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Why do we study animal toxins?



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The internationalization voyage of *Zoological Research*

Scientific journals usually mirror the development of a country or area in a specific period of time. With the transnational scientific communication model of today's world, the internationalization of scientific journals not only provides an avenue for researchers to attain their rights to receive and impart information and ideas openly and freely across a global platform, but the increased reach of such publications allows for a broader and stronger voice in regards to academic and scientific debates.

Therefore, countries in which English is a second language are making great strides in both hosting and inaugurating English-language journals and establishing cooperation with international publishing groups. Taking China as an example, before 1949, there were only three English-language journals, whereas of the current 4900+ scientific journals, more than 250 are in English and over 180 are published/co-published by international publishing groups. Especially in recent years, the numbers of the highly qualified journals are in a considerable increase to commensurate with the large role China plays as a global producer of research.

To stand out from the intense competition of emerging scientific journals and increasing access to readily available information, each journal must explore many paths and encounter much uncertainty and ambiguity as it finds its own way. Quite a few have made impressive and remarkable progress in the keen competition, such as *Microsystems & Nanoengineering*, *Light: Science and Applications*, and *National Science Review*, among others.

Adaptation and evolution are journeys that never end and are filled with countless obstacles. For example, other than the language barrier itself, unprofessional conduct and partiality such as citation bias, i.e., publications of Chinese scientists are most likely to be cited by other Chinese authors rather than by authors from other countries, citation preference of articles from journals with high impact factors, fake peer-review procedures, and improper cross-citation among some journals have all hindered the promotion and internationalization of scientific publications.

Zoological Research (ZR) has a 35-year history enriched with highs and lows, and is currently on its own voyage of rebranding from a local, regional leading Chinese journal to a prominent international one. During this transition, ZR has encountered many inevitable predicaments such as a reduction in qualified submissions, submission withdrawal after rounds of revisions, and evaluation bias as the Science Citation Index (SCI) is still regarded as the golden criterion. However, through deep appreciation of the devoted generations of scientists over the past few decades and sincere respect for its traditions, ZR

is facing these difficult challenges with great enthusiasm and optimism. The exciting step ZR took in 2014 to switch from a Chinese to English-only publication is obviously an initial startup of this exciting venture.

With a group of international top-tier scientists as our editorial members, we will continue to protect the quality of our published research, promote the spirit of fairness and free-speech to spur innovations and solicit contributions, and maintain a strictly professional and respected publishing platform. We would like to take this opportunity to thank you for your constant support, and more importantly encourage you all to join us in this voyage of new possibilities for the promising future of ZR.

Sincerely yours,

Su-Qing LIU, Editorial Director
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Zoological Research is recognized as a core journal by the Research Center of Chinese Science Evaluation (RCCSE)

We are pleased to announce that *Zoological Research* (ZR) has been recognized as a core journal (level A, top 5%-20%) and ranked 15th among 78 biology academic journals, according to the “RCCSE Chinese Academic Journal Evaluation Report – Ranking List of Authoritative Journals and Core Journals”. This evaluation report was released by RCCSE in cooperation with the Library of Wuhan University and the Network of Science & Education Evaluation in China, and included a total of 6 201 academic journals, with ZR among the 1 572 selected core journals. The major evaluation indexes included total citations, two-year impact factors, immediacy index, ratio of funded papers, Web immediacy downloads, database indexing and quantitative assessment.

Why do we study animal toxins?

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ABSTRACT

Venom (toxins) is an important trait evolved along the evolutionary tree of animals. Our knowledges on venoms, such as their origins and loss, the biological relevance and the coevolutionary patterns with other organisms are greatly helpful in understanding many fundamental biological questions, i.e., the environmental adaptation and survival competition, the evolution shaped development and balance of venoms, and the sophisticated correlations among venom, immunity, body power, intelligence, their genetic basis, inherent association, as well as the cost-benefit and trade-offs of biological economy. Lethal animal envenomation can be found worldwide. However, from foe to friend, toxin studies have led lots of important discoveries and exciting avenues in deciphering and fighting human diseases, including the works awarded the Nobel Prize and lots of key clinic therapeutics. According to our survey, so far, only less than 0.1% of the toxins of the venomous animals in China have been explored. We emphasize on the similarities shared by venom and immune systems, as well as the studies of toxin knowledge-based physiological toxin-like proteins/peptides (TLPs). We propose the natural pairing hypothesis. Evolution links toxins with humans. Our mission is to find out the right natural pairings and interactions of our body elements with toxins, and with endogenous toxin-like molecules. Although, in nature, toxins may endanger human lives, but from a philosophical point of view, knowing them well is an effective way to better understand ourselves. So, this is why we study toxins.

Keywords: Toxins; Survival competition; Evolution; Disease mechanism; Drug development

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INTRODUCTION

Struggle for existence in nature created toxins

A basic issue for a living organism is that how to adapt to the environments, to keep “homeostasis” facing various ecological conditions and noxious stimuli, and to win in the survival competitions (Darwin, 1859). Prey-predator interaction and prevention of pathogenesis while maintaining symbiosis in coexistence with enormous microbes are the key biological challenges (Cortez & Weitz, 2014; Lazzaro & Rolff, 2011; Yoshida et al, 2003). Accordingly, toxins are widely produced by all forms of life, including animals, plants and microbes, to interfere and disrupt the physiological processes of other organisms and on the other hand, favor their own struggles for existence. Toxins can be small molecular compounds, proteins and peptides. Toxic animals can be broadly classified into two categories (Mebs, 2002): (1) venomous species possess a specialized venom system and are able to produce their own venom, which is a mixture of gene-encoded proteins and peptide toxins; (2) species acquire and accumulate small molecular and poisonous metabolites and toxins from their environments while maintaining relative resistance to the toxins’ poisonous effects, such as poison-dart frogs (Daly et al, 2005), New Guinean Pitohui birds (Dumbacher et al, 1992) and African crested rats (Kingdon et al, 2012). In this review, we focused on the venomous animals and their gene-encoded proteins and peptide toxins.

Evolution links animal toxins with humans

Humans originated and live together with venomous animals. During evolution, the ancestors of humans and animal toxins were tightly associated with each other in terms of evolutionary conservation as well as mutual interactions. The natural and inherent links of animal toxins with humans were determined by the origin, biological relevance and biochemical properties of animal toxins. Although, at present stage, humans are generally neither the prey nor the predator of venomous animals, each year, numerous cases of animal envenomation are reported worldwide, which have caused substantial morbidity and mortality and has become a serious global public health problem (Balhara & Stolbach, 2014; Isbister & Bawaskar, 2014; Kasturiratne et al, 2008).

Molecular diversities of animal key physiological elements

Genome sequences of animals, including those of humans,

have revealed huge molecular diversity of key physiological elements, such as cell membrane ion channels and receptors, non-membrane factors, etc. For example, human genome comprises approximately 400 genes encoding pore-forming ion channels of plasma membranes, which can be broadly classified as either voltage or ligand gated depending on the primary factors determining channel opening and/or closing. Receptors and ion channels of cell membranes play vital roles in various malfunctions and diseases, and function as major drug targets (Bagal et al, 2013; Bradley et al, 2014; Wickenden et al, 2012; Wootten et al, 2013).

Coevolution results in the huge molecular diversities of animal toxins

Living strategies for prey capture and defense have evolved venom from venomous animals. Venom, typically a mixture of proteins and peptide toxins, can be broadly defined as a secretion, produced in a specialized gland in one animal and delivered to a target animal through the infliction of a wound, which contains molecules that disrupt normal physiological or biochemical processes so as to facilitate feeding or defense by the producing animal (Casewell et al, 2013; Fry et al, 2009a). Long-term coevolution has created extensively diversified proteins and peptide toxins, which specifically act on key physiological elements of the target organisms, such as cell membrane ion-channels and receptors. Selective pressure and long-term coevolution have endowed animal toxins with strong activity (act in pmol/L and nmol/L), high specificity (effective on the subtypes of receptors and ion channels) and huge molecular diversity (multiple-gene copy families).

Animal toxin study was originally driven by the motivation of understanding animal envenomation and clinical treatments. As early as 1781, an Italian naturalist Felice Fontana investigated the disturbances of snake venoms on blood coagulation. However, from foe to friend, venom toxins are being treated as invaluable and powerful pharmacological research tools, as well as important clinic therapeutics both in history and nowadays. By reviewing the historical contributions and the impacts of animal toxins on life sciences, in this article, we addressed the major aspects of toxin study and particularly, we summarized the known venomous animals in China, and emphasized on the studies of toxin knowledge-based physiological toxin-like proteins/peptides (TLPs), the patho-physiological relevance, as well as the similarities shared between toxins and immune effectors from the natural attack and defense systems.

BIOLOGY OF ANIMAL TOXINS

Venom system (apparatus) as a special trait in animals

Venoms have evolved on numerous occasions in animals. The venom (mixture of toxins) of a venom system typically stores in a discrete gland and a specialized delivery system. The common and well-known venomous animals include cnidarians (jellyfishes, sea anemones and hydra), molluscs (cone snails), annelids (leeches), arthropods (spiders, scorpions, centipedes, bees and wasps, ants, ticks and horseflies, crustaceans), echinoderms (sea urchins and starfishes), vertebrates (fishes, snakes and lizards, as well as mammals) (Figure 1). A wide

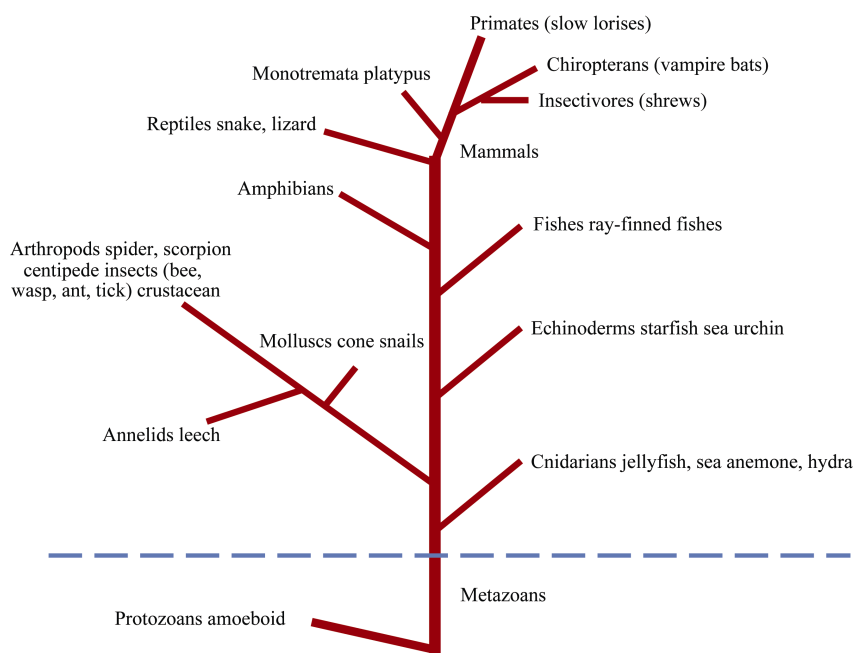


Figure 1 Venom (a mixture of toxins) evolved along the evolutionary tree of animal kingdom

As a special trait in animal kingdom, venom system has evolved in nature for survival competition, which plays important roles in predation, defense, competition, antimicrobial and even communication in given ecological contexts. The common and well-known venomous animals are shown. Toxins are produced from single cell protozoans to metazoan primates.

range of innovative structures (venom delivery systems) have evolved to facilitate the delivery of venoms, including fangs or modified teeth, harpoons, nematocysts, pincers, proboscises, spines, sprays, spurs and stings.

Nematocysts of cnidarians

Cnidarians (corals, sea anemones, jellyfish and hydra) are morphologically simple animals surviving in an aquatic environment with potential predators, competitors and pathogenic microbes. Most of the cnidarians are also active venomous predators feeding on arthropods and fish. Their diversified proteins and peptide toxins are stored and delivered into the preys through the highly developed and specialized stinging cells, the nematocysts. In spite of the large variations in size and morphology, nematocysts share a common organelle, which comprises a cylindrical capsule containing a long hollow thread attached to it. During the discharge of nematocysts following a chemical or mechanical stimulus, the thread is expelled from within the capsule matrix in a harpoon-like fashion (Beckmann & Ozbek, 2012; Mariottini & Pane, 2013; Ozbek et al, 2009; Rachamim & Sher, 2012). Although representing one of the most complex organelles in animals, the evolutionary origin of the nematocyst remains largely unknown.

Molluscs (cone snails)

The molluscs (cone snails) include more than 750 species of venomous predatory marine gastropods. During the past 50 million years, cone snails have evolved into three general

feeding groups based on their prey preference: fish-hunters, worm-hunters and mollusc-hunters (Duda et al, 2001). The proboscis, which is a long, flexible, hydrostatically-supported appendage, is used by cone snails to sense and locate preys (Greene & Kohn, 1989) and is subsequently functions as a conduit to deliver immobilizing venom. To envenomate preys, cone snails inject a harpoon-like radular tooth into their preys, allowing toxins to be delivered through the hollow central canal of the tooth (Salisbury et al, 2010).

Arthropods

Except for their ingenious exploitation of silk, another remarkable evolutionary success of spiders is the evolution of pharmacologically complex venom that ensures rapid subjugation of preys. Spiders produce venom in paired glands that reside either in the basal segment of the chelicerae in primitive mygalomorph spiders or in the anterior of the prosoma in modern araneomorph spiders. A duct from each venom gland leads to a small opening near the tip of the corresponding fang. Compression of the muscles encircling each venom gland forces venom along the duct and out through the opening in the fang tip (King, 2004; King & Hardy, 2013).

Scorpions are one of the most ancient groups of terrestrial animals belonging to the class Arachnida within the phylum Arthropoda. Scorpions represent a basal branch of arachnids and have a relatively distant relationship with Acari (mites) and Araneae (spiders), the other two groups of the class Arachnida. Scorpion stings are specialized tools that are sharp enough to penetrate cutaneous tissue, and strong enough to withstand the

stress of making the puncture. The sharply pointed aculeus of the telson inflicts the wound. The expanded bulb houses a pair of venom glands, each with an exit duct leading to an aperture just before the tip of the aculeus (Berkov et al, 2008; Hjelle, 1990).

Centipedes have the modification of the first pair of walking legs into venomous appendages often called poison claws, forcipules, or maxillipeds. Venom is secreted through a pore located on the outer curvature near the tip of each claw, which again is connected to each maxilliped's venom gland through a chitinous venom duct. The venom-injecting forcipules of centipedes represent an evolutionary novelty that appeared in the centipede stem lineage more than 400 million years ago (MYA). No other lineage of arthropods (or indeed of animals) has evolved claws for injecting venom from a pair of walking legs (Dugon & Arthur, 2012; Undheim & King, 2011).

Hymenoptera are the large group of insects which includes bees, wasps and ants. Female hymenoptera possess specialized stinging apparatus with which they inject their venom into preys or intruders. Hymenopteran venom glands are epidermal glands that have evolved from female accessory reproductive glands. The venom apparatus of European honey bees (*Apis mellifera*) comprises a sting and a venom gland. A honey bee venom gland is a simple, long, thin, distally bifurcated structure, opening into an ovoid reservoir (Bridges & Owen, 1984; Kheyri et al, 2013). The basic morphology of the venom apparatus is quite uniform among Vespidae. The proper glandular portion consists of two relatively long tubules that drain, independently or through a short common tract, into a muscular sac-like structure, the gland reservoir. A single duct eventually conveys the venom from the reservoir to the sting. The elongated accessory Dufour's gland, directly connected with the sting, completes the venom apparatus (Petrocelli et al, 2014). The venom apparatus of the fire ant (*Solenopsis saevissima*) has been described with the aid of light and electron microscopy techniques, which mainly consists of a sting and venom sac (Fox et al, 2010).

Crustaceans are the only major traditional arthropod group of which no venomous species were known. Recently, von Reumont et al (2014a) provided the first conclusive evidence that the aquatic, blind, and cave-dwelling remipede crustaceans are venomous, indicating the evolving of venoms in all four major arthropod groups. Analysis of the venom delivery apparatus of the remipede *Speleonectes tulumensis* showed that remipedes can inject venom in a controlled manner. Synchrotron radiation micro-computer tomography (SR-μCT) was used to prepare the first three-dimensional reconstruction of the venom delivery apparatus of the remipede *S. tulumensis*. The anterior trunk of *S. tulumensis* contains two equally sized venom glands, which connect via ducts to reservoirs located in the terminal segments of a robust pair of legs (maxillules) in the head (von Reumont et al, 2014a).

Vertebrate venom systems

In vertebrates, venom systems have evolved several times independently. Besides well-known venomous snakes, lizards

and fishes, venom systems can also be found in mammals. However, the venom systems in mammals were neglected by scientists for centuries. The mammalian animals known or suspected to be venomous come from the species of Insectivora, Monotremata, Chiroptera, as well as primates, including Haitian solenodons (*Solenodon paradoxurus*), European water shrews (*Neomys fodiens*), American short-tailed shrews (*Blarina brevicauda*), platypus (*Ornithorhynchus anatinus*), vampire bats (such as *Desmodus rotundus*) and the slow lorises of Southeast Asia (*Nycticebus spp.*) (Ligabue-Braun et al, 2012; Nekaris et al, 2013).

Fish venomous spines Venomous ray-finned fishes are diverse and with habitats ranging from freshwater to seas. The known venomous fishes are mainly distributed among the catfishes (*Siluriformes*) and six groups of "acanthomorphs" or spiny-rayed fishes, like toadfishes and scorpionfishes, in which several thousand of species are presumed to be venomous (Smith & Wheeler, 2006; Wright, 2009). So, venomous fish may outnumber the combined diversity of all the other venomous vertebrates. Diverse phylogenetic distribution of venomous fishes results in variation in the morphology of fish venom apparatuses. Many fish species with venomous dorsal spines have distinct anterolateral grooves on the lateral surfaces of the fin spines, where the venom gland is situated. While venomous toadfishes have distinct venom glands surrounding their dorsal spines, the anterolateral grooves are absent. There are venomous grooved teeth in the lower jaw of saber-toothed blenny fishes, which deliver the venom (Smith & Wheeler, 2006). The venom glands of catfishes are found in association with sharp, bony spines along the leading edge of the dorsal and pectoral fins. When a spine enters a potential predator, the integument surrounding the venom gland cells is torn to deliver venom into the wound (Wright, 2009). It was proposed that the venom glands of fishes are originated from epidermal secretory cells. The toxic peptides of fish venoms may be derived from and are highly homologous to protein components in epidermal secretions (Tamura et al, 2011; Wright, 2009).

Snake fangs Snakes are the masters of venom delivery systems in terms of sophistication, efficiency, and diversity (Jackson, 2003). Elapids, viperids, and atractaspidids possess a large post-orbital gland in which venom is secreted and stored. It is enclosed in a fibrous sheath for the attachment of muscles. It has been suggested that all venom glands are the homologs of the Duvernoy's gland coined to refer to the venom gland of colubrid snakes, which appeared early in colubroid evolution and subsequently specialized independently into venom glands (Jackson, 2007). Many venomous snakes use tubular fangs, which are specialized teeth associated with a venom gland and are positioned either anterior or posterior in the upper jaw. Tubular fangs have a completely enclosed venom canal for the conduction of venom into a bite wound. An elegant study has been carried out by using the sonic hedgehog gene as a marker, and by three-dimensionally reconstructing the development of snake embryos from different species. Their findings put forward a new model for the evolution of snake fangs. The

developmental uncoupling of the posterior from the anterior tooth region could have allowed the posterior teeth to evolve independently and in close association with the venom gland. Subsequently, the posterior teeth and venom gland could have become modified and formed the fang-gland complex (Vonk et al, 2008).

Grooved teeth of lizards and insectivores The closest relatives of snakes are the anguimorphs (which include the venomous helodermatids) and iguanian lizards. The anguimorphs, iguanians and snakes, which form a well-resolved clade, are shown to be the only lineages possessing protein-secreting mandibular and/or maxillary glands (Fry et al, 2012). In contrast to venomous snakes, the venom of the venomous lizards in the genus *Heloderma* is produced by multi-compartmentalised glands on the lower jaw from which ducts lead onto grooved teeth along the length of the mandible. Recently, anguimorph lizards other than helodermatids and iguanian lizards have been shown to be venomous (Fry et al, 2009b; 2010a). This new perspective revealed that *Heloderma* and snake venom systems are homologous but highly differentiated descendants of an early-evolved venom system in squamates which possessed incipient venom glands in both the mandibular and maxillary regions, with snakes favouring the development of the maxillary venom gland and secondarily reducing the mandibular components, while the anguimorph lizards did the reverse (Fry et al, 2009b; 2012). In mammals, mildly toxic salivary secretions are associated with grooved teeth in some insectivores. The venomous species of insectivora have significantly enlarged and granular submaxillary salivary glands from which the toxic saliva is produced.

Platypus spurs and slow loris brachial glands Mammalian platypus has the bizarre crural venom system. Rather than delivering venom through a bite, as do shrews and vampire bats, male platypuses have venomous spurs on each hind leg, which is connected via a duct to venom glands evolved from modified sweat glands. The study on the platypus reveals strong convergence between reptile and mammal venomous systems (Whittington et al, 2008). The slow lorises are the only primates, which harbour toxins. It has been proposed that the venom is a mixture of fluid of its brachial gland located in the ventral side of the elbow with saliva, and is applied to the top of the head for defense or kept in the mouth to bite (Nekaris et al, 2013). Knowledge of mammalian venom is only in its infancy, and that even more species of mammals may harbour venomous adaptations. The study of chemical and genetic aspects of venom can help to elucidate the evolution of this trait in mammals (Ligabue-Braun et al, 2012; Nekaris et al, 2013).

Biological roles of venoms

The ecological advantages conferred by the possession of a venom system are evident from the extraordinarily diverse range of animals that have evolved venoms. Animals' venoms serve a variety of functions. The three most common uses are predation or resource acquisition, defense and reduction of

competition.

Predation

The evolution of animal venoms is thought to be a typical predatory adaptation (Daltry et al, 1996; Fry et al, 2009a). First, selection for immobilization favors venoms that are fast acting and directly influence mobility and coordination. For this reason, many types of venom include neurotoxic components that disrupt information transfer in nerves or muscles. Snakes, scorpions, spiders, centipedes, and cone snails all produce different neurotoxins that act on key physiological elements of neurotransmission, such as cell membrane ion channels and receptors.

Second, disrupting blood coagulation system is another effective way to disturb the key physiological process and facilitate predation, which is a strategy used by many viperid snakes. Accordingly, their venoms contain numerous haemotoxins, which act on almost all the elements of blood coagulation and fibrinolytic systems (Kini, 2011). Vampire bats are highly specialized mammals, with their entire physiology modified to use blood as their only source of food and water. To do so, the bats have modified sharp teeth, anticoagulants in their saliva and a specialized tongue. The evolution of anticoagulants in the saliva of the three different vampire bat species revealed transitions in their preferred preys (Ligabue-Braun et al, 2012).

Third, excessive and uncontrolled proinflammatory and immune reactions can also cause heavy toxicity, even death (Medzhitov, 2010a; Palm et al, 2012). Even often neglected, the immune system of prey must be an important target of venoms. This is the strategy of some type of venomous animals used, and the related components called "immunotoxins" exist in their venoms. In fact, manipulating host defense mechanisms by venoms has been reported for some venomous animals like ticks (Cabezas-Cruz & Valdés, 2014). For successfully and effectively sucking blood, horsefly and leech venoms contain many components acting on blood coagulation and immune systems (Ma et al, 2009; Min et al, 2010). This notion is further supported by natterins and nattering-like toxins in fishes, and the action of these toxins on immune system (Lopes-Ferreira et al, 2014).

Defense

Venoms serve a defensive role, but this function is thought to be secondary (Brodie, 2009; Fry et al, 2009a). A range of venom components could be used for defensive purpose, like peptide toxins, alkaloids, protease inhibitors that prevent digestion, and other compounds that cause organism insult. Interestingly, some compounds elicit specific behaviors in predators, like the peptides in the skin mucus of *Xenopus* frogs that stimulate uncontrollable yawning and gaping that allow the frogs to crawl out of the mouth of snakes (Brodie, 2009).

Traditionally, venomous animals are thought to inject the same combination of toxins for both predation and defense. However, recent studies showed that cone snails can rapidly switch between distinct venoms in response to predatory or defensive stimuli. Predation- and defense-evoked venoms

originate from the distal and proximal regions of the venom duct, respectively, explaining how different stimuli can generate two distinct venoms (Dutertre et al, 2014a). Geography cone (*Conus geographus*) is the most dangerous cone snail species known, with reported human fatality rates as high as 65%. To study the venom that is directly relevant to human envenomation, the defense-evoked venom of several specimens of *C. geographus* was analyzed. The molecular composition of individual defense-evoked venom showed significant intraspecific variations (Dutertre et al, 2014b).

Competition

The role of ant venoms in ecological competition among ant species has been reported. The raspberry crazy ant (*Nylanderia fulva*) applies abdominal exocrine gland secretions to detoxify fire ant (*Solenopsis invicta*) venom. This capacity to detoxify a major competitor's venom probably contributes substantially to its ability to displace fire ant populations (Lebrun et al, 2014).

The platypus venom gland is seasonally active and secretes venom only during the short annual breeding season, suggesting that it has evolved primarily as an offensive weapon for use in conspecific aggression to assert dominance over other male platypuses (Grant & Temple-Smith, 1998). Most of the evidence now supports the proposition that the venom is used by males as a weapon when competing for females, taking part in sexual selection (Ligabue-Braun et al, 2012).

The venom of slow lorises can cause death in small mammals and anaphylactic shock and death in humans. Wild field and laboratory studies have been conducted for attempting to understand the function and ecological role of loris venom. The least evidence is found for the hypothesis that loris venom is evolved to kill preys. It was suggested that the venom's primary function in nature seems to be as a defense against parasites and conspecifics. It may also serve to threat olfactory-orientated predators (Nekaris et al, 2013). Further detailed studies on the ecology, habitat use and phylogenetic relationships of slow lorises may shed light on this topic.

Concerning the biological function of the venoms in insectivores, the venom as a weapon for intraspecific competition should be considered (Ligabue-Braun et al, 2012). Scleractinian coral colonies and many actinarians (anemones) use venom for predation and defense, but also possess specialized tentacles to attack other nearby colonies, thereby protecting and expanding their own territory in the context of intraspecific and interspecific competition for space (Nelsen et al, 2014; Williams, 1991)

Antimicrobial defense

Recent studies showed that there are many venom components that possess strong antimicrobial activity. Defensin and cathelicidin are the two main families of naturally occurring antimicrobial peptides, which exhibit potent microbicidal properties against bacteria, fungi, and some viruses. Cathelicidin-type antimicrobial peptides have been identified from elapid snake venoms (Zhao et al, 2008). OH-CATH30 peptide exerted potent antibacterial activity, selective immunomodulatory properties, and low toxicity to eukaryotic

cells (Li et al, 2012; 2013). In scorpion venoms, many peptides with antimicrobial, antiviral, antimalarial, immuno-modulating activities were also identified (Almaaytah & Albalas, 2014; Ortiz et al, 2015). In predation, numerous microbes exist in preys. It is reasonable to speculate that the existence of antimicrobial agents in the venom may function to prevent the potential infection caused in the process of predation. In addition to the use of venom for self and/or colony defense, some hymenopterans also spray their 'venom' to keep their broods free of parasites in the context of hygiene (Oi & Pereira, 1993).

Communication

Ants use many different chemical compounds to communicate with their nestmates. Foraging success depends on how efficiently ants communicate the presence of food and thus recruit workers to exploit the food resource. Trail pheromones, produced by different exocrine glands, are a key part of ant foraging strategies. In the subfamily *Myrmicinae*, trail pheromones are mostly produced in the venom gland (Cerdá et al, 2014). Fire ant venom components act as key attractants for the parasitic phorid fly, *Pseudacteon tricuspis* (Diptera: Phoridae), indicating the role of ant venom as attractants for their natural enemies (Chen et al, 2009). Many trail pheromones identified in the venom glands of ants are small molecular organic compounds, such as alkaloids, etc. The possible role of venom proteins and peptide toxins in ant communication is an interesting open question for further investigation.

Venom loss

Because venoms are protein-rich, they come with a considerable metabolic and biochemical price. The "venom optimization hypothesis" postulates that venom is metabolically expensive and therefore is used frugally through behavioral control (Wigger et al, 2002). The metabolic cost of venom is sufficiently high to result in secondary loss of venom whenever its use becomes non-essential to survival of the animal (Morgenstern & King, 2013). There are multiple examples of secondary loss of venom in the evolution processes of animals. It has been reported that a dinucleotide deletion in the only expressed toxin gene in sea snakes (*Aipysurus eydouxii*), resulting in an inactive form of the toxin. This is a result of the change in its dietary habit from fish to fish eggs, showing how the change in ecology subsequently significantly affected the composition of the venom (Li et al, 2005). All spiders are predators and have venom glands, with the exception of the hackled orbweavers (*Uloboridae*) and certain species of primitive mesothelids. Venom has also been secondarily lost in uloborid spiders which instead kill their prey by wrapping them tightly in hackled silk (King, 2004).

Toxins in animal venoms

Selection pressures and animal toxins

Ecological conditions play important roles in the natural selection of toxin compositions. The evolution of venom molecular components is often linked to diet and trophic

ecology through an evolutionary arms race between predators and preys. Specific resistance to snake venoms has evolved in both natural preys and predators of snakes (Biardi & Coss, 2011; Heatwole & Powell, 1998; Jansa & Voss, 2011). Venom resistance in natural preys and predators provides a selective pressure on snakes to develop venom that is of higher toxicity, which in turn selects for increased resistance in sympatric prey populations. This continuum results in a predator-prey co-evolutionary 'arms race' synonymous with Van Valen's "red queen hypothesis" (Richards et al, 2012).

As mentioned above, an additional evolutionary challenge for venomous animals is that toxins synthesis appears to carry an appreciable metabolic cost, which leads to the optimization of venom toxins to adapt different preys and predators in different ecological conditions. Thus, the variations of ecological context and long-term coevolution have created extensive diversified proteins and peptide toxins, which specifically act on targeted organisms. Significant variation in venom compositions and toxin molecules in the same animal species has been often detected from the venom samples collected from different places and/or times.

Evolutionary origin and genetic basis of animal toxins

In the immune system of vertebrates, three major gene families, namely the MHC, T cell receptor (TCR), and Ig gene families, play an important role in identifying and removing invading microbes like virus, bacteria, and eukaryotic parasites. These immune genes are believed to evolve via the 'birth and death' process of gene evolution (Nei, 1969; Nei et al, 1997). In this model of evolution, duplicate genes are produced by various mechanisms, including tandem and block gene duplication, and some of the duplicate genes diverge functionally but others become pseudogenes owing to deleterious mutations or are deleted from the genome. The end result of this mode of evolution is a multigene family with a mixture of divergent groups of genes and highly homologous genes within groups plus a substantial number of pseudogenes (Nei et al, 1997).

Similarly, many venom toxins are believed to be originated through evolutionary process by which a gene encoding a normal 'physiological' body protein, usually one involved in key regulatory processes or bioactivity, is duplicated and a duplicate copy selectively expressed in the venom gland, resulting in large multilocus gene families that encode toxins exhibiting a variety of functional activities and potencies (Casewell et al, 2013; Fry et al, 2009a).

Venom toxins are often characterized by accelerated evolution and positive selection, especially on amino acid residues that are surface-exposed on the protein macromolecular structure (Jiang et al, 2011; Kordis & Gubenšek, 2000). Thus, gene duplication, positive selection, and protein neofunctionalization are major genetic elements to work in unison to provide the evolutionary novelty that allows adaptation of venom toxins to different requirements under various biological contexts. Gene duplication is not a prerequisite for toxin recruitment. Some identified toxins are simply modified, alternatively spliced, or generated through

alterations in the structure of domains of gene loci that are physiologically expressed in non-venomous taxa and therefore appear to have been 'hijacked' for a role in venom (Casewell et al, 2013).

Huge molecular diversity of animal toxins

In recent years, the information on molecular diversity of animal toxins is explosively increasing because the applications of modern techniques. It has become clear that the animals' proteins and peptide toxins are considerably more complex than previously realized via proteomic and transcriptomic analyses of the venom compositions of venomous animals. The extensive diversification of toxins may have been driven by extreme diversification of physiological elements of potential preys and predators in evolutionary processes (Figure 2).

Cnidarians The toxicity of *Cnidaria* is a subject of concern for its influence on human activities and public health, as well as a potential source of natural bioactive compounds useful to develop new drugs or biomedical materials (Mariottini & Pane, 2013).

Jellyfish Jellyfish *Stomolophus meleagris* is one of the most dangerous jellyfish in China sea. People stung by the jellyfish would suffer itch, edema, myalgia, dyspnea, hypotension, shock, and even death. The venom of *S. meleagris* contains various toxins including serine protease inhibitors, PLA2, potassium channel inhibitors, metalloproteases, C-type lectins, hemolysins, cytotoxins, cardiotoxins and neurotoxins. The identified toxins are probably related to the sting caused by the jellyfish (Li et al, 2014a).

Sea anemone Sea anemone toxins comprise mainly proteins and peptides, including different ion channel modulators, cytolytic, protease inhibitors and PLA2s, which are efficient in targeting different preys (Frazão et al, 2012). The neurotoxic fractions from the exudates of *Stichodactyla helianthus* and *Bunodosoma granulifera* were analyzed by reversed-phase chromatography and mass spectrometry. The resulting fractions were analyzed by their toxicity to crabs. The first peptide fingerprints of these sea anemones were assessed, revealing the largest number of peptide components (about 156 peptides) so far found in sea anemone species (Rodríguez et al, 2012).

Hydra Like in other cnidarians, hydra polypeptide toxins are expressed mainly in nematocysts and represent a highly complex array of effector molecules aimed at paralyzing a prey and disintegrating its tissue (Rachamim & Sher, 2012). The proteome of nematocysts from the freshwater polyp *Hydra magnipapillata* has been reported, which revealed an unexpectedly complex secretome of 410 proteins, from which 55 toxin-related sequences were found to be homologous with toxins in other venomous animals. These include neurotoxins, cytolytic, toxic phospholipases, many peptidases, and proteins of the SCP_GAPR-1-like family. The molecular masses of the toxins mainly range from 25 to 100×10³

(Balasubramanian et al, 2012). Small peptide toxins affecting ion channels identified in many sea anemones have not been determined in this analysis.

Molluscs (cone snails) Cone snails (*Conus* species) are predatory molluscs that inhabit tropical and subtropical shallow seawater. The systematic mining of fish-hunting cone snail toxins began 30 years ago. Extensive studies revealed that their venom ducts produce a mixture of peptides, generally known as conotoxins, having exquisite specificity for different ion channels, receptors, and transporters (Olivera et al, 1985; 1990). They are mostly short disulfide-rich peptides of 10 to 40 amino acids with remarkable structural diversities. An emerging enigma concerning conotoxins is their striking diversity. It was estimated that each *Conus* species could produce more than 1 000 different conotoxins (Biass et al, 2009; Davis et al, 2009). Conotoxin-encoding transcripts are diversified by hypermutation, fragment insertion/deletion, and mutation-induced premature termination, and a single mRNA species can produce multiple toxin products through alternative post-translational modifications and alternative cleavages of the translated precursor (Lu et al, 2014).

Annelids (leeches) Leeches are hematophagous annelids. They penetrate the body surface of the host and have to take measures to inhibit the normal reactions in host tissues to blood vessel damage, including blood coagulation, swelling, pain and inflammation. Long term evolution made leeches have acquired the ability to control these processes in their hosts by transferring various bioactive substances to the host through tiny salivary ductile (Baskova et al, 2008; Lemke et al, 2013). An expressed sequence tag (EST) library-based analysis of the salivary transcriptome of the North American medicinal leech (*Macrobdella decora*) revealed a complex cocktail of anticoagulants and other bioactive secreted proteins, including saratin, bdellin, destabilase, hirudin, decorsin, endoglucuronidase, antistatin, and eglin, as well as to other previously uncharacterized serine protease inhibitors, lectoxin-like c-type lectins, ficolin, disintegrins and histidine-rich proteins (Min et al, 2010).

Arthropods

Spiders Spiders (order *Araneae*) are the most successful venomous animals in term of their species and toxin diversification, and spider venoms have been intensively investigated. The major components of most spider venoms are small disulfide-bridged peptides, and more than 1 000 spider toxins have been characterized from about 90 species (Herzig et al, 2011). From Chinese bird spider (*Ornithoctonus huwena*), 626 toxin precursor sequences in total were retrieved from the transcriptomic data and were clustered into 16 gene superfamilies, including six novel superfamilies and six novel cysteine patterns (Zhang et al, 2014). Many spider toxins described to date contain an unusual structural motif known as an inhibitor cystine knot, which is typically highly resistant to proteases, acidic pH, high temperatures and organic solvents (Saez et al, 2010). Spider toxins mainly target on various ion

channels and exhibit a range of pharmacological activities, including Ca^{2+} , K^+ , Na^+ channels, transient receptor potential (TRP) channels, mechanosensitive channels, acid-sensing ion channels (ASICs), glutamate receptors and glutamate transporters (King & Hardy, 2013).

Scorpions Though scorpions are a small arachnid group, they constitute a very well adapted order of predatory animals that have been living in the Earth for nearly 400 million years (Polis, 1990). Individual scorpion venoms often contain as many as several hundred components (Almeida et al, 2012; Xu et al, 2014), and by coupling with measures of taxonomic diversities of scorpions, this has led to estimates of ~100 000 bioactive peptides in the venoms of scorpions (King, 2011). Scorpion cysteine-stabilised α/β (CS α/β) toxins are disulfide-bridged peptides with a significantly constrained structure, possess pharmacological action on ion channels, including Ca^{2+} , Na^+ , K^+ , Cl^- channels (Ortiz et al, 2015). Non-disulfide-bridged peptides constitute an important group of scorpion venom components. The pharmacological properties of these linear peptides include antimicrobial, cytolytic, antiviral, antimalarial, bradykinin potentiating and immuno-modulating activities (Almaaytah & Albalas, 2014). Interestingly, it has been shown that a majority of CS α/β toxin scaffolds have experienced episodic influence of positive selection, while most non-CS α/β linear toxins evolve under the extreme influence of negative selections (Sunagar et al, 2013).

Centipedes Centipedes are excellent predatory arthropods. Recently, centipede *Scolopendra subspinipes dehaani* venom was systematically investigated by transcriptomic and proteomic analysis coupled with biological function assays. In total, 543 venom proteins and peptides were cloned, and 50 proteins/peptides were purified from the venom (Liu et al, 2012). In another report, 26 neurotoxin-like peptides belonging to 10 groups were identified from the venom of *Scolopendra subspinipes mutilans* (Yang et al, 2012). The purified toxins mainly possessed various ion channel modulating properties. Most of them showed no significant sequence similarity to other proteins and peptides deposited in the known public database. These works provide a novel reservoir of mining ion channel modulating agents. Furthermore, a selective $\text{Na}_v1.7$ inhibitor (named $\mu\text{-SLPTX-Ssm6a}$) with analgesic efficacy as assayed in rodent pain models was discovered, which might be a promising lead molecule for the development of novel analgesics targeting $\text{Na}_v1.7$ (Yang et al, 2013).

Bees and wasps An in-depth study of honeybee (*Apis mellifera*) venom proteome revealed an unexpectedly rich venom composition, in which in total of 102 proteins and peptides were found. A group of 33 putative toxins is proposed to contribute to venom activity by exerting toxic functions or by playing a role in social community (Van Vaerenbergh et al, 2014). There are two major forms of honeybee venom used in pharmacological applications: manually extracted glandular venom, and venom extracted through the use of electrical

stimulation. A proteome comparison data demonstrated that these two venom forms are different in their compositions, which are important in their use as pharmacological agents (Li et al, 2013a). An optimized experimental protocol was used for the detection of peptides in the venom of the social wasp *Polybia paulista*. The results revealed a surprisingly high level of intra- and inter-colonial variability for the same wasp species, which detected 78-108 different peptides in the venom of different colonies of *P. paulista* with molecular mass range from 400 to $3\,000 \times 10^3$; among those, only 36 and 44 common peptides were observed in the inter- and intra-colony comparisons, respectively (Dias et al, 2014).

Ants Ants (Hymenoptera, Formicidae) represent a taxonomically diverse group of arthropods comprising more than 10 000 of species. Ant venom components exhibit a variety of biological activities, including antimicrobial, haemolytic, cytolytic, paralytic, insecticidal and pain-producing activities (Aili et al, 2014). Transcriptomic analysis for Brazilian ant (*Tetramorium bicarinatum*) venom revealed a high diversification of the venom components, including venom allergens, distinct isoforms of PLA1 and PLA2, serine proteases, hyaluronidases, protease inhibitors, secapin, waprins-like and agatoxins (Bouzid et al, 2014). About 40% of the generated sequences have no hits in the databases, emphasizing the existence of many new unknown molecules. From the venom gland of the predatory giant ant *Dinoponera quadricaps*, inhibitor cysteine-knot (ICK)-like toxins, insect allergens, enzymes, and lethal toxins were determined (Torres et al, 2014). Ant venoms, similar to those of bees and wasps, contain many allergens, which are the most frequent elicitors of anaphylaxis in humans.

Sharing some common toxins in venoms, each species of ants appears to have a number of unique components. Interestingly, the nesting habits of ants have deeply influenced their venom toxicity and composition. In ant genus *Pseudomyrmex*, the venom of the ground-dwelling species, *Pseudomyrmex termitarius* is composed of 87 linear peptides. However, the venoms of the arboreal and the plant-ant species, *P. penetrator* and *P. gracilis*, contain 26 and 23 peptides with disulfide bonds, respectively (Touchard et al, 2014). The large number of peptides in *P. termitarius* venom is likely related to potential prey diversity plus the antibacterial peptides required for nesting in the ground.

Ticks and horseflies As haematophagous arthropods and for biological success, ticks use their salivary constituents to successfully obtain a blood meal by targeting major physiological pathways involved in host defense mechanisms. The resulting feeding site also becomes a favorable environment for many pathogens to exploiting ticks to facilitate their transmission to the host (Wikel, 2013). It has been reported that tick salivary gland extract inhibits host complement activation and depresses macrophage function by inhibiting lipopolysaccharide (LPS)-induced nitric-oxide synthesis and proinflammatory cytokine production (Cabezas-Cruz & Valdés, 2014; Stibrániová et al, 2013).

In traditional Eastern medicine, horseflies are used as anti-thrombosis material for hundreds of years. Similar to other hematophagous arthropods, such as mosquitoes (Arcà et al, 1999), several families of proteins or peptides, which act mainly on the hemostatic system or immune system of the host, were identified in the horsefly *Tabanus yao* salivary glands. These include fibrinogenolytic enzymes, RGD-containing anti-platelet aggregation disintegrins, thrombin inhibitors, vasodilator peptides, peroxidase and apyrase (Ma et al, 2009; Xu et al, 2008). The diversity of anti-thrombosis components in horsefly saliva reflects the molecular basis of its blood-sucking living strategy.

Echinoderms

Starfishes and sea urchins

Starfishes and sea urchins are the popular name for marine invertebrates that belong to the phylum Echinodermata. Comparatively speaking, studies on their venoms are still in a primitive stage. Some species of starfishes and sea urchins are dangerous to humans. When stung by the venomous spines on the surface of crown-of-thorns starfish (*Acanthaster planci*), various pathological symptoms, such as severe pain, redness, swelling, and protracted vomiting, are induced (Sato et al, 2008). The crude venom extracted from the spines exhibits diverse biological effects, including hemolytic, mouse lethal, edema-forming, PLA2, anticoagulant and cytotoxic activities (Butzke & Luch, 2010; Lee et al, 2014). In the case of sea urchins, envenomations are caused by stings from either pedicellariae or spines (Balhara & Stolbach, 2014). A galactose-binding lectin SUL-I was isolated from the venom of sea urchin *Toxopneustes pileolus*, which showed mitogenic, chemotactic, and cytotoxic activities through binding to carbohydrate chains on cells (Hatakeyama et al, 2014). Cathepsin B/X was found to be secreted by *Echinometra lucunter* sea urchin spines, a structure rich in granular cells and toxins, which was thought to participate in the inflammatory response to the accident (Sciani et al, 2013).

Venomous vertebrates

Fishes Despite the large number of species, compared with other groups of venomous organisms, the study on fish venoms is still in a relatively preliminary state and fish venoms are neglected source of bioactive proteins and peptides. Protein toxins natterins were characterized from Brazilian venomous fish *Thalassophryne nattereri* (Magalhães et al, 2005). Natterins and their analogues might be widely distributed in the fish venom glands, thereby forming one family of fish venom toxins (Tamura et al, 2011). The difficulty in the study of fish venoms is that the venoms are sensitive to heat, pH, and lyophilization, as well as are often contaminated with mucus components. A novel protein-handling protocol has been developed recently, upon which the investigation of fish venom composition using barb tissue from the blue-spotted stingray (*Neotrygon kuhlii*) was carried out. The results revealed a variety of protein types that are novel to animal toxins. Putative venom toxins identified include cystatin, peroxiredoxin and galectin (Baumann et al, 2014).

Amphibians Amphibians might not be considered as typical venomous animals due to the lack of a venom delivery system. Amphibian skin is naked to fulfill special physiological requirements, such as respiration and water-salt balance (Campbell et al, 2012; Duellman & Trueb, 1994). Thus, the skin has to form a special defense system to withstand constantly confronted injurious mechanical, chemical and biological factors. Defensive (innate immunity) responses against potential invading of pathogens and repairing capacity of the disrupted surface layer of cells are essential (Voyles et al, 2009). Amphibian skin contains an arsenal of bioactive molecules to fulfill the related functions (König et al, 2014; Zhang, 2006). Indeed, there are many poisonous frogs, including *Dendrobatidae*, *Mantellidae*, *Bufo* and *Myobatrachidae*, which are very "toxic" to mammals and caused by alkaloids sequestered from dietary alkaloid-containing arthropods (Daly et al, 2005; Hantak et al, 2013). The toxicity of some amphibian species to mammals results from physiological proteins and peptides secreted in the skin mucus (Lai et al, 2002a; 2002b; Liu et al, 2008; Qian et al, 2008a; 2008b). Many amphibian skin peptides are related to mammalian hormones or neurotransmitters, as well as antimicrobial peptides (Xu & Lai, 2015; Zhang, 2006).

Several hundreds of peptides were identified from Chinese odorous frogs (Li et al, 2007; Yang et al, 2012b). The function of frog skin peptides are diverse, including antimicrobial, antioxidant, immunomodulatory, and metabolic regulatory activities (Conlon et al, 2014; Yang et al, 2012b). Under environmental pressure, gene duplication, rapid mutation at the amino acid level, domain shuffling and conversion are among the major forces in the formation of heavy diversification of peptides in frog skin (Duda et al, 2002; Lee et al, 2005; Roelants et al, 2013). This evolution pattern is very similar to those of toxins in venomous animals.

Snakes Snake venoms comprise a diverse array of toxins that have a variety of pharmacological and toxicological effects, and are conveniently classified as hemotoxic and neurotoxic (Du, 2006; Kini, 2011; Kularatne & Senanayake, 2014). Most of the snake toxins were recruited or derived from the normal body proteins in the common ancestor of venomous squamates (Toxicofera) or advanced snakes (Caenophidia) during 100–200 MYA (Fry, 2005; Fry et al, 2009b; 2012). By using cutting-edge proteomic and transcriptomic approaches, the venomomics of various venomous snake species have been conducted (Brahma et al, 2015; Calvete, 2014).

The toxin profiles of elapid snakes *Naja naja* and *Bungarus multicinctus* were analyzed by sequencing their venom gland transcriptomes (Jiang et al, 2011). Totally 1 092 valid expressed sequences tags (ESTs) for *B. multicinctus* and 1 172 ESTs for *N. atra* were generated. The major components of *B. multicinctus* venom are neurotoxins, including long chain alpha-neurotoxins and recently originated beta-bungarotoxin, whereas, *N. atra* venom mainly contains 3FTs with cytotoxicity and neurotoxicity (short chain alpha-neurotoxins). A recent expansion of alpha-neurotoxins genes in *N. atra* was observed. Tandem duplications contributed the most to the expansion of toxin

multigene families. Furthermore, not only the multigene toxin families but also the less abundant toxins were under rapid adaptive evolution (Jiang et al, 2011).

Lizards The lizards of genus *Heloderma*, which live in the south-western part of the North American continent, have been recognized as venomous for more than a century. Envenomations of humans by helodermatid lizards may cause complicated symptoms including extreme pain, acute local swelling, nausea, fever, hypotension, and inhibition of blood coagulation (Koludarov et al, 2014). Lizard venoms contain a cocktail of different proteins and peptides including hyaluronidase, PLA2s, kallikrein-like proteases, helokinestatin, helofensin, as well as bioactive peptides including hormone-like exendin peptides (Fry et al, 2010a; 2010b). In a recent study attempting to characterize the gila monster (*Heloderma suspectum suspectum*) venom proteome, a total of 39 different proteins were identified out of the 58 selected spots that represent the major constituents of the venom. A neuroendocrine convertase 1 homolog was identified, which is likely to convert the proforms of exendins into the mature and active forms (Sanggaard et al, 2015).

Venomous mammals

The northern short-tailed shrew (*Blarina brevicauda*) saliva contains blarina toxin (Kita et al, 2004) showing kallikrein-like protease activity. This toxin cleaves kininogens to release kinins, including bradykinin, which are inflammation mediators. Blarina toxin shows sequence homologous to gila toxin and horridum toxin, two toxins from the Mexican beaded lizard. Blarina toxin and gila toxin have served as nice molecular models to study the structural basis of transition from a non-toxic to a toxic kallikrein, which is also a good example of convergent evolution at the molecular level (Aminetzach et al, 2009). Two distinct classes of anticoagulants are found in the saliva of vampire bats, i.e., plasminogen activators and inhibitors of proteinases (Ligabue-Braun et al, 2012).

The platypus venom contains natriuretic peptides, defensin-like peptides, nerve growth factors, isomerases, hyaluronidase, proteases, mammalian stress response proteins, cytokines, and other immune molecules (Wong et al, 2012). Gene duplication and subsequent functional diversification of beta-defensins gave rise to platypus *Ornithorhynchus* venom defensin-like peptides (Whittington et al, 2008). The brachial gland exudates of primate slow lorises contain a new member of the secretoglobulin family, which is a 17.6×10^3 heterodimeric protein homologous to Fel 1d, the major allergen from domestic cat (Nekaris et al, 2013). This is in accordance with the variable sensitivity to loris bites and the onset of anaphylaxis caused.

Neglected Venomous animals

Recent technological advances dramatically accelerate research into neglected or even completely unstudied venomous taxa. A transcriptomic profile analysis of the venom glands of the remiped crustaceans (*Speleonectes tulumensis*) showed that they express a unique cocktail of transcripts coding for known venom toxins, including a diversity of enzymes and a

probable paralytic neurotoxin very similar to one described from spider venom (von Reumont et al, 2014a). Glycerids are marine annelids commonly known as bloodworms, which prey on invertebrates, and their venom glands produce compounds that can induce toxic effects in animals. The transcriptomic profiles of the venom glands of three species of bloodworm, *Glycera dibranchiata*, *G. fallax* and *G. tridactyla* have been reported (von Reumont et al, 2014b). The toxins represent five functional categories: pore-forming and membrane-disrupting toxins, neurotoxins, protease inhibitors, other enzymes, and CAP domain toxins. The vast majority of neglected venomous taxa are invertebrates. The study of neglected venomous taxa is necessary both for understanding the full diversity of venom systems that have evolved in the animal kingdom, and to robustly answer fundamental questions about the biology and evolution of venoms (von Reumont et al, 2014c).

Genome of venomous animals

Recently, the whole genome information of many venomous animals has become available. These include the genome of sea anemone *Nematostella vectensis* (Putnam et al, 2007), *Hydra magnipapillata* (Chapman et al, 2010), leech *Helobdella robusta* (Simakov et al, 2013), Western honey bee (*Apis mellifera*), Asian honey bee (*Apis cerana*) (Park et al, 2015), five ant species and three solitary hymenopterans in the parasitoid jewel wasp genus (Fischman et al, 2011), scorpion *Mesobuthus martensii* (Cao et al, 2013b), king cobra snake (*Ophiophagus Hannah*) (Vonk et al, 2013) and centipede *Strigamia maritima* (Chipman et al, 2014). The advances should greatly help in understanding the molecular diversity of animal venomics. However, it should be emphasized that in the annotation of the genome sequence, the information from venom transcriptoms and proteomics is very important and even crucial, especially in the cases that many potential venom proteins and peptides are complete new. In addition, data collected from transcriptomic approach are needed to be validated whether the transcripts indeed code for active venom toxins.

Venomous animals in China

China has a vast territory with highly diversified topography, climate and vegetation, and a wealth of animal and plant resources. China is ranked eighth in the world and first in the Northern Hemisphere on richness of biodiversity. China is the home for approximately 10% of the world's biodiversity (Zhang & Ge, 2007), which provides rich resources for studying venomous animals.

Marine venomous animals

Jellyfish belongs to the phylum Cnidarians. The phylum is subdivided into five classes: *Staurozoa* (*Stauromedusae*), *Scyphozoa* (true jellyfish), *Hydrozoa* (fire corals and hydroids), *Cubozoa* (box jellyfish), and *Anthozoa* (sea anemones and true corals), and is composed of about 10 000 species, with 100 of them known to be dangerous to humans (Kayal et al, 2013; Cegolon et al, 2013). Sea anemones (order *Actiniaria*) are among the most diverse and successful members of the

anthozoan subclass *Hexacorallia*, occupying benthic marine habitats across all depths and latitudes. *Actiniaria* comprises approximately 1 200 species of solitary and skeleton-less polyps and lacks any anatomical synapomorphy (Rodríguez et al, 2014). The investigation of the actual distribution and species diversification of cnidarians in China is still in its infancy, and according to the present data, there are roughly 200 and 110 species described for jellyfishes and sea anemones, respectively (Li, 2013; Liu, 2008; Liu, 2013a; Pei, 1998;).

The gastropod family *Conidae*, commonly known as cone snails, includes the widely distributed, mainly tropical *Conus*, a relatively young genus first appearing in the Early Eocene. *Conidae* is one of the most diverse animals in the marine environment, with more than 760 valid species currently recognized in the World Register of Marine Species (WoRMS, 2013) (Puillandre et al, 2014). All cone snails whose feeding biology is known inject venom into large prey animals and swallow them whole. Works based on cone snail specimen collected along Chinese coastal waters described roughly 100 species, and most of them were found in the South China Sea (Li, 1999; Liu, 2008). Obviously, the record is largely incomplete due to the lack of systematic investigation of marine biodiversity in China seas (Liu, 2011).

Land venomous animals

Spiders are common in daily human experience because of their biodiversity, wide distribution and abundance in favorable seasons. Emerged about 400 MYA and developed along the long evolutionary course of insects, spiders prey essentially on insects for subsistence. Spiders are the most speciose venomous animals and are the most successful terrestrial predators, with over 45 000 extant species described to date according to the record in World Spider Catalog (version 16) (<http://www.wsc.nmbe.ch>) of American Museum of Natural History. Until now, there are about 2 600 spider species described in China, in which about 700 are found in Yunnan Province (Song et al, 1999; Yang, 2006).

Scorpions are a small arachnid group. Until now, there are 15 families, 197 genera and 2 069 species recorded in the world. About 30 of them are recognized as potentially dangerous for humans. They all belong to the family of *Buthidae* which includes nearly 80 genera distributed in both the old and new worlds (Chippaux & Goyffon, 2008). The recorded scorpion fauna of China consists of 53 species and subspecies belonging to 12 genera, 5 families, including 33 species (62.3%) and 1 genus recorded as endemic (Di et al, 2014).

Centipedes (*Chilopoda*), one of the four major lineages of myriapods, are an important group of predatory arthropods in many terrestrial habitats. They comprise approximately 3 300 species belonging to the five extant orders: *Scutigromorpha*, *Lithobiomorpha*, *Craterostigmomorpha*, *Geophilomorpha* and *Scolopendromorpha* (Edgecombe & Giribet, 2007). In China, 30 species belonging to 5 genera of 3 families in the order *Scolopendromorpha* have been recorded (Song, 2004). In the order *Lithobiomorpha*, 83 species belonging to 15 genera, among which one genus and 25 species are new to science, have been described (Ma, 2007).

Bees are arguably the most important group of angiosperm-pollinating insects. They arose in the early to mid-Cretaceous approximately 140 to 110 MYA, roughly coincident with the origins and early diversification of flowering plants. Bees comprise nearly 20 000 described species (Danforth et al, 2013). There are about 4 200 described vespidae species currently classified into 6 subfamilies based on morphological evidence (Hines et al, 2007). The current known species of bees and wasps (*Vespidae*) in China are about 1 000 and 200, respectively (Li, 1985; Wu, 2000).

According to The Reptile Database website (<http://reptile-database.reptarium.cz/>), there are about 3 500 snake species that exist in the world, in which about 750 are venomous. In China, there are about 220 snake species recorded, and about 60 are venomous snakes (Cai et al, 2012; Zhao, 2006). Currently, there are 360 known amphibian species distributed in China, including 210 species (58.3%) are recorded as endemic. Three Chinese regions are particularly rich in amphibian diversity: Hengduan, Nanling, and Wuyi mountains, and habitat loss, pollution, and over-harvesting are the most serious threats to Chinese amphibians (Fei, 1999; Xie et al, 2007).

Less than 0.1% of toxins have been explored

The approximate number of venomous animals known in China at present time is listed for each group, as well as the estimated number of proteins and peptides in their venoms based on present data of venom proteomic and/or transcriptomic analysis (Table 1). Exploring molecular diversities of animal toxins in China has been expanded rapidly. According to the 2014 report of Chinese 973 project term, the venomomics of 49 venomous animals have been investigated by transcriptomic and proteomic analysis in the past five years, in which about 5 000 toxin sequences of good quality were obtained and about 1 000 proteins and peptide toxins were purified and characterized biochemically. An online Animal Toxin Database has been established (ATDB 2.0, <http://protchem.hunnu.edu.cn/toxin/>), which embodies the updated information concerning toxin structure, biological function and their targets. In terms of the protein and peptide molecules estimated in Chinese venomous animals (Table 1), less than 0.1% of toxins have been explored.

FROM FOE TO FRIEND, TOXINS AND HUMAN HEALTH

Animal envenomation of humans is a serious public health hazard

Envenomation by venomous reptiles, scorpions, and insects are a common worldwide occurrence, which is an important, but neglected, public health hazard in many parts of the world, particularly in the tropics. It has been estimated that the actual numbers, as the authors suggested, could be as high as 1.8 million envenomings and 94 000 deaths each year due to snakebite worldwide, and the highest burden exists in South Asia, Southeast Asia, and sub-Saharan Africa (Kasturiratne et al, 2008). The envenomations by venomous fishes cause at least 50 000 reported injuries annually with symptoms, such as blisters, intense pain, fever, and even death (Church & Hodgson, 2002; Lopes-Ferreira et al, 2014). Each year more

than a million cases of scorpion envenomation occur worldwide, causing substantial morbidity and, among children, a risk of death (Isbister & Bawaskar, 2014). The bite by northern short-tailed shrew causes burning sensation at the wound, swelling and intense pain in humans. Platypus envenomation results in immediate and acute pain and swelling. Slow loris bites have a wide variety of effects upon humans, from none to death, with most of the reported cases resembling allergic reactions (Ligabue-Braun et al, 2012).

Toxicology of animal envenomation in humans

Block neurotransmission

Snakebites caused by the families Viperidae and Elapidae snakes are very dangerous to humans. The fatal effects include widespread bleeding, muscle paralysis, and tissue necrosis around the bite site. Neurotoxins are particularly important (Chippaux, 2008; Harrison et al, 2009). The venom of elapid snakes is rich in PLA2 and 3FTs, which are potent neurotoxins affecting the neuromuscular transmission at either pre- or post-synaptic levels. Pre-synaptic-acting neurotoxins (β -neurotoxins) inhibit the release of acetylcholine, while post-synaptic-acting neurotoxins (α -neurotoxins) cause a reversible blockage of acetylcholine receptors (Jiang et al, 2011). Recent data have challenged the traditional concept of neurotoxicity in snake envenoming, and highlighted the rich diversity of snake neurotoxins (Ranawaka et al, 2013). Though the disruption of blood coagulation system is a common envenomation outcome in victims bitten by viperid snakes like *Trimeresurus spp.*, neurotoxicity has also been well described in the victims (White, 2005; Warrell, 2010). So far, antivenom (mixtures of antibodies that neutralize venoms) is the only validated treatment for snakebite (Gutiérrez et al, 2014).

The venom of the bark scorpion (*Centruroides sculpturatus*) can cause serious and potentially fatal neurotoxicity, with young children most vulnerable to its effects. The most common symptoms of envenomation of patients included local pain, restlessness, and roving eye movements (Skolnik & Ewald, 2013). Highly species-specific antivenom is needed to treat severe envenomation, which is lacking for resource-limited areas, and poorly refined antivenom may induce severe side effects (Megarbane et al, 2014). Latrodectism resulted from bites by widow spiders (*Latrodectus spp*) causes local, regional, or generalized pain associated with non-specific symptoms and autonomic effects. Antivenoms are an important treatment for spider envenomation but have been less successful than have those for snake envenomation (Isbister & Fan, 2011). Envenomation by centipedes such as *Scolopendra subspinipes* typically leads to extreme localized pain, erythema, induration, and tissue necrosis. Mortality is uncommon and may result from secondary infection or anaphylaxis (Veraldi et al, 2014).

Disrupt blood coagulation system

Hematologic abnormalities are the most common effects of snake envenoming, especially in victims bitten by viperid snakes (Warrell, 2010; White, 2005). Venom-induced coagulopathy is a venom-induced activation of the clotting

Table 1 Major venomous animals in China and the estimated number of proteins/peptides in their venoms

Animals	Numbers of species (<i>n</i>)	Numbers of Proteins/peptides (<i>n</i>)	References
Cnidarians			
Jellyfishes	200	40 000	Li et al, 2014; Liu, 2008; Liu, 2013;
Sea anemones	110	20 000	Frazao et al, 2012; Li, 2013; Pei, 1998; Rodríguez et al, 2012
Hydra (<i>Hydridae</i>)	10	1 000	Balasubramanian et al, 2012; Rachamim & Sher, 2012; Zhang et al, 2012
Molluscs			
Cone snails	100	100 000	Biass et al, 2009; Davis et al, 2009; Li, 1999; Liu, 2008
Annelids			
Leeches	90	10 000	Lemke et al, 2013; Min et al, 2010; Yang, 1996
Arthropods			
Spiders	2 500	750 000	Song et al, 1999; Yang, 2006; Zhang et al, 2014
Scorpions	50	10 000	Cao et al, 2013; Di et al, 2014
Centipedes	110	30 000	Liu et al, 2012; Ma, 2007; Song, 2004
Bees	1000	50 000	Dias, 2014; Li, 1985; Wu, 2000
Wasps	200	20 000	Dias, 2014; Li, 1985; Wu, 2000
Ants	200	20 000	Bouzid et al, 2014; Torres et al, 2014; Zhou, 2012
Ticks	120	20 000	Cabezas-Cruz & Valdés, 2014; Yang, 2007
Horseflies	300	30 000	Ma et al, 2009; Wang, 1994; Xu et al, 2008
Crustaceans*	ND	ND	
Echinoderms			
Starfishes	150	ND	Liu, 2011
Sea urchins	100	ND	Liu, 2011
Vertebrates			
Mammals	ND	ND	
Snakes	60	20 000	Jiang et al, 2011; Zhao, 2006
Amphibians**	360	25 000	Fei, 1999; Xie et al, 2007; Yang, 2012; Zhang, 2006
Fishes	500	20 000	Baumann et al, 2014; Liu, 2008; Smith & Wheeler, 2006; Wright, 2009

The approximate number of venomous species known in China at present time is listed for each group of animals, and the estimated number of proteins and peptides in their venoms based on venom proteomic and/or transcriptomic analysis. *: Crustaceans are considered as non-venomous, but a recent report has described the venom system and venom components of a crustacean (von Reumont et al, 2014a); **: Amphibians are not typical venomous animals, but they are listed here because that their naked skin forms a special defense system and rich proteins and peptides exist in their skin secretions (Xu & Lai, 2015; Zhang et al, 2006); ND: no actual and reliable data available.

pathway by procoagulant toxins, resulting in clotting factor consumption and coagulopathy. It is a significant cause of both morbidity and mortality in these patients, either directly, or indirectly. The enzymatic toxins interfering with coagulation are procoagulant proteases (prothrombin activator, thrombin-like enzymes, factor X and factor V activators) and anticoagulant proteases (factor IX and X inhibitors, protein C activator, anticoagulant PLA2s). The venom components acting on fibrinolysis are fibrinolytic enzymes and plasminogen activators (Du et al, 2006; Kini, 2011). The major complication of venom-induced consumption coagulopathy is hemorrhage, including intracranial hemorrhage which is often fatal (Maduwage & Isbister, 2014). Metalloproteinases are widely distributed in

snake venoms and play important roles in haemostatic disorders and local tissue damage that follows snakebite. Some metalloproteinases induce hemorrhage by directly affecting capillary blood vessels. They also induce skeletal muscle damage and myonecrosis (Gutiérrez et al, 2014).

Trigger type 2 immunity

Most of the hymenoptera stings are well tolerated and result in only small local reactions with erythema, swelling, and pain in humans. However, the stings can result in severe systemic medical complications, including toxic and potentially fatal allergic reactions, mediated by venom-specific IgE antibodies (Brehler et al, 2013; Mingomataj et al, 2014). Bee and wasp

stings cause various types of allergic reactions, which contribute to the fatal outcome. Proteome and allergenome analysis of Asian wasp (*Vespa affinis*) venom and IgE reactivity of the venom components has been conducted. The results showed that the major allergenic proteins that reacted to IgE of >50% of the wasp allergic patients included PLA1, arginine kinase, heat shock protein (70×10^3), venom allergen-5, enolase, magnifin, glyceraldehyde-3-phosphate dehydrogenase, hyaluronidase, and fructose-bisphosphate aldolase (Sookrung et al, 2014). When there is a history of anaphylaxis from a previous hymenoptera sting and the patient has positive skin tests to venom, at least 60% of adults and 20%-32% of children will develop anaphylaxis with a future sting (Koterba & Greenberger, 2012). Though carrying a small but significant risk of systemic adverse reaction, venom immunotherapy is commonly used for preventing further allergic reactions to insect stings in people who have had a sting reaction (Incorvaia et al, 2011).

Contributions of toxins in deciphering human pathophysiology

Animal toxins show high specificity and potency for particular molecular targets. These features, which are difficult to replicate in the form of small molecules, have made animal toxins extremely valuable pharmacological tools. With animal toxins as irreplaceable molecular probes and research tools, many exciting discoveries that have significantly influenced life

science and medical fields were made (Figure 3).

Discovering non-membrane physiological elements

Snake venom played an important and fortuitous role in the discovery of nerve growth factors (Cohen & Levi-Montalcini, 1956). By utilizing snake venom and mouse salivary gland extract, purification of nerve growth factor and production of the antibodies against it became possible. For this pioneering work, Stanley Cohen and Rita Levi-Montalcini were awarded the 1986 Nobel Prize. When studying the toxicology of *Bothrops jararaca* snake envenoming, bradykinin was discovered (Rocha e Silva et al, 1949), which contributed greatly to our understanding of human pathophysiology in cardiovascular and immune systems.

Probing ion channels

The flow of ions across the cell membrane is essential to many life processes, and ion channels are transmembrane pore-forming proteins that create a gated, water-filled pore to allow the movement of ions across cell membranes (Gouaux & Mackinnon, 2005). Given the essential functions of ion-channels in neuronal signaling and muscle contractility, it is not surprising that many toxins have evolved to block or activate ion channels. Animal venoms provide a virtually untapped reservoir of millions of bioactive peptides with highly diverse structures to target on ion-channels (Dutertre & Lewis, 2010).

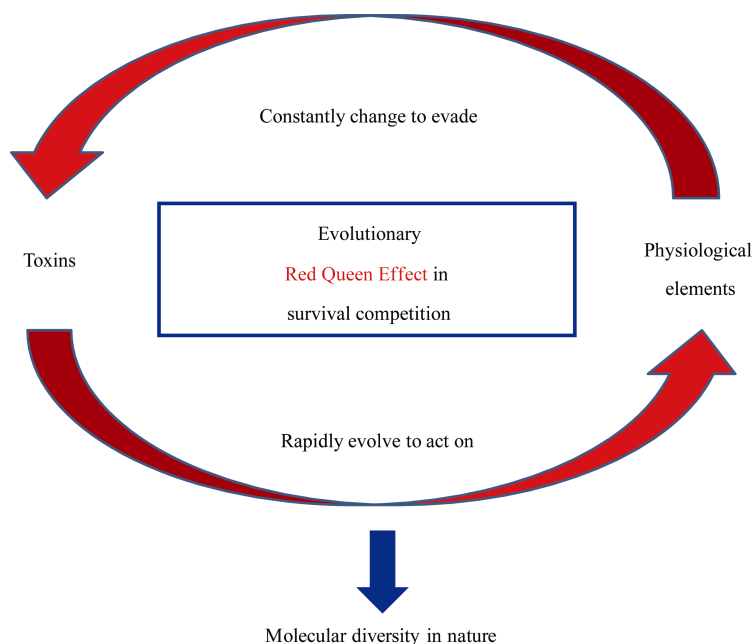


Figure 2 Evolutionary diversification of toxins

Dobzhansky (1973) stated in a classic article that nothing in biology makes sense, except in the light of evolution. The extensive diversification of toxins may have been driven by extreme diversification of physiological elements of potential preys and predators in evolutionary processes. Toxins may be subject to evolutionary Red Queen Effect (Van Valen, 1974), in which toxins must evolve rapidly to effectively act on diversified biological targets. On the other hand, it is possible that the physiological elements, which are critical for the survival of organisms, have to constantly change them to evade being targeted by toxins.

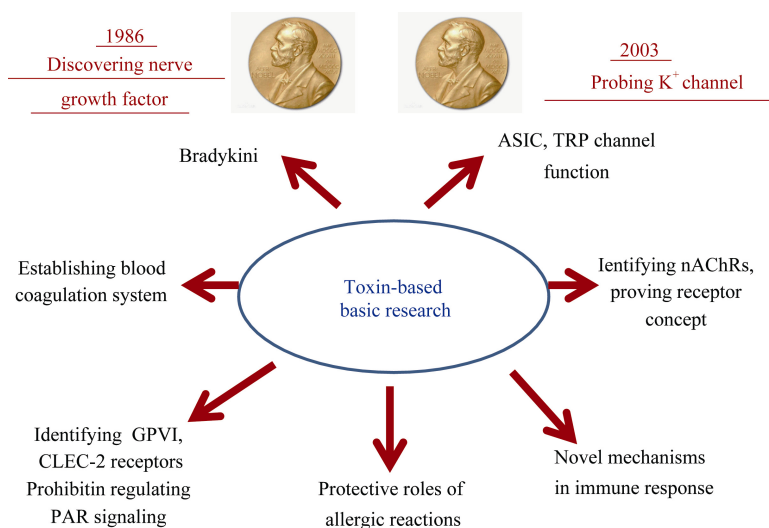


Figure 3 Contribution of toxins in deciphering human patho-physiology and diseases mechanisms

With animal toxins as irreplaceable molecular probes and research tools, many exciting discoveries have been made, which have significant impact on life sciences and medical fields. See description in detail for each story and references cited in the text.

Potassium (K⁺) channels Pore-blocking toxins from scorpion venoms, such as charybdotoxin, have profoundly impacted research in the K⁺ channel field primarily in two ways. First, they have enabled purification of specific novel K⁺ channels such as the BK channel, a Ca²⁺ and voltage-gated K⁺ channel (Banerjee et al, 2013). Second, they provided knowledge about channel subunit stoichiometry and the shape of the extracellular K⁺ pore entryway at a time when no 3-D structure was available for any ion channel (Hidalgo & MacKinnon, 1995; MacKinnon, 1991). In 2003, Prof MacKinnon was awarded the Nobel Prize for the structural and mechanistic study of ion channels. In his Nobel lecture, Prof MacKinnon emphasized the role of scorpion charybdotoxin in his studies. The toxin was used to probe the "pore" of K⁺ channels, leading to important conclusion concerning the architecture of the channels (MacKinnon, 2003).

Acetylcholine receptors Acetylcholine receptors (AChRs) consist of two major subtypes: the metabotropic muscarinic receptors (mAChRs) and the ionotropic nicotinic receptors (nAChRs). Both could be activated by the endogenous neurotransmitter acetylcholine. The muscarinic receptors are G protein-coupled seven-transmembrane proteins, which are activated by muscarine, a toxin from the mushroom *Amanita muscaria*, and inhibited by atropine, a toxin from *Atropa belladonna* as well as a widely used clinic drug (Albuquerque et al, 2009). α -bungarotoxin is the first snake venom 3FT that binds muscle-type nAChRs with near covalent affinity to inhibit their function and promote debilitating paralysis (Chang & Lee, 1963). At the time of the discovery of α -bungarotoxin, the nAChR, although physiologically and pharmacologically well defined, was a molecular enigma. Even the question of whether it was a protein was disputed (Hall, 1999). Affinity columns of α -bungarotoxin allowed separation of nAChRs from other proteins in detergent-solubilized electric organs, which led to the

identification, cloning, and sequencing of genes responsible for encoding these receptors. These advance resulted in nAChR at the most advanced stage for any type of receptor (Dolly & Barnard, 1984). The α -conotoxins from marine cone snails were used for discriminating among the subtypes of nAChRs (Lewis et al, 2012).

Acid-sensing ion channels Acid-sensing ion channels (ASICs) are voltage-independent proton-gated cation channels that are largely expressed in the nervous system as well as in some non-neuronal tissues. Six protein isoforms exist in rodents: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 (Deval et al, 2010). Several toxins targeting ASICs can discriminate between the subtypes of ASIC1- and ASIC3-containing channels (Baron et al, 2013). Snake toxin MitTx consists of a heteromeric complex between Kunitz- and PLA2-like proteins that together function as a potent, persistent and selective agonist for ASICs, eliciting robust pain-related behaviors in mice (Bohlen et al, 2011). A new class of 3FTs (mambalgins) from snake black mamba is able to abolish pain through inhibition of ASICs expressed either in central or peripheral neurons. Blockade of heteromeric channels made of ASIC1a and ASIC2a subunits in central neurons and of ASIC1b-containing channels in nociceptors is involved in the analgesic effect of mambalgins (Diochot et al, 2012). Taken together, these findings highlight an unexpected contribution of ASIC channels to nociception and identify new potential therapeutic targets for pain. The cocrystal structure of chicken ASIC1a with MitTx has been determined, which defines the structure of the selectivity filter of voltage-independent, sodium-selective ion channels, and captures the open state of the ASIC (Baconguis et al, 2014).

TRP channels The mammalian transient receptor potential (TRP) channel family consists of >30 members, many of which

are known to form tetrameric cation channels. Though several TRP channels are known to contribute to sensory signaling, like thermosensation, nociception, and pain, the physiological roles of many TRP channels remain enigmatic (Venkatachalam & Montell, 2007). A peptide toxin from the earth tiger tarantula spider that selectively and irreversibly activates the capsaicin- and heat-sensitive channel, TRPV1. This “double-knot” toxin (DKTx) traps TRPV1 in the open state by interacting with residues in the presumptive pore-forming region of the channel, which highlights the importance of conformational changes in the outer pore region of TRP channels during activation (Bohlen et al, 2010). The toxin was further used as a probe to determine structures of two activated states of TRPV1. The study revealed that TRPV1 opening is associated with major structural rearrangements in the outer pore, including the pore helix and selectivity filter, suggesting a dual gating mechanism. These findings revealed differential gating mechanisms for TRPs and voltage-gated ion channels (Cao et al, 2013a).

Discovering and probing membrane receptors

Glycoprotein VI (GPVI) Snake venoms contain a vast number of toxins, in which C-type lectins are fascinating due to their diverse binding specificities to platelet surface proteins and their complex targeting mechanisms. These proteins have made great contribution to the understanding of thrombosis and haemostasis (Du et al, 2006; Lee et al, 2003). Platelets play key roles in haemostasis and thrombus formation. From 1980s, GPVI emerged as a candidate receptor for collagen through investigation of patients with an auto-immune thrombocytopenia, as well as the signaling events that underlie platelet activation by collagen (Watson et al, 2010). However, the molecular identity of GPVI remained elusive at that time. Convulxin is a snake venom C-type lectin purified from the tropical rattlesnake *Crotalus durissus terrificus* (Prado-Franceschi & Vital-Brazil 1981; Vargaftig et al, 1983). It is able to activate platelets by binding specifically to GPVI (Polgár et al, 1997). As a critical step, GPVI was isolated from platelets using affinity chromatography of convulxin, which identified that GPVI is actually a member of the immunoglobulin superfamily (Clemetson et al, 1999). The downstream signaling cascade, which leads to the activation of $\alpha IIb\beta 3$ and thrombus formation was elucidated (Watson et al, 2010).

C-type lectin-like receptor 2 (CLEC-2) Rhodocytin (also called aggrexin) is a heterodimeric C-type lectin and was purified from snake *Calloselasma rhodostoma* venom in 1990s (Huang et al, 1995; Shin & Morita, 1998). It stimulates platelet aggregation independently of the collagen receptor GPVI/FcR γ -chain complex (Navdaev et al, 2001). Using rhodocytin affinity chromatography, a novel C-type lectin receptor (CLEC-2) in platelets was identified, which represents the first C-type lectin receptor found on platelets and represents a novel signaling pathway in platelets (Suzuki-Inoue et al, 2006). Soon afterwards, podoplanin, a type I transmembrane sialomucin-like glycoprotein, was identified as an endogenous ligand for CLEC-2. Subsequent works illustrated that platelets regulate tumour

metastasis, lymphangiogenesis, and dissemination of HIV through interaction between CLEC-2 and its endogenous ligand podoplanin (Suzuki-Inoue et al, 2011).

Interaction of trefoil factors with protease-activated receptors (PARs)

Trefoil factors (TFFs) are characterized by one to four trefoil domains, which are highly conserved among TFF proteins, from frogs to humans. TFFs are believed to be initiators of mucosal healing and being greatly involved in tumorigenesis (Lefebvre et al, 1996; Mashimo et al, 1996). However, the first hand actions and the mechanisms involved by which TFFs exert their biological activities are still largely unknown (Kjellev, 2009; Zhang et al, 2011). Bm-TFF2 is a two-domain single chain TFF isolated from frog *B. maxima*, which is able to trigger human platelet activation (Zhang et al, 2005). Unexpectedly, it bound and activated PAR1 on human platelets, which is independent of the receptor cleavage and tethered-ligand unmasking. Further results showed the capacity of human TFF2 to act on PAR4 to promote cell migration *in vitro*. The findings suggested the interaction of a PAR with a TFF (Zhang et al, 2011). Their possible interaction *in vivo*, with TFFs acting as either agonists or antagonists of PARs and physiological relevance are certainly worthy of further studying.

Prohibitins as novel regulators of PAR signaling

In the process of identifying Bm-TFF2 receptor(s) in human platelets, both prohibitin 1 (PHB1) and PHB2 were detected on the surface of human platelets and were found to be involved in PAR1-mediated platelet aggregation (Zhang et al, 2012b). PHBs are ubiquitously expressed and highly conserved. The membrane PHBs have been reported to be involved in inflammation, obesity and cancer metastasis (Thuau et al, 2013). The finding uncovered that PHBs are hitherto unknown regulators of PAR1 signaling. Targeting PHBs might be a useful therapeutic approach for anti-platelet therapy. Further study revealed that PHB1 participates in PAR1 activated internalization, Erk1/2 phosphorylation and degradation, but these regulatory roles are aberrant in cancer cells (Wang et al, 2014a). A crucial role of PHB1 in IgE-mediated activation and degranulation of mast cells was also identified afterwards (Hajime & Krishnaraj, 2013).

Establishment of basic concept in blood coagulation system

Snake venoms were used to obtain data that was the basis for considering that blood coagulation is primarily promoted by proteolytic enzymes. In the development of the general understanding of blood coagulation, venom proteases were proved very useful in clarifying some basic concepts (Serrano, 2013). These intriguing toxins are generally variants of normal mammalian physiological proteins. TSV-PA is a specific plasminogen activator from Chinese snake *Trimeresurus stejnegeri* venom (Zhang et al, 1995; 1997). Its 3D-structure, as the first one determined for snake venom serine proteases, was elucidated by the group of Profs Bode and Huber (the 1988 Nobel Laureate) (Parry et al, 1998), which displays a typical trypsin-like fold.

Deep understanding of immunity

Revealing novel mechanisms in immune responses

Venoms frequently trigger host immune responses. The illustration of their action may provide insight into novel inflammatory and immune pathways. It was found that bee venom-derived PLA2 activates T cells through generation of small neoantigens, such as free fatty acids and lysophospholipids, from common phosphodiacylglycerides. Subsequent studies in patient showed that injected PLA2 generates lysophospholipids within human skin *in vivo*, and polyclonal T cell responses are dependent on CD1a protein and the PLA2. These findings support a previously unknown skin immune response based on T cell recognition of CD1a proteins and lipid neoantigen generated *in vivo* by phospholipases, revealing mechanisms underlying phospholipase-dependent inflammatory skin disease (Bourgeois et al, 2013).

Elucidating protective roles of allergic reactions in innate immunity

Diverse components from animal venoms, plants, parasites, foods and environments can activate allergic responses, including fatal anaphylaxis (Gutierrez & Rodewald, 2013). Allergies have been considered misguided T helper type 2 cell responses (Artis et al, 2012). The mechanisms of innate immune recognition of parasitic worms, as well as allergens, are largely unknown (Medzhitov, 2010b; Licona-Limon et al, 2013). Bee venom PLA2 induces a T helper type 2 cell-type response and group 2 innate lymphoid cell activation. Interestingly, the IgE response to PLA2 could protect mice from future challenge with a near-lethal dose of PLA2, indicating that the innate immune system can detect the activity of a conserved venom component and induce a protective immune response against a venom toxin (Palm et al, 2013). Marichal et al (2013) also found protective rather than allergic immune responses in mice repeatedly challenged by bee venom or its components. These findings support the hypothesis that IgE, which also contributes to allergic disorders, has an important function in the protections of host against noxious substances.

Toxin knowledge guides physiological toxin-like protein/peptide (TLP) studies

The idea that most toxin molecules have evolved from endogenous genes operating in normal physiological processes and cellular pathways suggests the existence of endogenous counterparts of toxin genes. Rapid expansion of gene and protein information, uncovered especially by 3D-structural determination, revealed that numerous TLPs are expressed in non-venomous animals and/or in non-venom systems with unknown physiological functions, including in mammals. Knowledge obtained in the study of toxins could certainly help to illustrate the role and mechanism involved of these endogenous TLPs (Figure 4).

Ly6/neurotoxin family

Snake venom 3FTs and lymphocyte antigen 6 (Ly6) proteins have a variety of biological activities, but their three-finger folding combines them in one Ly6/neurotoxin family (Tsetlin, 2014). Identifying TLPs by applying homology search methods

have mostly failed, and ClanTox (classifier of animal toxins) was developed for identifying TLPs from mammalian complete proteomes (Tirosh et al, 2013). In the murine proteome, there are about 60 such proteins that belong to the Ly6/neurotoxin family. They are either secreted or anchored to the cell membrane.

Mammalian Lynx1 in nervous system Lynx1, a murine protein of Ly6/neurotoxin family, is highly expressed in several discrete neuronal populations in the brain. Based on the well characterized properties of snake venom 3FTs in nAChR, the possible action of lynx1 was tested. The results showed that lynx1 is a novel protein modulator for nAChRs (Miwa et al, 1999). Their works further indicate that lynx1 colocalizes with nAChRs on CNS neurons and physically associates with nAChRs. These results established direct interaction of lynx1 with nAChRs, indicating that this endogenous TLP plays important roles *in vivo* by modulating functional properties of their cognate CNS receptors. Lynx1 expression maintains stability of mature cortical networks in the presence of cholinergic innervations (Morishita et al, 2010).

Mammalian SLURPs in immunity Secreted mammalian Ly-6/uPAR-related protein 1 (SLURP-1) is another mammalian Ly6/neurotoxin family member. Structural similarity between SLURP-1 and snake venom 3FTs suggests that this protein might interact with nAChRs. This hypothesis led to the demonstration that SLURP-1 regulates epidermal calcium homeostasis and cutaneous inflammation through nAChRs (Chimienti et al, 2003). SLURP-1 binds to the conventional ligand binding site on keratinocyte 7 nAChRs and exhibits a proapoptotic effect (Chernyavsky et al, 2010). SLURP-2, another member of Ly6 family, was then shown to bind to 3 nAChRs, thereby delaying keratinocyte differentiation and preventing apoptosis (Arredondo et al, 2006). Both SLURP-1 and SLURP-2 are expressed in various immune cells and organs (Moriwaki et al, 2007). These findings illustrated that SLURPs act as an autocrine and/or paracrine factor via AChRs on epithelial cells and immune cells to modulate immune function.

Mammalian β -defensins

Many toxins share a striking degree of conservation with defensin-like antimicrobial peptides that contain a ganna-core motif (Yeaman & Yount, 2007). Crotonamine is a toxin from the snake *Crotalus durissus terrificus* venom. Computational docking suggests direct interactions of the peptide with Kv channels in eukaryotic but not prokaryotic cells (Yount et al, 2009). Later, it was shown that crotonamine selectively inhibits Kv1.1, Kv1.2, and Kv1.3 channels with an IC_{50} of ~ 300 nmol/L (Peigneur et al, 2012). Human β -defensin 2 (hBD2) is an antimicrobial peptide that protects hosts from microbial infection by killing bacteria, fungi and viruses, and recruits memory T cells through interacting with CCR6 (Pazgier et al, 2006). 3D-alignment between hBD2 and crotonamine revealed a striking degree of identity (Yount et al, 2009), suggesting that hBD2 might be a toxin-like modulator of Kv channels. hBD2 was then found to be able to inhibit human Kv1.3 channel (Yang et al, 2014). In another work, hBD2 was found to be a novel opener via

interacting with human $\beta 1$ subunit coexpressed with mouse α subunit of large conductance Ca^{2+} -activated K^+ channel (Liu et al, 2013b). These studies opened new possibilities to explore the path-physiological roles of hBD2 via actions through K^+ channels.

Apoptotic Bcl2 proteins

Bacteria have developed sophisticated virulence factors such as pore-forming toxins (PFTs) to mount their attack against their hosts. An essential feature of PFTs is their ability to convert from a water-soluble form to a transmembrane form via oligomerization step that is followed by membrane insertion and channel formation (Bischofberger et al, 2012; Iacovache et al, 2008). PFTs are classified into two large families, the α - and the β -PFT according to the type of structures they use to insert into the lipid bilayer upon pore formation via α -helices (α -PFT) or β -sheets (β -PFT), respectively (Iacovache et al, 2010). The Bcl-2 family proteins regulate programmed cell death. Despite their physiological importance, the biochemical functions of Bcl-2-related proteins had remained elusive until the structure determination. The arrangement of the α -helices in Bcl-xL, a member of Bcl-2 family member, was found to be reminiscent of the membrane translocation domain of bacterial toxins, in particular diphtheria toxin and the colicins (Muchmore et al, 1996). Subsequent investigation illustrated that Bcl-xL and other Bcl-2 members can insert into either synthetic lipid vesicles or planar lipid bilayers and form an ion-conducting channel, uncovering the possible mechanism involved of mammalian Bcl2 family members via inserting into membranes to favor or inhibit apoptosis (Minn et al, 1997; Schendel et al, 1997).

Venomous animals are important medicinal animals Traditional medicine and modern practice

Medicinal animals have a long history as a source of clinic therapeutics worldwide, in which venomous animals take a key position. Tarantulas were used by indigenous populations of Mexico and Central and South America to treat a variety of ailments ranging from asthma to cancer (Machkour-M'Rabet et al, 2011). Cobra snake venom has been used to treat cancer and moderate to severe pain, as well as multiple sclerosis and rheumatism (Reid, 2007; 2011). Medicinal leech therapy became less used toward the end of 19th century but now has emerged again as a widely useful therapy. Leech therapy is effective in establishing venous outflow in congested flaps and replants, and has been shown to be effective for symptomatic treatment of osteoarthritis of the knee (Michalsen et al, 2008). Some modern indications for leech therapy have been proposed by American Food and Drug Administration (FDA) (Nouri et al, 2012). Apitherapy is an effective and safe treatment for recalcitrant localized plaque psoriasis, when other topical or physical therapies have failed (Eltaher et al, 2014). Dried toad (*Bufo bufo*) skin secretions (Chan Su) has been used in traditional Chinese medicine as a cardiogenic, analgesic and anesthetic agent, and as a remedy for ulcers, as well as an anti-cancer agent (Meng et al, 2009; Wang et al, 2014b).

Snakes (*Zaocys dhumnades*, *B. multicinctus*, *Agkistrodon acutus*), amphibians (*Rana temporaria*, *B. bufo*), fishes

(*Hippocampus histrix*, *Solenognathus hardwickii*), scorpions (*Buthus martensii*), insects (*Eupolyphaga sinensis*, *Mylabris phalerata*), centipedes (*S. subspinipes*), leeches (*Whitmania pigra*, *W. acranulata*, *Hirudo nipponica*) are listed in national Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2010). They are used in treatments of various cardiovascular, nervous and immune related diseases. The first version of the pharmacopoeia was published in 1953. After about 60 years and several major revisions, these medicinal animals are still embodied in the pharmacopoeia, indicating their confirmed treatment effects in clinic. These long-term clinic practice and success are invaluable experience and resources for modern drug development, especially upon the challenges of complicated human diseases caused by multi-element disorders.

Toxins and TLPs in medicinal animals play key roles in their pharmacological effects

Although effectively used in clinic, the material basis of medicinal animals is still enigmatic. Many toxins and TLPs are low molecular weight peptides rich in disulfide bridges, which are highly stable molecular scaffolds resistant to heat and degradation by proteases (Fry et al, 2009a; Harvey, 2014; King, 2011). Numerous endogenous counterparts of toxin genes, namely physiological TLPs, are expressed in non-venomous animals and/or in non-venom systems. Centipedes are important venomous medicinal animals in traditional Chinese medicine, and the whole animal is used in clinic. Approximately 400 novel protein/peptide molecules have been found from the venom of centipede *S. subspinipes* (Liu et al, 2012). In addition, comprehensive transcriptomic analysis of body (without venom glands) and venom glands of the centipede revealed a substantial overlapping of transcripts expressed (Lee et al, unpublished observation). It is reasonable to speculate that peptide toxins in the venom glands and TLPs in the body tissues are mainly responsible for the pharmacological effects obtained in clinic because of their biochemical stability and biological activity.

Modern clinic drugs

The potency, specificity, and stability of toxins have made them a valuable source of natural products for drug discovery (Harvey, 2014; King, 2011). In the 1970s, antihypertensive drug captopril was developed from a bradykinin potentiating peptide (BPP) discovered in the venom of the Brazilian viper *Bothrops jaracaca* (Cushman & Ondetti, 1991). This important achievement marked the beginning of modern toxins-based drug discovery. Several important drugs derived from venom peptides or proteins (Figure 5) have been approved and widely used in clinic. There are still tons of molecules in clinical trials and many more in various stages of preclinical development.

Cardiovascular diseases

Hypertension In 1965, BPPs were identified by Prof Sergio Ferreira (Ferreira, 1965) from snake venoms, which are inhibitors of angiotensin-converting enzymes (Camargo et al, 2012). The structure determination and synthesis of BPP9a, namely teprotide, were performed by Ondetti et al (1971). The teprotide, when injected, was able to lower blood pressure.

However, developing classical peptides for oral antihypertensive drugs were proved difficult. The strategy to develop non-peptidic inhibitors from peptides directed the synthesis of captopril by Cushman et al (1977). Based upon the model proposed by Byers & Wolfenden (1972), when a succinyl radical was added to the carboxy terminal proline of BBP5a, a weak specific angiotensin-converting enzyme inhibitor with oral activity resulted, leading to the invention of captopril and a new class of antihypertensive clinic therapeutics (McCleary & Kini 2013). Profs Cushman and Ondetti shared the 1999 Albert Lasker Award in clinical medical research.

Thrombosis and haemostasis Excessive platelet aggregation is associated with myocardial infarction and other thrombotic diseases. Integrin $\alpha\text{IIb}\beta 3$ plays key roles in platelet aggregation, serving as a rational target for antithrombotic therapy (Bledzka et al, 2013). In order to discover $\alpha\text{IIb}\beta 3$ antagonists, 62 snake venoms were screened, leading to the identification of barbourin, a 73-amino acid disintegrin from the venom of *Sistrurus miliarius barbouri* (Scarborough et al, 1991). Eptifibatide (Integrilin) is a cyclic heptapeptide (6 amino acids) designed from barbourin (Scarborough et al, 1993; Scarborough, 1999). It has a relatively long half-life in plasma (about 2.5 hours), and cyclizes the peptide via a disulfide bond greatly enhancing its potency. Tirofiban (Aggrastat) is a non-peptide mimetic of $\alpha\text{IIb}\beta 3$ inhibitor, which was designed based on a RGD peptide from snake venom (Lynch et al, 1995). Based on the distance separating the side chains of Arg and Asp in the RGD motif of echistatin, a disintegrin isolated from the venom of *Echis carinatus* (Saudek et al, 1991), a lead was identified and optimized (Egbertson et al, 1994) to produce tirofiban. These two drugs received FDA approval in clinic for antithrombotic therapy, like acute coronary syndromes since 1998.

Thrombin-like enzymes (TLEs) are serine proteinases reported from many different crotalid, viperid and colubrid snakes that share some functional similarity with thrombin. Unlike thrombin, most TLEs are neither inhibited by heparin-antithrombin III complex, nor are they able to activate FXIII. Ancrod and batroxobin are the most well-known examples of TLEs from the venoms of *Agkistrodon rhodostoma* and *Bothrops atrox*, respectively (Nolan et al, 1976; Stocker & Barlow, 1976). They rapidly catalyze the formation of soluble clot (that can be easily broken down by plasmin) and deplete the level of circulating fibrinogen, preventing formation of insoluble clots in acute thrombosis events. These TLEs are used in clinic for treatment of many thrombosis events with beneficial outcomes. A mixture of two enzymes from the venom of *B. atrox*, a TLE and a thromboplastin-like enzyme, forms a clot-promoting product called Haemocoagulase, which has procoagulant effects only at the sites of injury or surgery and is used as a haemostatic agent in clinic primarily in China (Koh & Kini, 2012).

Hirudin, consisting of 65 amino acids, is a direct thrombin inhibitor from the saliva of the medicinal leech *H. medicinalis* (Petersen et al, 1976). By molecular modeling and design, a novel class of bivalent peptide inhibitors of thrombin hirulogs were developed (Maraganore et al, 1990). Bivalirudin (20 amino

acids) was developed from hirulog-1, which combines a C-terminal segment of 12 amino acids derived from native hirudin binding site to an active site-binding tetrapeptide sequence at its N-terminus, linked together by four glycine residues. It specifically binds to both the active catalytic site and anion binding exosite of thrombin, with a short half-life of 25 min *in vivo*. Bivalirudin lacks immunogenicity and has a wider therapeutic index than recombinant hirudin. It has become one of most widely used antithrombotics in clinic (Coppens et al, 2012).

Neurogenic diseases

Pain relief Omega-conotoxin MVIIA from *Conus magus* selectively blocks N-type Ca^{2+} ion channels (Olivera et al, 1987). Ziconotide, a 25-amino acid polypeptide, is the synthetic version of the peptide. When administered intrathecally, it produces potent analgesia by interruption of Ca^{2+} channel-dependent transmission of pain signals in the spinal cord, and was approved by the FDA in 2004 for treating patients with intractable pain (Pope & Deer, 2013). Early in the 1930s, the relief of pain was found to be the dominant pharmacological activity when cobra snake venom was used in cancer patients in clinic. Using cobra venom as an analgesic in clinic was adopted by Macht in the United States (Macht, 1936). In the early 1980s, a cobra venom neurotoxin preparation isolated from Chinese cobra (*N. naja atra*) venom, named ketongning or cobratide, was developed as a drug in clinic for pain killing by Kunming Institute of Zoology of the Chinese Academy of Sciences, which has been primarily used in China for almost 35 years. Later, the investigators from the same institute developed the oral tablets "Kelluoqu" by combining cobratide with tramadol hydrochloride and ibuprofen, which was approved by the Chinese FDA in 1998 for the treatment of moderate to severe pain (Lu et al, 2010).

Metabolic diseases

Glucagon-like peptide 1 (GLP-1) is a hormone that stimulates insulin and suppresses glucagon secretion. It exerts its actions by acting on G-protein-coupled GLP-1 receptor (Drucker & Nauck, 2006). The pleiotropic actions of GLP-1 and GLP-1 receptor on the control of blood glucose have fostered considerable interests in the use of GLP-1 and GLP-1 receptor agonists for the treatment of type 2 diabetes. GLP-1 has a very short half-life *in vivo*. So, GLP-1 receptor agonists with peptide degradation-resistant and more suitable pharmacokinetic properties should be better for the long-term treatment of type 2 diabetes. Exendins are hormone-like peptides found in the lizard *Heloderma* venoms (Irwin, 2012). Exendins-3 and -4 exhibit sequence similarity (56%) and have biological functions most similar to mammalian GLP-1 by acting on mammalian GLP-1 receptors (Eng et al, 1992; Göke et al, 1993). Exenatide (synthetic exendin-4) has a circulating half-life of 60–90 min, leading to its development as an anti-diabetic agent in 2005. Recently, evidence suggested that agonists for GLP-1 receptors may have biological properties relevant to Parkinson's disease. Exenatide may serve as a neuroprotective candidate and be used in the treatment of Parkinson's disease in clinic (Foltynie & Aviles-Olmos, 2014).

Infectious diseases

Peptide antibiotics Traditional antibiotics have been widely used, resulting in the emergence of many antibiotic-resistant strains worldwide. Thus, there is a vital need for new effective therapeutics to conquer infections caused by drug-resistant bacteria (Fischbach & Walsh, 2009). Naturally occurring antimicrobial peptides, owing to their unique mechanisms that differ from the conventional clinical drugs, are considered to be excellent templates for the design of novel antibiotics with promising therapeutic effects, especially for drug-resistant microbes (Hancock & Sahl, 2006). Cathelicidins are cationic host-defense peptides that play important roles in innate immune system, which have also been identified from elapid snake venoms (Zhao et al, 2008). In animal models, OH-CATH30, isolated from king cobra venom, protects mice from lethal sepsis due to its direct antimicrobial activity and selective immune-modulatory properties. Treatment with OH-CATH30, alone or in combination with levofloxacin, significantly improves the clinical outcomes of rabbit antibiotic-resistant *Pseudomonas aeruginosa* keratitis. These results suggest that OH-CATH30 is an excellent candidate for infectious disease caused by drug-resistant bacteria (Li et al, 2012; 2013b; 2014b).

SIMILARITIES SHARED BY VENOMS AND IMMUNE SYSTEMS

Numerous species of microbes, plants and animals are able to produce toxins. Besides well-developed venom systems in metazoan animals, some amoeboid protozoans are facultative or obligate parasites in humans and they produce PFTs for invasion (Leippe, 2014). On the other hand, all organisms have developed immune systems to defend against the threats of potential parasites and pathogens early during evolution.

Besides well-known innate and adaptive immunity, the ancient and ubiquitous cell-autonomous immunity operates across all three domains of life (Randow et al, 2013). The basic similarities of venoms and immune systems are reflected by their primary biological tasks, attacks and defenses (Figure 6). They may also function as ways of peaceful co-existence among organisms. However, the biological significance and mechanisms involving these two systems are not well appreciated and understood nowadays, i.e., the prevention of pathogenesis while maintaining symbiosis in the coexistence of enormous microbes.

Toxins and immune effectors share similar evolutionary patterns

In animals, venom system is considered to evolve for feeding or defense by toxin producing animals, which mainly functions among prey-predator interactions of animals. Defenses against microbial invasions and malignant cells are major missions of immune systems. As discussed earlier in this review, the genetic and evolution origination of venom toxins and host immune effectors are believed to evolve via the 'birth and death' process of gene evolution (Casewell et al, 2013; Nei et al, 1997). Recruitment of a proper gene and duplication and rapid mutation created diversified innovative toxins or immune effective molecules, which are selectively expressed in venom glands or immune related organs (Figure 6).

Toxins and immune effectors share common protein folds

Accumulated evidence have uncovered a fact that many similar proteins and common protein folds, which were previously identified by primary sequences but now by 3D-structures, have been used and engineered into conductors of immune effectors as well as venom toxins. This is exemplified by cases

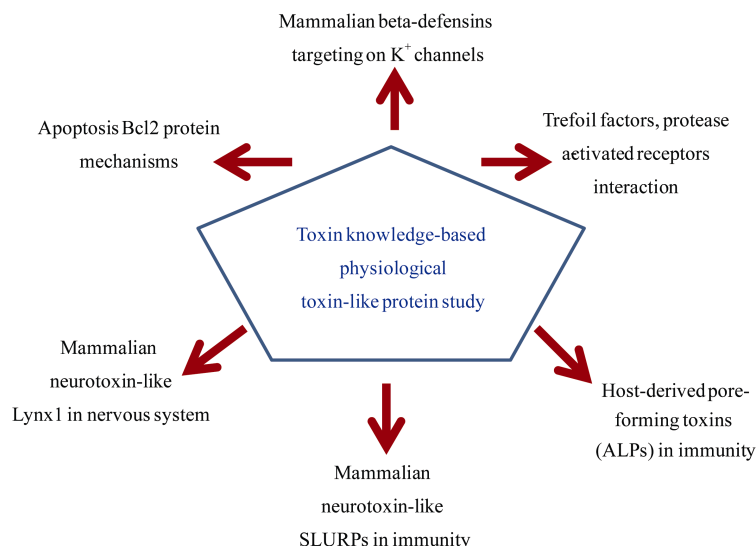


Figure 4 Toxin knowledge guides physiological toxin-like protein/peptide (TLP) study

There are numerous TLPs expressed in non-venomous animals and/or in non-venom systems with unknown physiological functions, including those in mammals. Knowledge obtained in the study of toxins has greatly facilitated uncovering the functions and mechanisms involved of these endogenous TLPs. See description in detail for each story and references cited in the text.

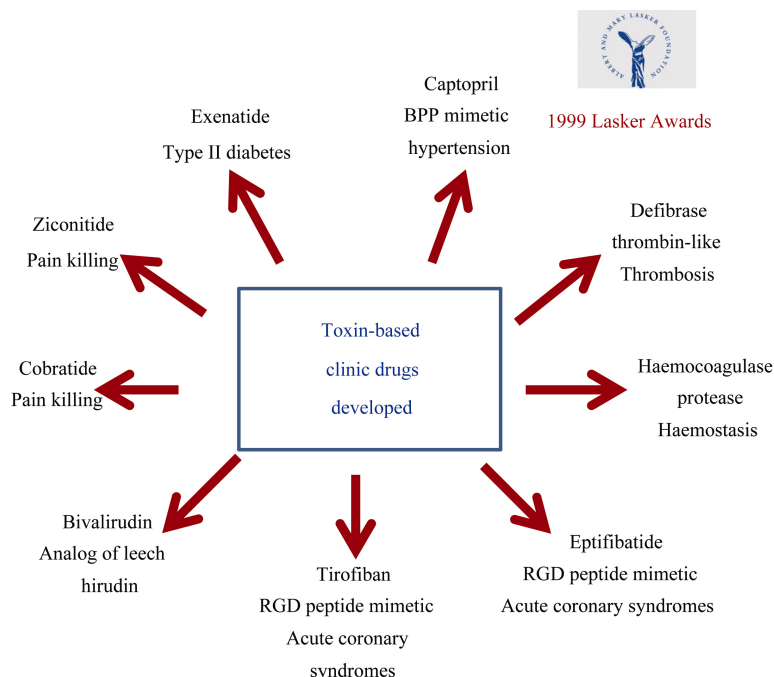


Figure 5 Modern toxin-based drugs developed

The potency, specificity, and stability of toxins have made them an invaluable source of natural products for drug discovery. The approved and widely used drugs derived from venom peptides or proteins are listed here. See description in detail for each example and references cited in the text. There are still tens of molecules in clinical trials and many more in various stages of preclinical development.

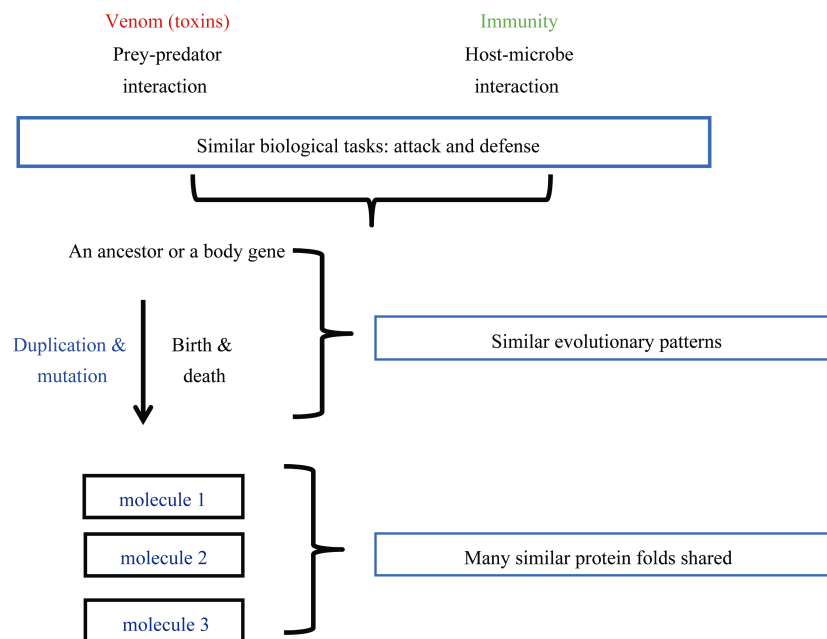


Figure 6 Similarity shared by venom and immune systems

The basic similarity of venom and immune systems is reflected by their primary biological tasks, attacks and defenses. The genetic and evolution origin of toxins and immune effectors are believed to evolve via the 'birth and death' process of gene evolution (Casewell et al, 2013; Fry, 2005; Nei, 1997). Recruitment of a proper gene and duplication and rapid mutation created diversified innovative toxin or immune effective molecules, which are selectively expressed in the venom glands or immune related organs.

mentioned earlier in this review about the structural similarity and conversion between snake neurotoxins and immune active SLURPs, crotoamine toxins and defensins and their targeting on ion channels. These molecules were once thought to be distinct in form and function now appear to be members of a same family, probably descended from archetype predecessors that emerged in the early time of life on earth (Yeaman & Yount, 2007).

Cellular membranes are crucial for the survival of organisms. Pore-formation is frequently used in toxic attack on cells, as it can lead to efficient disruption of cell functions and even cell death. Many major pathogenic bacteria employ PFTs as virulence factors, representing some 30% of all known bacterial toxins (van der Goot, 2014). Aerolysin, a toxin produced by the Gram-negative bacterium *Aeromonas hydrophila* and related species, belongs to the β -PFT group and shares a common mechanism of action involving β -barrel structures resulting from the assembly of β -hairpins from individual toxin monomers into a heptamer (Bischofberger et al, 2012; Iacovache et al, 2008). The aerolysin domain is defined according to its structural similarity to the transmembrane domain of aerolysin toxins (Szczesny et al, 2011).

As discussed below, aerolysin domain with membrane activity and pore-forming capacity has been discovered in virulence factors of pathogenic microbes for attacking, immune effectors of vertebrates (Xiang et al, 2014), as well as venom toxins in fishes (Magalhães et al, 2005) and centipedes (Liu et al, 2012) for prey-predator interaction.

Aerolysin-like proteins (ALPs) in venoms and immunity

Large-scale sequencing and bioinformatics analyses have revealed that proteins with an aerolysin fold, namely aerolysin-like proteins (ALPs), can be found in all forms of life. Particularly, a diverse array of proteins harboring an aerolysin domain fused with other domains has been identified in various animal and plant species (Szczesny et al, 2011; Xiang et al, 2014). In vertebrate species, ALPs are widely expressed in various body tissues of animals, including embryonic epidermis, skin, blood, gastrointestinal tract, spleen and kidney (Liu et al, 2008). However, little is known about their biological functions and mechanisms involved. The vast majority of these proteins have low sequence similarity (<20%). 3D-structures are crucial in revealing structural conservation that is elusive at sequence level (Moran et al, 2012).

ALPs in venoms

Aerolysin fold has been recruited to venom systems in venomous animals as toxin components in their venoms, as revealed in those of fishes and centipedes (Liu et al, 2012; Szczesny et al, 2011). Natterins, which are a class of protein toxins characterized from *T. nattereri* fish venom (Magalhães et al, 2005). In-depth bioinformatics analyses indicated that natterins are actually ALPs (Szczesny et al, 2011). Their toxicological effects are known to cause nociception and edema (Lopes-Ferreira et al, 2014). Two nattering-like proteins have been characterized from skin secretions of oriental catfish (*Plotosus lineatus*). In the same fish, which also possess a venom system, immunocytochemical approaches have

established that the venom gland toxins of oriental catfish are natterin-like proteins (Tamura et al, 2011). The origin of these fish venoms was closely associated with skin glands and epidermal secretions, which normally play physiological roles in wound healing and innate immune defense (Al-Hassan et al, 1985; Wright, 2009).

ALPs in immunity

Direct killing/inhibiting pathogens Biomphalysin is an ALP protein in the snail *Biomphalaria glabrata*. The exclusive expression of Biomphalysin in hemocytes, the immune cells of *B. glabrata*, consolidates the role of Biomphalysin in immunity. In the presence of plasma, recombinant Biomphalysin is highly toxic toward parasitic *Schistosoma mansoni*, suggesting that one or more unknown plasma factors could act together with Biomphalysin. These results provide the first functional description of a mollusk immune effector protein involved in killing *S. mansoni* (Galinier et al, 2013).

Lysenin or lysenin-like proteins are ALPs from earthworm. One subgroup of earthworm immune cells (so called coelomocytes) express the highest amount of lysenin, and its expression can be enhanced by Gram-positive bacterial exposure. It has been suggested that lysenin appears to display sphingomyelin-dependent and sphingomyelin-independent activities to kill various foreign intruders of the earthworm's coelomic cavity (Bruhn et al, 2006; Oppen et al, 2013). But the direct killing or inhibiting effects of lysenin on microbes have not been well-characterized, and further study is necessary to well illustrate its real actions in antimicrobial responses.

Acting together with TFF to trigger host innate immunity

Frog *B. maxima* lives in very harsh environments, such as pools containing microorganism-rich mud, and its skin is very "toxic". Comprehensive transcriptome analysis of the frog skin and blood suggested that the frog can live well in harsh environments owing to its nearly parallel innate and adaptive immune systems to mammals (Zhao et al, 2014). Interestingly, a heteromeric protein complex was recently identified and isolated from the frog skin secretions, which is responsible for the lethal toxicity of the frog on mammals (Gao et al, 2011; Liu et al, 2008; Qian et al, 2008a;). This heteromeric protein consists of a $\beta\gamma$ -crystallin fused ALP, namely *B. maxima* aerolysin-like protein 1 (Bm-ALP1), and a three domain trefoil factor (Bm-TFF3). It was named $\beta\gamma$ -CAT to reflect its domain composition (Figure 7A insert).

The rich expression of Bm-ALP1 and Bm-TFF3 in the frog blood and immune-related tissues, and the induction of its presence in peritoneal lavage by bacterial challenge were detected, raising the possibility of their involvement in antimicrobial infections. Indeed, subsequent *in vivo* assays illustrated that the complex of Bm-ALP1 and Bm-TFF3 ($\beta\gamma$ -CAT) was able to significantly accelerate bacterial clearance, thus reduce the mortality rate in frog *B. maxima* and mouse peritonitis models (Xiang et al, 2014). In contrast to small molecular-weight antimicrobial peptides from the same frog species (Lai et al, 2002b; Lee et al, 2005), $\beta\gamma$ -CAT neither directly kills bacteria nor inhibits their growth. The rapid

maturation and release of IL-1 β triggered by $\beta\gamma$ -CAT were detected both *in vivo* and *in vitro* and may have resulted from the oligomerization of and pore formation by Bm-ALP1 within cellular endo-lysosomes, which partially explain the robust and effective antimicrobial responses observed (Xiang et al, 2014). Figure 7A shows the mechanisms involved and action models proposed for $\beta\gamma$ -CAT in host antimicrobial responses. The mechanism by which the complex of Bm-ALP1 and Bm-TFF3 ($\beta\gamma$ -CAT) activates inflammasome is completely different from that of aerolysin, which was found to activate inflammasome via pore formation on the plasma membrane (Gurcel et al, 2006).

Importantly, preliminary assays revealed that the biological activities of $\beta\gamma$ -CAT were inhibited by free sialic acids, and were largely attenuated by eliminating sialic acid residues in cell membranes with sialidases (Guo & Zhang, unpublished observation), which suggests that either Bm-TFF3 or Bm-ALP1 $\beta\gamma$ -crystallin domains interact with oligosaccharide chains (glycans), and sialic acids are essential in its binding to cells. Sialic acids are a diverse family of monosaccharides widely expressed on all cell surfaces of vertebrates and "higher" invertebrates, and on certain bacteria. Different modified forms of sialic acids can be attached to underlying glycans by means of various linkages from the C2 position. The remarkable diversity is expressed in a cell-type and developmentally regulated manner, and often changes in response to environmental cues, which plays important roles in pathogen infection, inflammation and immunity (Hart & Copeland, 2010; Varki, 2006, 2007). The in-depth studies of the interaction of $\beta\gamma$ -CAT with glycans containing sialic acids, which might act as its putative membrane receptor(s), and biological relevance will certainly help to illustrate the roles of ALPs and TFFs in host immunity as well as the mechanisms involved.

ALPs and TFFs may consist of novel pathways and effectors in immunity The eradication of invading microorganisms is essential for the survival of multicellular organisms. Innate antimicrobial responses play a key role in host defense against many infections (Beutler, 2004). Profs Bruce Beutler and Jules Hoffmann were awarded the Nobel Prize in 2011 for the discovery of toll-like receptors (TLRs), sensors of microbes and a kind of pattern-recognition receptors (PRRs), which made great progress in our understanding of innate immunity. On the other hand, the interface of animals with the microbial world is characterized by the necessity to peacefully coexist with symbiotic microorganisms (the microbiota) (Chu & Mazmanian, 2013; Duerkop & Hooper, 2013). Thus, the basic puzzle here is how the host does to prevent pathogenesis while maintaining symbiosis. The strategies and molecular effectors of host endogenous regulators that ensure rapid, effective and controllable antimicrobial responses are incompletely understood. In addition, parasitic worms and allergens induce type 2 immune responses through mechanisms that appear to be independent of PRRs and remain largely unknown (Iwasaki & Medzhitov 2015; Licona-Limon et al, 2013; Medzhitov, 2010b; Medzhitov et al, 2012; Sansonetti, 2014; Strowig et al, 2012).

Interestingly, the membrane attack complex of the

complement system shares a common core fold with bacterial cholesterol-dependent cytolysin-like PFTs (Rosado et al, 2008). The complement system is an evolutionarily well-conserved system, which constitutes a highly sophisticated body defense machinery. The current complexity in mammals consists of more than 30 components, while some components of the most primitive complement system can be identified in cnidaria (Nonaka, 2014). Three activation pathways of complement system are well-defined: the classical pathway, the lectin pathway, and the alternative pathway (Holers, 2014).

Here comes ALPs, another kind of bacterial PFT-like proteins and their possible roles in host immunity. $\beta\gamma$ -CAT is the first example of an ALP and a TFF complex. Present data suggests that in contrast to microbial TLR ligands, which represent first signal of potential microbial infection, host-derived ALP and TFF complex primarily acts as a secondary signal, which might be necessary to initiate and trigger rapid and effective immune actions for eliminating dangerous microbial infections (Figure 7A). Thus, we hypothesized that ALPs and TFFs may consist of novel pathways and effectors in inflammation and immunity. The composition, regulation and effective actions of the pathways with ALPs and/or TFFs as potential sensors and effectors should have evolved diversification and variation along evolutionary processes in different lineages of organisms including vertebrates, which are certainly interesting and important subjects for future investigation.

In light of many well-defined innate immune pathways, such as those mediated by TLRs, the inflammasome-related NOD-like receptors (NLRs) as well as the components of complement system, which might be the functional positions and biological necessities of ALPs and TFFs in immunity? There are many possibilities, but in our opinion, the following aspects should be emphasized and studied in detail: (1) the clearance of intracellular microbial invasion of bacteria, virus or parasites; (2) the initiating of type II immunity to against parasitic infection as well as in allergic reactions; (3) the wound healing and tissue repairing. Further studies on their actions upon noxious stimuli, regulatory networks and underlying mechanisms will elucidate unknown pathways and effectors in inflammation and immunity, and eventually help to illustrate human pathophysiology, disease mechanisms and to provide novel drug targets and to develop novel therapeutics for related diseases.

Numerous ALPs mainly contain a membrane active and pore-forming aerolysin domain that undergoes fusion with agglutinin, jacalin, tachylectin, DM9, $\beta\gamma$ -crystallin, and Ig-like domains have been found (Szczesny et al, 2011; Xiang et al, 2014), and could be readily identified by blast in Genbank from diverse plant and animal species, such as rice, grapes, fishes, amphibians, reptiles as well as birds (Figure 7B). Some of these additional domains fused to aerolysin membrane insertion domain might be carbohydrate-related, such as agglutinin, jacalin, tachylectin domains. Whether the ALPs containing these lectin-like domains represent an unknown type of Sugar-binding Oligomerization Proteins (SOPs), which could be regulated by sugar recognition and binding, are worthy of further elucidating in detail. Interestingly, TFF domains are found to widely exist in diverse glycosidases (Genbank data),

implying their interaction with sugar, which might represent their original and ancient functions. These domains have been recruited in immune system in host defense, and the sugar binding capacity of human TFF2 has been identified recently (Hanisch et al, 2014).

FUTURE DIRECTIONS AND CHALLENGES

Toxins are natural evolutionary products of living organisms for special biological purposes. They are often gene-coded proteins and peptides, and are different from simple chemical toxic substances (toxicants). People may often be confounded by "toxinology" and toxicology. Although there is some overlapping, significant difference exists between them. Toxinology, or more accurately toxin biology and toxin medicine, is the specialized area that deals scientific disciplines with microbial, plant and animal venoms, poisons and toxins. Besides the chemistry and mode of action, toxicological effects in other organisms, it deals also with the biology of toxin-producing and toxin-targeting organisms, the venom apparatus, as well as the ecological roles and bio-economy of toxins.

Toxins to answer basic biological questions

The primary biological role of venom system concerns struggle for existence and environmental adaptation. Consequently, venom toxins are tightly associated with specific ecological contexts and environmental conditions. The formation and loss of venom systems and coevolutionary patterns with other organisms and biological relevance related in various animal classes provide nice models to understand fundamental biological questions concerning the strategies for environmental adaptation, genetic basis, evolutionary mechanisms and biological economy. It has been suggested that the genetic and functional diversity of animal toxins make them ideal systems for testing the models postulated to underlie gene evolution and adaptive change in organisms (Innan & Kondrashov, 2010).

Zoological and ecological issues

Investigations concerning the distributions and ecological environments and living conditions of venomous animals, the species taxonomy as well as venom system anatomy and characterization are no doubt the basic fields needed to be substantially reinforced. This is particularly important in areas that are rich in biodiversity, such as China and countries in Southeast Asia. Even though there has been a strong research community in China in documenting the flora and the fauna of animals distributed in China, and remarkable achievements have been made over the past decades, the actual animal diversity, especially venomous animals, in China are still incomplete and many new venomous animals are waiting for discovery. We are now pressed for time and the situation is very serious facing continued decline and extinction of animal species worldwide. Data on animal feeding behavior, prey-predator relationship, distinct microbiota should be collected, which are important clues for directing the related toxin study.

Structure-function of toxins

The elucidation of molecular diversity of toxins and their

biochemical properties, including post-translational modification, 3D-structure and family classification are another basic aspect of toxin study. The biological activity and toxicological effect of toxins in other organisms should be carefully assayed. Living strategy of a given animal is an important guiding principle in the assays. Blood coagulation and neuromuscular transmission are well-known key physiological networks targeted by venomous animals for rapid and effective immobilization of prey and/or defense. The haematotoxic and neurotoxic effects associated with venom exposure are widely recognized. In addition, immune and metabolic systems are also important targets of animal toxins, which is the strategy used by many venomous animals.

Natural pairing hypothesis

The most concerned issue right now and in the future is the molecular targets of toxins. It is quite difficult to fully understand the mutual interactions and mechanisms involved of the novel toxin molecules. Needless to say, this is a long-lasting task. The evolutionary origin and conservation of animals determine the inherent links among animals. Despite the huge species diversification, significant similarities and conservations share among animal physiological elements. Here, we propose the "natural pairing hypothesis" is that: (1) each animal key physiological element has been targeted by toxins in evolution process, and there has been at least one toxin molecule acting on it; (2) for the interactions of physiological elements and toxins, there are endogenous similarities and conservation, which occur among physiological elements and endogenous toxin-like molecules and play roles in physiological processes. This speculative idea is supported by accumulated and emerging identification of the interactions among animal physiological elements with toxins, as well as with endogenous toxin-like molecules. Traditionally, the study of toxin targets is mainly focused on protein components. In light of the critical roles of oligosaccharide chains (glycans) in physiological processes (Hart & Copeland 2010; Varki 2007), the specific glycans as toxin targets *in vivo* and biological relevance are worthy of noting and studying, especially for venom lectin-like proteins.

Although humans are generally neither the prey nor the predator of venomous animals, numerous toxins are able to actively and specifically interact with human physiological elements. This phenomena is hard to be either simply explained by conservations between humans and other animals or be seen as an interesting event by chance. The basic biological principles, which tightly link humans with toxins, are not recognized and understood well yet. We are now simply viewing a brief window of biological time, which represents the present status of trade-offs reached by currently living organisms subject to a number of evolutionary forces (Varki, 2006).

As reviewed above, it has been revealing that numerous physiological body proteins and peptides of various animals, including those of mammals, are homologous of toxins (TLPs). It would be speculated that the natural pairing and interaction of toxins and animal physiological elements are imitated and

conserved in normal physiological processes, which are conducted by endogenous interactions between body TLPs with their pairing physiological elements, especially in mammals, including humans. The similarity shared among normal endogenous interactions (TLPs with their pairing elements) in humans and exogenous interactions (toxins with their pairing elements) might be another explanation for unexpected tight links of toxins and humans.

Origin and loss, evolution and economy of toxins

As discussed extensively above, venom system is a special and complex trait shaped by evolution in struggles for existence in animals. However, the biological strategies of different animals, the origin and related genetic basis, the developmental regulation, as well as the underlying evolutionary mechanisms are not fully understood yet and are needed to be addressed in detail. Furthermore, the influence of specific prey-predator interaction (including those against microbial invasion) on the variation of toxins and coevolutionary patterns between toxins and body key physiological elements are interesting and important future challenges.

The possession of a venom system obviously has conferred the animal ecological advantages. However, there are also numerous non-venomous species in the same animal class, like non-venomous snakes. Many examples of venom loss in animals have been observed, supporting the notion that venom system occurs at a considerable cost in animals. The cost-benefit trade-offs of a given trait is optimized by evolutionary process, like that of inflammatory response in innate immunity (Okin & Medzhitov, 2012). Similarly, the trade-offs between beneficial and cost aspects of venoms may account for the origin and loss of a venom system, which is associated with the physiology and living strategy of a specific animal species. The biological philosophy and secrets underlying are important subjects worthy of investigating.

These studies, combining with principles and knowledge obtained in social and economic sciences, should certainly help biologists to better understand how evolution shaped development, change and balance among key factors (complex traits) in the struggle for existence of animals, such as venom (toxins), immunity, body power and intelligence, as well as genetic basis involved, inherent association and cost-benefit trade-offs of biological economy (Figure 8).

Toxins in fighting human diseases

Toxin related study in biomedicine may generally be conducted through two ways depending on the working fields, interests and technique skills of researchers. People mainly working in basic research fields or pharmaceutical industry focus on scientific questions of human patho-physiology and/or a clinic disease, and the works with the help of toxins have contributed substantially to human health. Studies starting with toxins are an alternatively way. Though being much more difficult, working with novel toxins or TLPs, which have novel actions and mechanisms, may eventually lead to novel clues and/or ways for fighting human diseases.

Deciphering human patho-physiology and diseases mechanisms

One of the most fascinating and important works is that using toxins as molecular probes to decipher the physiological functions and patho-physiological relevance of human physiological elements, and eventually disease mechanisms.

First, generally speaking, the issue on one hand is the determination of molecular interaction of a toxin molecule with a human physiological element, which could be an ion-channel, a receptor or a non-membrane protein. Work on the other hand is pharmacological and toxicological activities of the toxin, especially those of *in vivo*. The data obtained provide first indication and suggestion on the biological function of the human physiological elements. These assays should be conducted in both cellular level and in animal models. The obtained results should preferably be validated in animal model deficient in the targeted proteins. Mechanisms involved in the interactions between the toxin and the targeted protein should be investigated in detail. Collectively, these works will hopefully illustrate the unknown function and patho-physiological relevance of the targeted protein, and eventually the disease mechanisms. There are extensive molecular diversities of potential molecular targets of animal toxins, such as approximately 400 genes for pore-forming ion channels in human genome. Only a small part of them have been characterized until now. Screening of specific toxin molecules that act on those "unknown" or "orphan" ion-channels and/or receptors may be very interesting and important. Very often, the *in vivo* target of a given toxin is totally elusive, and data obtained from animal models should serve as an important guiding principle. In addition, the signs and symptoms during animal envenoming in humans could provide nice suggestions.

Second, immune system is an important target of animal toxins. Animal envenomation in humans often leads serious inflammatory responses and allergic reactions, which are one of the major causes of death. As reviewed above, the using of bee venom PLA2 revealed the protective roles of allergic reactions in innate immunity, which is contrast to the traditional view of allergy as a misguided and detrimental immune response (Palm et al, 2013). There are accumulative evidences showing conservation and similarity in venom toxins and immune effectors from these two systems. Thus, to understand immune systems, future studies are necessary to witness the facts from venom toxins.

Third, many components in animal venoms are mammalian hormone-like peptides, which could deeply affect metabolism systems. A nice example is exendins from the venom of lizards, which are homologous of human GLP-1 and are able to stimulate insulin and suppress glucagon secretions. Amphibian skin is also enriched with bioactive peptides, which may stimulate or inhibit metabolic activities. Starting with a focused metabolic disease, like diabetes, the screening of venom toxins preferably in animal models may lead to the discovery of novel metabolic active components modulating metabolic systems. Such works may result in uncovering the unknown regulatory pathways in human metabolism and disease mechanisms involved.

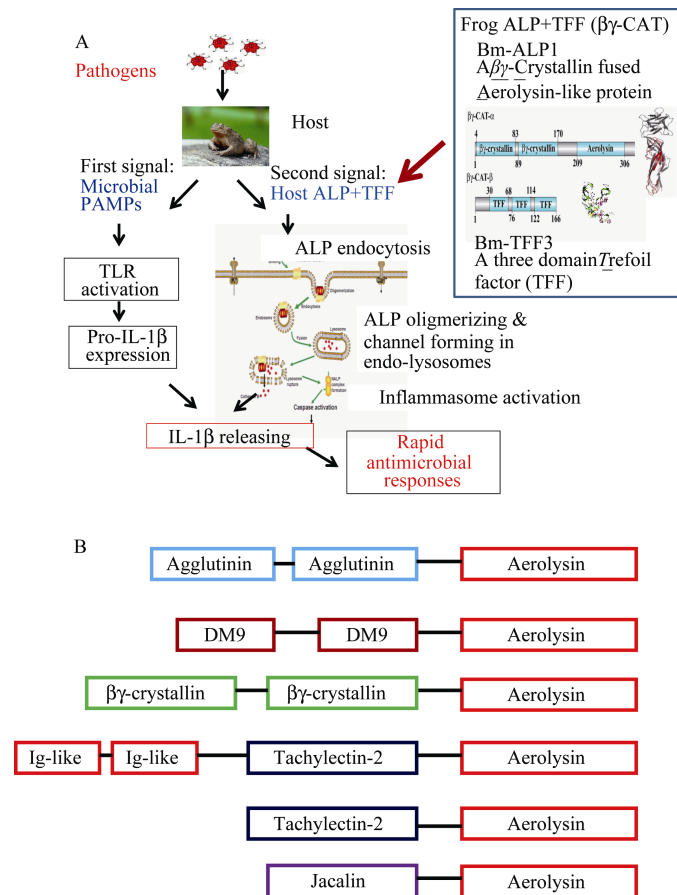


Figure 7 Aerolysin-like proteins (ALPs) and trefoil factors (TFFs) may consist of novel pathways and effectors in immunity

A: $\beta\gamma$ -CAT, a heteromeric complex consists of Bm-ALP1 and Bm-TFF3 (a three domain TFF), was identified from *Bombina maxima* (insert). Upon bacterial infection, pathogen-associated molecular patterns (PAMPs) induce the activation of Toll-like receptors (TLRs), which subsequently trigger the intracellular production of pro-IL-1 β . Additionally, $\beta\gamma$ -CAT was endocytosed via membrane receptor mediation. Bm-ALP1 was found to oligomerize along endo-lysosome pathways to trigger lysosome destabilization, and led to IL-1 β maturation and secretion via inflammasome activation, resulted in host rapid and effective antimicrobial responses (Xiong et al, 2014). B: ALPs mainly contain a pore-forming aerolysin domain that undergoes fusion with agglutinin, jacalin, tachylectin, DM9, $\beta\gamma$ -crystallin, and Ig-like domains have been found (Szczesny et al, 2011; Xiang et al, 2014), and could be readily identified by blast in GenBank from diverse plant and animal species, such as rice, grapes, fishes, amphibians, reptiles as well as birds. The schematic domain composition of representative ALPs is cited and modified from Szczesny et al (2011). We speculated that some of these ALPs might be sugar-binding oligomerization proteins (SOPs), which could be regulated by sugar recognition and binding.

Fourth, numerous physiological TLPs, especially in terms of their 3D-structures, with unknown functions are expressed in non-venomous animals and in tissues not related to venom systems, such as ALPs. Knowledge obtained in toxin study are helping to illustrate their functions *in vivo*, the mechanisms involved as well as the path-physiological relevance in humans. Technological advances on the determination of protein 3D-structure will greatly accelerate the uncovering of body physiological TLPs. Another important work is that once the possible function of an "unknown" or "orphan" ion-channel or a receptor has been elucidated via its interaction with toxins, people will immediately search for its potential unknown endogenous ligand(s). In many cases, these works are difficult

due to the lack of significant structural similarities.

Drug development

Based on their interactions with human targets, toxin molecules have stimulated many drug development projects. Thus, another attractive aspect in toxin study is that as the clinic therapeutics, toxins may be used in either direct or after toxin-based drug designs. Focusing on a properly selected disease and its clinic indications, the evaluation of druggability and pharmaceutical properties of a given toxin molecule is the most important work. Even though with high costs, these assays should preferably be conducted on animal models of human diseases, which are more productive than been simply

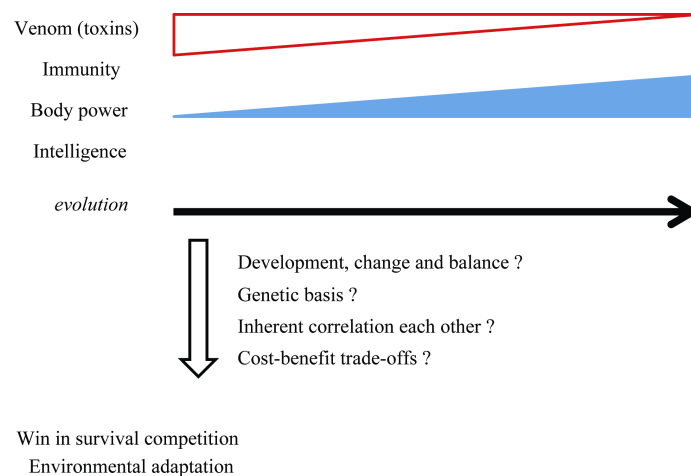


Figure 8 Venom systems provide nice models to understand fundamental biological questions

The cost-benefit trade-offs of a given trait is optimized by evolutionary process. In depth understanding of the origin and loss of venoms, biological relevance and coevolutionary patterns with other organisms provides nice models to investigate how evolution has shaped development, change and balance among key factors in the struggles for existence of animals, such as venom (toxins), immunity, body power and intelligence, as well as cost-benefit trade-offs of biological economy and the genetic basis related.

analyzed on molecular and cellular levels. The *in vivo* efficacy, potential antigenicity, unfavorable pharmacokinetics, costs, side-effects and advantages compared with present clinic drugs are major concerns in toxin-based drug development. In many cases, toxin molecules serve as templates in small molecule design for optimizing drug development directed by structural information of the mutual interaction between a toxin in its native or modified forms and its specific targets, and the pharmacological activity resulted.

There are many medicinal animals widely and effectively used in clinic as therapeutics, which are often venomous animals. The traditional practice and experience of these medicinal animals, especially clinic indications related, provide invaluable information in modern drug development. We should not forget that there are many TLPs expressed in the non-venom tissues of both venomous and non-venomous medicinal animals. It is highly interesting to investigate in detail whether the effective components in these medicinal animals are venom toxins and/or body TLPs. Such work will greatly promote the establishment of medicinal animal standards in industry and markets and enhance the modernization of traditional medicine.

As reviewed by Harvey (2014), drug discovery and development are an inherently risky business. Although with some notable successes, there have been many more disappointments on the road from toxin discovery to approval of a new medicine. Some products have been dropped in clinic trials due to side-effects and toxicity in human encountered. The failure may also be often caused by the discrepancy of toxin targets determined *in vitro* and those actually targeted by the compound *in vivo*, leading to unexpected and unwanted effects in clinic. After efforts of many years, US FDA did not approve marketing of pexiganan, an analog of magainin-2 from frog skin for treatment of infected foot ulcers in diabetic patients because of not enough efficacies demonstrated in clinic. Consequently, alternative

therapeutic applications need to be explored (Conlon et al, 2014).

Problems encountered

First, toxins are traditionally isolated from crude venoms by classic chemical techniques. Obtaining pure enough toxin sample with sufficient quantity is the first key factor that limits toxin study. Minor contamination often resulted in wrong conclusions in the interpretation of pharmacological and toxicological activities of a specific toxin. On the other hand, the majority of toxins revealed by genome and transcriptomic analysis are almost impossible to obtain by classical purification processes. New advances in chemical synthesis and recombinant expression of a toxin polypeptide have been greatly accelerating toxin study, which allow sufficient and pure enough toxin sample obtained (Cui et al, 2013; Schroeder et al, 2014).

Second, a serious concern of animal toxins in biomedical research and drug development is the specificity of a toxin on its molecular targets. Facing the diversity of physiological elements in prey and/or predator, selection pressure and coevolution have made creation of diversified toxins with relatively high specificity and potency, especially compared with those of small compounds from plants. The interaction of a toxin with an ion channel or a receptor depends on its binding affinity. Taking K^+ channels as an example, it was estimated that there are nearly 100 K^+ channel members (Ashcroft, 2006) in humans. High specificity means the difference of binding affinity to a kind of K^+ channels or to the subtypes of K^+ channels is high, which does not exclude the possible action on other K^+ channels or channel subtypes. Furthermore, the interaction of a toxin with an ion channel was usually determined *in vitro*, and often assayed or screened with targets in hands, which are unfortunately only corresponding to a small part of those *in vivo*. The interactions discovered might not really reflect its optimal targets, especially *in vivo*. This discrepancy of toxin action

determined *in vitro* and those actually conducting *in vivo* may either mislead the use of the toxin as a tool to deciphering patho-physiology or result in the failure of toxin-based drug development (Harvey, 2014).

We should recognize that to human health, venomous animals are neglected strategic resources. The exploitation involves investigation and collection of animals, the well-organized preservation of toxin genes before the extinction of specific animal species, and toxin-related works, which is a typical systematic engineering. Obviously, tight collaboration of researchers in different fields, including with people in industry is a preferable way for effective outcome prospected. Establishment of toxin center(s) or working networks, if possible in national level and international level, should greatly prompt the advance of toxin studies. If we plan to obtain the genome sequences of 10 000 people, why not try to obtain those of 100 venomous animals too. These works will substantially help to illustrate human patho-physiology and disease mechanisms, potential drug finding, rational new drug design, as well as clinic utilization.

CONCLUDING REMARKS

As a special trait in animals, venom system has evolved in nature for survival competitions, which plays important biological roles in predation, defense, competition and even communication in given ecological contexts. Facing the huge molecular diversity of key physiological elements of animals including humans, such as cell membrane ion channels and receptors, long-term coevolution has evolved extensive and diverse peptide toxins in the venom of venomous animals, which are able to specifically target on these key physiological elements.

Defenses against microbial invasion and self-malignant cells are major missions of immune system. Attack and defense are the basic commonality of venom and immune systems. It would be reasonable to predict and hypothesize that both systems share substantial conservation and similarity in their biological strategies and molecular effectors, as supported by many

conserved protein folds used in both systems. The investigation with this notion in mind should certainly benefit mutually and facilitate the in-depth understanding of these two systems. Venom (toxins), immunity, body power and intelligence are evolved along the evolutionary tree of animals and play key roles in animal survival competitions. Toxin study will hopefully provide useful information and will help to illustrate the basic biological issue that how a living organism adapt environments, keep "homeostasis" facing various ecological conditions and noxious stimuli, and win in struggles for existence.

According to "The Medical Classic of the Yellow Emperor (Huangdi Neijing)", one of the earliest theoretical classics on Chinese medicine, a disease is an unbalanced state of human body. Drugs *per se* are agents causing another unbalanced state, which are used to recover the given unbalanced state of a human disease. So, drugs are inherently more or less toxic, and can be viewed as special poisons/toxins. Theoretically, each poison/toxin molecule created by nature could potentially serve as a pharmacological tool and/or a clinic therapeutics in either its native or modified forms in conditions that the toxin is used at a right time, in a right place, with a right dosage and for proper purposes or clinic indications.

Evolution links animal toxins with humans, providing natural basis of animal envenomation as well as for animal toxins being used as pharmacological research tools and/or clinic therapeutics. Our goal is to reveal the right natural pairings and interactions between our body elements and toxins. Starting with focused scientific questions and/or a clinic disease, or starting with toxins themselves, through diligent work and/or serendipity, work with toxin molecules has led, and will lead in future, to new discoveries and exciting avenues for deciphering and fighting human diseases. Biomedical researches in humans and in model animals have made great advances in the understanding of our physiology and diseases. In depth understanding of toxins is an effective way to better know ourselves. All in all, this is why we study toxins (Figure 9).

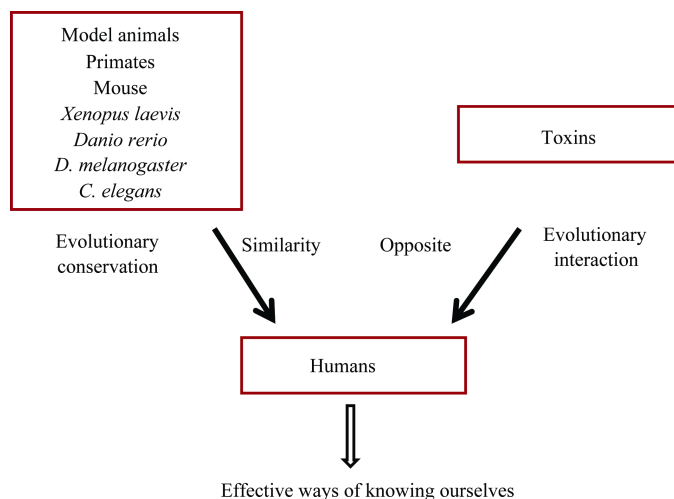


Figure 9 Recognizing us from toxins is an effective way to better understand ourselves

To understand human physiology and diseases, studies in model animals rely on the similarity and conservation shared by humans and model animals in evolution. In nature, toxins are against humans, and studies depending on the evolutionary interaction between humans and toxins are an alternative and effective way to better understand ourselves.

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Population genetic studies in the genomic sequencing era

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ABSTRACT

Recent advances in high-throughput sequencing technologies have revolutionized the field of population genetics. Data now routinely contain genomic level polymorphism information, and the low cost of DNA sequencing enables researchers to investigate tens of thousands of subjects at a time. This provides an unprecedented opportunity to address fundamental evolutionary questions, while posing challenges on traditional population genetic theories and methods. This review provides an overview of the recent methodological developments in the field of population genetics, specifically methods used to infer ancient population history and investigate natural selection using large-sample, large-scale genetic data. Several open questions are also discussed at the end of the review.

Keywords: Population genetic inference; Allele frequency spectrum; Linkage disequilibrium; Natural selection; Demography

INTRODUCTION

A central goal of evolutionary biology is to understand the mechanisms of how natural selection and other factors, such as random drift and mutation, drive the evolutionary process. Population geneticists address these questions quantitatively by building mathematical models, developing statistical methods for inferring parameters of ancestral processes, and testing hypotheses based on the analysis of real data. With the development of sequencing technologies in recent years, large-sample, large-scale genetic polymorphism data from humans and other species have increased greatly (Mardis, 2008), bringing valuable resources to address evolutionary questions. However, the computational capacities of traditional population genetic methods only allow their applications to small samples and/or local chromosome regions. Methodological development in population genetics has reacted to the emergence of these large-sample, large-scale genetic data. This review provides a summary of methodological advances that aim to answer two

fundamental questions in population genetics, specifically ancient demography inference and natural selection detection.

LEARNING ANCIENT DEMOGRAPHY

In genetic studies of human evolution, mitochondrial DNA (mt-DNA) and the Y chromosome have been invaluable markers for reconstructing the history of modern humans (Cavalli-Sforza & Bodmer, 1971). In their seminal paper, Cann et al (1987) constructed a phylogenetic tree of 147 individuals from five human populations using mt-DNA sequences, and inferred the time of the common female ancestor to be 200 Kyr. Since mt-DNA is maternally inherited, Cavalli-Sforza and colleagues sequenced the non-recombining region of the Y chromosome (NRY) and developed a system for investigating paternal origins (Underhill et al, 2001). mt-DNA and NRY are especially useful for constructing population history, since the non-recombining regions of mt-DNA or NRY share a single gene genealogy, enabling the inference of a common phylogenetic tree. Using mt-DNA and NRY, population geneticists can determine the migration route and dispersal of global human populations. Many successful applications of this approach can be found in studies of East Asian populations (Jin & Su, 2000; Ke et al, 2001; Kong et al, 2003; Yao et al, 2002; Zhang et al, 2013; Zhao et al, 2009).

Most genetic polymorphisms are, in fact, stored in autosomal regions. Due to recombination along chromosomes, two distant autosomal regions from a sample are likely to evolve with independent genealogies. These gene genealogies represent independent realizations of the evolutionary process given the underlying demographic history, and thus are more informative for demographic inference than mtDNA or NRY, which only carry information of single gene genealogy. One approach to exploring population structure using autosomal polymorphisms is principal component analysis (PCA). This approach decomposes the covariance matrix that summarizes the correlations among individuals or populations, and efficiently extracts the main structure of the data. It then presents the

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population structure by plotting a few leading principal components against each other. This method was introduced by Cavalli-Sforza & Bodmer (1971) to the field of population genetics, and was very useful for investigating population structure using abundant genomic polymorphism from multiple populations (Patterson et al, 2006).

Recently, other than the exploratory analysis of population structure using PCA, population genetic studies have gained more insights into demographic history from population genetic models. Methods for population history inference have been developed using different aspects of genomic polymorphism, mostly based on: (1) the allele frequency spectrum (AFS, alternatively "site frequency spectrum", SFS), and (2) linkage disequilibrium (LD) or haplotype structure (Table 1).

Allele frequency spectrum-based methods

The AFS is a sampling distribution of alleles in a finite sample (Chen, 2012), and focuses on the allele frequency distribution of a single locus, ignoring the correlation among nearby loci. Such approximation greatly simplifies theory and methodology development. AFS theory was developed in two parallel frameworks: the diffusion (Kimura, 1955) and coalescent processes (Fu, 1995).

Theoretical studies on a single-population AFS started from stationary populations of constant size, and later were extended for populations with time-varying size (Griffiths & Tavaré, 1998), including exponentially growing populations (Evans et al, 2007; Polanski & Kimmel, 2003; Wooding & Rogers, 2002) and piecewise constant populations (see the N-epoch model describing population size change in Marth et al, 2004). The AFS for populations with time-varying size (non-equilibrium populations) was applied to real data to infer ancient Asian and European demography (Keinan et al, 2007).

Recently, the AFS of one population was extended to the joint allele frequency spectrum (JAFS) of multiple populations. Gutenkunst et al (2009) obtained the numerical solution of a three-dimensional diffusion equation, and applied the method to infer the joint demographic history of three world populations: West European (CEU), East Asian (HAN) and West African (YRI). Their software $\partial\alpha\partial i$ has been extensively applied to analyze genomic data, and uses the finite difference approach to obtain a numerical solution, and thus computational complexity is a function of population size and increases exponentially with population number. The computation becomes intensive when the population number is large or the population is under rapid growth. Lukić et al (2011) reduced computational complexity by using spectral methods, and successfully applied their method to infer the history of four world populations (Lukić & Hey, 2012).

The above two JAFS approaches rely on numerical solutions of diffusion equations. Others have used coalescent simulations to approximate the JAFS of two or several populations (Excoffier et al, 2013; Li & Stephan, 2006). Chen (2012) derived an analytical form of the JAFS for multiple populations using coalescent theory. Their method incorporated various scenarios including time-varying population size, instantaneous migration

and the hitch-hiking effect. Compared to the diffusion-based approach, computational complexity of the coalescent-based method is reduced to a function of sample size, which is much more efficient.

Haplotype structure-based methods

Another group of methods consider linkage disequilibrium, or the correlation of gene genealogies of adjacent sites. For example, the pairwise sequential Markovian coalescent method (PSMC, Li & Durbin, 2011) uses a hidden Markov model to approximate the dependency of the coalescent times of two haplotypes between adjacent loci, and further infers the detailed ancient population size from coalescent times. The limitation of the PSMC method is that it can only analyze one diploid genome. Burgess & Yang (2008) inferred ancient population size by analyzing multiple sequences, with each sequence representing one population. They developed a Markov chain Monte Carlo approach (MCMCCoal) to sample gene genealogies. Gronau et al (2011) modified MCMCCoal to allow two sequences from each sampled population, and applied their G-PhoCS method to infer the joint demography of four world populations. The Coal-HMM method (Hobolth et al, 2007) can also analyze multiple genomes from several populations. Instead of sampling over gene genealogies as per MCMCCoal, Coal-HMM treats the unobserved gene genealogy at each genomic position as unobservable latent states in a hidden Markov model. The method in Mailund et al (2011) is similar to the PSMC method, but can analyze two sequences from two populations.

Extending the above methods to the analysis of a large or even medium number of individual genomes is more challenging. Lohse et al (2011) used a probability generation function to infer the coalescent times of multiple individuals, and then population demography. Harris & Nielsen (2013) investigated the extent of shared IBD (identical by descent) tracts between each pair of haplotypes, and applied their method to a two-isolated-population model with migration. The *diCal* method in Sheehan et al (2013) was built on the sequential Markovian coalescent process and improved computation by proposing more efficient importance-sampling proposal distributions. Recently, Schiffels & Durbin (2014) extended Li and Durbin's PSMC method to the multiple sequential Markovian coalescent (MSMC) method, which can deal with multiple individual genomes from two populations. The possible number of gene genealogies increases dramatically when multiple sequences are included, and computation again becomes intensive. MSMC tackles the problem by focusing only on some summary statistics of the genealogies, such as first coalescent time of any two sequences and total length of all singleton branches of the genealogy.

Overall, the existing haplotype-based methods can analyze a small number of individual genomes, but are quite powerful for inferring ancient history. For example, the PSMC method works well for learning population size between 20–200 Kyr (Li & Durbin, 2011). The remaining challenge is to efficiently approximate the sequential Markovian coalescent or the ancestral recombination graph for larger samples.

Table 1 Methods for inferring demographic history using genomic polymorphism

Method	Data	Inferred demography	Notes
Wooding & Rogers (2002)	Allele frequency spectrum of a single population	Exponential growth rate of a single population	Coalescent, numerically
Marth et al (2004)	As above	N-epoch model for a single population	Coalescent, analytically
Polanski et al (2003)	As above	Arbitrary size of a single population	Coalescent, analytically
Evans et al (2007)	As above	Arbitrary size of a single population	Numerical solution of diffusion
PSMC (Li & Durbin, 2011)	One personal genome	As above	Sequential coalescent
diCal (Sheehan et al, 2013)	Multiple genomes	As above	-
Dadi (Gutenkunst et al, 2009)	Joint allele frequency spectrum of multiple populations	Demography of three populations	Numerical solution of diffusion
Lukić et al (2011)	As above	Demography of four populations	As above
JAFS (Chen, 2012)	Joint allele frequency spectrum of multiple populations	Demography of multiple populations	Coalescent, analytically
Excoffier et al (2013)	As above	As above	Coalescent, simulation
MCMCCoal (Burgess & Yang, 2008)	Individual genomes from multiple populations	Demography of multiple populations	MCMC
Coal-HMM (Hobolth et al, 2007)	As above	As above	-
G-PhoCS (Gronau et al, 2011)	Individual genomes from multiple populations	Demography of multiple populations	A variant of MCMCCoal
Harris & Nielsen (2013)	IBD tracks of two populations	Divergence and migration of two populations	-
MSMC (Schiffels & Durbin, 2014)	Individual genomes from two populations	Demographic history of two populations	-

Demography of East Asian populations

The AFS and haplotype-based methods have been applied to genomic data to infer demographic histories of humans and other species. In human studies, the demographic history of Western Europeans was the main focus due to the abundance of sequence data for European populations. Several studies inferred East Asian demographic history using the HapMap or the Thousand Genomes Project data (Gravel et al, 2011; Gronau et al, 2011; Li & Durbin, 2011; Schaffner et al, 2005;

Schiffels & Durbin, 2014). The inferred parameters of these studies are detailed in Table 2. These studies identified major events in East Asian history; for example, Keinan et al (2007) analyzed the AFS of the HapMap HCB samples and estimated a severe bottleneck in East Asian populations with an intensity (defined as $T/2N$) of 0.123 ± 0.015 . While detailed knowledge on Asian population history is still limited, understanding East Asian demographics at a finer level is useful for disease studies, and relies on the availability of genomic polymorphism data.

Table 2 Inferred demographic history of East Asians by different studies

Parameter	Schaffner (2005)	Gravel (2011)	Gronau (2011)	Schiffels (2014)
Current pop size	100 000	45 521	4 100	>1 000 000
Growth rate	-	0.48%	-	-
Eurasian pop size	7 700	1 861	1 000	~1 200
Eurasian split time	50.0 Kyr	23.0 Kyr	38.0 Kyr	20-40 Kyr
African effective size	24 000	14 474	141 000	~15 000 (at 110 Kyr)
Out-of-Africa time	87.5 Kyr	51.0 Kyr	49.0 Kyr	60-80 Kyr

DETECTING NATURAL SELECTION

Methods

Detecting the effects of natural selection and identifying the selected loci in the genome is another research hotspot in population genetics. Natural selection, especially positive selection (aka, selective sweeps) generates distinctive genetic polymorphism patterns in contemporary populations. Statistical approaches have been constructed using some informative aspects of these patterns.

Allele frequency spectrum When natural selection drives a selected mutant to fixation in a population, the allele frequencies of SNPs in the vicinity of the selected mutant are also affected. Their frequencies can be increased, if the alleles are linked to the selected mutant, or decreased otherwise. This is known as the hitch-hiking effect. Smith & Haigh (1974) provided a deterministic equation to approximate this effect (Fay & Wu, 2000). Recent theoretical studies on the selective coalescent process derived more accurate sampling formulas for modeling the hitch-hiking effect (Durrett & Schweinsberg, 2004; Etheridge et al, 2006). The sampling formulas can be used to derive the AFS of a linked SNP under hitch-hiking. Methods for detecting selection can then be constructed by combining information of multiple SNP loci with a composite-likelihood approach. As an approximation of the full likelihood, the composite likelihood of multiple loci is obtained by taking the product of marginal likelihood of individual loci. The AFS-based methods include SweepFinder for a single population (Nielsen et al, 2005), and the JAFS method of multiple populations (Chen, 2012).

Summary statistics, such as, Tajima's D (Tajima, 1989), Fu and Li's F^* (Fu & Li, 1993) and Fay and Wu's H (Fay & Wu, 2000) are known as neutral tests for selection. These statistics are summaries derived from a single-population AFS. For example, a negative D value indicates a skewed AFS with

overrepresented singletons, while a positive D indicates an enrichment of segregating sites with medium frequencies. A likelihood approach using a detailed AFS has more power than neutral tests based on summary statistics.

AFS methods are powerful for detecting selection, but have several limitations: (1) population history can confound the effect of selection on the AFS. For example, a recent rapid population growth increases the relative abundance of rare alleles, which is similar to the effect of negative selection. However, since demographic effects are genome-wide, it is possible to control for these effects by explicitly modeling demographic history in the methods (Li & Stephan, 2006; Williamson et al, 2005); (2) AFS methods are not robust to SNP ascertainment bias (Clark et al, 2005). SNP data generated from platforms designed under complex ascertainment schemes, such as SNP arrays, are unfit for AFS methods. With more ascertainment-free genomic data from NGS technology, AFS-based methods are expected to become more applicable.

Haplotype structure When a selected allele is increased to high frequency, the ancestral haplotypes carrying the selected alleles are also increased. The time interval for the selection process is short enough that the ancestral haplotypes are not broken by recombinations, and thus long extent haplotypes in the vicinity of the selected allele can be observed. Such a haplotype structure can be used to test for recent positive selection when the selected allele is still segregating. Several methods were developed based on such haplotype structure, including the EHH test (Sabeti et al, 2002), iHS test (Voight et al, 2006), and the hidden Markov model approach (Chen et al, 2015). The idea was further extended to the comparison of haplotype homozygosity between two populations (e.g., XPEHH test, Sabeti et al, 2007; Tang et al, 2007). These haplotype-based methods are robust to SNP ascertainment schemes, and are very useful for SNP array data designed under complicated ascertainment schemes.

Population differentiation Using population differentiation to detect selection is based on the fact that if the gene is under local adaptation in one population, its frequency divergence among populations should be highly beyond the genomic level. The fixation index, *F_{st}*, which measures the divergence between two populations at a single locus, was adopted by Lewontin & Krakauer (1973) to detect selection. A moments-based estimator of *F_{st}* developed by Weir & Cockerham (1984) has been commonly used when sample sizes from two populations are unbalanced and has been applied to a genome scan for selected loci (Akey et al, 2002).

F_{st} is calculated for a single locus and has a large variance. The random fluctuation of *F_{st}* values across SNP loci causes high false positive rates. Combining the incremental effects of selection on multiple loci helps reduce the false positive rates and increase power. The XP-CLR test by Chen et al (2010) was developed in light of this principle. It explicitly models the decay of population differentiation as a function of genetic distance between the neutral marker and selected mutant, and uses a composite likelihood scheme to combine the effects of multiple loci. Other methods that make use of population differentiation include the locus-specific branch test (LSBL) and its variants (Shriver et al, 2004; Yi et al, 2010), though they are single locus-based.

The above three classes of methods utilize different aspects of data patterns. Grossman et al (2010) attempted to combine multiple genetic signals to reduce the false positive rates in detecting selection (composite of multiple signals test, CMS).

Natural selection in East Asians

Modern humans faced environmental changes and infectious diseases when they migrated out of Africa and colonized other places in the world. Natural selection was very likely essential during this process. Specifically, East Asian environments were extremely divergent in terms of climatic factors, such as UV light, temperature and altitude, making Asian populations ideal for studying natural selection (Shi & Su, 2011).

Shi et al (2009) hypothesized that the *P53-MDM2* pathway may be important for the adaptation to low temperature when East Asians moved from the south towards high-latitude areas. Tibetans have lived on the Himalayan Plateau for tens of thousands of years. Recent genomic studies identified several genes conferring hypoxia adaptation, among which *EPAS1* shows the strongest signal of Tibetan-specific selection (Beall et al, 2010; Peng et al, 2011; Simonson et al, 2010; Wang et al, 2011; Xu et al, 2011a; Yi et al, 2010). Xiang et al (2013) recently identified one functional mutant in another gene *EGLN1*. Interestingly, *EGLN1* and *EPAS* are both of the hypoxia pathway with direct interaction.

EDAR shows an extremely strong signal of recent positive selection in East Asian (Sabeti et al, 2007). Kamberov et al (2013) used mice to show that a non-synonymous mutation could cause phenotypical changes in the skin. The mechanism underlying selection on *EDAR* remains unclear, though it was hypothesized to be due to the high humidity in Eastern Asia.

Life styles, such as the transition from hunter-gather to an agricultural society might also be important driving forces. One

gene related to lifestyle transition is *ADH* (Li et al, 2008; Peng et al, 2010). The *ADH1B*47His* allele in East Asians shows signals of selection and its geographic frequency distribution is consistent with cultural relic sites of rice domestication in China, indicating that the *ADH* gene might be related to the potential benefits of fermented beverages and food.

Artificial selection in domestication

Domestication and animal and plant breeding have been ongoing since the origin of agriculture around 10 000 years ago. Artificial selection shaped the traits of domesticated species to meet human demands during this process (Li & Zhang, 2009). Investigating selection on domestication traits helps identify the genetic architecture of these traits and improve breeding (Doebley et al, 2006). In recent years, such endeavors have been facilitated by genomic sequencing technology (Doebley et al, 2006; Fang et al, 2009; Huang et al, 2012; Hufford et al, 2012; Lyu et al, 2013, 2014; Qi et al, 2013a; Xu et al, 2011b; Zhou et al, 2015).

Most such studies identified a list of selected loci, and then checked their overlap with domestication QTL loci or some meaningful GO categories and pathways. Xia et al (2009) sequenced 40 domesticated and wild silkworms. They identified signals of selection at 354 candidate genes possibly important during domestication, some of which have enriched expression in the silk gland, midgut and testis. Wang et al (2013) studied natural selection in dog genomes, and identified genes related to starch digestion and metabolism, including nutrient transport and regulation of the digestion process. This reflects an agricultural living condition during the domestication history of dogs.

Zhou et al (2015) analyzed genomic sequences of 62 wild soybeans, 130 landraces and 110 improved cultivars. They identified 121 domestication-selective sweeps and 109 improvement-selective sweeps. Among these selected targets, some were related to morphological features, such as seed size and color, seed weight, stem determinacy, flower color, seed coat color and pubescence form. In addition to morphological traits, more than 90 sweep targets were located within known oil QTL regions.

Results from these genomic studies shed light on the fundamental mechanisms of the artificial selection process. For example, Hufford et al (2012) analyzed genomic sequences of 75 wild landraces and improved maize, and found evidence for stronger selection during domestication than improvement and that artificial selection was common in regulatory regions, which was confirmed by transcriptome analysis.

Inferring selection intensity, allele age and fixation time

In addition to identifying loci under selection, population geneticists are also interested in knowing further details of the selection process, such as, when natural selection initialized and how to quantify selection intensity. A detailed portrait of the selection process provides hints for deciphering the mechanism of selection, and validates anthropological hypotheses. For example, in studies on Tibetan high altitude adaptation, Peng et al (2011) and Xiang et al (2013) inferred the allele ages of

EPAS1 and *EGLN1* using haplotype structure. Although both genes were under strong selection, the estimated times were different: selection on *EPAS1* started around 20 Kyr ago and selection time of *EGLN1* was only about 7 Kyr. Interestingly, the two selection times are consistent with the two waves of population migrations to the Tibetan Plateau (Qi et al, 2013b; Zhao et al, 2009). Based on this, Xiang et al (2013) proposed a two-step hypothesis on the development of Tibetan adaptation to high altitude.

For selected alleles still segregating in the population, e.g., *EPAS1* and *EGLN1*, Chen & Slatkin (2013) proposed a method for inferring selection intensity and allele age using haplotype structure. Their method relies on importance sampling algorithms to sample from the genealogical space and allele frequency trajectories, which requires very intensive computation. The method can only analyze a local region for a small number of individuals. Chen et al (2015) developed a hidden Markov model for investigating the haplotype structure around the selected mutant, and provided a simplified population genetic model for inferring the parameters. The simplified model is much more efficient, and can be applied to genome-wide analysis for large samples.

If selection occurred anciently, the selected allele may have been fixed in the population. The parameter of interest for ancient selection is the time since fixation. For example, the genetic changes underlying the emergence of speech and language in modern humans, believed to be under strong selection, were inferred to be fixed during the last 200 Kyrs (Enard et al, 2002). Linnen et al (2009) studied cryptically colored deer mice living on the Nebraska Sand Hills and showed that their light coloration was caused by a cis-acting mutation closely linked to a single amino acid deletion in *Agouti*. The fixation time of the mutant was 8-10 Kyrs ago.

To date, only a few methods for inferring fixation time of selection have been proposed. The Bayesian approach by Przeworski (2003) simulates samples under selection, and matches a list of summary statistics between simulated and real data with rejection sampling. The method integrates over all possible values of selection intensity, and provides the posterior distribution of fixation time. Linnen et al (2009) used a two-step scheme to infer fixation time, first assuming the fixation time was 0 and estimating the selection intensity using the AFS of all SNPs from the gene region (Kim & Stephan, 2002). Fixing selection intensity to the estimated value, they used the Bayesian approach of Przeworski (2003) to obtain the posterior distribution of fixation time. However, the above two methods fail to jointly estimate selection intensity and fixation time. Chen (2012) modeled the pattern of the allele frequency spectrum of SNPs linked to a selected mutant as a function of selection intensity and fixation time, and efficiently estimated both parameters.

CHALLENGES AND FUTURE STUDIES

With the rapid development of sequencing technology, large-sample or even population-scale sequencing data have become available. For example, Coventry et al (2010)

sequenced two genes (*KCNJ11* and *HHEX*) for 10 422 European Americans and 3 293 African Americans. Nelson et al (2012) and Fu et al (2013) conducted exome sequencing for several thousand individuals. Large sample genomic data provide valuable resources for population genomic studies. However, the computational capacities of traditional population genetic methods only allow their application to local regions and/or small samples. Developing computationally efficient methods capable of analyzing large-sample, large-scale genomic data is necessary and challenging. Some computational issues, such as controlling for data quality, especially for sequencing data with low coverage, are important for population genetic inference, but beyond the scope of this review. Discussions on this topic can be found in the literature (e.g., Han et al, 2014; Jiang et al, 2009; Johnson & Slatkin, 2006; Liu et al, 2010).

Computational challenges

One main obstacle prohibiting existing population genetic methods from application to large genomic data is intensive computation. The likelihood function of most population genetic methods has gene genealogy as a nuisance variable. These methods evaluate the likelihood function by adopting Markov chain Monte Carlo (MCMC) or importance sampling (IS) to integrate over the gene genealogy space. It is computationally very intensive, and only works for a small sample of haplotypes from a local chromosome region (Griffiths & Tavaré, 1994). Such methods cannot be directly scaled up to large-sample genomic data even with high performance computers. Developing efficient computing algorithms is necessary.

Another issue is numerical instability. For example, an essential component in coalescent-based methods is the distributions of coalescent time and ancestral lineage numbers. Both equations are expressed as a function of alternating hypo-geometric series. When the sample size is large (e.g., $n > 100$), the coefficient of each individual term of the series becomes so large that it is beyond the capacity of double-precision variables of any computer language. One scheme to avoid such numerical overflow is to use a high-precision arithmetic library (HPAL) in programming. This significantly increases programming difficulty and computing time with only a limited improvement in performance. A more applicable solution is to replace the exact distributions with their asymptotic distributions (Chen & Chen, 2013; Griffiths, 1984). Asymptotic formulas are usually in simple analytical form and easy to calculate.

Methods for detecting soft sweeps and polygenetic selection

Some questions are raised from a biological view instead of computational issue. One such issue is the revision of our views on the general forms of natural selection. After more than ten years of genome-wide studies on selective sweeps in humans, only a few genes have been identified under strong selection due to extreme environmental factors (*EPAS1*) or infectious diseases (*G6PD*). This is in conflict with our traditional understanding and urges us to reflect and explore the actual general form of adaptation in nature. Recently, population

geneticists hypothesized that other forms of selection, such as, soft sweeps and polygenetic selection, are likely to be more common in nature and are under the radar of existing genomic approaches (Pritchard et al, 2010; Wollstein & Stephan, 2015).

Most conventional methods for detecting selective sweeps were developed by assuming selection starts from a *de novo* mutation. Such a selective process is called a hard sweep. If selection starts from a standing mutant, which has been in the population under neutrality for a long time and is in high frequency, it is a soft sweep (Hermisson & Pennings, 2005). Researchers hypothesized that soft sweeps are more prevalent than hard sweeps (Pritchard & Di Rienzo, 2010). The chance for a new advantageous mutant to occur is very small, and it is also very unlikely that the new advantageous mutant can survive the effect of random drift in the early stage of the selective process to finally reach high frequency in the population. Ohta & Kimura (1975) already noticed this in their seminal paper on the hitch-hiking effect: "It is likely that the new advantageous allele will be chosen, in response to environmental changes, from the pre-existing alleles rather than occurring by mutation".

Although soft sweeps are more common, it is not trivial to propose a powerful method for detecting soft sweeps. The genetic polymorphism pattern caused by soft sweeps is indistinguishable from that under neutrality in many aspects, including the allele frequency spectrum, reduction of genetic diversity, and linkage disequilibrium. This explains very few methods for detecting soft sweep so far (Przeworski et al, 2005; but see Garud et al, 2015).

To date, genomic studies on selection have focused on a single locus, for example, lactase persistence. Some traits, such as skin pigmentation, are determined by several major genes and show an evolutionary mode similar to the single-gene cases. However, most traits are quantitative and determined by multiple genes with minor effects and complex interactions. "It seems likely to us that, as in traditional quantitative genetic models, many -- possibly even most -- adaptive events in natural populations occur by polygenic adaptation" (Pritchard & Di Rienzo, 2010). Such traits, when under natural selection, tend to evolve in a polygenic mode: one could expect that multiple functional loci shift their allele frequencies without being fixed when the population fitness is improved by natural selection (Hancock et al, 2010). Our understanding of polygenic selection is in the early stages, and as pointed out by Pritchard & Di Rienzo (2010), empirical study and theoretical modeling are both needed to understand the mechanism of polygenic selection.

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The breeding biology of Red-Whiskered Bulbul (*Pycnonotus jocosus*) in Xishuangbanna, southwest China

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ABSTRACT

To fill the gap in breeding biology information about the Red-Whiskered Bulbul (*Pycnonotus jocosus*), in 2013, we studied the breeding biology of this species in Xishuangbanna, southwest China. The breeding began from February and continued until early August. The breeding strategy of *P. jocosus* was more flexible and their nests were only built in cultivated landscapes, whereas, no nest building in native tropical rain forest was found. Small open cup nests were built on 50 different plant species, and at heights ranging from 2.1 ± 0.6 m above the ground ($n=102$). The mean clutch size was 2.53 ± 0.51 eggs ($n=40$) and the mean egg mass was 2.81 ± 0.25 g ($n=60$). The average incubation period was 11.1 ± 0.5 days ($n=14$), and the average nestling period was 11.0 ± 0.8 days ($n=31$). The overall nest success was 34.22%. The hatching and fledging showed either asynchrony or synchrony. Invertebrate food decreased with nestling age, whereas, plant food increased with nestling age. Moreover, distinct parental roles of the parents in nestling period were found. Compared with other passerine species, *P. jocosus* spent less time in incubating (58%). The clutch size, incubation and nestling period of the *P. jocosus* in southwest China were different from those of the *P. jocosus* in India.

Keywords: Red-Whiskered Bulbul; Breeding biology; Incubation; Nestling; Parental roles

INTRODUCTION

The Red-Whiskered Bulbul (*Pycnonotus jocosus*) is a small passerine in the *Pycnonotidae* family, distributed in India, Southeast Asia and South China (Mark, 2009). In South China

it ranges from Southeast Tibet to South Yunnan (MacKinnon & Phillipp, 2011). It inhabits wooded habitats, secondary forests, urban areas, parks, and gardens (Fishpool & Tobias, 2005; Mark, 2009), commonly close to human populations.

P. jocosus mainly eats fruits and seeds, and smaller amounts of invertebrates (Yang et al, 2004). It is one of the most common and important seed dispersal agents in anthropogenic open habitats in tropical Asia (Corlett, 1998). However, so far, its breeding biology is quite limited (Appendix 1, available online) (Chidkrua, 1999; Liu, 1992). The information regarding its incubation, parental roles, and nestling food are still lacking. The previous study found the different nesting ecology in the *P. jocosus* inhabiting downtown or periphery of India (Mazumdar & Kumar, 2007), suggesting the flexible breeding strategy of this species.

In this study, we presented details of the breeding biology of *P. jocosus* in Xishuangbanna, including the duration of the breeding season, nest morphology, nest site, clutch size, egg mass, incubation rhythm, length of incubation and nestling periods, parental roles, nestling food, feeding frequency, nestling development, and nesting success. We aimed to collect basic information about its breeding biology in this area, and explore possible reasons for its high abundance in human-dominated environment from its breeding ecology characters.

MATERIALS AND METHODS

Study area

The study was conducted in Xishuangbanna Tropical Botanical

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Garden (XTBG, centered at N21°41', E101°25', 570 m a.s.l.), which lies in central Xishuangbanna, southwest China. The dry and wet seasons span from November to April and from May to October, respectively (Zhang & Cao, 1995). In 2013, XTBG experienced an average monthly temperature of 22.1 °C and average monthly precipitation of 139.9 mm (Appendix 2, available online). XTBG is 11.25 km² in area, within which 2.5 km² is native tropical rain forest, and the remainder is largely cultivated landscapes.

Nest searching and monitoring

The study was conducted in 2013 from the middle of February (before breeding) to the middle of August (until no newly built nests were found). During this period, nest surveys were conducted every day by an experienced field assistant. Nests were found at different stages (nest building, egg laying, incubation period, or nestling period). Once a nest was located, the following data were collected: nest characteristics (size, height above the ground, and nesting plant species), egg laying date, clutch size, egg mass, incubation rhythm, hatching date, the number of hatchlings, fledging date, and the number of fledglings.

If the nest was found during the egg laying stage, clutch size was recorded by counting the number of eggs every day until no more eggs were laid. Nests that were found during the incubation period weren't included in the clutch size calculation, because some eggs may have been lost before nest discovery. Egg mass was weighed to 0.01 g before incubation started (Ohaus SPS202F pocket scale, Ohaus Corp). Nestling mass was weighed every day at 0800h-0830h from day 0 (the first day that the egg hatched) to day 8 or day 9. Beyond day 9, perturbations to the nest may cause fledglings to leave prematurely. In order to decrease the risk of predation due to our visits, rubber gloves were worn when weighing the nestlings (Whelan et al, 1995).

The feeding visits and fledging behaviors of 16 broods were observed by using video recorders (SONY HDR-PJ760E). Other broods were checked once daily. The feeding visits were recorded from the day that the first egg hatched until the last nestling fledged or the nests failed. Recordings started between 0800-0900h, and ended between 1700h-1800h. Cameras were set up at a distance of 1-4 m away from the nest, pointing down at an angle of approximately 30° (Smith & Montgomerie, 1991). The parents usually fed nestlings within a few minutes of camera set-up and recording; the camera did not interfere with nest activity.

Incubation

The incubation period was the time between the last egg laid and the first egg hatched (Auer et al, 2007). We used temperature data loggers (Model Lu Ge L91) to monitor the onset of incubation and incubation rhythm (Badyaev et al, 2003; Cooper & Mills, 2005; Hartman & Oring, 2006). Some broods initiated incubation at the end of egg-laying and all the young were then hatched together, which is defined as "hatching synchrony". Others began incubation before all the eggs were laid, thus some young were hatched before the others, often on

successive days, which is termed as "hatching asynchrony" (Clark & Wilson, 1981). A sudden increase or decrease in temperature indicates the arrival or departure of the parents. This assumption was verified by comparing videotape recordings and temperature logger data collected at one nest, observing high concordance between the two data types. The data loggers were installed as far as possible before clutch completion to determine the onset of incubation. One of the probes was placed in nests lightly covered with existing nest materials, and another probe was placed outside of the nest (Hartman & Oring, 2006). The temperature was recorded once each minute.

We evaluated the following variables characterizing incubation behavior from the data logger recordings: the onset of incubation, and night resting period (the time between the last evening arrival and the first morning departure from nest). The daily active period was the length of time remaining after the night resting period. We then calculated the mean on-bout duration (mean time for each incubation bout) and mean off-bout duration (mean time interval between two continues incubation) (Balakrishnan, 2010; Kovařík et al, 2009).

Feeding frequency, nestling food and parental roles

The nestling period was defined as the time between the first egg hatching to the last member of the brood fledging (Temba et al, 2014). Feeding frequency was defined as the number of feeding visits each hour and the number of visits without food was not used to calculate feeding frequency. Food was divided into two categories: invertebrate or plant food, which could be clearly identified from video recordings. We used feeding volume to compare invertebrate and plant food composition in the nestlings' diet, which was more precise than feeding frequency. Food volume was scored by comparing the item volume to the volume of parents' bill, and divided into four classes: 0.25, 1, 1.75, and 2.5 bill-equivalents (Dearborn, 1998). The contributions of invertebrate and plant food were compared as below:

$$IP=IV/FV \quad (1)$$

$$PP=PV/FV \quad (2)$$

In which, IP represents the percentage of invertebrate food, IV represents the total volume of invertebrate food provided by each parent during the nestling period, FV represents the total food volume provided by each parent during the nestling period, PP represents the percentage of plant food, PV represents the total volume of plant food provided by each parent during the nestling period.

Male and female *P. jocosus* are usually difficult to distinguish due to an absence of marked sexual dimorphism. However, we found that in two nests the adult birds have different color patterns on their faces (Appendix 3, available online). Although the male and female still could not be distinguished, it offered the possibility to analyze whether the male and female adults contributed equally to nestlings in brooding nestlings after feeding visits, feeding frequency, contribution of invertebrate and plant food (Adler & Ritchison, 2011), time spent at the nest (including the time spend on brooding nestlings and feeding visits), and fecal sac clearing (the number of feces cleaned by every parent in the nestling period) (Dell'Omo et al, 1998).

Breeding success

A nest was considered failed if there were the following occurrences: egg loss during the incubation period, broken eggs, cold eggs, no hatchling emergence even when the incubation time had exceeded the maximum for other observations, and signs of predation (i.e., disturbed nests with the nestlings disappearing before fledging, nestlings that died in the nest with wounds produced by parasites, or nestlings found on the ground with nests upturned after a rainstorm).

Successful nests were defined as those where at least one nestling fledged. Hatching success was defined as the total number of hatchlings from the total number of eggs, fledging success as the total number of fledglings from the total number of hatchlings, and nesting success as the total number of fledglings from the total number of eggs (Buij et al, 2013; Bensouilah et al, 2014).

Data analysis

ANOVA was used to calculate whether feeding frequency and invertebrate composition changed with age. For plant food, nonparametric tests (Kruskal-Wallis rank sum test) were used, as the variances did not exhibit homoscedasticity. Nestling growth rate constant (k) and asymptote mass (A) were calculated using a logistic growth equation $y = A / (1 + e^{-(k \cdot (t - t_i))})$ (Ricklefs, 1976), where t_i is the inflection point of the growth curve. All of the mean values are reported with the standard deviation (mean \pm SD).

RESULTS

We found 102 nests from February to August in 2013. Among them, 40 nests were found at the egg laying period, 47 were found at the incubation period, and 15 were found at the nestling period. *P. jocosus* laid eggs from February to July and the breeding season ended in early August. The breeding peak lasted from April to June (Figure 1).

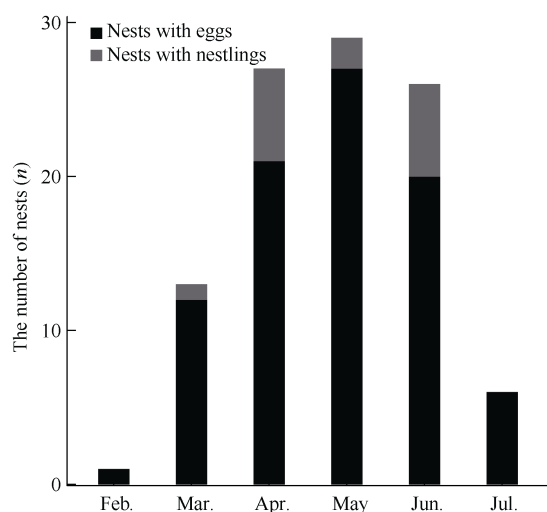


Figure 1 Nesting season of *P. jocosus* at XTBG, 2013

Nest placement and measurement

All nests were built in cultivated landscapes. Nests were found in 50 different plant species. Ten nests were found on the *Areca catechu*, which was the most frequently used nesting plant species. Nest height was 2.1 ± 0.6 m above ground ($n=102$, range=0.9-3.8 m). Nests were shallow and bowl-shaped, built of thin grass leaves, fine roots, and spider silk outside of the nest. The inside diameter of nests was 6.5 ± 0.7 cm ($n=75$, range=4.5-9.0 cm), outside diameter was 9.1 ± 1.2 cm ($n=75$, range=6.9-14.5 cm), inside depth was 4.8 ± 0.7 cm ($n=75$, range=3.3-7.0 cm), and outside height was 6.6 ± 1.0 cm ($n=75$, range=4.5-8.6 cm).

Clutch size and egg mass

Eggs were oval in shape, and had a pale-white base color with dense pink speckles towards the broad end (Figure 2). Females laid eggs on consecutive days and one egg per day. The mean clutch size was 2.53 ± 0.51 , mostly ranging from 2-3 eggs ($n=40$), however, one nest had four nestlings when discovered. Egg mass was 2.81 ± 0.25 g ($n=60$, range=2.24-3.59 g).



Figure 2 Eggs and nest of the *P. jocosus*

Incubation

The mean incubation period was 11.1 ± 0.5 days ($n=14$, range=10-11 days). From video recording we found that only one adult incubated the eggs ($n=2$). *P. jocosus* incubated continuously over the nights (night nesting period) from 1903h \pm 49 min to 0647h \pm 37 min ($n=8$). The parent spent an average of $58 \pm 13\%$ of daylight hours on incubating. The average on-bout lasted 17.0 ± 9.3 min ($n=8$). Parents left nests at an average of 2.9 ± 1.2 times/hour., and the average off-bout was 10.9 ± 4.0 min ($n=8$).

Five temperature data loggers were installed prior to clutch completion, thus the onset of incubation could be clearly determined. Two broods were completed before incubation began while the other 3 were incubated before the clutch was completed. The rate of hatching asynchrony and synchrony were 82% and 18%, respectively, in the 50 nests.

Nestling period, feeding frequency, nestling food and parent roles

The average nestling period was 11.0 ± 0.8 days ($n=31$, range=10-13 days). Based on the video observation, the average nest feeding frequency was 7.5 ± 3.5 times/hour ($n=20$), and the feeding frequency differed among nestling ages,

gradually increasing until hitting a peak at day 8 (ANOVA $F_{12, 124}=3.37$, $P<0.01$; Figure 3).

Both invertebrate and plant food were provided to nestlings, but invertebrates significantly declined with nestling age (ANOVA $F_{12, 119}=8.26$, $P<0.001$), while plant food significantly increased with nestling age (nonparametric tests $\chi^2=80.12$, $df=12$, $P<0.001$) (Figure 4).

From the two nests where the parents could be distinguished, we found that parents have different parental roles over the nestling period (Table 1). Both parents fed nestlings and cleaned feces by consumption or removal. However, only one parent would brood nestlings after feeding. We defined the parent who

brooded nestlings as A, and the other as B. Parents A and B were different in feeding frequency. During the whole nestling period, parents A and B finished $31.04\pm16.25\%$ and $65.04\pm18.93\%$ feeding visits, respectively. In 4.75% of the 2 190 feeding visits, the two parents could not be distinguished. The contributions in food provision, especially in invertebrates were different in parent A and B. Parent A spent more time on brooding nestlings and feeding visits during the nestling period (average feeding visit of Nest I: parent A: 71 ± 46 s, parent B: 37 ± 21 s; Nest II: parent A: 114 ± 24 s, parent B: 37 ± 16 s) (Figure 5). The feces cleaning effort in parent A was lower than parent B. Nest II had only one nestling at day 7, as one of them died from parasite attack.

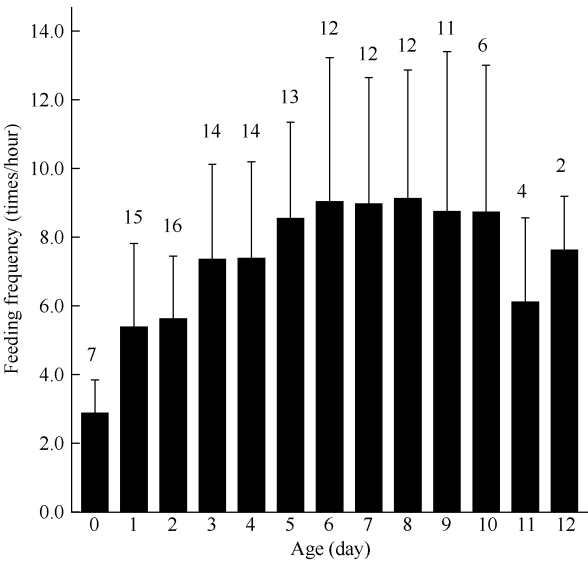


Figure 3 Relationship between feeding frequency and nestling ages for *P. jocosus* nestling birds at XTBG, 2013
Numbers above error bar are sample size (*n*); Day 0 is the day the first nestling was hatched.

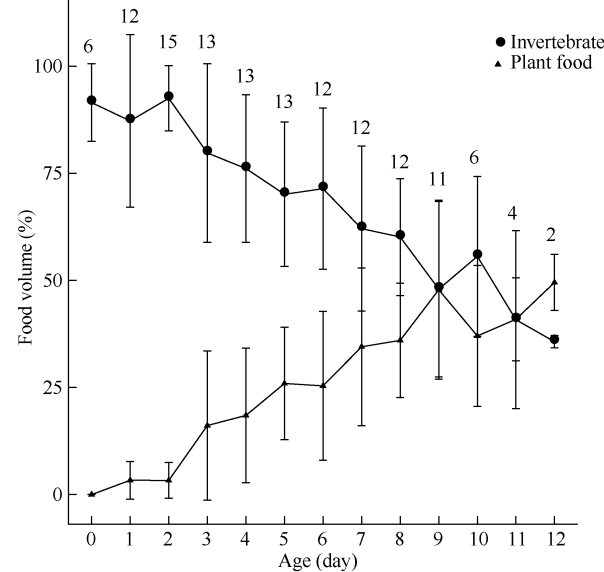


Figure 4 Age-related invertebrate and plant food volume percentage (mean±SD) change of *P. jocosus* at XTBG, 2013
Numbers above error bar are sample size (*n*); Day 0 is the day the first nestling was hatched.

Table 1 Relative contributions of parent A and B during the nestling period in XTBG, 2013

Nest	Brood size	Parents	Feeding frequency (times/hour)	Invertebrate food (%) ^a	Plant food (%) ^a	Feces cleaning effort ^b	Brood nestling
I	3	A	2.53	11.14	14.48	90	Yes
		B	3.80	47.90	22.57	203	No
II	2	A	1.53	17.39	3.39	56	Yes
		B	2.95	56.94	17.39	167	No

^a: Food type of 3.91% and 4.89% of the food volume from nest I and II could not be distinguished, respectively; ^b: Feces cleaning efforts are defined as the number of feces consumed or transported by adult birds over the nestling period.

Nestling development

Nestling mass growth exhibits an “S” curve (Figure 6), with a good fit for the logistic growth equation. The growth rate constant (*k*) was 0.45, and the asymptote (*A*) was 18.10 g.

Nest leaving

Eleven of 46 broods were recorded by video, the nestlings of most broods (*n*=44) fledged on the same day (synchrony). Only

two broods fledged asynchronously (fledged over three days). In these cases, after the older offspring fledged, the parents continued to feed the remaining nestlings until they fledged. The rate of fledging synchrony and asynchrony were 95.65% and 4.35%, respectively (*n*=46).

Nest success

For a total of 225 eggs (*n*=87 nests) laid, hatching success

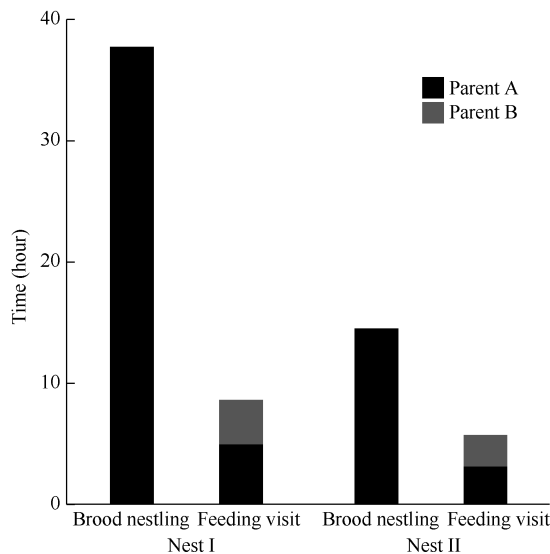


Figure 5 Time spent at the nest of parent A and B of *P. jocosus* during nestling period at XTBG, 2013 (Video recording time: nest I: 100.46 hours; nest II: 96.99 hours)

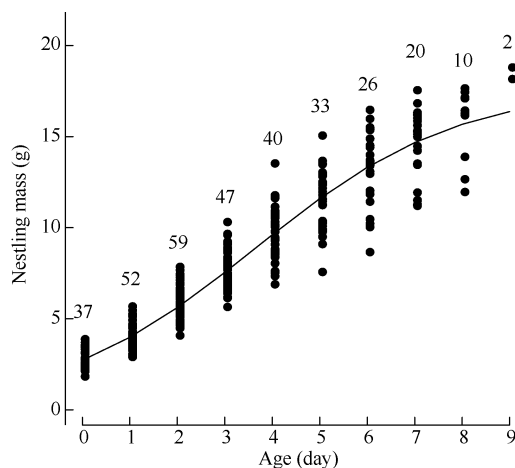


Figure 6 Individual mass growth of *P. jocosus* nestling at XTBG, 2013

Day 0 is the day the first nestling was hatched; The logistic function was $y=18.10/(1+e^{(-0.45*(t-4.10)})}$, $r^2=0.85$; Numbers above black dot are sample size (n).

Table 2 Nest ($n=87$) failure factors of *P. jocosus* in XTBG, 2013

Stages	Reasons	Number of eggs or nestlings lost (n)	Percentage (%)
Eggs	Infertile	12	11.54
	Predation	65	62.5
	Desertion	24	23.08
	Strom	3	2.88
Nestlings	predation	30	68.18
	Parasite	12	27.27
	Storm	2	4.55

was 53.78% ($n=50$ nests) while fledging success was 63.63% ($n=34$ nests), and overall nest success was 34.22%. The average number of fledglings per successful nest was 2.26 ± 0.67 ($n=34$ nests, range=1-3).

Nest failure

Many factors contributed to nest failure (Table 2). Eggs failed due to infertility, storms, parent desertion, and predation; nestlings died from nest parasites (larvae of an unknown dipteran species), storms, and predation. Storms are a threat to the nests of *P. jocosus* as they are shabby and are not robust to bad weather. Reasons for nest desertion during the incubation period are unknown.

Predators are the main factor resulting in nest failure (Table 2, eggs stages: 62.5%, nestlings stages: 68.18%), however direct observations of predators were limited and mainly included one Himalayan striped squirrel (*Tamias maclellandi*) which predated a nestling, and one tree shrew (*Tupaia sp.*) which predated a nestling. One squirrel (*Sciuridae sp.*) was also observed attacking a new fledgling.

DISCUSSION

In this study, we obtained basic breeding biology information about the nest, clutch size, incubation, feeding frequency, nesting success, parental roles, and nestling food of *P. jocosus* in Xishuangbanna. All nests were observed in cultivated landscape. *P. jocosus* can use large numbers of plant species as nest building sites, indicating that they can adapt to human-dominated habitats.

Nests

P. jocosus were only observed building nests in cultivated landscape. Nests were built in wide range of plant species, compared to research on *Pycnonotus sinensis*, which built nests in 11 plant species ($n=99$ nests) (Lan et al, 2013), and some other open-cup passerine species such as *Sporophila caerulea*, which built nests in 8 plant species ($n=41$ nests) (Francisco, 2006) and *Garrulax henrici* which built nests in 13 plant species ($n=91$ nests; Lu et al, 2008), *P. jocosus* has plasticity with respect to nesting plant species.

Incubation and nest leaving

Similar to most bulbul species (Fishpool & Tobias, 2005), only one parent performed incubation in our population. Parent *P. jocosus* only spent $58 \pm 13\%$ of daylight hours to incubate, less

than the incubation time of some passerine species such as *Junco phaeonotus* (76.9%) (Weathers & Sullivan, 1989), *Dendroica caerulescens* (64%) (Joyce et al, 2001), and *Anthus pratensis* (77.19%) (Kovářík et al, 2009). The shorter incubation time may result from the high ambient temperature, as the mean temperature was 28.07 °C (range: 17.3 °C–45.9 °C) during 0700–1900h in our study site. Previous research suggests that below 26 °C chicken embryos will suspend development (Conway & Martin, 2000). Temperature fluctuations within an appropriate range during incubation would increase hatching success (Sun, 2001). The embryo would not die from exposure when the parent left the nest in high ambient temperature, and it would benefit *P. jocosus* by permitting the parent to spend less time on incubation and more time on foraging in the study site.

Although previous study reported that *P. jocosus* begins incubating immediately after the first egg was laid (Fishpool & Tobias, 2005), in our study, the incubation was initiated either before or after the clutch was completed. The high asynchrony (82%) of hatching indicates that the incubation of most of the broods was initiated before the clutch was completed. However, except two broods (4.35%), most of the broods fledged on the same day (95.65%). The low asynchrony in fledging than that in hatching is in accordance with previous study (Viñuela & Bustamante, 1992). Hatching synchrony is considered to be favorable, because the nestlings may be more easily cared when they are fledged (Lack, 1954, 1968). On the other hand, asynchronous birth can also be adaptive under some conditions (Podlas & Richner, 2013). Previous studies indicate that asynchronous hatching could reduce the competition between chicks and energy wasted by begging offspring, and also decrease the reproductive loss to parents (Cotton et al, 1999; Mainwaring et al, 2012). Thus parents may use a brood reduction strategy in unpredictable environments. If predation risks or inadequate food resources restrict the feeding behavior, parents may prefer older nestlings and starve younger ones to reduce overall reproductive losses (Lack, 1954; Morandini & Ferrer, 2015; Williams et al, 1993). In the two broods that fledged asynchronously, the older chicks fledged earlier and received more food, but there was no difference between the nestlings in broods that fledged synchronously (Li, unpublished data).

Cutch size, incubation period and nestling period

The clutch size of most African and Asian bulbuls is usually 2–3 eggs, but some species also lay 4–5 eggs (Kitowski, 2011). The clutch size of *P. jocosus* in Xishuangbanna was similar, but the mean clutch size was smaller than *P. jocosus* in India (Mazumdar & Kumar, 2007). The differences in clutch size could be influenced by parent's feeding ability, predation pressure, and food resource abundance (Lack, 1954; Eggers et al, 2006). The nesting success (34.22%) in our population was lower than that in India (city center: 72.2%; city outskirts: 80.5%), and predation was a major reason of nest failure (eggs stages: 62.5%, nestlings stages: 68.18%) (Table 2). Therefore, we assume that high predation pressure was the major reason of the small clutch size in our population. The incubation and

nestling period of *P. jocosus* were also shorter than that in India (Mazumdar & Kumar, 2007). Previous study suggests that increased nest predation risk favors a shorter nestling period (Martin, 1995), thus, the short nestling period in our population may be the result of high predation pressure.

High predation pressure limits food provision, restrains nestling development, and prolongs nestling period (Martin, 1992, 1995; Ricklefs, 1976), whereas, long nestling period may increase the risk of being predated. Thus, a smaller clutch size resolves this contradiction (Martin, 1992, 1995).

Nest success

Predation was the major factor limiting the hatching and fledging success in our study. The total nest success of *P. jocosus* in our population was lower than that in India (Mazumdar & Kumar, 2007), but higher than other *Pycnonotus* species (Kitowski, 2011; Rao et al, 2013). In our study site, the level of nest success was similar to the Chinese Bulbul (*Pycnonotus sinensis*; Lan et al, 2013).

Although the observed signs were consistent with predation in a large number of nests, little is known about the nest predators. We recorded that one Himalayan striped squirrel was predated a nestling, and one squirrel was attacking a fledgling. Potential predators in our study area included but were not limited to: squirrels, stray cats and predatory birds (*Buteo buteo*, *Spilornis cheela*, *Pernis ptilorhynchus*, *Centropus sinensis*, *Urocissa erythrorhyncha*, and *Accipiter trivirgatus* are common in our study site). Another study carried out at the same study location reported that snakes (*Oligodon cyclurus*) were also nest predators (Quan & Li, 2015).

Breeding season and nesting cycle

The breeding season in our study area was 1–2 months later than that in Changwat Loei of Thailand (Chidkrua, 1999). The breeding season may start earlier in lower latitudes (Conover & Present, 1990), and the latitude of our area was higher than Thailand. The peak of the egg laying period matched the peak of temperature, but was offset from the peak of precipitation. *P. jocosus* spends 5–7 days to construct the nest (Mazumdar & Kumar, 2007). The female lays eggs in 2–3 days. The sum of incubation period and nestling period was about 22 days. Fledglings still required parental care for 34 days (Fishpool & Tobias, 2005). Given the long breeding season (Feb.–Aug.), it seems that adults have time for producing more than one brood each breeding season.

Parental roles

Differences in parental roles between the sexes in the nestling period are a common phenomenon in passerines (Neudorf et al, 2013). However, little information about parental roles has been reported for bulbuls. From our observations of individually recognizable birds in this monomorphic species, we observed that *P. jocosus* had parental roles in incubation and nestling periods. Only one parent incubated the eggs, and the partner might be responsible for guarding. Both males and females provisioned nestlings and cleaned feces, but the tasks were not equally shared. Only one of the adults would incubate nestlings

after feeding visits.

Nestling food

The parents of *P. jocosus* brooded nestlings and provided more invertebrates when nestlings were younger. As altricial nestlings are poikilotherms, for several days after hatching they need parents to keep them warm (Ricklefs & Hainsworth, 1968; Watson, 2013). The age-related changes in nestling diet might be because that the adults of *P. jocosus* eat more plant foods in their diet (Yang et al, 2004). Or that was a trade-off for parents (Trivers, 1974) as there was a significant positive correlation between invertebrate biomass and faster growth rate in wood thrush (*Hylocichla mustelina*) nestlings (Duguay et al, 2000). The rich proteins in invertebrates promote faster growth, but they are more difficult to obtain than plant food (Morton, 1973). As the food demanding increases with nestling age, the parents might not be able to find enough invertebrates alone.

These obvious variances indicate that *P. jocosus* is highly flexible in their breeding strategies. As their nests can be easily found and accessed, we suggest that *P. jocosus* could be regarded as a model species in future studies of Asian bird nesting ecology.

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Effects of frugivorous birds on seed retention time and germination in Xishuangbanna, southwest China

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ABSTRACT

The dispersal of many plants depends on transportation by birds as seed dispersers. The birds play an important role in long distance seed dispersal and may also affect seed germination. However, for plants who have many bird dispersers, the influence of dominant and non-dominant dispersers on retention time (dispersal distance) and germination remains poorly understood. In this study, we performed experiments with captive frugivorous birds and fruiting plant species to study the effects of dominant and non-dominant dispersers on seed retention time (SRT) and germination (seed germination percentage and germination speed). Our study showed a great interspecific variation in the effects of frugivorous birds on both SRT and germination. Some birds enhance the germination of a given plant species, but others do not. Generally, the dominant visitors improved the seed germination and performed longer seed retention time.

Keywords: Dominant visitors; Frugivores; Interaction; Seed germination; Xishuangbanna

INTRODUCTION

Seed dispersal by frugivores is a critical ecosystem process found throughout the world (Jordano, 2000; Schleuning et al, 2011). Benefits of seed dispersal include avoiding inbreeding depression as well as avoiding a high density of predators and pathogens near their parent trees (Janzen-Connell hypothesis) (Bell et al, 2006; Connell, 1971; Janzen, 1970; Mangan et al, 2010; Swamy et al, 2011). Seed retention time (SRT), the time elapsed from fruit ingestion to seed elimination/defecation (Fukui, 2003), dictates the effective seed dispersal distance (Wotton et al, 2008). Some researchers have shown that SRT usually varies among different plant species due to differences in seed size (Chang et al, 2012; Figuerola et al, 2010; Fukui, 2003). There is also a positive correlation between SRT and the body size of the frugivores (Murphy et al, 1993; Spiegel & Nathan, 2007).

However, comparing SRT between different bird species has been rarely studied, and existing studies have produced inconsistent results. For instance, Spiegel & Nathan (2007) reported that the SRT of *Ocbradenus baccatus* consumed by Tristram's grackle (*Onychognathus tristramii*) was much longer than those seeds consumed by Yellow-vented bulbul (*Pycnonotus xanthopygos*). On the other hand, Figuerola et al (2010) showed no differences between the SRT for the plants *Scirpus litoralis* and *S. maritimus* ingested by four waterfowl species.

Apart from avoiding potential Janzen-Connell effects, frugivores can also change seed germination success through the digestive processing of the seed. Many studies have shown that the digestive systems of frugivores significantly influence seed germination (Robertson et al, 2006; Samuels & Levey, 2005; Traveset & Verdú, 2002). For example, digestion of frugivores can either increase (Reid & Armesto, 2011) or decrease (Lieberman & Lieberman, 1986) the percent of seed germinated or the germination speed (and can reduce predator detection and consumption of seeds as well as attacks by pathogens) (Fricke et al, 2013). The effects of gut passage on seed germination usually differs between pairs of bird and plant species (Barnea et al, 1991; Traveset et al, 2001b).

Frugivorous birds are considered to be important seed dispersers in ecosystem processes (Shanahan et al, 2001). The interaction between frugivorous birds and fruiting plants has been examined in a range of studies (Jordano, 1995). But the relationship between frugivorous birds and fruiting plants has often been asymmetrical (McCann et al, 1998; Paine, 1992) and not all frugivores are effective dispersers to the plant species consumed (Bradford & Westcott, 2011). For example, for a given species of plant, not all the visiting birds but only one or two bird species had a mutualistic and compact relationship

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with it (Silveira et al, 2012). As such, in our study, we classified birds into “dominant” and “non-dominant” visitors according to their observed visiting frequency to seven plant species. Then we compared the different effects between the two groups of birds on SRT and seed germination for these plant species.

MATERIALS AND METHODS

Study areas and species

This study was conducted in Xishuangbanna Tropical Botanical Garden (570 m a.s.l., centered at N21°55', E101°16'), in Yunnan Province, southwest China. Our study area lies within the Indo-Burma biodiversity hotspot and contains a high diversity of fruiting

plant species (Myers et al, 2000). Four-hundred-thirty-four bird species have been recorded in this region, constituting 36.3% of the avian richness in China (Jiang et al, 1998).

To better understand the effect of dominant birds and non-dominant birds on fruiting plants, we selected seven common fleshy fruited species that are visited by birds and non-avian frugivores (Table 1). The seeds of one of the species, *Ophiopogon bodinieri*, were divided into two types: *O. bodinieri* (big) and *O. bodinieri* (small), as their fruits were significantly different in size (mean±SD, 9.19±0.55 mm vs 8.19±0.47 mm, $t=7.608$, $P<0.001$). To minimize the intraspecific variation in fruit features, fruits were collected from either a single mother tree for woody plants or a cluster of individuals for herbaceous

Table 1 Fruiting period and fruit traits of our study species

Plant species	Life form	Fruiting period	Fruit size (mm)	Seed size (mm)	Pulp weight (g)
<i>Litsea glutinosa</i>	Xylophyta	Sep-Oct	8.47±0.48	6.07±0.27	0.28±0.07
<i>Syzygium hainanense</i>	Xylophyta	Jul-Aug	8.00±0.47	5.43±0.48	0.22±0.07
<i>Polyalthia suberosa</i>	Xylophyta	Jun-Dec	8.68±0.52	6.40±0.23	0.23±0.04
<i>Microcos paniculata</i>	Xylophyta	Oct-Dec	10.34±0.97	8.18±1.11	0.26±0.11
<i>Dendrophthoe pentandra</i>	Shrub	Dec-Jun	11.96±0.80	7.71±0.65	0.25±0.04
<i>Ardisia squamulosa</i>	Shrub	Nov-Mar	7.44±0.57	3.87±0.37	0.14±0.03
<i>Ophiopogon bodinieri</i> (big)	Herbage	Aug-Oct	9.19±0.55	6.39±0.22	0.15±0.04
<i>Ophiopogon bodinieri</i> (small)	Herbage	Aug-Oct	8.19±0.47	5.36±0.32	0.12±0.03

plants within the same day (Fukui, 2003).

Six common frugivorous bird species were selected for the experiment: Red-whiskered Bulbul (*Pycnonotus jocosus*), Sooty-headed Bulbul (*Pycnonotus aurigaster*), Black-crested Bulbul (*Pycnonotus melanicterus*), Blue-throated Barbet (*Megalaima asiatica*), Plain Flowerpecker (*Dicaeum concolor*), and Crested Myna (*Acridotheres cristatellus*). These species were selected because they are the most common and abundant species in our study site, and consume large amounts of fruits from a wide variety of plant species (Yang, 1994, 2004). All individuals were kept in separate cages (30 cm×30 cm×40 cm, length×width×height) and fed daily diets consisting of apple, pear, bananas, and mealworms. Water was supplied *ad libitum* throughout the experiment. All Plain Flowerpecker individuals were released at the beginning of the experiment because they did not consume any fruits in captivity.

Field observation

Field observations were conducted from July 2012 to July 2013, during the fruiting period of the seven target species. Three trees or clusters for each plant species were selected to record the number of individuals of each bird species that visiting the plant species; field observation was carried out at peak times of avian activity (0700-0830h, 1600-1730h). During the observation, the entire tree or cluster of plants was scanned once every 10 minutes with a 10×42 binocular (Olympus). Each tree or cluster was observed for 3 days in total with 3 hours in each day. We defined the dominant visitors as the species with the largest number of individuals during the observation period.

Seed retention time

Thirty-three individuals of birds were used in the SRT experiment, including 8 Red-whiskered Bultuls, 6 Sooty-headed Bultuls, 8 Black-crested Bultuls, 5 Blue-throated Barbets and 6 Crested Mynas, respectively. To ensure that birds would consume fruit from the start of the trial onwards, and thereby standardize the SRT, the maintenance diet was removed the night before trials (Tewksbury et al, 2008). The experiments began in the morning (0800h) and the birds were given 30 fruits of a single plant species. The fruits were subsequently removed 10 minutes after the first fruit was eaten and a moveable plastic tray was placed at the bottom of the cage to collect the seeds defecated every 5 minutes (Spiegel & Nathan, 2007). The experiment ended when no seed was observed in the faeces for 60 minutes. We used the midpoint of the first 10 free feeding minutes (5 minutes after the first fruit was eaten) as the beginning of the test. The mean SRT for each fruit species was defined as the mean retention time for all defecated seeds from a single bird.

Seed germination

Twenty fruits of each plant species were given to each bird. In total, 7 plant species, 4-5 bird species, and 5 individuals for each bird species were used for this experiment. After all of the seeds of the 20 fruits were excreted, they were extracted from the faeces and dried at room temperature. Some individuals of Crested Myna appeared ill during the experiment, and they were released before the experiment ended. Therefore, for the myna we have consumption and germination trials for 3 plant

species only (*P. suberosa*, *M. paniculata* and *A. squamulosa*). Furthermore, the seeds of *D. pentandra* were collected from defecations of Plain Flowerpecker in the field. Collecting these seeds/defecations in the field was possible because our field observation showed that the flowerpecker faeces (including *D. pentandra* seeds) is easily identified and found. We also conducted a control treatment for each plant species by removing the fruit pulp manually. To standardize the manual removal with the bird digestion treatments, there were 5 repetitions in the control treatments and 20 seeds in each repetition.

Seeds were placed in Petri dishes with agar medium in a constant temperature incubator (27 °C, 14 h light, 10 h darkness) (Reid & Armesto, 2011). Seeds that germinated were counted and removed daily to reduce their possible effects on non-germinated seeds (Mandon-Dalger et al, 2004). Seeds were considered germinated when the radicle emerged (Traveset et al, 2001a). Seed germination checks would stop when no seeds germinated for 2 consecutive months. These non-germinated seeds were all unviable due to fungus infection or rot. Two indices were introduced to estimate the germination differences between the different treatments (digested by various bird species and control seeds): final germination percentage (GP) and germination speed (GS). The indices were computed are as follows:

$$GP = (N_1 + N_2 + \dots + N_n) / 20 \times 100\% \quad (1)$$

$$GS = N_1 / 1 + N_2 / 2 + \dots + N_n / n \quad (2)$$

Where, N_n expresses the number of germinated seeds in day n .

Data analysis

A one-way ANOVA with Tukey's honest significant difference (HSD) multiple comparisons was used to analyze the differences of GP, GS and mean SRT among various bird species for each plant species. A square root transformation was applied to make the mean SRT values exhibit homoscedasticity and normality. All analyses were performed in R 3.1.1 (The R Core Team, 2014).

RESULTS

Visiting frequency of birds to each plant species

There were 27 hours of observation for each plant species and 216 (because *O. bodinieri* were divided into two types) hours in all. Eleven species and 1 021 individuals of bird were observed in total. No birds visited *O. bodinieri* (small and big) and *A. squamulosa*. The Red-whiskered Bulbul was the dominant visitor of *L. glutinosa*, *S. hainanense*, *P. suberosa* and *M. paniculata* with visiting frequencies of 80.34%, 43.33%, 45.61% and 79.17%, respectively (Figure 1). The Plain Flowerpecker was the dominant visiting species for *D. pentandra* (75.25%) (Figure 1).

Seed retention time

Overall, 181 experiments of 7 plant species were conducted; this was less than anticipated because not all the plant seeds were eaten within the regulation time (10 minutes) by some of

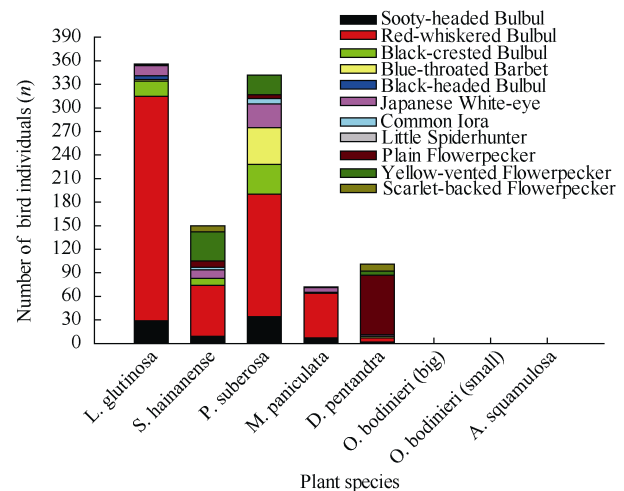


Figure 1 Frequency of different visitors to each plant species

the bird species (Appendix 1). The mean SRTs varied significantly between bird species for the plants *P. suberosa* ($F_{4, 21}=6.64$, $P=0.001$), *M. paniculata* ($F_{4, 28}=3.37$, $P=0.023$), *O. bodinieri* (big) ($F_{3, 18}=4.08$, $P=0.023$) and *O. bodinieri* (small) ($F_{3, 18}=4.25$, $P=0.020$), but not for *L. glutinosa* ($F_{3, 21}=1.42$, $P=0.266$), *S. hainanense* ($F_{3, 20}=2.19$, $P=0.121$) and *A. squamulosa* ($F_{4, 24}=1.37$, $P=0.274$) (Figure 2). Seeds of *P. suberosa* and *M. paniculata* digested by their dominant visitors showed a longer mean SRT (Figure 2C, D).

Seed germination

No seeds of *L. glutinosa* and *M. paniculata* germinated from any treatment. The GP significantly differed among treatments for both *P. suberosa* ($F_{5, 24}=4.90$, $P=0.003$) and *D. pentandra* ($F_{5, 24}=64.8$, $P<0.001$) (Figure 3B, C). No differences in GP were found between treatments for *S. hainanense* ($F_{4, 20}=0.80$, $P=0.539$), *O. bodinieri* (big) ($F_{4, 20}=0.37$, $P=0.825$), *O. bodinieri* (small) ($F_{4, 20}=2.16$, $P=0.111$) and *A. squamulosa* ($F_{5, 24}=1.24$, $P=0.323$) (Figure 3A, D, E, F). For *P. suberosa*, the dominant visitors (i.e. Red-whiskered Bulbul) significantly increased GP, and some non-dominant species (Crested Myna and Black-crested Bulbul) also improved GP (Figure 3B). For *D. pentandra*, the GP of seeds digested by the dominant visitors was significantly larger than that of any other treatments (Figure 3C).

The GS differed significantly between treatments for both *P. suberosa* ($F_{5, 24}=3.94$, $P=0.010$) and *D. pentandra* ($F_{5, 24}=106.20$, $P<0.001$) (Figure 4B, C). No differences of GS were found among treatments for *S. hainanense* ($F_{4, 20}=1.85$, $P=0.159$), *O. bodinieri* (big) ($F_{4, 20}=0.35$, $P=0.842$), *O. bodinieri* (small) ($F_{4, 20}=2.64$, $P=0.064$) and *A. squamulosa* ($F_{5, 24}=1.10$, $P=0.386$) (Figure 4). The GS of *P. suberosa* was enhanced by both the dominant visitors (Red-whiskered Bulbul) and some other non-dominant species (Crested Myna and Black-crested Bulbul) (Figure 4B). For *D. pentandra*, the GS of seeds digested by the dominant visitors was significantly larger than any other treatments (Figure 4C).

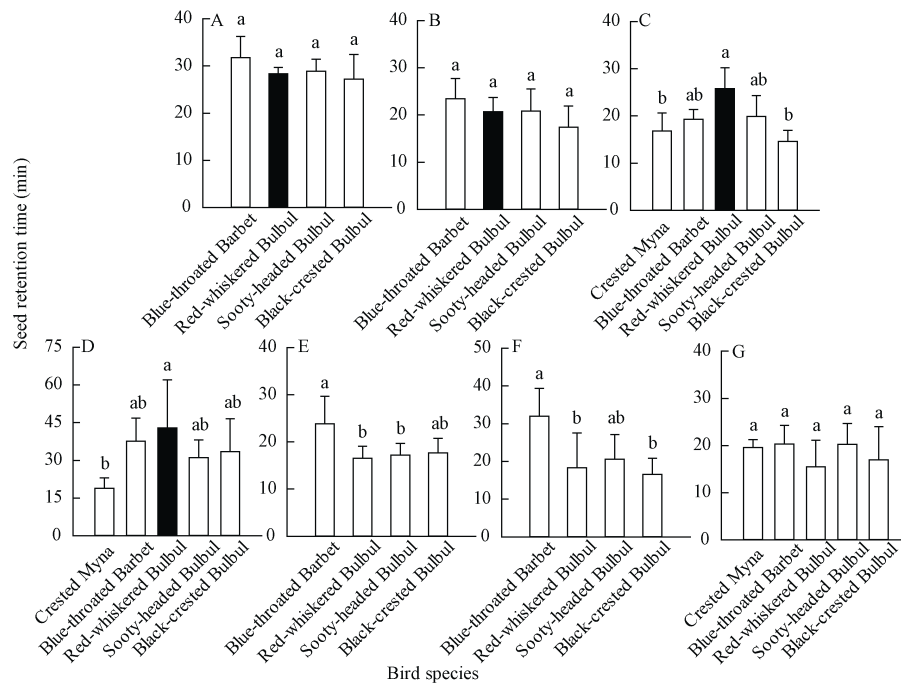


Figure 2 Seed retention time of the six plant species

A: *L. glutinosa*; B: *S. hainanense*; C: *P. suberosa*; D: *M. paniculata*; E: *O. bodinieri* (big); F: *O. bodinieri* (small); G: *A. squamulosa*; Different lowercase letters indicate a significant difference ($P \leq 0.05$); Black bars represent the dominant visitor for the given plant species.

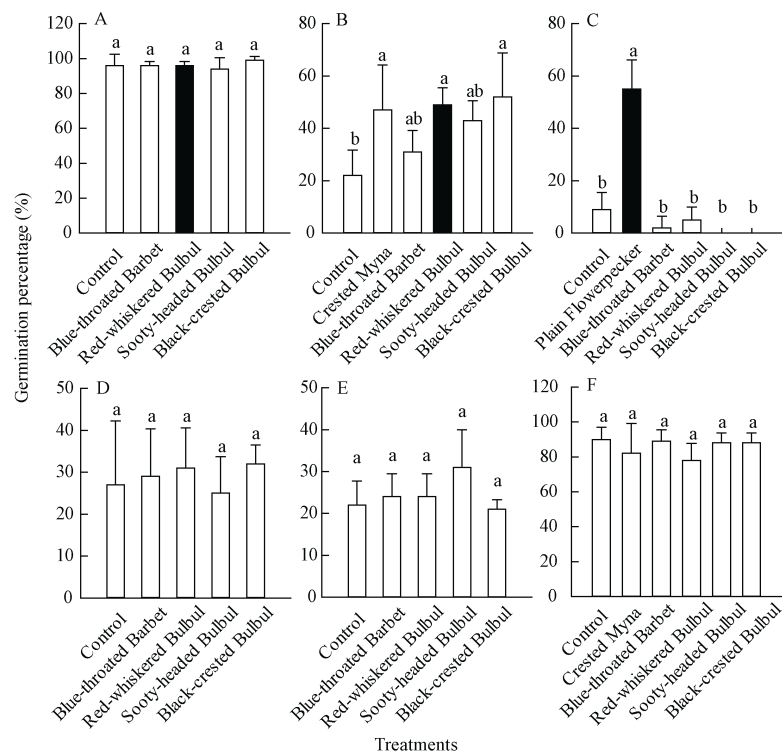


Figure 3 Germination percentage of digested and control seeds of the five plant species

A: *S. hainanense*; B: *P. suberosa*; C: *D. pentandra*; D: *O. bodinieri* (big); E: *O. bodinieri* (small); F: *A. squamulosa*; Different lowercase letters indicate a significant difference ($P \leq 0.05$); Black bars represent the dominant visitor for the given plant species.

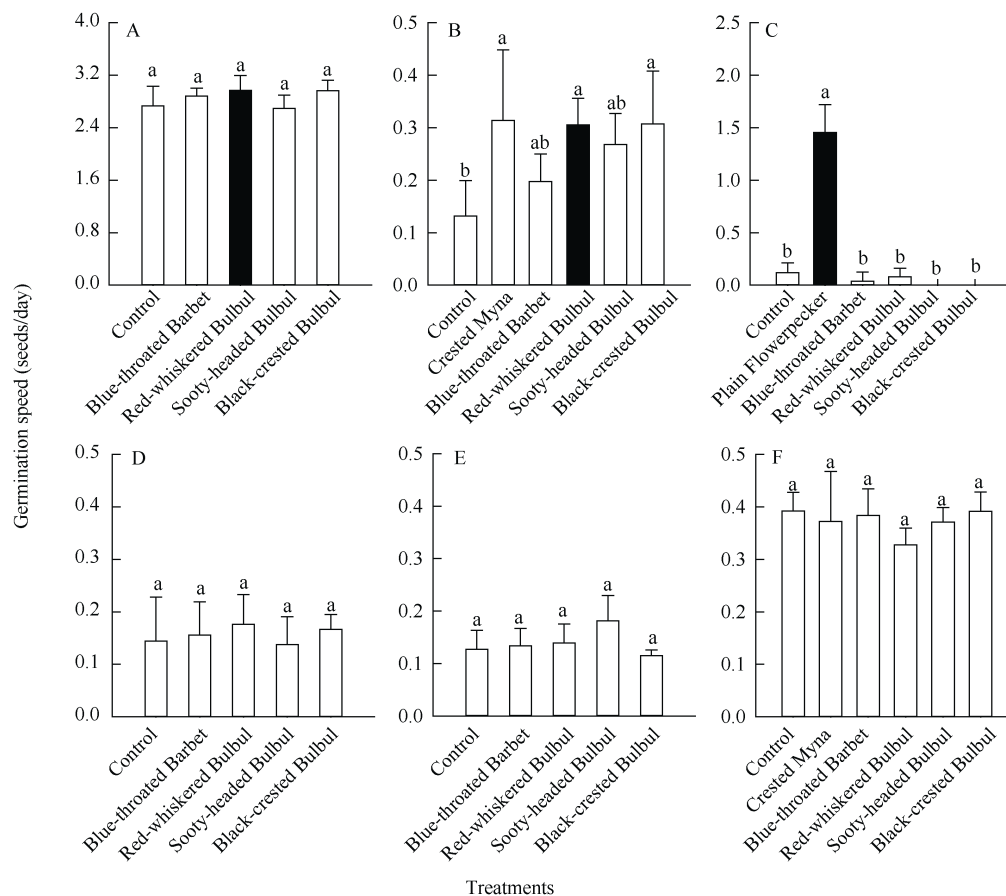


Figure 4 Germination speed of digested and control seeds of the five plant species

A: *S. hainanense*; B: *P. suberosa*; C: *D. pentandra*; D: *O. bodinieri* (big); E: *O. bodinieri* (small); F: *A. squamulosa*; Different lowercase letters indicate a significant difference ($P \leq 0.05$); Black bars represent the dominant visitor for the given plant species.

DISCUSSION

Previous studies have usually chosen one or a few species of birds that consume a particular plant species and have then compared differences between these bird species on SRT and germination (Barnea et al, 1991; Charalambidou et al, 2003; Figuerola et al, 2010; Fukui, 2003; Jordaan et al, 2011). However, the effects of birds on plants varies between both bird and plant species (Traveset & Willson, 1997; Yagihashi et al, 1999). As such, our study considered a range of avian frugivore and plant species pairs.

In general, the process of seed passage through the avian digestive tract is a key aspect of endozoochory, and the SRT is a significant factor for both plants and birds (Fukui, 2003). Our results showed that SRT exhibited significant variation between avian frugivores visiting four plant species (*P. suberosa*, *M. paniculata*, *O. bodinieri* (big), *O. bodinieri* (small)), similar to previous studies of Jordaan et al (2011), but inconsistent with the result of Figuerola et al (2010). The variation of SRT may be due to differences in disperser body size (Spiegel & Nathan, 2007), seed size consumed (Figuerola et al, 2010; Fukui, 2003),

or some combination thereof. Some studies have suggested that seed dispersal distance is a function of SRT (Fukui, 2003). Thus, the variation of SRT among bird species may lead to wide variation in seed dispersal distance.

Once ingested by birds, seeds may experience both chemical and mechanical processing in the digestive system. As a result, the seed coats or endocarp may be altered, thereby affecting germination (Barnea et al, 1990). However, whether digestion increases seed germination is unclear (Traveset, 1998). In our study, digestion significantly affected both GP and GS for some species but not for others. For plants which had bird visitors in the field (*S. hainanense*, *P. suberosa*, *D. pentandra*), both GP and GS were improved through bird digestion. For the three species which had no bird visitors in the field (*O. bodinieri* (big), *O. bodinieri* (small), *A. squamulosa*), the GP and GS were not influenced by birds ingesting. For the plant species that have bird consumers, it looks that birds are not only seed dispersers but also germination enhancers, suggesting that their relationship is a mutualism.

By dividing the visitors into dominant and non-dominant ones, we found that the SRT of a given plant species digested by

dominant visitors was either the same as or longer than that consumed by non-dominant visitors. The GP and GR for *P. suberosa* and *D. pentandra* were much higher after consumption by the dominant visitors than by some non-dominant visitors as well as control ones. Therefore, compared to non-dominant visitors, the action on seed retention time and germination of dominant visitors is more prominent. Perhaps the different effects on seed germination and retention time by dominant and non-dominant visitors can also help to explain why some previously studies reported inconsistent results.

In conclusion, this study supports findings in the literature that frugivorous birds have positive effects on seed retention time and ultimately seed germination. Moreover, dominant visitors seem to confer more benefits than non-dominant visitors to a plant species, suggesting that future studies should consider the visiting frequency of frugivorous birds as an important ecological factor when studying gut passage time of seeds and how birds affect seed germination.

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Appendix 1 The effective sample size of SRT experiment

Plant species	Bird species				
	Blue-throated Barbet	Red-whiskered Bulbul	Sooty-headed Bulbul	Black-crested Bulbul	Crested Myna
<i>L. glutinosa</i>	5	6	6	8	
<i>S. hainanense</i>	5	5	6	8	
<i>P. suberosa</i>	5	5	6	5	5
<i>M. paniculata</i>	5	8	6	8	6
<i>O. bodinieri</i> (big)	5	6	6	5	
<i>O. bodinieri</i> (small)	5	6	6	5	
<i>A. squamulosa</i>	5	6	6	7	5

Extraction and identification of membrane proteins from black widow spider eggs

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ABSTRACT

The eggs of oviparous animals are storehouses of maternal proteins required for embryonic development. Identification and molecular characterization of such proteins will provide much insight into the regulation of embryonic development. We previously analyzed soluble proteins in the eggs of the black widow spider (*Latrodectus tredecimguttatus*), and report here on the extraction and mass spectrometric identification of the egg membrane proteins. Comparison of different lysis solutions indicated that the highest extraction of the membrane proteins was achieved with 3%-4% sodium laurate in 40 mmol/L Tris-HCl buffer containing 4% CHAPS and 2% DTT (pH 7.4). SDS-PAGE combined with nLC-MS/MS identified 39 proteins with membrane-localization annotation, including those with structural, catalytic, and regulatory activities. Nearly half of the identified membrane proteins were metabolic enzymes involved in various cellular processes, particularly energy metabolism and biosynthesis, suggesting that relevant metabolic processes were active during the embryonic development of the eggs. Several identified cell membrane proteins were involved in the special structure formation and function of the egg cell membranes. The present proteomic analysis of the egg membrane proteins provides new insight into the molecular mechanisms of spider embryonic development.

Keywords: *Latrodectus tredecimguttatus*; Egg; Membrane protein; Extraction; Identification

INTRODUCTION

In oviparous species, the egg is a storehouse of maternal proteins required for fertilization and the initiation of zygotic development (Calvert et al, 2003; Yue et al, 2011). During

hatching, nutrients are transported from the egg components to the developing embryo. The proteins in the egg, which usually form lipoproteins with lipids, are crucial for the development, growth, and survival of the embryo (Laino et al, 2013; Wu et al, 2009; Zhao et al, 1994). Identification and molecular characterization of such proteins can provide insight into the regulation of embryonic development. Therefore, egg proteome analysis has become a hotspot in the field of proteomics. Up to now, many proteomic studies have been performed on eggs of the domestic chicken (Mann 2007; Rose & Hincke, 2009). Mann et al (2008) identified 119 proteins in egg yolk, 78 proteins in egg white, and 528 proteins in the decalcified eggshell organic matrix, whereas Farinazzo et al (2009) identified 255 yolk proteins in their study.

Cell membranes and cellular inner membranes are critical components of egg structure and function, and are involved in the partitioning of organelles, protecting the integrity of the genome and proteome, and providing defense against foreign molecules and external conditions that may damage or destroy the eggs. Membrane proteins are not only the main components of biological membranes but also the main executors of membrane functions. It has been estimated that membrane proteins account for about 30% of cellular proteins (Wu et al, 2003). Some proteomic analyses have focused on the membrane proteins of eggs. Due to the fact that most membrane proteins are hydrophobic and difficult to dissolve in aqueous buffer, the extraction of egg membrane proteins is often performed with the help of detergents and/or other

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additives (Mann et al, 2006; Rose & Hincke, 2009). For example, SDS-containing lysis solution was used to solubilize proteins in the hen eggshell membrane (ESM), followed by SDS-PAGE and LC-MS/MS, revealing 62 proteins, including 53 not previously reported (Kaweewong et al, 2013). Egg proteomes of other species, such as the silkworm (Fan et al, 2013) and snail (Sun et al, 2012), have also been analyzed. However, reports on spider egg proteomes are limited.

The black widow spider is an oviparous animal. The adult female constructs 7-8 egg sacs containing about 450 eggs each from June to October, with 1-3 weeks in between (Bonnet 2004). Compared with the eggs of other species, the eggs of the black widow spider received particularly early attention due to their inherent toxicity (Kobert 1889; Buffkin et al, 1971; Russell & Maretć, 1979). In our previous work, we conducted a proteomic analysis of the water-soluble proteins in the eggs of the black widow spider (*Latrodectus tredecimguttatus*) and identified 157 proteins, concluding that the molecular basis and mechanism for egg toxicity were different from those of spider venom (Li et al, 2012). Our present work analyzed the membrane proteins in the spider eggs based on detergent-assisted extraction and mass spectrometric identification to more comprehensively understand the egg proteome of the spider *L. tredecimguttatus*.

MATERIALS AND METHODS

Reagents

Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, SDS, 3-[(3-cholamidopropyl) dimethylamino] propanesulfonate (CHAPS), acrylamide, bisacrylamide, glycine and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium laurate (SL) was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Trypsin was from Promega (Madison, WI, USA). Protein assay kit was from Solarbio (Beijing, China). All other reagents were products of the highest grade available.

Extraction of egg membrane proteins

The eggs were homogenized in ddH₂O with a mortar and pestle and the resulting homogenate was centrifuged at 15 400 g for 15 min at 4 °C. After the supernatant was removed, the pellet (membrane debris) was repeatedly homogenized and extensively washed to eliminate the soluble components, and was then lyophilized. For extracting proteins from the egg membrane debris, different lysis solutions (40 mmol/L Tris-HCl/4% CHAPS/2% DTT, containing 1%-4% of SDS or SL, pH 7.4) were used and compared. Briefly, 50 mg aliquots of the lyophilized membrane debris were separately placed in eight Eppendorf tubes, with the eight different lysis solutions added, respectively. After intermittent oscillation with a vortex oscillator for 30 min, the mixtures were centrifuged at 15 400 g for 15 min. The supernatants were separately collected and the pellets were extracted twice. The obtained supernatants were separately pooled and the total volume of the extraction for each tube was adjusted to 2.5 mL. Protein content was determined with the protein assay kit using BSA as the standard according to the manufacturer's instructions.

SDS-PAGE of egg membrane proteins

SDS-PAGE of the egg membrane proteins was performed according to Laemmli (1970) under denaturing conditions on a 10% polyacrylamide separating gel overlaid with a 5% stacking gel. The sample solution (6 µL) was mixed with 3 µL of loading sample buffer (500 mmol/L Tris-HCl, 4% SDS, 100 mmol/L DTT, 20% glycerol, a trace of bromophenol blue, pH 6.8,) and then centrifuged at 10 000 g for 10 min. The proteins in the supernatants were loaded and separated through gel electrophoresis, which was run at 20 mA on the stacking gel and at 40 mA on the separating gel. After completion of electrophoresis, the resolved proteins in the gel were fixed with 10% acetic acid/40% methanol and visualized by staining with Coomassie brilliant blue G-250.

In-gel digestion

Digestion of the separated proteins in the gel was performed according to Chen et al (2006). Briefly, the lane gel was cut into slices about 2-3 mm wide and then broken into small pieces, followed by washing with 25 mmol/L NH₄HCO₃ and 50% ACN/25 mmol/L NH₄HCO₃ sequentially. The gel pieces were dehydrated in 100% ACN and then dried in a Speed Vac. In-gel digestion was performed with trypsin (1 µg enzyme/slice) in 25 mmol/L NH₄HCO₃ containing 10% ACN with incubation overnight at 37 °C. The released peptides were extracted twice by adding 100 µL of 67% ACN containing 5% formic acid with ultrasonication. The supernatants were pooled and then concentrated in a Speed Vac.

nLC-MS/MS analysis

Tryptic digests prepared by in-gel digestion were analyzed by an Easy-nLC system (Proxeon Biosystems, Odense, Denmark) coupled with a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) and HCTultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an autosampler and a C18 reverse phase column (PepMap, 75 µm i.d., 15 cm long, Sunnyvale, CA, USA). For nLC separation, solvent A (0.1% formic acid) and solvent B (0.1% formic acid in ACN) were used. The peptides were eluted using a gradient from 0 to 35% B in 38 min, 35% to 90% B in 15 min, and 90% to 100% B in 5 min. The flow rate was 200 nL/min. The peptides eluted from the column were online directed into the mass spectrometer. The LC-MS system was controlled by Data-Dependent Automatic Acquisition (DDA), using positive ion detection mode. Peptide ions were detected in the MS scan, and the seven most abundant ions in each MS scan were selected for collision-induced dissociation (CID) using data-dependent MS/MS mode over the m/z range of 350-1800.

Data processing and protein identification

Raw mass spectrometry data were processed with Xcalibur v.2.1 (Thermo Scientific) and Proteome Discoverer v.1.3 beta (Thermo Scientific). For protein identification, database searches were performed using the in-house sequence algorithm in the Proteome Discoverer software against UniProt/Swiss-Prot and UniProt/TrEMBL, with the parameters set as follows: enzyme, trypsin; allowance of up to two missed

cleavages; MS accuracy, 0.015; MS/MS accuracy, 0.05; fixed modification, carbamidomethylation (C); variable modification, oxidation (Met). Proteins were identified at the 95% confidence level ($P < 0.05$). The theoretical molecular weight (MW) and isoelectric point (pI) of the identified proteins were retrieved from the output files of Proteome Discoverer. Further information on the subcellular location and function of identified proteins were retrieved from <http://www.uniprot.org/uniprot>.

RESULTS

Extraction and separation of egg membrane proteins

Due to the water-insolubility of the membrane proteins in black widow spider eggs, we used eight mixed lysis solutions containing different concentrations (1%-4%) of SL or SDS to extract this set of proteins. The protein extraction efficiencies were

compared and demonstrated different extraction abilities, as shown in Table 1. As the concentration of the detergent increased, the protein extraction rate gradually increased. SL-containing lysis solutions achieved extraction rates varying from 1.28% to 2.33%, whereas the protein extraction rate of SDS-containing lysis solutions was 1.33% to 1.97%. Figure 1 shows the dynamic changes in extraction efficiency based on the protein concentration of the extract. Comparatively, when the concentrations of SDS and SL were 1% and 2%, the protein extraction efficiencies of the two lysis solutions were not significantly different ($P > 0.05$). However, when the concentrations of the detergents increased to 3% and 4%, the extraction efficiency of the SL-containing mixed lysis solutions was higher than that of the SD-containing mixed lysis solutions ($P < 0.05$). The highest extraction of egg membrane proteins was obtained with the mixed lysis solution containing 3%-4% SL.

Table 1 Comparison of the extraction efficiencies of different mixed lysis solutions

Item	SL concentration (%)				SDS concentration (%)			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
Membrane sample (mg)	50	50	50	50	50	50	50	50
Extract volume (mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Proteins extracted (mg)	0.638	0.803	1.105	1.165	0.663	0.673	0.853	0.985
Extraction rate (%)	1.28	1.61	2.21	2.33	1.33	1.35	1.71	1.97

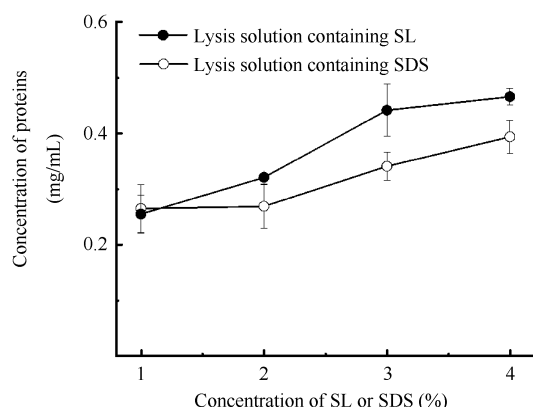


Figure 1 Effect of different mixed lysis solutions on the extraction of egg membrane proteins

The experiments were performed in triplicate.

Prior to nLC-MS/MS analysis, the extracted membrane protein mixtures were separated in parallel lanes with SDS-PAGE (Figure 2), which showed that the protein distribution profiles were similar between the two extraction conditions. As the concentration of the detergents increased, the protein content of the extract exhibited an increasing trend. However, when the concentrations of the detergents were greater than 2%, the extraction of proteins greater than about 60×10^3 was obviously enhanced while that of the proteins below 60×10^3 remained relatively stable, indicating that the efficient extraction of higher molecular weight proteins, which are generally more hydrophobic, needed detergents at higher concentrations.

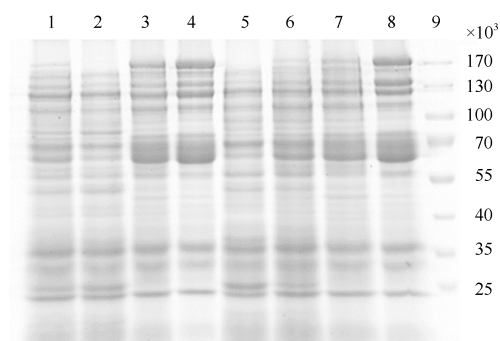


Figure 2 SDS-PAGE image of egg membrane proteins extracted by different mixed lysis solutions

Lanes 1-4: proteins extracted by lysis solutions containing 1%, 2%, 3%, and 4% SDS, respectively; Lanes 5-8: proteins extracted by lysis solutions containing 1%, 2%, 3%, and 4% SL, respectively; Lane 9: protein molecular weight marker.

Identification the membrane proteins

After the proteins in the gel slices were in-gel digested and the recovered tryptic peptides were analyzed with nLC-MS/MS, the acquired data were used to search against protein databases. As a result, a total of 39 proteins with membrane-localization information were identified after de-redundancy and removal of the proteins with no membrane localization information (Table 2). The data in Table 2 and Figure 3 show that the identified membrane proteins were distributed in the MW range of 14.6×10^3 - 352.9×10^3 , with more than 60% of the identified membrane proteins in the MW range of 20×10^3 - 80×10^3 . The

39 membrane proteins were distributed in the pI range of 4.74-10.04. About 77% of the proteins were acidic (pI<7), with proteins with a pI value between 5 and 6 accounting for 53.85% (Figure 4). Table 2 also lists the number of unique peptides identified for each protein. The proteins keratin, hornerin, and ATP synthase had relatively higher numbers of unique peptides.

According to their biological functions, the 39 identified

membrane proteins could be divided into four groups, though this functional classification was not strict as proteins usually have multiple functions: (i) Metabolic enzyme; (ii) Structure; (iii) Regulation; and (iv) Transport (Figure 5; Table 2). Nineteen membrane proteins (accounting for 48.72%) were metabolic enzymes, involved in substance and energy metabolism, including acetyl-coenzyme A carboxylase carboxyl transferase,

Table 2 Information on the membrane proteins identified from the spider eggs

Protein name	MW ($\times 10^3$)	pI	No. of pep
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	35	6.34	1
ATP synthase subunit alpha	55.2	5.62	7
ATP-dependent Clp protease ATP-binding subunit ClpX	46.8	5.22	1
ATP synthase subunit beta	50.4	5.1	1
ATP synthase gamma chain	31.4	8.34	2
Carbamoyl-phosphate synthase large chain	117.5	5.26	1
ATP synthase epsilon chain	14.6	5.11	1
Succinate dehydrogenase flavoprotein subunit	64.4	6.27	1
Glycerol kinase	55.5	5.49	2
Serine hydroxymethyltransferase 1	44.9	6.11	1
Coproporphyrinogen-III oxidase, aerobic	35.3	5.8	1
Adenylate kinase	23.3	6.42	1
Aspartate carbamoyltransferase.	36.3	7.27	1
Nucleoside diphosphate kinase	15	5.57	1
Adenylosuccinate synthetase	46.9	5.21	1
Probable polyketide synthase 18	339.3	6.77	1
Aspartate--tRNA ligase	66.2	5.54	1
Dual oxidase	174.4	6.99	2
Polyribonucleotide nucleotidyltransferase	74.9	5.4	7
Keratin, type I cytoskeletal 10	58.8	5.21	11
Keratin, type I cytoskeletal 9	62	5.24	27
Keratin, type II cytoskeletal 6A	60	8	4
Keratin, type II cytoskeletal 2 epidermal	65.4	8	32
PREDICTED: microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 isoform 1	352.9	5.68	3
PREDICTED: microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 isoform 2	332.6	5.89	3
Hornerin	282.2	10.04	10
Plakophilin-1	80.1	8.95	1
Desmoglein-1	113.7	5.03	1
Junction plakoglobin	81.7	6.14	2
Desmocollin-1	99.9	5.43	1
Dynamin-like 120 kDa protein, mitochondrial	111.6	7.87	1
Keratin, type II cytoskeletal 1	66	8.12	17
14-3-3 protein epsilon	29.2	4.74	1
Ras-related protein Rab-3A	24.9	5.03	1
Neuronal growth regulator 1	37.9	6.52	1
60 kDa chaperonin	56.8	5.08	7
Protein translocase subunit SecY	47.8	9.82	1
Protein translocase subunit SecA	102.8	5.74	1
AP-4 complex subunit beta-1	82.3	5.44	1

No. of pep: Number of unique peptide for an identified protein.

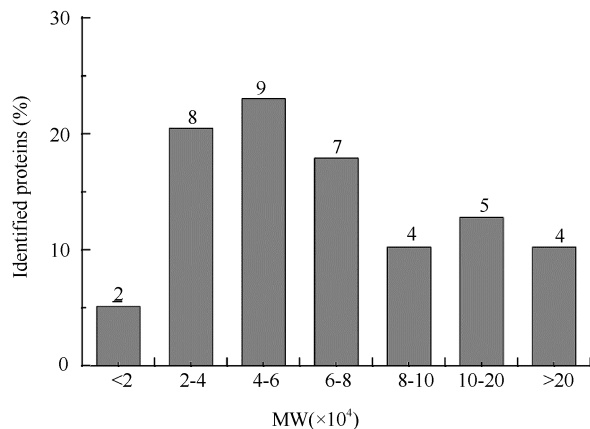


Figure 3 Distribution of the identified membrane proteins as a function of molecular weight (MW)

Values on the top of the column are the number of identified proteins.

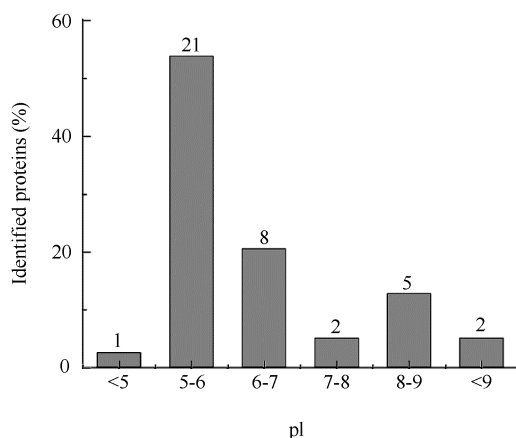


Figure 4 Distribution of identified membrane proteins as a function of isoelectric point (pI)

Values on the top of the column are the number of identified proteins.

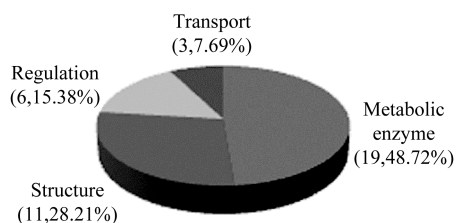


Figure 5 Functional classification of the identified membrane proteins

ATP synthase and carbamoyl-phosphate synthase. Eleven proteins (28.21%) were classified into the "Structure" group, and included membrane-localized cytoskeleton components and cell membrane proteins related to cell adhesion and cell junction, such as hornerin, plakophilin-1, junction plakoglobin, and some special kinds of keratins. Most proteins in this group not only acted as a structural component but also exerted other biological functions (see Discussion). Six regulatory proteins and three transport proteins were unambiguously identified, and

were primarily involved in the regulation of enzyme activity and signal pathways as well as transmembrane transport of proteins.

Of the 39 identified membrane proteins, 14 had unambiguous cell membrane-localized annotation in the protein databases. These cell membrane proteins were involved in catalytic, structural and regulatory functions. At least eight identified cell membrane proteins, including hornerin, plakophilin-1, junction plakoglobin and neuronal growth regulator 1, were involved in membrane cornification and recognition and communication between eggs, suggesting that the cell membrane of these spider eggs had specificities.

DISCUSSION

Contemporary proteomic techniques provide an effective means to comprehensively analyze all proteins in a cell, tissue or organism. However, membrane protein analysis has comparatively lagged behind that of soluble proteins due to their poor solubility in aqueous buffers, which limits their solubilization, extraction and enzymolysis (Rabilloud 2003; Santoni et al, 2000; Wu & Yates, 2003). To improve the extraction of membrane proteins, particularly high-hydrophobicity integral membrane proteins, a series of additives can be supplemented to the buffer alone or in combination, including detergents, chaotropes, aqueous-organic solvents, and organic acids (Masuda et al, 2008; Speers & Wu, 2007). SDS is the most often used detergent because it has a stronger ability than other commonly used detergents to disrupt biological membranes and extract membrane proteins (Masuda et al, 2008; Botelho et al, 2010). However, SDS is difficult to remove from the samples and therefore interferes with the following proteolysis and mass spectrometric analysis, thus limiting its application to membrane proteomics (Yu et al, 2003; Botelho et al, 2010). Recently, another detergent, sodium laurate (SL), was found not only to lyse membranes and extract membrane proteins as efficiently as SDS, but was also compatible with proteases and mass spectrometry as it can be conveniently removed from samples by phase transfer after acidification (Lin et al, 2013). In the present study, we used SDS- and SL-containing lysis solutions to extract the membrane proteins from black widow spider eggs. Because the eggs used were near hatching, their cell membranes were cornified to a certain extent, which further hampered the lysis of membranes and the extraction of membrane proteins. Therefore, to enhance extraction efficiency, other additives (CHAPS and DTT) were added to the Tris-HCl buffer to constitute a basic lysis solution. The results demonstrated that the mixed lysis solution containing higher concentrations of SL showed a certain advantage over the SDS-containing mixed lysis solution in the extraction of egg membrane proteins.

Membrane proteins perform a variety of cellular functions, including catalyzing metabolic reactions, transporting molecules and ions across the membrane, relaying signals in metabolic regulation and allowing cells to identify and interact with each other (Almén et al, 2009). In the present study, we identified 39 membrane proteins with corresponding functions. These proteins were distributed in wide MW (14.6×10^3 – 352.9×10^3) and

pl (4.74-10.04) ranges, suggesting diversity in both structure and function. Nearly half of the identified membrane proteins were metabolic enzymes, the majority of which were involved in energy metabolism and synthetic metabolism and included ATP synthase, succinate dehydrogenase, adenylate kinase, nucleoside diphosphate kinase, acetyl-coenzyme A carboxylase carboxyl transferase, aspartate carbamoyltransferase, and adenylosuccinate synthetase. The identification of these metabolic enzymes indicated that the relevant metabolic processes were active during the embryonic development of the spider egg. All metabolic processes in a cell are elaborately regulated and many membrane proteins participate in these regulation processes.

Six identified proteins had regulatory activities and were classified into the "Regulation" group, although they also had other biological functions. For example, 14-3-3 protein epsilon in this group is a member of the 14-3-3 protein family and is involved in the regulation of a large spectrum of cellular processes, including metabolism, signal transduction, and cell development (Dunaway et al, 2005; Paul & van Heusden, 2005). Many experiments have demonstrated the importance of 14-3-3 proteins in embryonic development. Wu & Muslin (2002) used an unphosphorylated peptide inhibitor of 14-3-3, R18, to determine the role of 14-3-3 proteins in *Xenopus* embryonic development, and demonstrated the requirement for 14-3-3 in mesodermal specification. Inhibition of 14-3-3 resulted in embryos with axial patterning defects and reduced expression of mesodermal marker genes. These phenotypic defects were caused by impaired fibroblast growth factor signaling in R18-injected embryos. To evaluate the role of individual 14-3-3 proteins in vertebrate embryonic development, Lau & Muslin (2009) utilized an antisense morpholino oligo microinjection technique in *Xenopus laevis* embryos, and showed that embryos lacking specific 14-3-3 proteins displayed unique phenotypic abnormalities. Keratin, type II cytoskeletal 1 was another identified membrane protein classified into this group, which not only acts as a structural constituent of the cell membrane but also as a high affinity receptor (Pixley et al, 2011).

Eleven membrane proteins were categorized into the "Structure" group, including several keratins closely related to embryo development. Lu et al (2005) demonstrated that two type I and two type II keratin genes were already transcribed in the 2-cell stage embryo, and type II keratins preceded type I keratins during early embryonic development. Hornerin was a constituent of the cell membrane and was identified based on 10 unique peptides, only lower than that of several keratins, suggesting that the concentration of hornerin in the sample was fairly high. The relative concentration of a protein identified by mass spectrometry is directly related to the number of identified peptides, neglecting the possible effects of other factors such as enzymatic digestion constraint, detection mass range of the mass spectrometer and differential post-translational modification. Therefore, the number of identified unique peptides assembled into a protein may reflect the protein's relative abundance (Fan et al, 2013; Jin et al, 2008; Li et al, 2010). Hornerin can bind Ca^{2+} and was rich in the cell membrane in our present study, suggesting that it may play an

important role in the cornification of cell membrane because Ca^{2+} is known to trigger the process of cornification (Hennings et al, 1980). Another identified cell membrane protein, desmocollin-1, can also bind Ca^{2+} and is involved in cornification (Ishida-Yamamoto et al, 2011). During embryo development, calcium is a major nutritional requirement (Johnston & Comar, 1955). It is speculated that the existence of such Ca^{2+} -binding proteins in the cell membrane not only enrolls Ca^{2+} to strengthen the cell membrane, but also stores Ca^{2+} for later stages of embryo development. It is worth mentioning that we identified several cell membrane proteins with cell adhesion and cell junction functions, including plakophilin-1, desmoglein-1 and junction plakoglobin, suggesting recognition and communication between eggs in the same egg sac, although the eggs existed dispersedly. In addition, the identification of polyribonucleotide nucleotidyltransferase and several proteins with protein transport activity, as well as chaperonin, suggested that processing and translation of mRNA was active during the embryonic development of the eggs. A batch of ribosomal proteins and regulatory factors for protein synthesis were identified, together with membrane proteins from the eggs, supporting this speculation (data not shown).

In summary, to efficiently extract and identify the membrane proteins of black widow spider eggs, we comparatively employed different lysis solutions to lyse the biological membranes and extract membrane proteins, followed by SDS-PAGE and nLC-MS/MS analysis. The mixed lysis solution containing SL showed a certain advantage over that containing SDS when the concentrations of the detergents were higher (3%-4%). A total of 39 membrane proteins involved in structure, catalysis, metabolism regulation, signal transduction or cell communication were identified, which is consistent with the functions of biological membranes. Nearly half of the identified membrane proteins were metabolic enzymes involved in various cellular processes, particularly energy metabolism and biosynthesis, suggesting that relevant metabolic processes were active during the embryonic development of the eggs. The identification of cell membrane proteins is helpful for revealing the special structure and functions of egg cell membranes.

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