like protein IQGAP1, partial [Meleagris gallopavo]	1.91E-04	1.37E-04	5.99E-05	7.50E-05	31.42	54.67
203	4	gi|733894129	PREDICTED: long-chain specific acyl-CoA dehydrogenase, mitochondrial, partial [Meleagris gallopavo]	1.37E-03	1.38E-03	3.18E-04	4.02E-04	23.30	29.17
204	3	gi|971425096	PREDICTED: rho GDP-dissociation inhibitor 1 isoform X1 [Gallus gallus]	1.67E-03	1.07E-03	4.29E-04	3.07E-04	25.67	28.69
205	4	gi|733872716	PREDICTED: superoxide dismutase [Cu-Zn], partial [Meleagris gallopavo]	6.87E-03	6.22E-03	2.21E-03	1.65E-03	32.13	26.54
206	4	gi|733906457	PREDICTED: uncharacterized protein LOC100540290 [Meleagris gallopavo]	5.34E-04	1.00E-03	2.60E-04	5.89E-04	48.67	58.82
207	2	gi|971380110	PREDICTED: heterogeneous nuclear ribonucleoproteins A2/B1 isoform X5 [Gallus gallus]	6.17E-04	7.78E-04	1.69E-04	1.73E-04	27.34	22.28
208	1	gi|45384344	hematopoietic prostaglandin D synthase [Gallus gallus]	2.05E-04	2.35E-04	1.23E-04	1.67E-04	59.89	70.98
209	3	gi|733899364	PREDICTED: phosphoglycerate kinase 1 [Meleagris gallopavo]	4.45E-04	6.93E-04	2.69E-04	4.00E-04	60.46	57.77
210	1	gi|733894813	PREDICTED: heterogeneous nuclear ribonucleoprotein A3 isoform X7 [Meleagris gallopavo]	1.68E-04	1.91E-04	4.44E-05	3.86E-05	26.40	20.19
211	2	gi|733902933	PREDICTED: ATP-dependent 6-phosphofructokinase, liver type, partial [Meleagris gallopavo]	9.49E-04	7.20E-04	1.48E-04	5.83E-04	15.64	81.01
212	3	gi|52138693	WD repeat-containing protein 1 [Gallus gallus]	8.16E-04	6.63E-04	1.03E-04	1.23E-04	12.66	18.63
213	4	gi|733928317	PREDICTED: phosphoenolpyruvate carboxykinase [GTP], mitochondrial-like [Meleagris gallopavo]	3.97E-03	3.32E-03	3.19E-04	5.66E-04	8.04	17.02
214	2	gi|971373773	PREDICTED: prohibitin-2 isoform X1 [Gallus gallus]	7.65E-04	9.39E-04	4.03E-04	3.01E-04	52.59	32.07
215	8	gi|326917357	PREDICTED: cytochrome b5 [Meleagris gallopavo]	1.03E-02	7.99E-03	3.48E-03	1.33E-03	33.63	16.62
216	1	gi|733904382	PREDICTED: peptidyl-prolyl cis-trans isomerase B, partial [Meleagris gallopavo]	5.98E-04	5.71E-04	9.13E-05	3.32E-04	15.26	58.09
217	3	gi|733870176	PREDICTED: leukotriene A-4 hydrolase [Meleagris gallopavo]	9.11E-04	1.01E-03	4.24E-04	2.70E-04	46.55	26.78
218	1	gi|733868114	PREDICTED: ATP synthase subunit gamma, mitochondrial isoform X3 [Meleagris gallopavo]	2.36E-04	1.88E-04	1.04E-04	2.50E-04	43.95	133.22
219	2	gi|733888723	PREDICTED: catenin delta-1 isoform X4 [Meleagris gallopavo]	1.53E-04	3.15E-04	4.73E-05	1.50E-04	30.82	47.44

220	1	gi|971426102	PREDICTED: complement component 1 Q subcomponent-binding protein, mitochondrial [Gallus gallus]	1.98E-04	1.56E-04	9.51E-05	1.02E-04	48.06	65.46
221	5	gi|71896025	UMP-CMP kinase [Gallus gallus]	1.38E-03	1.28E-03	3.31E-04	1.04E-04	23.92	8.11
222	4	gi|733873020	PREDICTED: amine oxidase [flavin-containing] A-like [Meleagris gallopavo]	2.46E-03	2.51E-03	2.46E-04	8.86E-04	9.97	35.27
223	1	gi|733907491	PREDICTED: 2'-5'-oligoadenylate synthase-like protein [Meleagris gallopavo]	1.42E-04	1.95E-04	8.39E-05	7.28E-05	59.14	37.26
224	1	gi|119331076	xaa-Pro dipeptidase [Gallus gallus]	2.71E-04	2.23E-04	6.34E-05	2.01E-05	23.45	9.04
225	3	gi|733882785	PREDICTED: epidermal retinol dehydrogenase 2-like isoform X2 [Meleagris gallopavo]	1.11E-03	1.30E-03	3.67E-04	5.80E-04	33.23	44.61
226	2	gi|326928043	PREDICTED: peroxisomal acyl-coenzyme A oxidase 2 [Meleagris gallopavo]	6.44E-04	5.43E-04	1.14E-04	1.45E-04	17.61	26.76
227	2	gi|733911996	PREDICTED: short-chain specific acyl-CoA dehydrogenase, mitochondrial [Meleagris gallopavo]	4.04E-04	5.62E-04	7.68E-05	1.55E-04	19.00	27.62
228	1	gi|733901867	PREDICTED: carnitine O-palmitoyltransferase 2, mitochondrial, partial [Meleagris gallopavo]	5.81E-04	6.07E-04	2.26E-04	1.15E-04	38.89	18.95
229	1	gi|323690835	cytochrome c oxidase subunit II (mitochondrion) [Meleagris gallopavo]	2.70E-04	4.26E-04	1.69E-04	2.30E-04	62.74	54.03
230	1	gi|733883413	PREDICTED: polyadenylate-binding protein 1 [Meleagris gallopavo]	8.54E-05	1.29E-04	8.40E-05	1.23E-04	98.39	95.43
231	6	gi|733912388	PREDICTED: SEC14-like protein 2 [Meleagris gallopavo]	2.14E-03	2.10E-03	2.86E-04	3.33E-04	13.39	15.86
232	3	gi|971435124	PREDICTED: puromycin-sensitive aminopeptidase [Gallus gallus]	8.37E-04	7.56E-04	1.08E-04	1.44E-04	12.93	19.06
233	2	gi|326923501	PREDICTED: inorganic pyrophosphatase [Meleagris gallopavo]	3.43E-04	4.13E-04	7.04E-05	7.07E-05	20.52	17.13
234	3	gi|733931977	PREDICTED: medium-chain specific acyl-CoA dehydrogenase, mitochondrial, partial [Meleagris gallopavo]	5.84E-04	1.17E-03	1.34E-04	8.72E-04	23.04	74.68
235	3	gi|733883379	PREDICTED: ribonuclease UK114 [Meleagris gallopavo]	7.10E-04	7.11E-04	3.77E-04	2.68E-04	53.03	37.65
236	1	gi|71897035	14-3-3 protein zeta [Gallus gallus]	8.79E-05	1.15E-04	6.97E-05	7.63E-05	79.19	66.35
237	3	gi|733871004	PREDICTED: L-lactate dehydrogenase B chain [Meleagris gallopavo]	9.67E-04	9.02E-04	3.56E-04	2.23E-04	36.82	24.69
238	4	gi|733880125	PREDICTED: protein disulfide-isomerase A6 [Meleagris gallopavo]	9.92E-04	1.40E-03	2.91E-04	3.89E-04	29.36	27.75
239	4	gi|733872231	PREDICTED: sodium/potassium-transporting ATPase subunit beta-1 [Meleagris gallopavo]	2.49E-03	2.77E-03	1.88E-04	5.98E-04	7.52	21.62
240	4	gi|733906854	PREDICTED: adenine phosphoribosyltransferase [Meleagris gallopavo]	2.20E-03	2.11E-03	1.88E-04	3.77E-04	8.51	17.92
241	1	gi|733912766	PREDICTED: serine dehydratase-like [Meleagris gallopavo]	3.24E-04	1.53E-04	5.96E-05	6.00E-05	18.40	39.28
242	1	gi|733908033	PREDICTED: dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1, partial [Meleagris gallopavo]	2.40E-04	2.75E-04	1.07E-04	3.33E-05	44.80	12.11
243	2	gi|733921638	PREDICTED: aminopeptidase B [Meleagris gallopavo]	5.11E-04	3.04E-04	1.69E-04	2.20E-04	33.06	72.25
244	3	gi|50800573	PREDICTED: 60S acidic ribosomal protein P2 [Gallus gallus]	3.30E-03	3.00E-03	6.73E-04	4.08E-04	20.41	13.59
245	1	gi|971397746	PREDICTED: mucin-2 [Gallus gallus]	4.51E-04	6.85E-04	1.35E-04	3.03E-04	29.96	44.21
246	1	gi|733918594	PREDICTED: dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit [Meleagris gallopavo]	1.45E-04	1.72E-04	5.68E-05	5.23E-05	39.09	30.34

247	2	gi|733884061	PREDICTED: fibrinogen beta chain [Meleagris gallopavo]	1.69E-04	2.97E-04	7.80E-05	7.00E-05	46.21	23.53
248	3	gi|733928247	PREDICTED: antimicrobial peptide NK-lysin-like, partial [Meleagris gallopavo]	3.12E-03	2.66E-03	7.29E-04	9.42E-04	23.35	35.37
249	3	gi|733885734	PREDICTED: alcohol dehydrogenase class-3 [Meleagris gallopavo]	1.21E-03	1.37E-03	2.59E-04	2.66E-04	21.37	19.39
250	2	gi|326912625	PREDICTED: peptidyl-prolyl cis-trans isomerase FKBP4 [Meleagris gallopavo]	6.15E-04	6.08E-04	1.71E-04	1.00E-04	27.76	16.47
251	4	gi|734703826	solute carrier family 15 (oligopeptide transporter), member 1 [Meleagris gallopavo]	1.25E-03	1.00E-03	2.27E-04	1.59E-04	18.12	15.88
252	2	gi|733879227	PREDICTED: prolyl endopeptidase isoform X2 [Meleagris gallopavo]	3.41E-04	3.82E-04	6.55E-05	7.87E-05	19.21	20.59
253	5	gi|733892638	PREDICTED: anterior gradient protein 2 homolog [Meleagris gallopavo]	4.83E-03	6.09E-03	2.43E-03	2.19E-03	50.19	35.93
254	4	gi|971418811	PREDICTED: hydroxyacylglutathione hydrolase, mitochondrial isoform X2 [Gallus gallus]	1.02E-03	9.92E-04	4.01E-04	3.08E-04	39.32	31.07
255	2	gi|733898577	PREDICTED: 40S ribosomal protein S4, X isoform [Meleagris gallopavo]	6.27E-04	7.58E-04	3.09E-04	2.59E-04	49.27	34.17
256	1	gi|733921537	PREDICTED: selenium-binding protein 1-A isoform X2 [Meleagris gallopavo]	1.15E-04	3.55E-04	1.02E-04	9.85E-05	89.20	27.77
257	1	gi|326919104	PREDICTED: cytochrome P450 4V2 [Meleagris gallopavo]	3.25E-04	2.27E-04	2.04E-04	2.90E-05	62.71	12.75
258	2	gi|733901649	PREDICTED: 40S ribosomal protein S8 [Meleagris gallopavo]	9.37E-04	1.17E-03	3.17E-04	4.43E-04	33.86	37.73
259	1	gi|733901896	PREDICTED: fatty-acid amide hydrolase 1 [Meleagris gallopavo]	6.78E-04	3.05E-04	7.33E-04	3.68E-04	108.17	120.82
260	2	gi|326927948	PREDICTED: coatomer subunit gamma-1 [Meleagris gallopavo]	5.42E-04	6.54E-04	8.81E-05	8.84E-05	16.27	13.51
261	1	gi|733875970	PREDICTED: 40S ribosomal protein S3 [Meleagris gallopavo]	3.86E-04	5.04E-04	2.27E-04	1.38E-04	58.71	27.40
262	2	gi|733922839	PREDICTED: GTPase IMAP family member 1-like [Meleagris gallopavo]	2.67E-04	2.84E-04	1.72E-04	1.66E-04	64.29	58.38
263	2	gi|768311628	lambda-crystallin homolog [Gallus gallus]	3.61E-04	4.74E-04	1.55E-04	1.65E-04	43.07	34.73
264	1	gi|733930562	PREDICTED: LOW QUALITY PROTEIN: retinol dehydrogenase 7-like [Meleagris gallopavo]	4.05E-04	4.33E-04	1.55E-04	1.43E-04	38.32	32.96
265	1	gi|971385840	PREDICTED: UTP--glucose-1-phosphate uridylyltransferase isoform X1 [Gallus gallus]	1.78E-04	2.20E-04	1.14E-04	1.34E-04	63.99	61.02
266	3	gi|733925057	PREDICTED: granzyme A-like [Meleagris gallopavo]	1.26E-03	1.23E-03	3.71E-04	5.74E-04	29.41	46.61
267	1	gi|971371700	PREDICTED: phosphate carrier protein, mitochondrial isoform X1 [Gallus gallus]	2.79E-04	2.51E-04	1.16E-04	7.87E-05	41.45	31.30
268	5	gi|971428647	PREDICTED: cell division control protein 42 homolog isoform X1 [Gallus gallus]	1.73E-03	2.11E-03	9.12E-04	5.68E-04	52.67	26.89
269	2	gi|733920301	PREDICTED: adenylyl cyclase-associated protein 1 [Meleagris gallopavo]	2.92E-04	3.75E-04	2.01E-04	8.93E-05	68.58	23.81
270	1	gi|733877058	PREDICTED: 3'(2'),5'-bisphosphate nucleotidase 1 [Meleagris gallopavo]	9.29E-04	9.76E-04	3.77E-04	2.24E-04	40.60	22.94
271	1	gi|733873176	PREDICTED: ATP-dependent RNA helicase DDX3X [Meleagris gallopavo]	6.10E-04	3.65E-04	8.55E-05	9.37E-05	14.02	25.68
272	1	gi|733889970	PREDICTED: ectonucleoside triphosphate diphosphohydrolase 5 isoform X2 [Meleagris gallopavo]	1.23E-04	1.96E-04	1.08E-04	1.27E-04	87.73	65.08
273	3	gi|478486799	60S ribosomal protein L12 [Gallus gallus]	6.61E-04	7.92E-04	3.78E-04	2.53E-04	57.15	31.94
274	2	gi|733885423	PREDICTED: UDP-glucuronosyltransferase 2C1-like isoform X1 [Meleagris gallopavo]	9.36E-04	8.89E-04	6.81E-04	4.90E-04	72.74	55.17
275	3	gi|733922773	PREDICTED: alpha-2-macroglobulin-like protein 1 [Meleagris gallopavo]	4.82E-04	7.62E-04	2.54E-04	5.32E-04	52.57	69.82

276	1	gi|733906933	PREDICTED: cytochrome c oxidase subunit 4 isoform 1, mitochondrial [Meleagris gallopavo]	4.63E-03	5.10E-03	5.87E-04	7.44E-04	12.69	14.59
277	2	gi|493794686	pyridoxal kinase isoform 2 [Gallus gallus]	9.96E-04	9.34E-04	9.10E-05	2.67E-04	9.13	28.60
278	3	gi|45383816	protein S100-A6 [Gallus gallus]	1.88E-03	1.98E-03	1.48E-03	1.41E-03	78.70	71.38
279	3	gi|733925869	PREDICTED: retinal dehydrogenase 1 [Meleagris gallopavo]	3.36E-03	3.00E-03	1.15E-03	9.99E-04	34.13	33.31
280	1	gi|733893111	PREDICTED: acyl-CoA dehydrogenase family member 11 [Meleagris gallopavo]	3.69E-04	1.41E-04	1.27E-04	1.48E-04	34.37	105.12
281	3	gi|762006008	60S ribosomal protein L11 [Gallus gallus]	7.34E-04	1.04E-03	3.92E-04	3.24E-04	53.48	31.22
282	3	gi|60592998	succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial [Gallus gallus]	2.69E-04	1.80E-04	9.05E-05	9.00E-05	33.59	50.15
283	3	gi|326914133	PREDICTED: S-formylglutathione hydrolase [Meleagris gallopavo]	7.27E-04	8.46E-04	2.28E-04	2.10E-04	31.32	24.77
284	3	gi|326918974	PREDICTED: fatty acid-binding protein, intestinal [Meleagris gallopavo]	3.89E-03	2.68E-03	1.29E-03	1.13E-03	33.29	42.04
285	1	gi|733877540	PREDICTED: regulator of microtubule dynamics protein 2 [Meleagris gallopavo]	1.56E-04	5.61E-05	8.51E-05	5.63E-05	54.61	100.41
286	2	gi|971437479	PREDICTED: tropomyosin beta chain isoform X7 [Gallus gallus]	7.06E-04	6.53E-04	1.37E-04	1.31E-04	19.45	20.04
287	5	gi|83745135	histone H4 [Gallus gallus]	2.30E-03	2.70E-03	1.53E-03	1.91E-03	66.48	70.82
288	4	gi|733898322	PREDICTED: thioredoxin-dependent peroxide reductase, mitochondrial, partial [Meleagris gallopavo]	2.64E-03	3.32E-03	7.13E-04	7.14E-04	26.96	21.50
289	2	gi|487442401	succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial isoform 2 precursor [Gallus gallus]	5.66E-04	1.01E-03	3.04E-04	4.01E-04	53.81	39.92
290	3	gi|326933399	PREDICTED: coatomer subunit delta [Meleagris gallopavo]	6.74E-04	5.89E-04	2.91E-04	1.39E-04	43.25	23.58
291	4	gi|733925055	PREDICTED: granzyme K-like [Meleagris gallopavo]	7.27E-04	9.80E-04	2.42E-04	4.08E-04	33.34	41.65
292	3	gi|733888683	PREDICTED: carnitine O-palmitoyltransferase 1, liver isoform [Meleagris gallopavo]	7.67E-04	9.98E-04	2.01E-04	2.20E-04	26.20	22.09
293	2	gi|733870043	PREDICTED: 3-mercaptopyruvate sulfurtransferase isoform X2 [Meleagris gallopavo]	3.74E-04	5.99E-04	3.13E-04	1.42E-04	83.57	23.66
294	1	gi|733923318	PREDICTED: synaptic vesicle membrane protein VAT-1 homolog [Meleagris gallopavo]	2.25E-04	2.49E-04	1.71E-04	1.01E-04	75.75	40.48
295	2	gi|733887622	PREDICTED: pepsin A [Meleagris gallopavo]	9.62E-04	4.31E-04	2.77E-04	4.32E-04	28.77	100.20
296	2	gi|733923173	PREDICTED: interferon-induced 35 kDa protein [Meleagris gallopavo]	2.52E-04	2.67E-04	7.99E-05	2.89E-05	31.67	10.82
297	1	gi|733888183	PREDICTED: proteasome subunit alpha type-1, partial [Meleagris gallopavo]	1.29E-04	1.19E-04	1.83E-05	4.86E-05	14.22	40.93
298	1	gi|733932295	PREDICTED: T-complex protein 1 subunit gamma [Meleagris gallopavo]	2.48E-04	2.92E-04	6.84E-05	1.10E-04	27.60	37.50
299	6	gi|733876725	PREDICTED: destrin [Meleagris gallopavo]	3.14E-03	3.21E-03	1.23E-03	3.21E-04	39.17	10.00
300	1	gi|733868350	PREDICTED: nicotinamide phosphoribosyltransferase [Meleagris gallopavo]	5.93E-05	7.29E-05	1.85E-05	2.64E-05	31.24	36.19
301	3	gi|971376395	PREDICTED: pseudouridine-5'-phosphatase [Gallus gallus]	6.06E-04	5.95E-04	1.25E-04	1.82E-04	20.56	30.62
302	2	gi|733893846	PREDICTED: UDP-glucuronosyltransferase 1-1-like isoform X4 [Meleagris gallopavo]	4.42E-04	6.69E-04	2.57E-04	9.67E-05	58.24	14.45
303	4	gi|461496478	40S ribosomal protein S2 [Gallus gallus]	4.15E-04	6.23E-04	1.75E-04	2.37E-04	42.01	38.02

304	2	gi|733906455	PREDICTED: uncharacterized oxidoreductase C663.09c-like [Meleagris gallopavo]	1.10E-03	8.62E-04	5.40E-04	4.41E-04	49.20	51.11
305	3	gi|733872652	PREDICTED: T-complex protein 1 subunit theta isoform X2 [Meleagris gallopavo]	2.62E-03	1.16E-03	1.52E-03	7.10E-04	57.73	61.25
306	2	gi|326920873	PREDICTED: maleylacetoacetate isomerase [Meleagris gallopavo]	5.13E-04	1.01E-03	1.39E-04	5.49E-04	26.98	54.29
307	1	gi|733893848	PREDICTED: UDP-glucuronosyltransferase 1-1-like isoform X5 [Meleagris gallopavo]	5.75E-04	6.45E-04	5.49E-05	9.59E-05	9.55	14.87
308	2	gi|326936041	PREDICTED: delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial, partial [Meleagris gallopavo]	5.69E-04	6.27E-04	3.84E-04	3.53E-04	67.49	56.34
309	1	gi|478621112	cytochrome b-c1 complex subunit 6, mitochondrial [Gallus gallus]	2.92E-04	3.14E-04	1.22E-04	2.08E-04	41.84	66.29
310	1	gi|733915364	PREDICTED: acyl-CoA synthetase family member 2, mitochondrial isoform X2 [Meleagris gallopavo]	9.77E-05	1.00E-04	2.98E-05	4.46E-05	30.48	44.46
311	3	gi|733906602	PREDICTED: arylamine N-acetyltransferase, pineal gland isozyme NAT-3 [Meleagris gallopavo]	8.25E-04	1.03E-03	6.50E-05	4.54E-04	7.87	43.88
312	2	gi|733921934	PREDICTED: LOW QUALITY PROTEIN: polymeric immunoglobulin receptor [Meleagris gallopavo]	3.54E-04	4.19E-04	1.61E-04	1.99E-04	45.54	47.37
313	4	gi|57530226	Na(+)/H(+) exchange regulatory cofactor NHE-RF1 [Gallus gallus]	9.99E-04	9.80E-04	3.85E-04	3.37E-04	38.57	34.39
314	1	gi|330417943	fructose-bisphosphate aldolase C [Gallus gallus]	2.24E-04	2.98E-05	2.37E-04	1.67E-05	105.69	56.02
315	1	gi|971435122	PREDICTED: importin subunit beta-1 [Gallus gallus]	7.16E-05	4.25E-05	1.64E-05	2.66E-05	22.94	62.62
316	2	gi|733876524	PREDICTED: epithelial cell adhesion molecule [Meleagris gallopavo]	1.48E-03	1.34E-03	3.79E-04	4.70E-04	25.55	35.10
317	1	gi|971428168	PREDICTED: 40S ribosomal protein S21 [Gallus gallus]	1.49E-04	1.56E-04	9.61E-05	5.24E-05	64.59	33.54
318	3	gi|326918338	PREDICTED: microsomal glutathione S-transferase 2 [Meleagris gallopavo]	1.19E-02	1.36E-02	2.79E-03	2.93E-03	23.40	21.48
319	4	gi|733894380	PREDICTED: 10 kDa heat shock protein, mitochondrial [Meleagris gallopavo]	5.60E-04	1.19E-03	1.49E-04	4.37E-04	26.67	36.58
320	2	gi|971378941	PREDICTED: hemopexin [Gallus gallus]	8.75E-04	2.21E-03	6.57E-04	9.81E-04	75.16	44.39
321	2	gi|733922477	PREDICTED: 60S ribosomal protein L10a [Meleagris gallopavo]	2.60E-04	4.14E-04	9.33E-05	2.43E-04	35.86	58.68
322	3	gi|733869052	PREDICTED: 60S ribosomal protein L18a [Meleagris gallopavo]	3.58E-04	4.49E-04	1.85E-04	2.06E-04	51.80	45.96
323	5	gi|733896659	PREDICTED: prosaposin [Meleagris gallopavo]	1.45E-03	1.96E-03	6.51E-04	4.12E-04	44.79	21.08
324	1	gi|733915436	PREDICTED: ATP synthase subunit d, mitochondrial [Meleagris gallopavo]	1.25E-04	1.68E-04	5.54E-05	1.19E-04	44.24	70.70
325	2	gi|733877367	PREDICTED: mitochondrial amidoxime reducing component 2-like [Meleagris gallopavo]	1.38E-04	4.45E-04	6.18E-05	3.06E-04	44.87	68.79
326	1	gi|166091440	serine/arginine-rich splicing factor 1 [Gallus gallus]	2.08E-04	3.60E-04	5.90E-05	5.35E-05	28.35	14.87
327	1	gi|733928350	PREDICTED: LOW QUALITY PROTEIN: isochorismatase domain-containing protein 1-like [Meleagris gallopavo]	2.35E-04	2.10E-04	6.75E-05	9.43E-05	28.75	44.80
328	3	gi|733900436	PREDICTED: microsomal glutathione S-transferase 3 isoform X3 [Meleagris gallopavo]	1.23E-03	7.03E-04	8.42E-04	2.68E-04	68.75	38.17
329	1	gi|971428635	PREDICTED: protein deglycase DJ-1 isoform X1 [Gallus gallus]	4.71E-04	4.61E-04	1.80E-04	1.24E-04	38.15	26.90
330	1	gi|733911356	PREDICTED: ubiquitin-conjugating enzyme E2 L3 [Meleagris gallopavo]	2.23E-04	3.34E-04	7.81E-05	4.55E-05	34.97	13.61

331	2	gi|733902994	PREDICTED: IgGFc-binding protein-like [Meleagris gallopavo]	8.11E-04	6.01E-04	1.57E-04	3.71E-04	19.36	61.66
332	1	gi|326923011	PREDICTED: acyl-CoA-binding protein [Meleagris gallopavo]	2.57E-04	1.18E-04	7.54E-05	1.35E-04	29.37	114.50
333	2	gi|733896591	PREDICTED: LOW QUALITY PROTEIN: phenazine biosynthesis-like domain-containing protein [Meleagris gallopavo]	6.25E-04	7.09E-04	2.75E-04	5.41E-05	44.03	7.63
334	2	gi|733878183	PREDICTED: acetyl-CoA acetyltransferase, cytosolic [Meleagris gallopavo]	2.79E-04	2.90E-04	1.53E-04	1.94E-04	55.00	67.01
335	1	gi|733896884	PREDICTED: protein transport protein Sec24C isoform X3 [Meleagris gallopavo]	7.82E-04	6.52E-04	1.28E-04	1.58E-04	16.31	24.22
336	1	gi|733882250	PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial [Meleagris gallopavo]	1.23E-03	1.12E-03	3.31E-04	3.63E-04	26.90	32.29
337	1	gi|326927860	PREDICTED: acyl-CoA dehydrogenase family member 9, mitochondrial [Meleagris gallopavo]	1.73E-04	1.24E-04	1.38E-04	1.03E-04	80.01	83.02
338	2	gi|733877776	PREDICTED: saccharopine dehydrogenase-like oxidoreductase, partial [Meleagris gallopavo]	2.44E-04	3.62E-04	4.65E-05	6.85E-05	19.02	18.90
339	1	gi|733922209	PREDICTED: putative adenosylhomocysteinase 2 [Meleagris gallopavo]	4.46E-05	8.86E-05	4.32E-05	8.41E-05	96.79	94.91
340	1	gi|733928504	PREDICTED: pyruvate carboxylase, mitochondrial-like, partial [Meleagris gallopavo]	1.17E-04	2.55E-04	4.71E-05	6.90E-05	40.38	27.08
341	1	gi|733898394	PREDICTED: glutaredoxin-3 [Meleagris gallopavo]	3.73E-05	9.15E-05	3.87E-05	4.68E-05	103.80	51.19
342	1	gi|733929989	PREDICTED: lamin-A-like, partial [Meleagris gallopavo]	2.54E-04	2.65E-04	4.73E-05	5.29E-05	18.58	19.94
343	1	gi|513167767	PREDICTED: kinesin-1 heavy chain isoform X2 [Gallus gallus]	1.28E-04	1.41E-04	8.72E-05	6.84E-05	68.41	48.39
344	2	gi|337730623	glutathione S-transferase mu 3 [Meleagris gallopavo]	3.76E-04	4.46E-04	1.37E-04	1.21E-04	36.50	27.09
345	1	gi|733871192	PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial [Meleagris gallopavo]	3.21E-05	1.37E-04	2.16E-05	1.17E-04	67.21	85.68
346	1	gi|482661640	coronin-1C isoform 1 [Gallus gallus]	1.39E-04	1.10E-04	2.81E-05	1.43E-05	20.25	12.93
347	1	gi|733927465	PREDICTED: fatty acyl-CoA hydrolase precursor, medium chain-like [Meleagris gallopavo]	1.52E-03	1.54E-03	5.35E-04	1.08E-03	35.15	70.10
348	1	gi|733902288	PREDICTED: coatomer subunit beta' [Meleagris gallopavo]	1.16E-04	1.36E-04	5.98E-05	2.25E-05	51.66	16.53
349	1	gi|57525400	60S ribosomal protein L3 [Gallus gallus]	1.20E-04	1.08E-04	4.70E-05	3.68E-05	39.33	33.95
350	3	gi|733884582	PREDICTED: glutathione reductase, mitochondrial [Meleagris gallopavo]	4.72E-04	3.41E-04	2.04E-04	1.29E-04	43.33	37.82
351	1	gi|971432776	PREDICTED: tropomyosin alpha-3 chain isoform X17 [Gallus gallus]	1.97E-04	1.38E-04	8.71E-05	8.84E-05	44.11	63.94
352	2	gi|971428665	PREDICTED: capping protein (actin filament) muscle Z-line, beta isoform X1 [Gallus gallus]	3.25E-04	4.31E-04	1.35E-04	9.03E-05	41.72	20.96
353	1	gi|733930991	PREDICTED: keratin, type II cytoskeletal 8 isoform X2 [Meleagris gallopavo]	6.85E-04	8.39E-04	1.13E-04	3.45E-04	16.45	41.12
354	1	gi|733908162	PREDICTED: succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial [Meleagris gallopavo]	1.01E-04	1.35E-04	6.57E-05	9.84E-05	65.07	72.65
355	2	gi|733925751	PREDICTED: 40S ribosomal protein S6 [Meleagris gallopavo]	5.35E-04	7.72E-04	1.32E-04	8.33E-05	24.72	10.79
356	2	gi|733868745	PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 [Meleagris gallopavo]	3.62E-04	3.21E-04	4.53E-05	4.66E-05	12.50	14.54

357	2	gi|326919083	PREDICTED: 3-hydroxybutyrate dehydrogenase type 2 [Meleagris gallopavo]	8.86E-04	4.13E-04	1.24E-04	2.58E-04	14.04	62.56
358	1	gi|971437108	PREDICTED: poly(rC)-binding protein 2 isoform X16 [Gallus gallus]	3.71E-04	3.99E-04	2.45E-04	2.67E-04	66.10	66.87
359	2	gi|971433158	PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial [Gallus gallus]	4.25E-04	3.80E-04	1.35E-04	7.98E-05	31.77	21.00
360	1	gi|733887735	PREDICTED: mitochondrial carrier homolog 2 isoform X2 [Meleagris gallopavo]	6.96E-05	1.56E-04	6.30E-05	2.92E-05	90.49	18.73
361	1	gi|971371289	PREDICTED: cullin-associated NEDD8-dissociated protein 1 [Gallus gallus]	4.42E-05	6.66E-05	6.67E-05	4.51E-05	150.80	67.67
362	2	gi|733887163	PREDICTED: succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial, partial [Meleagris gallopavo]	4.05E-04	3.47E-04	2.77E-05	1.28E-04	6.83	36.98
363	1	gi|733880345	PREDICTED: quinone oxidoreductase PIG3 isoform X2 [Meleagris gallopavo]	5.37E-04	6.14E-04	9.90E-05	1.53E-04	18.42	24.95
364	1	gi|733916532	PREDICTED: glyoxalase domain-containing protein 4 [Meleagris gallopavo]	1.09E-04	1.44E-04	1.16E-04	5.26E-05	106.55	36.44
365	1	gi|733909859	PREDICTED: enoyl-CoA delta isomerase 1, mitochondrial [Meleagris gallopavo]	2.49E-04	2.79E-04	3.15E-05	1.67E-04	12.66	59.78
366	1	gi|733872402	PREDICTED: Na(+)/H(+) exchange regulatory cofactor NHE-RF3-like [Meleagris gallopavo]	2.11E-04	2.27E-04	1.25E-04	6.77E-05	59.09	29.83
367	2	gi|733914177	PREDICTED: protein SET [Meleagris gallopavo]	6.49E-04	6.93E-04	8.53E-05	4.70E-05	13.14	6.78
368	1	gi|283765938	MHC class Ia2 antigen allele B, partial [Meleagris gallopavo]	2.89E-04	1.24E-04	6.85E-05	1.41E-04	23.68	114.47
369	1	gi|733926972	PREDICTED: coronin-2A [Meleagris gallopavo]	1.60E-04	3.02E-04	5.77E-05	6.56E-05	36.11	21.71
370	1	gi|733889717	PREDICTED: fibrinogen-like protein 1 isoform X1 [Meleagris gallopavo]	8.64E-05	5.44E-05	5.08E-05	1.95E-05	58.80	35.87
371	2	gi|733883345	PREDICTED: cytochrome b-c1 complex subunit 7 [Meleagris gallopavo]	3.24E-04	3.85E-04	1.72E-04	1.90E-04	53.00	49.51
372	4	gi|733899238	PREDICTED: glyoxalase domain-containing protein 5 isoform X1 [Meleagris gallopavo]	1.23E-03	9.42E-04	3.59E-04	1.37E-04	29.16	14.52
373	2	gi|57525040	glucosamine-6-phosphate isomerase 1 [Gallus gallus]	5.78E-04	3.68E-04	3.68E-04	2.58E-04	63.62	70.04
374	2	gi|397529551	ras-related protein Rab-1A [Gallus gallus]	1.52E-03	1.45E-03	5.88E-04	7.59E-04	38.58	52.36
375	1	gi|733917647	PREDICTED: proteasome subunit alpha type-7, partial [Meleagris gallopavo]	5.19E-04	4.16E-04	1.73E-04	1.26E-04	33.36	30.22
376	2	gi|733918036	PREDICTED: probable aminopeptidase NPEPL1 [Meleagris gallopavo]	2.25E-04	2.23E-04	9.12E-05	6.47E-05	40.61	29.04
377	1	gi|50729140	PREDICTED: cytochrome b5 reductase 3 isoform X1 [Gallus gallus]	3.45E-04	3.59E-04	4.98E-05	4.12E-05	14.44	11.48
378	1	gi|733900752	PREDICTED: calcium-binding mitochondrial carrier protein SCaMC-1 [Meleagris gallopavo]	1.55E-04	1.48E-04	1.70E-05	4.88E-05	11.01	33.10
379	2	gi|733907661	PREDICTED: ADP-ribosylation factor 4 [Meleagris gallopavo]	6.75E-04	6.57E-04	1.39E-04	1.56E-04	20.62	23.79
380	1	gi|733919423	PREDICTED: prenylcysteine oxidase 1 [Meleagris gallopavo]	1.47E-04	3.78E-04	1.13E-04	9.21E-05	77.20	24.38
381	1	gi|363738555	PREDICTED: ras-related protein Rab-7a [Gallus gallus]	1.73E-04	1.66E-04	7.57E-05	1.00E-04	43.85	60.29
382	1	gi|733929522	PREDICTED: 60S ribosomal protein L10, partial [Meleagris gallopavo]	3.92E-04	4.52E-04	1.83E-04	2.55E-04	46.83	56.49
383	2	gi|733923888	PREDICTED: basigin, partial [Meleagris gallopavo]	3.24E-04	2.32E-04	9.71E-05	1.36E-04	29.98	58.60
384	1	gi|733871985	PREDICTED: fatty acid amide hydrolase-like, partial [Meleagris gallopavo]	2.24E-04	2.35E-04	1.63E-04	3.97E-05	72.84	16.88

385	1	gi|733876406	PREDICTED: actin-related protein 2 [Meleagris gallopavo]	1.90E-04	1.65E-04	9.85E-05	9.09E-05	51.99	55.16
386	2	gi|45382431	myotrophin [Gallus gallus]	2.28E-04	3.04E-04	1.69E-04	1.73E-04	74.16	56.67
387	1	gi|733929362	PREDICTED: E3 ubiquitin-protein ligase TRIM39-like, partial [Meleagris gallopavo]	9.91E-05	1.18E-04	3.15E-05	1.70E-05	31.74	14.43
388	1	gi|733932305	PREDICTED: very long-chain acyl-CoA synthetase-like, partial [Meleagris gallopavo]	8.15E-05	9.12E-05	7.13E-05	1.35E-05	87.48	14.78
389	1	gi|733931170	PREDICTED: cysteine sulfinic acid decarboxylase [Meleagris gallopavo]	2.03E-04	2.28E-04	1.87E-04	1.51E-04	92.11	66.05
390	2	gi|733905605	PREDICTED: creatine kinase U-type, mitochondrial, partial [Meleagris gallopavo]	1.41E-03	2.44E-03	4.71E-04	1.19E-03	33.43	48.61
391	2	gi|733904386	PREDICTED: 40S ribosomal protein S17 [Meleagris gallopavo]	5.83E-04	5.35E-04	8.60E-05	1.11E-04	14.76	20.83
392	2	gi|733891573	PREDICTED: GTPase IMAP family member 6-like isoform X2 [Meleagris gallopavo]	7.37E-04	7.46E-04	7.79E-05	1.90E-04	10.57	25.53
393	1	gi|733890971	PREDICTED: proteasome subunit alpha type-3 [Meleagris gallopavo]	4.40E-05	8.58E-05	3.02E-05	4.18E-06	68.74	4.87
394	1	gi|356640682	glutathione S-transferase alpha class A1.2 [Meleagris gallopavo]	1.13E-04	1.54E-04	8.44E-05	5.60E-05	74.45	36.40
395	1	gi|971414771	PREDICTED: glycine cleavage system H protein, mitochondrial isoform X1 [Gallus gallus]	3.71E-04	1.72E-04	1.23E-04	1.04E-04	33.20	60.60
396	1	gi|733890098	PREDICTED: transmembrane emp24 domain-containing protein 10 [Meleagris gallopavo]	2.65E-04	1.81E-04	1.14E-04	7.49E-05	42.93	41.37
397	2	gi|733888825	PREDICTED: actin, alpha skeletal muscle B-like [Meleagris gallopavo]	3.82E-03	3.60E-03	2.04E-03	1.05E-03	53.40	29.11
398	1	gi|733877281	PREDICTED: leucine-rich PPR motif-containing protein, mitochondrial [Meleagris gallopavo]	4.55E-04	2.73E-04	1.61E-04	1.92E-04	35.43	70.50
399	1	gi|733870157	PREDICTED: ubiquitin-conjugating enzyme E2 N [Meleagris gallopavo]	4.47E-05	7.43E-05	2.99E-05	5.17E-05	66.98	69.57
400	1	gi|733893850	PREDICTED: UDP-glucuronosyltransferase 1-1-like isoform X6 [Meleagris gallopavo]	1.51E-04	1.44E-04	7.85E-05	3.32E-05	52.13	23.00
401	1	gi|733879115	PREDICTED: amine sulfotransferase-like [Meleagris gallopavo]	1.09E-04	2.84E-04	7.98E-05	1.93E-04	73.13	68.18
402	1	gi|760998679	tubulin alpha-8 chain [Gallus gallus]	9.99E-05	2.88E-04	5.75E-05	1.88E-04	57.58	65.13
403	2	gi|733927925	PREDICTED: retinal dehydrogenase 1-like, partial [Meleagris gallopavo]	5.17E-03	4.55E-03	1.53E-03	1.64E-03	29.69	35.98
404	1	gi|971392610	PREDICTED: electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial isoform X2 [Gallus gallus]	6.86E-05	1.48E-04	6.22E-05	1.48E-05	90.73	9.96
405	1	gi|733881947	PREDICTED: LOW QUALITY PROTEIN: endoplasmic reticulum resident protein 44, partial [Meleagris gallopavo]	2.95E-04	2.78E-04	5.74E-05	1.11E-04	19.49	39.80
406	1	gi|733884040	PREDICTED: nascent polypeptide-associated complex subunit alpha [Meleagris gallopavo]	7.92E-05	1.05E-04	3.22E-05	4.56E-05	40.64	43.42
407	1	gi|71895543	14-3-3 protein gamma [Gallus gallus]	1.44E-04	2.02E-04	1.08E-04	1.59E-04	75.21	78.72
408	1	gi|733912576	PREDICTED: phosphatidylethanolamine-binding protein 1 [Meleagris gallopavo]	4.71E-05	1.08E-04	5.69E-05	5.23E-05	120.98	48.17
409	1	gi|971420700	PREDICTED: septin-2 isoform X2 [Gallus gallus]	5.78E-05	9.84E-05	3.31E-05	1.02E-05	57.25	10.33
410	1	gi|733889053	PREDICTED: calcineurin B homologous protein 1 [Meleagris gallopavo]	3.08E-04	2.75E-04	9.23E-05	6.29E-05	29.98	22.88
411	1	gi|733872460	PREDICTED: cell surface A33 antigen [Meleagris gallopavo]	1.30E-04	1.68E-04	4.79E-05	2.04E-05	36.73	12.20
412	1	gi|733912901	PREDICTED: myelin-oligodendrocyte glycoprotein-like [Meleagris gallopavo]	3.15E-04	4.13E-04	2.18E-04	3.42E-05	69.17	8.28

413	1	gi|50755667	PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 [Gallus gallus]	1.11E-04	2.20E-04	4.84E-05	4.05E-05	43.70	18.39
414	1	gi|33339193	cytochrome c oxidase subunit VIIb precursor [Meleagris gallopavo]	1.42E-04	2.54E-04	9.20E-05	1.72E-04	64.70	67.48
415	1	gi|733907729	PREDICTED: solute carrier family 26 member 6 isoform X2 [Meleagris gallopavo]	3.69E-04	3.38E-04	6.84E-05	1.44E-04	18.54	42.68
416	1	gi|733929338	PREDICTED: E3 ubiquitin-protein ligase TRIM39-like isoform X1 [Meleagris gallopavo]	5.51E-05	5.58E-05	1.66E-05	1.64E-05	30.18	29.44
417	1	gi|758371923	cathepsin B precursor [Gallus gallus]	1.83E-04	1.94E-04	5.53E-05	6.65E-05	30.15	34.25
418	1	gi|971417755	PREDICTED: heterogeneous nuclear ribonucleoprotein A/B isoform X2 [Gallus gallus]	4.93E-05	1.66E-04	6.04E-05	6.15E-05	122.42	37.07
419	1	gi|733878191	PREDICTED: superoxide dismutase [Mn], mitochondrial [Meleagris gallopavo]	3.84E-04	4.55E-04	4.62E-05	1.13E-04	12.01	24.93
420	1	gi|733929904	PREDICTED: tubulin beta chain-like, partial [Meleagris gallopavo]	2.91E-05	8.50E-05	2.86E-05	7.21E-05	98.28	84.86
421	3	gi|60302796	SH3 domain-binding glutamic acid-rich-like protein [Gallus gallus]	8.63E-04	1.02E-03	3.68E-04	3.94E-04	42.59	38.46
422	1	gi|733915879	PREDICTED: zinc finger ZZ-type and EF-hand domain-containing protein 1 [Meleagris gallopavo]	5.06E-05	9.34E-05	1.98E-05	2.95E-05	39.15	31.58
423	2	gi|971393157	PREDICTED: long-chain-fatty-acid--CoA ligase 1 isoform X1 [Gallus gallus]	3.52E-04	4.05E-04	1.08E-04	1.42E-04	30.57	35.18
424	2	gi|971382053	PREDICTED: translocon-associated protein subunit alpha isoform X2 [Gallus gallus]	6.86E-04	7.21E-04	1.49E-04	1.99E-04	21.74	27.58
425	1	gi|326919277	PREDICTED: 60S ribosomal protein L9 [Meleagris gallopavo]	2.35E-04	2.61E-04	1.06E-04	8.99E-05	45.06	34.48
426	2	gi|733876979	PREDICTED: epoxide hydrolase 1 isoform X2 [Meleagris gallopavo]	6.05E-04	7.19E-04	1.91E-04	2.97E-04	31.61	41.35
427	1	gi|971380840	PREDICTED: gamma-glutamylcyclotransferase [Gallus gallus]	1.27E-04	8.66E-05	2.62E-05	1.39E-05	20.62	16.03
428	1	gi|971370745	PREDICTED: plasma membrane calcium-transporting ATPase 1 isoform X5 [Gallus gallus]	1.50E-04	2.07E-04	1.53E-04	8.38E-05	101.53	40.53
429	1	gi|971380302	PREDICTED: 60S ribosomal protein L15 isoform X1 [Gallus gallus]	6.56E-04	6.24E-04	7.54E-05	3.16E-04	11.50	50.69
430	1	gi|733930827	PREDICTED: neutral and basic amino acid transport protein rBAT-like [Meleagris gallopavo]	1.87E-04	1.85E-04	1.48E-04	2.23E-05	78.92	12.04
431	5	gi|71897241	D-dopachrome decarboxylase [Gallus gallus]	1.54E-03	1.63E-03	9.34E-05	1.86E-04	6.05	11.42
432	1	gi|733921095	PREDICTED: dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial [Meleagris gallopavo]	9.05E-05	1.23E-04	4.65E-05	2.44E-05	51.37	19.89
433	2	gi|971412140	PREDICTED: proteasome subunit alpha type-4 isoform X1 [Gallus gallus]	3.26E-04	3.66E-04	1.26E-04	1.76E-04	38.57	47.94
434	2	gi|971414914	PREDICTED: cadherin-1 isoform X1 [Gallus gallus]	8.31E-04	8.29E-04	3.55E-04	2.05E-04	42.74	24.77
435	2	gi|733906339	PREDICTED: cytochrome b-c1 complex subunit Rieske, mitochondrial [Meleagris gallopavo]	5.96E-04	5.36E-04	6.25E-05	1.04E-04	10.48	19.47
436	1	gi|50758444	PREDICTED: thioredoxin domain-containing protein 17 [Gallus gallus]	3.60E-04	4.03E-04	3.98E-05	7.45E-05	11.07	18.47
437	3	gi|733929367	PREDICTED: butyrophilin subfamily 2 member A1-like [Meleagris gallopavo]	7.57E-04	4.89E-04	1.45E-04	1.80E-04	19.11	36.73
438	1	gi|513231603	PREDICTED: S-adenosylmethionine synthase isoform type-2-like isoform X2 [Gallus gallus]	3.69E-05	1.40E-04	2.68E-05	2.79E-05	72.57	19.93
439	2	gi|971384254	PREDICTED: 60S ribosomal protein L7 isoform X1 [Gallus gallus]	1.16E-04	2.93E-04	5.82E-05	1.60E-04	50.07	54.57
440	1	gi|733926194	PREDICTED: histidine triad nucleotide-binding protein 1 [Meleagris gallopavo]	1.37E-04	1.82E-04	8.19E-05	9.22E-05	59.85	50.60

441	2	gi|429903872	membrane-associated progesterone receptor component 1 [Gallus gallus]	7.61E-04	1.19E-03	3.52E-04	2.67E-04	46.31	22.37
442	2	gi|733876853	PREDICTED: aldose 1-epimerase [Meleagris gallopavo]	8.24E-04	6.52E-04	2.19E-04	3.48E-04	26.52	53.38
443	1	gi|326912357	PREDICTED: aldose reductase-like isoform X2 [Meleagris gallopavo]	1.05E-04	1.06E-04	6.61E-05	7.42E-05	63.21	70.14
444	1	gi|971437427	PREDICTED: talin-1 isoform X3 [Gallus gallus]	1.12E-04	1.79E-04	4.15E-05	4.75E-05	37.14	26.58
445	1	gi|971379304	PREDICTED: glycine--tRNA ligase isoform X1 [Gallus gallus]	1.58E-04	2.27E-04	9.56E-05	4.96E-05	60.69	21.83
446	1	gi|733883237	PREDICTED: 2,4-dienoyl-CoA reductase, mitochondrial [Meleagris gallopavo]	1.93E-05	8.51E-05	1.23E-05	5.46E-05	63.79	64.12
447	1	gi|479277982	60S ribosomal protein L8 [Gallus gallus]	2.06E-04	2.10E-04	9.55E-05	7.46E-05	46.30	35.49
448	1	gi|733920284	PREDICTED: hydroxymethylglutaryl-CoA lyase, mitochondrial isoform X2 [Meleagris gallopavo]	1.50E-04	1.01E-04	3.33E-05	7.45E-05	22.16	73.48
449	1	gi|971426468	PREDICTED: AP-2 complex subunit beta isoform X2 [Gallus gallus]	2.21E-04	1.94E-04	7.86E-05	2.42E-05	35.58	12.48
450	1	gi|733918680	PREDICTED: F-box only protein 6-like [Meleagris gallopavo]	1.91E-04	1.22E-04	5.87E-05	7.70E-05	30.67	63.16
451	1	gi|733888518	PREDICTED: 40S ribosomal protein S13 [Meleagris gallopavo]	1.71E-04	2.11E-04	1.48E-04	1.28E-04	86.73	60.53
452	1	gi|971375846	PREDICTED: glycerol kinase isoform X4 [Gallus gallus]	5.68E-05	1.20E-04	3.43E-05	2.57E-05	60.34	21.45
453	1	gi|971425996	PREDICTED: polyubiquitin-B isoform X1 [Gallus gallus]	8.28E-04	8.44E-04	8.23E-05	1.68E-04	9.94	19.85
454	1	gi|733872400	PREDICTED: Na(+)/H(+) exchange regulatory cofactor NHE-RF3-like [Meleagris gallopavo]	2.00E-04	2.10E-04	5.52E-05	5.70E-05	27.54	27.13
455	1	gi|733911548	PREDICTED: GTP-binding nuclear protein Ran [Meleagris gallopavo]	2.46E-04	2.89E-04	4.84E-05	7.77E-05	19.66	26.88
456	1	gi|733886057	PREDICTED: LOW QUALITY PROTEIN: acid ceramidase, partial [Meleagris gallopavo]	1.56E-04	1.95E-04	8.79E-05	7.30E-05	56.14	37.36
457	1	gi|971421079	PREDICTED: malectin [Gallus gallus]	2.13E-04	1.77E-04	5.39E-05	1.21E-04	25.28	68.50
458	1	gi|733885686	PREDICTED: caspase-6 [Meleagris gallopavo]	3.88E-05	1.80E-04	2.46E-05	9.01E-05	63.48	50.18
459	1	gi|733905607	PREDICTED: creatine kinase U-type, mitochondrial-like [Meleagris gallopavo]	1.87E-04	1.05E-04	2.00E-05	1.62E-05	10.73	15.48
460	1	gi|733917022	PREDICTED: glutathione synthetase [Meleagris gallopavo]	7.95E-05	1.16E-04	7.82E-05	6.86E-05	98.34	59.38
461	1	gi|971437044	PREDICTED: tubulin alpha-1C chain [Gallus gallus]	8.81E-04	1.88E-03	5.28E-04	1.14E-03	59.97	60.65
462	2	gi|733915527	PREDICTED: nucleoside diphosphate kinase B [Meleagris gallopavo]	6.14E-04	8.52E-04	2.90E-04	4.19E-04	47.22	49.17
463	1	gi|303227902	acetyl-CoA acetyltransferase, cytosolic [Gallus gallus]	2.46E-04	3.03E-04	2.02E-04	1.44E-04	82.02	47.51
464	1	gi|971423813	PREDICTED: 1-acyl-sn-glycerol-3-phosphate acyltransferase beta isoform X2 [Gallus gallus]	2.02E-04	2.13E-04	9.92E-05	3.58E-05	49.03	16.76
465	1	gi|733929326	PREDICTED: trans-1,2-dihydrobenzene-1,2-diol dehydrogenase [Meleagris gallopavo]	4.16E-05	1.37E-04	2.32E-05	2.23E-05	55.81	16.24
466	1	gi|475808004	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial [Gallus gallus]	3.34E-05	1.47E-04	2.84E-05	8.16E-05	84.86	55.44
467	1	gi|971419734	PREDICTED: 40S ribosomal protein S15a isoform X1 [Gallus gallus]	3.24E-04	2.96E-04	7.81E-05	7.44E-05	24.13	25.13
468	2	gi|733895942	PREDICTED: aspartate--tRNA ligase, cytoplasmic [Meleagris gallopavo]	1.40E-04	1.88E-04	4.15E-05	1.13E-04	29.71	59.98

469	1	gi|50746903	PREDICTED: CDGSH iron-sulfur domain-containing protein 2 [Gallus gallus]	3.83E-04	2.72E-04	6.30E-05	5.93E-05	16.43	21.81
470	1	gi|326918888	PREDICTED: immunoglobulin J chain [Meleagris gallopavo]	1.12E-04	3.33E-04	7.12E-05	1.77E-04	63.76	53.04
471	1	gi|733888407	PREDICTED: L-lactate dehydrogenase C chain [Meleagris gallopavo]	6.51E-04	8.58E-04	4.49E-04	9.27E-04	69.02	107.99
472	1	gi|733926850	PREDICTED: hydroxysteroid dehydrogenase-like protein 2, partial [Meleagris gallopavo]	4.12E-05	1.38E-04	4.95E-05	9.81E-05	120.18	70.94
473	1	gi|363733121	PREDICTED: peptidyl-prolyl cis-trans isomerase D [Gallus gallus]	7.47E-05	8.42E-05	4.14E-05	1.00E-05	55.42	11.89
474	1	gi|971408086	PREDICTED: calponin-3 isoform X2 [Gallus gallus]	3.75E-04	3.08E-04	2.57E-04	1.69E-04	68.38	54.78
475	1	gi|971409436	PREDICTED: E3 ubiquitin-protein ligase HECTD3-like isoform X1 [Gallus gallus]	2.11E-04	2.08E-04	6.36E-05	5.17E-05	30.11	24.87
476	2	gi|733908045	PREDICTED: actin-related protein 2/3 complex subunit 4 [Meleagris gallopavo]	5.80E-04	7.63E-04	5.19E-04	4.88E-04	89.48	63.93
477	1	gi|326920300	PREDICTED: FAS-associated death domain protein [Meleagris gallopavo]	2.25E-04	2.37E-04	5.85E-05	6.78E-05	26.03	28.62
478	1	gi|971387849	PREDICTED: rho GTPase-activating protein 18 isoform X2 [Gallus gallus]	8.37E-05	1.33E-04	4.29E-05	9.13E-05	51.22	68.51
479	1	gi|733893517	PREDICTED: 60S ribosomal protein L14 [Meleagris gallopavo]	9.28E-05	2.23E-04	7.42E-05	1.38E-04	80.02	62.01
480	1	gi|733931319	PREDICTED: NAD-dependent malic enzyme, mitochondrial-like [Meleagris gallopavo]	2.19E-04	2.94E-04	1.49E-04	2.62E-04	67.95	89.26
481	1	gi|733918251	PREDICTED: 60S ribosomal protein L22, partial [Meleagris gallopavo]	1.83E-04	3.77E-04	9.15E-05	1.60E-04	50.12	42.39
482	1	gi|733871657	PREDICTED: trypsin II-P29-like [Meleagris gallopavo]	5.28E-04	4.44E-04	5.12E-04	3.99E-04	96.96	89.97
483	1	gi|758818522	macrophage migration inhibitory factor [Gallus gallus]	1.10E-03	1.33E-03	6.05E-04	6.57E-04	55.25	49.54
484	1	gi|971426770	PREDICTED: glycerophosphodiester phosphodiesterase domain-containing protein 1 isoform X2 [Gallus gallus]	2.79E-04	1.93E-04	5.74E-05	1.13E-04	20.58	58.31
485	1	gi|733900697	PREDICTED: transmembrane emp24 domain-containing protein 5 [Meleagris gallopavo]	1.72E-04	2.03E-04	6.93E-05	3.80E-05	40.27	18.70
486	1	gi|733906298	PREDICTED: xaa-Pro dipeptidase [Meleagris gallopavo]	2.11E-04	1.80E-04	8.52E-05	1.46E-04	40.41	81.10
487	2	gi|733883824	PREDICTED: elongation factor 1-delta isoform X8 [Meleagris gallopavo]	2.45E-04	2.57E-04	7.13E-05	1.09E-04	29.09	42.25
488	1	gi|95772094	histone H1.01 [Gallus gallus]	6.48E-05	9.95E-05	7.88E-06	5.01E-05	12.17	50.33
489	1	gi|733895836	PREDICTED: LOW QUALITY PROTEIN: N-myc-interactor [Meleagris gallopavo]	1.10E-04	7.30E-05	4.27E-05	3.04E-05	38.96	41.67
490	1	gi|733902563	PREDICTED: kyphoscoliosis peptidase isoform X3 [Meleagris gallopavo]	8.76E-05	7.83E-05	3.07E-05	3.62E-05	35.01	46.23
491	1	gi|71895491	outer mitochondrial membrane cytochrome b5 [Gallus gallus]	1.53E-04	1.14E-04	4.23E-05	6.16E-06	27.58	5.39
492	1	gi|733901193	PREDICTED: LOW QUALITY PROTEIN: phosphoglucomutase-1 [Meleagris gallopavo]	1.44E-04	1.69E-04	4.21E-05	2.07E-05	29.13	12.25
493	1	gi|971373134	PREDICTED: tetratricopeptide repeat protein 38 [Gallus gallus]	9.63E-05	5.92E-05	4.34E-05	2.07E-05	45.04	35.04
494	1	gi|538917383	mitochondrial amidoxime-reducing component 1 [Gallus gallus]	2.42E-04	2.17E-04	1.36E-04	1.73E-04	56.10	80.00
495	2	gi|733884044	PREDICTED: retinol dehydrogenase 16 [Meleagris gallopavo]	3.57E-04	3.44E-04	1.61E-04	6.79E-05	45.17	19.74
496	1	gi|733888998	PREDICTED: spectrin beta chain, non-erythrocytic 4-like [Meleagris gallopavo]	3.54E-04	3.66E-04	6.41E-05	7.99E-05	18.09	21.81

497	1	gi|733930740	PREDICTED: hemopexin-like, partial [Meleagris gallopavo]	1.47E-04	3.94E-04	4.59E-05	3.49E-04	31.15	88.63
498	1	gi|118100151	PREDICTED: transmembrane protein 120A [Gallus gallus]	3.81E-04	3.02E-04	1.28E-04	6.55E-05	33.61	21.67
499	1	gi|971431933	PREDICTED: ATP synthase subunit g, mitochondrial [Gallus gallus]	5.71E-04	4.21E-04	1.68E-04	1.56E-04	29.43	37.13
500	1	gi|733898303	PREDICTED: deleted in malignant brain tumors 1 protein-like [Meleagris gallopavo]	3.80E-04	4.71E-04	7.42E-05	2.41E-04	19.51	51.18
501	1	gi|71897227	serine--tRNA ligase, cytoplasmic [Gallus gallus]	6.21E-05	1.35E-04	5.07E-05	4.98E-05	81.62	36.92
502	1	gi|971401065	PREDICTED: CDGSH iron-sulfur domain-containing protein 1 isoform X1 [Gallus gallus]	2.32E-04	1.88E-04	5.86E-05	4.47E-05	25.29	23.71
503	1	gi|733921796	PREDICTED: bcl-2-like protein 15 [Meleagris gallopavo]	1.55E-04	1.39E-04	6.34E-05	5.26E-05	40.82	37.69
504	1	gi|733912519	PREDICTED: phospholipase A2, minor isoenzyme-like [Meleagris gallopavo]	4.42E-04	2.63E-04	3.53E-04	3.15E-04	79.91	119.82
505	1	gi|971437368	PREDICTED: activated RNA polymerase II transcriptional coactivator p15-like [Gallus gallus]	2.84E-04	2.68E-04	3.62E-05	3.98E-05	12.76	14.83
506	1	gi|971390681	PREDICTED: platelet-activating factor acetylhydrolase isoform X1 [Gallus gallus]	1.40E-04	1.23E-04	2.71E-05	3.81E-05	19.31	30.96
507	1	gi|971384613	PREDICTED: protein LYRIC-like [Gallus gallus]	1.62E-04	2.08E-04	8.79E-05	3.62E-05	54.39	17.41
508	1	gi|733930232	PREDICTED: dehydrogenase/reductase SDR family member 4-like, partial [Meleagris gallopavo]	2.79E-04	3.27E-04	9.94E-05	8.54E-05	35.65	26.11
509	1	gi|733924467	PREDICTED: immediate early response 3-interacting protein 1 [Meleagris gallopavo]	2.46E-04	2.41E-04	7.52E-05	3.38E-05	30.59	14.02
510	1	gi|733916794	PREDICTED: tax1-binding protein 3 [Meleagris gallopavo]	8.67E-05	1.01E-04	6.24E-05	6.16E-05	72.01	61.05
511	1	gi|733910438	PREDICTED: ATP synthase subunit f, mitochondrial [Meleagris gallopavo]	4.44E-04	1.06E-03	1.45E-04	1.69E-04	32.63	16.00
512	1	gi|733908826	PREDICTED: sideroflexin-1 isoform X2 [Meleagris gallopavo]	1.71E-04	1.55E-04	7.27E-05	1.25E-04	42.52	80.27
513	1	gi|733886643	PREDICTED: grpE protein homolog 1, mitochondrial [Meleagris gallopavo]	2.01E-04	2.54E-04	1.75E-04	9.88E-05	87.06	38.83
514	1	gi|733869832	PREDICTED: adenylosuccinate lyase [Meleagris gallopavo]	5.67E-05	6.70E-05	2.72E-05	2.41E-05	47.88	35.99
515	1	gi|326914412	PREDICTED: vitelline membrane outer layer protein 1 homolog [Meleagris gallopavo]	1.29E-04	1.68E-04	9.10E-05	1.04E-04	70.61	62.19
516	1	gi|326913863	PREDICTED: lysosome-associated membrane glycoprotein 1 [Meleagris gallopavo]	1.05E-04	8.84E-05	4.28E-05	2.29E-05	40.78	25.94
517	1	gi|733928858	PREDICTED: LOW QUALITY PROTEIN: stress-induced-phosphoprotein 1-like, partial [Meleagris gallopavo]	1.05E-04	1.79E-04	6.62E-05	7.24E-05	63.27	40.39
518	1	gi|733915758	PREDICTED: transmembrane protein 120A, partial [Meleagris gallopavo]	1.49E-04	2.04E-04	9.79E-05	1.11E-04	65.82	54.39
519	1	gi|733926363	PREDICTED: sialic acid synthase [Meleagris gallopavo]	2.60E-05	2.95E-05	4.51E-06	1.73E-05	17.30	58.48
520	1	gi|733923267	PREDICTED: junction plakoglobin isoform X2 [Meleagris gallopavo]	6.54E-05	1.02E-04	4.87E-05	8.30E-05	74.50	81.22
521	1	gi|733896162	PREDICTED: uncharacterized protein LOC104911870 [Meleagris gallopavo]	9.23E-05	8.20E-05	9.12E-05	5.76E-05	98.84	70.23
522	1	gi|971379871	PREDICTED: dnaJ homolog subfamily B member 6 isoform X1 [Gallus gallus]	1.31E-04	1.37E-04	3.78E-05	5.83E-05	28.89	42.43
523	1	gi|743405597	probable ATP-dependent RNA helicase DDX4 [Gallus gallus]	4.07E-04	4.97E-04	7.71E-05	6.74E-05	18.93	13.55

5.5. Acute phase proteins mRNA changes in the liver, adipose tissue and jejunum of turkeys (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus

Results presented here are still preliminary and required further studies before they can be published.

Keywords: acute phase proteins, animal welfare, haemorrhagic enteritis virus, intestine, *Meleagris gallopavo*.

Abstract

The aim of this study was to investigate how four acute phase proteins (APPs), namely α1-acid glycoprotein (AGP), Serum Amyloid A (SAA), PIT54 and C-Reactive Protein (CRP), can be modified by a disease in turkey (*Meleagris gallopavo*). Heamorrhagic enteritis (HE) is a stressful disease in turkeys, caused by an adenovirus. HE disease is characterized by acute onset of depression, splenomegaly, intestinal haemorrhage and sudden death. Surviving birds exhibit HEV-induced immunosuppression condition, which facilitates secondary infections with opportunistic pathogens agents such as the avian pathogenic *Escherichia coli*, resulting in significant economic loss. A group of five BUT BIG 6 commercial hens infected with HE virus, which presented lesions at jejunum level, were included in this study and the quantitative gene expression of APPs in the liver, adipose tissue and jejunum was compared to that of healthy control group, composed by nine birds. The expression of AGP, PIT54 and SAA was found to be increased in sick animals. AGP and SAA mRNA expression were increased in both liver and adipose tissue, whereas PIT54 mRNA expression was increased in liver alone. CRP mRNA expression was decreased in HEV-infected birds. The results of this study are still preliminary but they show that HE disease affects APPs expression. The finding that AGP, PIT54 and SAA are upregulated in sick animals validates their use as welfare biomarkers in turkey.

1. Material and methods

1.1. Samples collection and preservation

Liver, visceral adipose tissue and jejunum samples were collected during routinely slaughtering procedures from nine 92 day-old clinically healthy BUT BIG 6 breeding line female turkeys and five BUT BIG 6 breeding line female turkeys PCR HEV positive (HEV-infected animals) and with lesions in intestine at jejunum level. Clinical status of the animals was also assessed by ante- and post-mortem inspection. Portions of liver, visceral adipose tissue and jejunum were removed immediately after slaughtering, snap frozen into liquid nitrogen and afterwards stored at −80ºC (for gene expression analyses).

1.2. Qualitative and quantitative mRNA expression

Total RNA was extracted using QIAzol lysis reagent according to the manufacturer's protocol (Qiagen) and treated with DNase I (Thermo Fisher Scientific, Fermentas). Total RNA was quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. Reverse transcription was carried out with 1 µg RNA using the iScript cDNA Synthesis Kit (BioRad). The cDNA was used as template for PCRs.

Qualitative PCR was performed in 10 µL final volume containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (LeGene Biosciences). PCR conditions were 35 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 45 sec (Eppendorf Mastercycler). PCR products were visualized on 1.6% agarose gel stained with ethidium bromide. The same primers were used in qualitative and quantitative PCR (Table 1).

Quantitative real time PCR (qPCR) was performed using 12 µL Eva Green Supermix (BioRad), 250 nM AGP and PIT54, 300 nM SAA and CRP and 400 nM GAPDH, YWHAZ and RPL4, using Eco Real-Time PCR System (Illumina). In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing fourfold serial dilution starting from the pooled sample composed by a liver cDNA mix from five healthy animals. Each sample was tested in duplicate. Non-reverse transcribed controls were performed by omitting

reverse transcription and no template controls were conducted by adding nuclease free water. The thermal profile used (95°C for 10 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec; for melting curve construction, 55°C for 15 sec and 80 cycles starting to 55°C and increasing 0.5°C each 10 sec) was the same for each target gene. The MIQE guidelines were followed (Bustin et al., 2009). For all genes studied, the standard curves derived from serial dilution of pooled sample gave correlation coefficients (R^2) greater than 0.990 and efficiencies greater than 94%. Results were compared using the comparative $^{\Delta\text{-}\Delta}$Cq method.

Table 1. List of the genes under study and their respective primers sequences, accession numbers and fragment lengths.

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length (bp)
AGP	XM_003211214	TCCCTGCCGAAATAGACAAC	TCCTTCATCTCAGCCATGTG	103
SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136
YWHAZ	XM_003205203.1	TTCCCTTGCAAAAACGGCTT	TTCAGCTTCGTCTCCTTGGG	148

AGP, alpha 1-acid glycoprotein; SAA, serum amyloid A; PIT54, scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog (haptoglobin-like); CRP, C-reactive protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL4, ribosomal protein L4; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.SAA, PIT54, CRP, GAPDH, RPL4 and YWHAZ were from Marques et al. (2016). AGP and OVT primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0).

1.3. Statistical analysis

Statistical analysis was performed using a General Linear Model procedure of SAS (SAS version 9.2 Inst. Inc., Cary, NC). Least significant difference was used to compare mean values. Statistical significance was accepted at p-value ≤ 0.05.

2. Results

2.1. HEV influences mRNA abundance of AGP, PIT54 and SAA in liver

APP mRNA abundance in liver from healthy and HEV-infected animals was determined by qPCR after normalization against reference genes. Results are presented in Figure 1. Liver AGP, PIT54 and SAA mRNA abundance was found to be statistically significantly increased sick animals (HEV-infected), as compared with healthy animals (p-value = 0.0438, 0.0232 and 0.0071, respectively). The mRNA abundance of CRP did not show any statistical significant difference.

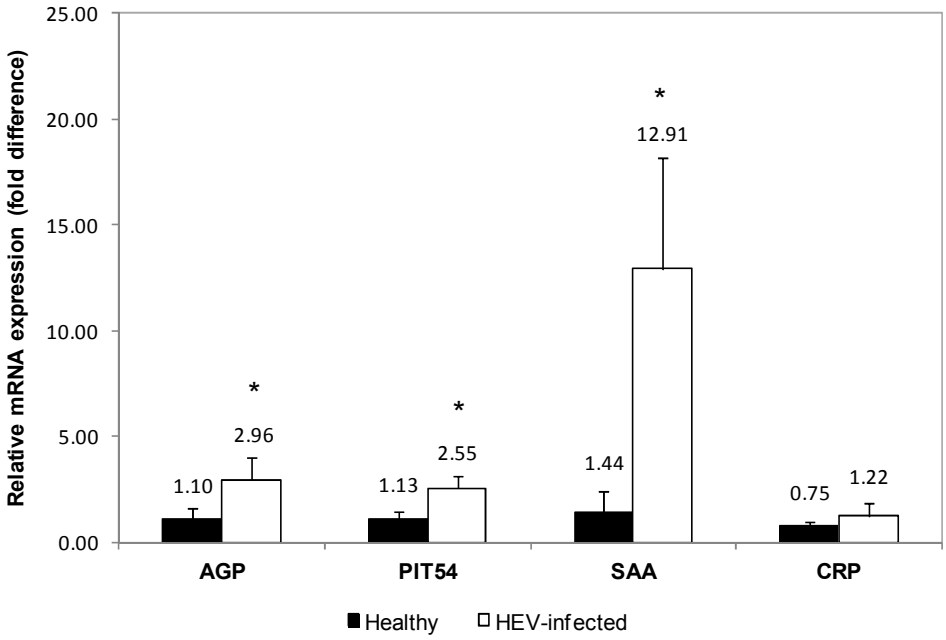

Figure 1. Relative expression of AGP, PIT54, SAA and CRP in liver of healthy and HEV-infected turkeys studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Data are means ± SEM of five HEV-infected and nine healthy animals. Folds relative to liver=1.

2.2. HEV influences mRNA abundance of AGP, PIT54 and SAA in adipose tissue

APP mRNA abundance in adipose tissue from healthy and HEV-infected animals was determined by qPCR after normalization against reference genes. Results are presented in Figure 2. Adipose tissue AGP and SAA mRNA abundance was found to be statistically significantly increased sick animals (HEV-infected), as compared with healthy animals (p-value = 0.0071 and 0.0016, respectively). The mRNA abundance of PIT54 and CRP did not show any statistical significant difference.

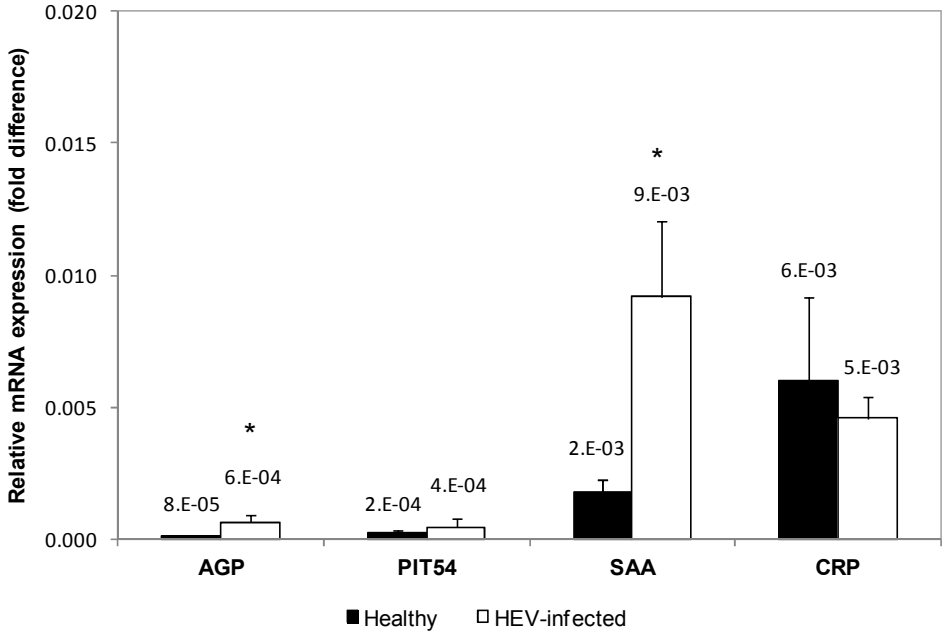

Figure 2. Relative expression of AGP, PIT54, SAA and CRP in adipose tissue of healthy and HEV-infected turkeys studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Data are means ± SEM of five HEV-infected and nine healthy animals. Folds relative to liver=1.

2.3. HEV influences mRNA abundance of AGP, PIT54 and SAA in jejunum

APP mRNA abundance in jejunum from healthy and HEV-infected animals was determined by qPCR after normalization against reference genes. Results are presented in Figure 3. Jejunum CRP mRNA abundance was found to be statistically significantly increased healthy animals, as

compared with HEV-infected animals (p-value = 0.0159). The mRNA abundance of AGP, PIT54 and SAA did not show any statistical significant difference.

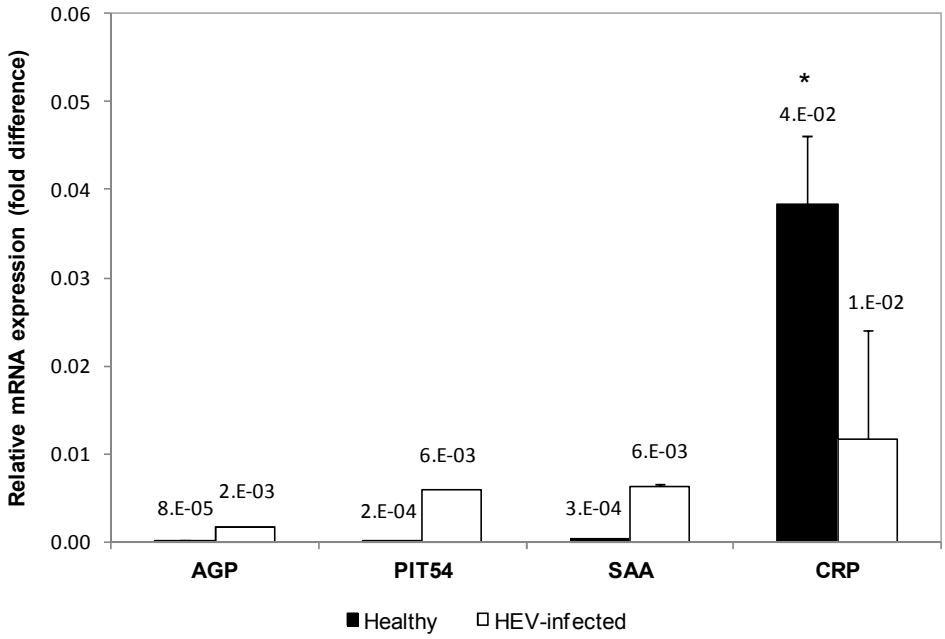

Figure 3. Relative expression of AGP, PIT54, SAA and CRP in jejunum of healthy and HEV-infected turkeys studied by qPCR. The results were normalized using the geometric mean of reference genes (GAPDH, YWHAZ and RPL4). Data are means ± SEM of five HEV-infected and nine healthy animals. Folds relative to liver=1.

3. Discussion

Although HEV related diseases have been known for many years, this is the first study demonstrating that APPs are more expressed in liver, adipose tissue and jejunum of HEV-infected turkeys, at least at mRNA expression. We found that the HEV infection modifies liver mRNA abundance of AGP, SAA and PIT54. Remarkably, adipose tissue was also found to be reactive to HEV disease, since AGP and SAA mRNA abundance was found also increased in sick animals.

AGP is one of the most widely measured APP in chicken (O'Reilly and Eckersall, 2014) and its role as APP in turkey have been demonstrated in this thesis and literature (Marques et al., 2016). In agreement with our results, elevated serum AGP levels have already been observed in chickens infected with viral pathogens (Inoue et al., 1997; Jang et al., 2013; Nakamura et al., 1996). AGP plays important anti-inflammatory and immunomodulatory roles to control inflammation (Luo et al., 2015) and its immunomodulatory activity is focused on dampening the side effects of inflammation, including inhibition of phagocytosis and monocyte apoptosis (Ceciliani et al., 2007; Lecchi et al., 2013). Although HEV disease is caused by an adenovirus, it has been proposed that the lesions in the intestinal epithelial cells is not due to the effects of the virus but, instead, due to an exacerbating cytokine-mediated reaction (Suresh and Sharma, 1996). Given that AGP mRNA was found to be statistically significantly overexpressed in both liver and adipose tissue, we speculate that the higher amount of AGP in HEV-infected animals is modulating the negative effects of the inflammation.

PIT54 is homologous to the mammalian haptoglobin – a hemoglobin-binding protein (Quaye, 2008; Wicher and Fries, 2006). PIT54 mRNA abundance was found to be increased only in liver and not in adipose tissue. Interestingly, we found in our previous work that PIT54 was overexpressed in adipose tissue in response to transport-induced stress. The role of PIT54 in adipose tissue still deserves further investigation. Under a HEV pathological condition, hemoglobin may leak into the blood in high concentrations that are toxic for the surrounding tissues. This hemoglobin can be sequestered by PIT54, inhibiting its oxidative activity (Nielsen and Moestrup, 2009). PIT54 has been recently associated with avian infectious bronchitis virus (IBV) infection (Asasi et al., 2013).

Among the other APPs included in the present study, SAA was the one that has shown the highest overexpression in HEV-infected animals as compared with control (9-folds higher in

liver and 4.5-folds higher in adipose tissue). SAA is a major APP in vertebrates and can easily increase its concentration up to 1000–fold as response to a disease. SAA belongs to a family of apolipoproteins, which are rapidly released into blood circulation when they are associated with high-density lipoprotein (HDL) (Benditt and Eriksen, 1977). SAA may serve as an immune opsonin for bacteria (Shah et al., 2006), and not only induces the expression of pro-inflammatory cytokines but also those exhibiting anti-inflammatory properties, such as IL-1 and IL-10 (Cheng et al., 2008; Ye and Sun, 2015). Supporting our results, SAA levels were also found as increased in chickens infected with IBV (Asasi et al., 2013). The finding that adipose tissue can express SAA is not new (Ceciliani et al., 2015), but it was found for the first time in turkey in the present investigation. SAA has been regarded as an adipokine and regulates the adipocyte cholesterol efflux (Poitou et al., 2009).

CRP mRNA abundance was not modified in liver and adipose tissue from sick animals. However, other studies have identified CRP increment in response to bacteria and viral infections in chicken (Patterson and Mora, 1964; Rauber et al., 2014; Seifi et al., 2014). Interestingly, CRP mRNA expression was statistically higher in jejunum from healthy animals. The results presented here are still preliminary and more studies and experimental conditions are required before we can conclude that CRP is a negative APP for HEV disease model. CRP has never been described as a negative APP in poultry. Nevertheless, it would not be the first description that one of the major APP for many species was described as negative APP. For example, AGP has recently identified as a negative APP in pigs (Heegaard et al., 2013).

4. Conclusions

In conclusion, the present study provides information on acute phase proteins behaviour during a disease model in turkey. On the background of mRNA abundance data, the concentration of AGP, PIT54 and SAA might be modified in liver and adipose tissue from turkeys infected with heamorrhagic enteritis virus, while CRP mRNA was decreased in jejunum. Although encouraging, these results must be considered as preliminary. More animals need to be included and the study must be paralleled by the measurement in serum of the respective transcript product, in order to confirm that AGP, PIT54 and SAA proteins are overexpressed in sick birds.

Chapter 6. Final remarks and conclusions

Poultry is one of the most consumed meat in the world. Stress and diseases play important biological roles, not only for animal health, but also for quality and gain in meat. A specific knowledge of methods to assess poultry welfare is of key importance for the management of animal well-being and thereby also for economic income and meat quantity and quality. Welfare biomarkers are of great help in understanding the physiological and molecular state of the birds.

The aims of this thesis was to identify the major acute phase proteins in turkey species, which have never been addressed until now, and characterized their expression using a stress model by road transport, one of the most stressful events in birds' life. Following the identification of welfare biomarkers, we have also studied the novel microRNAs as emergent biomarkers in other species but never addressed in turkey species. We have characterized their expression using the same stress model by road transport. Liver is the main organ to produce acute phase proteins, however extrahepatic expression have been described in many species. In poultry, this description has been poorly addressed, and we have studied for the first time the extrahepatic expression of five very well known APP in 20 chicken tissues under non-pathological condition. Moreover, turkey intestinal mucosa was determined by proteomic techniques and the local expression for some genes related with immune system was described. Gastrointestinal tract is regarded as one of the most important organ for poultry welfare.

With the experiments presented in this thesis, we have identified for the first time four APPs in turkey species – AGP, SAA, PIT54 and CRP – and demonstrated that they are involved with stress by road transport procedures and stress by heamorrhagic enteritis infection, which decrease the animals' welfare. AGP, CRP and PIT54 mRNA expression was related to stress, while AGP, PIT54 and SAA mRNA expression was related to disease in turkey. In addition, microRNAs involvement in stress-related procedures was also described for the first time in turkey species and three miRNAs – miR-22, miR-155 and miR-365 – were acknowledged was possible welfare biomarkers. The diagnostic value of these candidate miRNAs was confirmed by ROC curve.

It is known that APP can be found in extrahepatic tissues. We have described for the first time that 20 tissues in chicken can produce APP, even without any association with inflammation or diseases, contributing in this way for the local surveillance and immunity. The mRNA coding for

five APP – AGP, SAA, PIT54, CRP and OVT – was detected in our panel of tissues with a higher expression in gastrointestinal tract, respiratory system and lymphatic system.

Furthermore, we hereby presented the first proteomic study on turkey intestinal mucosa healthy and infected with haemorrhagic enteritis virus. The global proteome mapping allowed the identification of 523 unique proteins. In this thesis, we demonstrated that turkey intestine produce at least 64 proteins differentially expressed and involved in amino acid and protein metabolism, carbohydrate metabolism, fatty acids metabolism, cytoskeleton, immune system, nucleic acid biogenesis, ribosome, binding and transport and apoptosis, confirming the involvement of intestinal mucosa in these processes.

Since intestine has an intense capillary network, proteins can be produced by other organs and carried to intestine through blood. In addition, we demonstrated the expression by intestine of 11 proteins involved in inflammatory and immune processes, providing further insight in the immunological competence of intestine. However, little is so far known about these molecular mechanisms and further investigation are of great interest, also from an economical point of view.

In conclusion, this thesis provided information about biomarkers of stress in poultry. Beside the obvious implementation of the results of this study in the field of biomarkers, further investigations are required, in particular for what concerns the pathways regulated by miRNA in the specific activation of hypotahalamic-pituitary-adrenal axis and how it is its impact on the regulation of the innate immunity in poultry species.

List of papers and abstracts published, submitted or in preparation during PhD

During my PhD, I have been involved in other side projects, investigating different aspects of the innate immune response, goat diets, testicular maturation in rabbits and taste receptors in rabbits and ruminants. Part of the results have already been published or submitted for publication in peer-reviewed journals.

Andreia Tomás Marques, Sandra Anjo, Mangesh Bhide, Ana Varela Coelho, Cristina Lecchi, Guido Grilli, Bruno Manadas, Fabrizio Ceciliani. 2017. Proteome analysis of intestinal mucosa in turkey (*Meleagris gallopavo*) infected with haemorrhagic enteritis virus (HEV). [To be submitted to *J Proteomics*].

Andreia Tomás Marques, Laura Nordio, Cristina Lecchi, Guido Grilli, Chiara Giudice, Fabrizio Ceciliani. 2017. Widespread extrahepatic expression of acute phase proteins in healthy chicken (*Gallus gallus*) tissues. [Submitted to *Vet Immunol Immunopathol*].

Cristina Lecchi, Andreia Tomás Marques, Miriam Redegalli, Sarah Meani, Leonardo James Vinco, Valerio Bronzo, Fabrizio Ceciliani, 2016. Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkeys. *Animal*. Jul;10(7):1213-1217. doi: 10.1017/S1751731115003043.

Andreia Tomás Marques, Cristina Lecchi, Guido Grilli, Chiara Giudice, Sara Rota Nodari, Leonardo J. Vinco, Fabrizio Ceciliani, 2016. The effect of transport stress on turkey (*Meleagris gallopavo*) liver acute phase proteins gene expression. *Res Vet Sci*. Feb 104:92-95. doi: 10.1016/j.rvsc.2015.11.014.

Other Publications

Laura Restelli#, Andreia Tomás Marques#, Giovanni Savoini, Michela Carisetti, Cristina Lecchi, Emoke Bendixen, Fabrizio Ceciliani. 2016. Polyunsaturated and saturated fatty acids integration of maternal diets influence omental adipose tissue proteome of suckling goat-kids. [Submitted to *J Proteomics*].
# these two authors equally contribute to the manuscript

Barbara Banco, Guido Grilli, Chiara Giudice, Andreia Tomás Marques, Sara Cotti Cometti, Giuseppe Visigalli, Valeria Grieco. 2016. Immunophenotyping of Rabbit Testicular Germ and Sertoli Cells Across Maturational Stages. *J Histochem Cytochem.* Nov 64(11):715-726. doi: 10.1369/0022155416669918.

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Fabrizio Ceciliani, Laura Soler, Guido Grilli, Andreia Tomás Marques, Chiara Giudice, Cristina Lecchi. 2015. The localization and differential expression of Serum Amyloid A in bovine liver and adipose tissue depots. *Vet Immunol Immunopathol.* Nov 15;168(1-2):35-39. doi: 10.1016/j.vetimm.2015.08.004.

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Abstracts

Andreia Tomás Marques, Cristina Lecchi, Laura Nordio, Chiara Giudice, Guido Grilli, Helena Ariño-Bassols, Fabrizio Ceciliani, 2016. Widespread extrahepatic expression of acute-phase proteins in chicken (*Gallus gallus*) tissues. *Proceeding of Veterinary and Animal Science Days*, Vol 3, No 1s. doi: http://dx.doi.org/10.13130/2283-3927/7060.

Andreia Tomás Marques, Fabrizio Ceciliani, Miriam Redegalli, Michela Carisetti, Sarah Meani, Leonardo James Vinco, Valerio Bronzo, Cristina Lecchi, 2015. Serum miRNA disregulation during transport-related stress in turkey (*Meleagris gallopavo*). *Proceeding of Veterinary and Animal Science Days*, Vol 2, No 1s. doi: http://dx.doi.org/10.13130/2283-3927/5126.

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Savage Chickens
by Doug Savage
TURKEY.
WHAT DID YOU JUST CALL ME?
UH... UH... NOTHING!
CHICKEN.
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