

Neurochemistry as a bridge between morphology and behavior: Perspectives on aggression in insects

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Abstract Aggression is a common behavioral trait shared in many animals, including both vertebrates and invertebrates. However, the type and intensity of agonistic encounters and displays can vary widely both across and within species, resulting in complicated or subjective interpretations that create difficulties in developing theoretical models that can be widely applied. The need to easily and objectively identify quantifiable behaviors and their associated morphologies becomes especially important when attempting to decipher the neurological mechanisms underlying this complex behavior. Monoamines, neuropeptides, and pheromones have been implicated as important neuromodulators for agonistic displays in both invertebrates and vertebrates. Additionally, recent breakthroughs in insect research have revealed exciting proximate mechanisms important in aggression that may be broadly relevant, due to the relatively high conservation of these neurochemical systems across animal taxa. In this review, we present the latest research demonstrating the importance of monoamines, neuropeptides, and pheromones as neuromodulators for aggression across a variety of insect species. Additionally, we describe the stalk-eyed fly as a model system for studying aggression, which integrates physiological, morphological, and neurochemical approaches in exploring detailed mechanisms responsible for this common yet complex behavior. We conclude with our perspective on the most promising lines of future research aimed at understanding the proximate and ultimate mechanisms underlying aggressive behaviors [*Current Zoology* 60 (6): 778–790, 2014].

Keywords Monoamines, Aggression, Insects, Evolutionary physiology, Sexual Selection

Due to the potentially profound fitness benefits of gaining access to limited resources through competitions, both vertebrates and invertebrates commonly exhibit aggressive behavior. Precisely because of this central fitness role, aggression presents a valuable model to explore evolutionary connections between behavior, morphology, and physiology. Since fighting can be costly, animals have evolved a variety of morphological ornaments and armaments that may be employed in intricate signaling displays to convey aggressive intent and fighting ability without engaging physically (Emlen, 2008; Geist, 1966). Insects provide an important, underutilized model to study the connections between behavior, morphology, and physiology, because sexual selection has resulted in the evolution of extraordinary secondary sexual characters that are used in aggressive confrontations as both signals and weapons. Furthermore, research focusing on the neurobiological aspects

involved in aggression has uncovered intrinsic factors such as monoamines, neuropeptides, and pheromones as important modulators of this complex behavior. Importantly, many of these underlying neurochemical modulatory mechanisms appear to have similar functions in other taxa, including vertebrates.

To obtain a better understanding of the mechanisms driving complex behaviors, such as aggression, it is imperative to incorporate morphological and physiological information while also acknowledging underlying neurobiological factors that have a significant modulatory role on behavioral expression. However, gathering these three elements of information within a given taxonomic group can prove difficult, leading to omission of critical data that cannot be supplied by extrapolation from different species. This seems especially pertinent when considering factors such as neuromodulation that may shape proximate expression of individual

Received July 16, 2014; accepted Nov.14, 2014.

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behavior, which could in turn explain why an individual's responses can differ depending on the particular circumstance, e.g., facing a familiar versus unknown opponent. Given that the outcome of aggressive encounters often determines reproductive success, the variables mediating agonistic behavior in the proximate sense will provide the substrate for evolutionary selection. Therefore, the ability to quantify the interaction between morphology and neurophysiology in directing behavioral expression has the potential to allow rigorous testing of currently held hypotheses, such as why certain morphological traits continue to be selected for despite apparent cost in some species but have been lost in relatives. The aim of this review is to present the information from insect studies to demonstrate how neurochemistry can be leveraged to investigate the link between morphology and behavior. We show how these elements can be studied simultaneously by utilizing the stalk-eyed fly as a model system, which incorporates morphological and aggressive variability with highly specific neurochemical detection and manipulation methods. We then provide a perspective on possible future approaches using this model that could benefit our current understanding of complex animal behaviors.

2 Morphological Correlates of Aggression

The diversity and conspicuous nature of morphologies associated with aggressive displays has long attracted the attention of evolutionary biologists. The overwhelming majority of these examples come from sexually dimorphic species, which often experience strong sexual selection (Andersson, 1994). We will use the terms *armament* to refer to morphologies shaped by intrasexual selection (typically male-male competition) and *ornament* to refer to morphologies shaped by intersexual selection (usually female choice). The most plausible explanation for the evolution of armaments is their use in combat, and typically the individual with the largest armament is more likely to win aggressive encounters (Eberhard, 1987; Moczek and Emlen, 2000; Weislo and Eberhard, 1989). However, when armaments are an honest signal of status, they may actually reduce combat, since males can assess one another and an inferior male may choose not to fight in order to avoid unnecessary costs when winning is unlikely. Furthermore, while an ornament does not have to be used in combat, it may either directly or indirectly signal condition, and could potentially be used for assessment

of fighting ability by rival males. Therefore, armaments and ornaments are not necessarily mutually exclusive traits. This is supported by the observation that within many species that experience strong sexual selection, females benefit from choosing males bearing morphological features that also predict high success in intrasexual competition (Berglund et al., 1996; Conner, 1988; Suzaki et al., 2013; Watson and Simmons, 2010). Morphological traits conveying individual status to both sexes may also encompass different sensory modalities in insects, such as auditory information. This is exemplified by the songs of male crickets, which not only communicate competitive ability to rival males but also attractiveness to potential mates (Brown et al., 1996; Brown et al., 2006). Thus, perception of a range of morphological characters, such as armaments and ornaments, by individuals can be a straightforward way to assess fighting ability of potential rivals.

Developmental history is critical for the optimal growth of armaments that will withstand the rigors of combat and, thus, have value as an aggressive signal. Appropriately, this topic has been extensively studied (for review see Emlen and Nijhout, 2000). In the case of holometabolous insects (those that pupate, e.g., flies, beetles, wasps), all the resources necessary to express these traits must be obtained during a discrete larval developmental window, followed by the simultaneous formation of all adult structures during the quiescent pupal phase. Once emergent, adult individuals have little opportunity to alter the expression of morphological structures, which are static upon final eclosion to the adult form due to the rigid nature of the exoskeleton. Thus, resource limitations during development can have important fitness consequences and may ultimately prove the deciding factor for future agonistic encounters.

Two critical periods of development have been associated with the condition-dependent growth of secondary sexual characters in insects (Emlen et al., 2006). The first critical period applies to all insects, and occurs during the end of larval feeding, just before either the final molt to adulthood for hemimetabolous species (e.g., crickets) or the transition to the pupal stage for holometabolous species (e.g., flies). During this time, levels of juvenile hormone (JH) and ecdysone interact to signal the developmental transition from either pre-adult nymph or larva to the next stage (Emlen and Allen, 2003). Recent experiments indicate that JH regulates condition-dependent expression of mandibles in male stag beetles (Gotoh et al., 2011) and horned flour beetles (Okada et al., 2012). Since mandibles in these beetle

species also function as armaments, the fluctuation in circulating JH, as influenced by resources available during larval feeding, may also be a proximate factor in mediating intrasexual competition. For holometabolous insects, a second critical developmental period occurs once the larva has ceased feeding and has entered pupation. This developmental period spans the time during which growth and development of the adult structures actually occurs. During the second critical period, activity of the insulin/insulin-like growth factor signaling (IIS) pathway is associated with development of secondary sexual characters, including ornaments and armaments (Emlen et al., 2006). In beetles, horn growth during the pupal phase (following cessation of larval feeding) is particularly sensitive to circulating insulin levels compared to other developing organs, suggesting another opportunity for larval condition to influence armament expression in the adult (Emlen et al., 2012; Warren et al., 2013). In other words, nutrition accrued during larval feeding can continue to influence developmental signaling pathways in a condition-dependent manner when feeding is no longer occurring. The IIS pathway has also been implicated in determining adult polyphenism in hemimetabolous insects, as demonstrated by its role in the differentiation of termite soldier castes (Hattori et al., 2013). Most likely, the activities of JH and IIS influence each other, with both being affected by either nutrition or condition that will, in turn, depend on larval feeding experience (Abrisqueta et al., 2014; Perez-Hedo et al., 2014).

While most variation in insect armaments appears to be heavily condition-dependent, expression is also heritable to some degree (Gotoh et al., 2012; Unrug et al., 2004), as is social dominance (Moore et al., 2002). Candidate gene studies have revealed that the developmental gene *doublesex* (*dsx*), which is involved in many aspects of insect sexual dimorphism, has a dramatic impact on the development of armaments both within and between sexes of horned beetles (Kijimoto et al., 2012). Recent investigations have also shown that the *dsx* gene in male stag beetles is more sensitive to circulating JH levels, which promotes growth of enlarged male-type mandibles that are used during intrasexual competition (Gotoh et al., 2014). Sequencing of RNA suggests that several other undescribed genes involved in the expression of scarab beetle horns are under recent positive selection, most likely caused by sexual selection favoring larger armaments (Warren et al., 2014). Combined, this raises the possibility that genes such as *dsx* will infer some intrasexual heritability in arma-

ments to influence future success during agonistic conflicts. However, the degree to which genetic makeup will affect armament growth appears to be intimately linked with larval experience and expression of condition-dependent factors such as JH.

If competitors have similar developmental and/or genetic histories and so have comparable morphology, how can one predict the victor? In this case, it may be beneficial to consider individual social and environmental factors that affect adulthood. Within insects, sexually mature males typically show the most aggressive behaviors, and aggression is usually increased in larger males (Dixon and Cade, 1986; Moore et al., 2014). In cases where males defend territories, resident males often have an advantage over non-resident males in aggressive disputes (Simmons, 1986). Additionally, older individuals are more likely to show increased aggression in contests, both with age-matched and younger opponents, and have an increased probability of winning fights against younger opponents (Stockermans and Hardy, 2013; Tsai et al., 2014). Levels of aggression have also been shown to increase with social density (Wang and Anderson, 2010). Another contributing factor could be motivational asymmetry. For example, male crickets that have experienced restricted access to mating opportunities are both more aggressive and more likely to win contests against males that have continuous access to females (Brown et al., 2007). Furthermore, the act of winning or losing a contest in itself can affect future outcomes, although experimental evidence supporting either winner or loser effects has been mixed (see Chase et al., 1994) and may depend upon opponent familiarity as demonstrated for some vertebrate species (Forster et al., 2005; Ling et al., 2010). However, to fully understand the utilization of armaments by different species during actual competition, it is imperative to consider other intrinsic factors that have a role in the success of competitions. For example, what neurobiological factors influence the motivational state of opponents, and can this be enough to overcome morphological biases?

3 Neurochemical and Pheromonal Modulation of Aggression

Much of the pioneering work investigating the roles of various neurochemicals, namely monoamines, on invertebrate aggression was completed in arthropods, specifically crustaceans (Livingstone et al., 1980; Harris-Warrick and Kravitz, 1984; Huber et al., 1997). More

recently, the powerful genetic tools available to alter brain function in *Drosophila* (Baier et al., 2002; Miczek et al., 2007), as well as pharmacological interventions that alter the highly ritualized and characterized fighting behavior of crickets (Adamo and Hoy, 1995; Stevenson et al., 2000; Adamo et al., 1995), have provided insight into the neuromodulation of this complex behavior in insects. Taken as a whole, these studies have sometimes yielded contradictory or ambiguous results, which may arise from such factors as species differences, rearing conditions, subjective behavioral scoring, receptor subtype expression, and genetic background. Nevertheless, there appears to be a close relationship between the effects of the monoamines octopamine and serotonin, along with other less studied neuropeptides and pheromones in the modulation of aggressive displays, indicating a high degree of evolutionary conservation in the behavioral function of these systems across arthropods.

3.1 Octopamine

An important aspect in animal aggression is the decision to engage in a potentially costly, although sometimes valuable, antagonistic interaction. The physiological activity accompanying initial opponent assessment is generally referred to as the fight or flight response, mediated in mammals and other vertebrates by the sympathetic adrenergic/noradrenergic systems (Nelson and Trainor, 2007; Watt et al., 2007). In insects, however, there does not appear to be a physiological role for these catecholamines. Instead, insects rely on two other monoamines, tyramine (TA) and its hydroxylated metabolite, the norepinephrine analog octopamine (OA), to work in a physiologically similar manner (Roeder et al., 2003; Roeder, 2005; Verlinden et al., 2010; Farooqui, 2012).

The octopaminergic system has been convincingly implicated in the motivation and escalation of aggressive behavior in some insect species (Adamo et al., 1995; Stevenson et al., 2000; Baier et al., 2002; Stevenson et al., 2005; Hoyer et al., 2008; Zhou et al., 2008; Rillich and Stevenson, 2011; Stevenson and Schildberger, 2013). Much of this research was conducted in crickets and fruit flies, utilizing both pharmacological and genetic approaches. In male crickets, one of the first suggestions for the role of OA in modulating insect aggression came from the finding that OA increased in the hemolymph following agonistic encounters (Adamo et al., 1995). Subsequently, depletion of dopamine (DA) and OA in male crickets by hemocoel injections of the tyrosine hydroxylase inhibitor, alpha-methyl-p-tyrosine (AMT), was found to reduce the initiation, level and

duration of aggressive behaviors (Stevenson et al., 2000). The reduced aggressive responses were linked to the depletion of OA, rather than DA, since the behavior was rescued by treatment with the OA receptor agonist chlordimeform in OA-depleted crickets and suppressed in non OA-depleted crickets treated with the OA antagonist epinastine (Stevenson et al., 2005). However, it should be noted that while OA depletion in crickets reduces the intensity of fights, expression of aggressive behavior is not totally abolished (Stevenson et al., 2000). This suggests that for male crickets, the primary role of OA is to increase the individual's willingness to escalate the level of aggression once the fight has actually been initiated. In contrast, recent evidence suggests that DA, rather than OA, is necessary for the recovery of aggression in crickets that have been socially defeated (Rillich and Stevenson, 2014), possibly by modulating the motivation to initiate future agonistic encounters.

A similar role for OA in modulating *Drosophila* aggression has been suggested from work conducted in mutant flies. *Drosophila* mutants lacking the enzyme tyramine- β -hydroxylase (T β H), which catalyzes the synthesis of OA from TA, exhibit reduced aggression (Baier et al., 2002; Hoyer et al., 2008; Zhou et al., 2008). Conversely, both treatment with the OA receptor agonist chlordimeform and overexpression of T β H, independently increased aggression in socially reared flies (Zhou et al., 2008). These responses appear to be governed by a specific population of octopaminergic neurons found in the suboesophageal ganglion (Zhou et al., 2008). The role of OA in *Drosophila* aggression may involve neuromodulatory regulation of contextually appropriate behavioral responses to sensory cues conveyed by the opponent, since the absence of OA induces courtship behavior between males rather than aggression (Certel et al., 2007).

3.2 Serotonin (5-HT)

The evolutionarily ancient monoamine, serotonin (5-HT), has fundamental roles in a variety of physiological processes in both vertebrates and invertebrates. Although widely studied in other taxa, relatively few investigations have examined the role of 5-HT in insect aggression. As with OA, much of the pioneering work suggesting that 5-HT enhances aggression in arthropods was completed in crustaceans (Livingstone et al., 1980; Edwards and Kravitz, 1997; Huber et al., 1997). For example, both acute and constant infusion of 5-HT into the hemolymph of crayfish *Astacus astacus* increases the likelihood and duration for a smaller individual to fight, in a potentially costly agonistic interaction, with a

larger, dominant opponent (Huber et al., 1997). However, elevated 5-HT levels did not affect either the outcome of fights or the escalation pattern of fighting behaviors. In contrast, in a different species of crayfish *Procambarus clarkia*, reduced levels of aggression were observed following 5-HT injections (Tierney and Mangiamele, 2001) suggesting species specificity in neuromodulatory effects of 5-HT. Interpretations of these findings, along with others, led to the initial suggestion that 5-HT does not have a direct effect on arthropod aggression, but instead may have a modulatory role in the decision to retreat from a fight (Peeke et al., 2000; Kravitz and Huber, 2003).

In insects, specifically crickets and *Drosophila*, 5-HT was initially reported to have little influence on aggressive behavior (Stevenson et al., 2000; Baier et al., 2002). Pharmacologically depleting 5-HT in crickets by administration of the 5-HT synthesis inhibitor α -methyl-tryptophan (AMTP) failed to alter the expression of either aggressive or submissive behaviors (Stevenson et al., 2000). Similarly, aggressive behavior in *Drosophila* was not significantly altered either by selective depletion of 5-HT using the irreversible tryptophan hydroxylase inhibitor, p-chlorophenylalanine, or by enhancing 5-HT levels via administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP; Baier et al., 2002).

In contrast, other more recent studies using similar methods to alter 5-HT suggest that it may actually play a significant role in modulating aggressive behaviors in both crickets and *Drosophila*. Pretreatment of crickets with 5-HTP increased some components of cricket fighting behavior, such as fight duration, but decreased the number of attacks and did not appear to alter fight outcome (Dyakonova and Krushinsky, 2013). *Drosophila* either pretreated with 5-HTP or genetically modified to overexpress tryptophan hydroxylase to increase 5-HT, exhibit increased aggression (Dierick and Greenspan, 2007). However, depletion of 5-HT, either through genetic manipulation or by the administration of AMTP, did not significantly reduce aggressive behavior when compared to controls, suggesting that 5-HT modulates but is not necessary for the expression of aggression in *Drosophila* (Dierick and Greenspan, 2007). This role for serotonergic modulation of aggression is supported by the demonstration that *Drosophila* in which 5-HT neurons were selectively inhibited can exhibit aggression but show a limited ability to escalate the fight (Alekseyenko et al., 2010). Conversely, both fight intensity and rate of fight escalation were increased by selective serotonergic activation (Alekseyenko et al.,

2010).

Studies in our laboratory suggest an important role for 5-HT in aggression in a different dipteran species, the stalk-eyed fly *Teleopsis dalmanni*. Pharmacologically administering 5-HTP markedly increases the probability of winning an aggressive contest in sized-matched pairs, as well as increasing the incidence of high-intensity aggressive behaviors (Bubak et al., 2014). In accordance with the previously mentioned crayfish work, we also saw a reduction in the motivation to retreat with increases in brain 5-HT concentrations (Bubak et al., 2014). The function of 5-HT in influencing individual aggression and opponent assessment in stalk-eyed flies is discussed in more detail in Section IV.

Overall, these results suggest that increased activity in arthropod serotonergic systems contributes to species-specific expression of particular components of aggressive behaviors, but may not be required for the absolute display of aggression. These studies, particularly those using pharmacological manipulations to globally increase or decrease 5-HT, represent net serotonergic effects and do not provide the ability to differentiate more subtle serotonergic actions mediated by different 5-HT receptor subtypes. An approach that will be informative in future research will be to test behavioral outcomes following targeted manipulations of specific serotonergic receptors using pharmacological or genetic methods. Indeed, pharmacologically targeting different 5-HT receptors in socially isolated *Drosophila* indicates that 5-HT₂-like receptor activation decreases a subset of aggressive behaviors such as lunging, while activation of 5-HT_{1A}-like receptors increases specific behaviors such as wing threats (Johnson et al., 2009). This finding is important, revealing insights into potential molecular mechanisms responsible for specific aspects of aggression not provided by broader experiments focusing on whole system deprivation or elevation of 5-HT. Similarly, selective manipulation of each monoamine and subsequent association of its activity with specific aspects of aggressive behavior, (e.g., OA activity escalates aggressive intensity, while 5-HT activity either reduces motivation to retreat or modulates use of specific agonistic signals depending on receptor subtype) may be fruitful in unraveling how complex interactions among neurotransmitter systems can modulate proximate behavioral expression, which may in turn dictate fight outcome and reproductive fitness.

3.3 Pheromones and Neuropeptides

Another critical component in aggressive behavior is recognition of potential opponents. The ability of in-

sects to discriminate between potential rivals or mates is mediated, in part, by chemosensory communication. For example, detection of sex-specific pheromones by *Drosophila* males directs subsequent expression of social behavior, with either aggressive or courtship behavior evoked by male and female pheromones, respectively (Fernandez et al., 2010; Ferveur, 2005). The sexually dimorphic nature of these pheromones is evidenced by the elicitation of male attacks on females after masculinizing the females' pheromones (Fernandez et al., 2010). In *Drosophila* males, the volatile pheromone, 11-*cis*-vaccenyl acetate (cVA), is associated with the stimulation of male-male aggression, acting through olfactory receptor neurons (Wang and Anderson, 2010; Liu et al., 2011). Interestingly, acute exposure of socially-naïve male flies to cVA increases aggression, while chronic exposure such as prolonged social housing suppresses the behavior (Liu et al., 2011). These bidirectional actions of cVA are mediated through activation of two distinct olfactory receptor neurons, with aggression increased and suppressed by Or67d and Or65a receptors, respectively (Liu et al., 2011). This finding suggests a role for cVA in regulation of behavior according to social experience (Liu et al., 2011), and highlights the need to identify specific ligand substrates when determining mechanisms by which aggression is modulated, as discussed above for 5-HT.

Although the mechanisms by which pheromones modulate aggression are not completely understood, it is likely that pheromone reception is intimately linked to biogenic amine systems in some insect species. As mentioned above, recognition of male-specific sensory cues in *Drosophila* and subsequent expression of contextually appropriate behavior is disrupted by decreasing OA activity (Certel et al., 2007). Moreover, neurons expressing taste receptors that differentiate male and female pheromones connect functionally and synaptically with distinct OA neurons in male *Drosophila*, and both ablation of the taste receptor neurons and decreasing OA synthesis reduce male-male aggression (Andrews et al., 2014). Recent work in our laboratory has found different monoamine profiles in the brains of pavement ants *Tetramorium caespitum*, a species that exhibits social warfare, following exposure to hydrocarbons from nest-mate or non nest-mate colonies (Bubak, unpublished results). Specifically, when ants came in contact with a nest-mate or were exposed to beads saturated in nest-mate hydrocarbons, 5-HT was significantly elevated (Bubak, unpublished results). Conversely, exposure of ants to beads saturated in non nest-mate hydro-

carbons induced both mandible biting directed towards the beads and increased levels of brain OA (Bubak, unpublished results). Combined, the studies with *Drosophila* (Certel et al., 2007; Andrews et al. 2014) and our work with pavement ants suggest that identification of potential rivals and subsequent behavioral responses in these species is mediated by activation of 5-HT and OA systems following pheromone detection.

Neuropeptides have also been implicated in the expression of invertebrate aggression. Using the genetic tools available for *Drosophila*, studies have focused on the role of neuropeptides in modulating insect aggression, in an attempt to uncover phylogenetic similarities. For example, *Drosophila* possess male-specific neurons that express a gene encoding for the neuropeptide, Tachykinin (*Tk*) (Asahina et al., 2014). This peptide is homologous to Substance P, a mammalian peptide associated with increased aggression in a number of vertebrate species (Halasz et al., 2009; Katsouni et al., 2009; Siegel et al., 1997). Activation of *Tk*-containing neurons in *Drosophila* increases male-male aggression, while silencing *Tk* neurons decreases aggression (Asahina et al., 2014). Notably, neither manipulation altered male-female courtship behaviors (Asahina et al., 2014). Additionally, the invertebrate homolog to neuropeptide Y, neuropeptide F (NPF), has been posited to have an inhibitory role in aggression in *Drosophila*, with elevated aggression observed following genetic silencing of NPF circuits (Dierick and Greenspan, 2007). Aggression in male mice is similarly increased by decreasing neuropeptide Y activity through genetic deletion of neuropeptide Y receptors (Karl et al., 2004). In line with the conservation of neuropeptide function between vertebrates and invertebrates, transcription factors regulating neuropeptide signaling have been identified in the *pars intercerebralis* (PI) of the *Drosophila* brain (Davis et al., 2014). This brain area is thought to be functionally and structurally similar to the mammalian hypothalamus, which has been shown to play a role in aggression through modulation of neuropeptide signaling in several mammalian species (Hartenstein, 2006; Kruk et al., 1984; Gregg and Siegel, 2001).

The general interactive functions of monoamines, chemosensory signals and neuropeptide systems in modulating aggressive behavior appear to share a high degree of conservation across both arthropods and vertebrates. This highlights the value of utilizing animals with relatively simplified neural circuitries, such as insects, for future investigations directed towards gaining more in-depth perspectives on neurobiological factors

underlying this complex social behavior. Information gained from such studies can then be applied to understanding mechanisms governing aggression in higher order, more complex nervous systems. Similarly, using insect models to elucidate how a given neurobiological factor may have different behavioral effects depending on social ecology could provide insight into what factors will come under selection pressure to ultimately produce species differences in expression of behavior in other taxa, as has been proposed for applying parallels between neuropeptide signaling and sociality in birds to other vertebrates (Goodson et al., 2005). The ease of gathering such information in insect models can thus direct whether generalizations about neurochemical modulation of aggression may be applied broadly because of conservation in systems across taxa, or if specific nuances in natural history need to be taken into account to explain why departures from observed patterns have evolved.

4 The Stalk-Eyed Fly as a Model System for Studying Aggression

Stalk-eyed flies (Diptera; Diopsidae) have emerged as an excellent model system for understanding how sexual selection drives the evolution of showy male traits and associated behaviors (Burkhardt and de la Motte, 1988; Wilkinson et al., 1998), particularly for studies of morphology (Hingle et al., 2001; Wilkinson and Reillo, 1994; Worthington et al., 2012; Husak et al., 2013), physiology and performance (Swallow et al., 2000; Swallow et al., 2009; Ribak and Swallow, 2007; Ribak et al., 2009a), and neurobiology (Buschbeck and Hoy, 1998; Worthington and Swallow, 2010; Egge et al., 2011; Egge and Swallow 2011; Bubak et al., 2013; Bubak et al., 2014). Males and females of all species in the family Diopsidae are hypercephalic, with the eye bulbs displaced on the ends of long stalks. Recognized as an important feature in diopsid mating systems, eyestalks are used extensively as ornamental signals in both intra and intersexual interactions (Wilkinson and Dodson, 1997; Wilkinson and Johns, 2005). In sexually dimorphic species of stalk-eyed flies, such as *Teleopsis dalmanni* (Fig. 1), male mating success is positively correlated with eye span (Burkhardt et al., 1994; Wilkinson and Reillo, 1994; Cotton et al., 2010). Females also show a preference for males with longer eye spans (Wilkinson et al., 1998; Burkhardt and de la Motte, 1988). In addition, males compete for access to the limiting resources of food and mates by following a stereotyped fighting repertoire that typically begins with

the lining up of eyestalks (Lorch et al., 1993; Panhuis and Wilkinson, 1999, Egge et al., 2011). Such observations indicate that eye span is under current sexual selection.

Use of eye span as a communicative signal during male-male competition in stalk-eyed flies is best characterized in sexually dimorphic species such as *T. dalmanni* and *T. whitei*. Larger males with broader eye spans typically win agonistic contests, thereby excluding smaller rivals and gaining access to the contested limiting resources (Burkhardt and de la Motte 1987; Cotton et al 2010; Egge and Swallow 2011; Panhuis and Wilkinson, 1999). Males use multiple displays that escalate from lower to higher intensity behaviors in a stereotyped manner (Egge et al., 2011, but see Brandt and Swallow, 2009) in what appears to be mutual assessment, in order to compare asymmetries in ornament size and fighting ability. Conflicts typically begin with males facing each other and lining up their eyestalks in a parallel manner (de la Motte and Burkhardt, 1983; Panhuis and Wilkinson, 1999). In encounters where opponent eyestalks are evenly matched in size, the individuals will gradually progress to expression of low-intensity behaviors consisting of non-combative actions such as forearm flexing or rearing (see Fig. 2). If an opponent does not retreat at this point, high-intensity aggressive behaviors typically follow. These involve physical contact, and comprise elements such as lunges, jump attacks, or tussles (see Fig. 2). High-intensity physical fights are non-lethal and usually result in one opponent departing uninjured. Aggressive interactions can de-

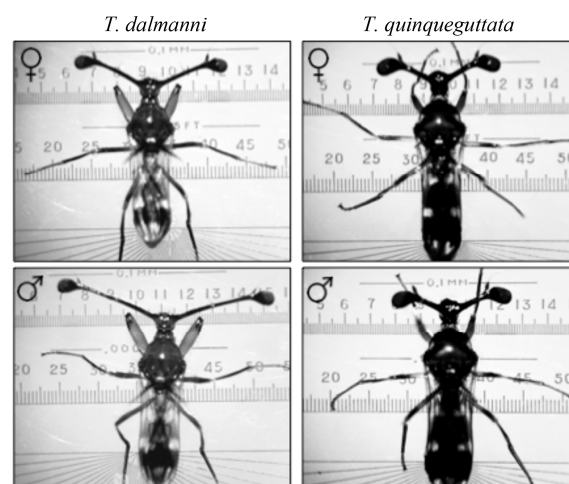


Fig. 1 Representative images of both male and female stalk-eyed flies from a sexually dimorphic species, *T. dalmanni*, and a sexually monomorphic species, *T. quinqueguttata*

Note the pronounced eyestalk elongation of the male *T. dalmanni* compared to the male *T. quinqueguttata*.

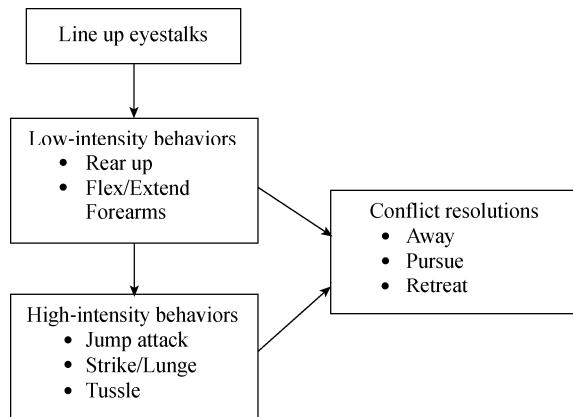


Fig. 2 Flow chart of aggressive behaviors exhibited by *T. dalmanni*

Encounters begin with lining up of eyestalks, which can then lead to low-intensity behaviors, followed by either conflict resolution or further escalation to high-intensity behaviors. Modified from Egge et al., 2011.

escalate at any stage of the conflict, and are terminated when one of the rivals capitulates and retreats (Egge et al., 2011).

Aggression in sexually monomorphic stalk-eyed flies has not been as well studied, but appears to differ from dimorphic species in terms of frequency and components. For example, males of *T. quinqueguttata* are morphologically indistinguishable from females in terms of eye span (Fig. 1), and initiate far less aggressive interactions than any of the dimorphic species that have been measured to date (Panhuis and Wilkinson, 1999). In addition, the repertoire of behaviors displayed by *T. quinqueguttata* includes a larger array of low-intensity, non-contact displays including wing threats and bobbing (P Johns unpublished). This difference in morphology and behavioral repertoires between sexually dimorphic and monomorphic species is useful for comparative analyses and may provide valuable insight into the evolution of signals used in this complex behavior.

Aggressive interactions in stalk-eyed flies are easily characterized and quantifiable. This offers an ideal platform for understanding how the neurobiological factors governing individual differences in behavioral expression and ornament use may provide a proximate mechanism to be acted upon by sexual selection. To investigate the neural mechanisms underlying individual variation in aggression in stalk-eyed flies, we developed a method sufficiently sensitive to detect and quantify a variety of different monoamines (including 5-HT, DA, and OA) from the brain of a single fly using high

performance liquid chromatography (HPLC) with electrochemical detection (Bubak et al., 2013). This quantification from an individual subject contrasts with other methods using smaller insects such as *Drosophila*, where it is common to pool brain samples to get accurate measurements. This ability to measure monoamines from a single fly has proven to be an important tool for understanding how individual differences in concentrations of monoamines, such as 5-HT, contribute to contest intensity and outcome. For example, we conducted a study in which some males had 5-HT levels elevated via oral administration of the 5-HT precursor, 5-HTP. When pitted in dyadic interactions against non-treated size-matched controls, 5-HTP-treated flies won 70% of the fights (Bubak et al., 2014), in line with the relationship between elevated 5-HT and increased aggression reported in *Drosophila* (Dierick and Greenspan, 2007; Alekseyenko et al., 2010). Consistent with this, a portion of the 30% of treated males that lost fights actually had 5-HT levels that were lower than the endogenous concentrations of their control opponents, despite having being treated earlier with 5-HTP. Reanalysis of fight outcome and brain 5-HT concentrations further showed that winners had higher mean brain 5-HT concentrations when compared to losers, regardless of pretreatment. In addition, high-intensity aggression was negatively correlated with the difference in 5-HT concentrations between opponents, such that individuals with more closely matched 5-HT engaged in more high-intensity behaviors and longer interactions before the conflict was resolved (Bubak et al., 2014). These results demonstrate that contest intensity and outcome are not simply a function of absolute levels in 5-HT, but are instead dependent upon the relative difference in 5-HT concentrations between size-matched opponents. Such a finding is reliant upon the ability to measure central monoamine levels from individual subjects, which can then be combined with pharmacology and correlated with behavioral expression. Issues such as this could have large implications in studies where the species is too small to be analyzed on the individual level, and further highlight the use of stalk-eyed flies as a model system for detecting potential relationships between neurophysiological mechanisms, morphology of armaments, and aggressive behavior.

While relative differences in 5-HT concentrations appear to dictate contests between size-matched stalk-eyed flies, a major component in winning an aggressive encounter between two conspecifics is size discrepancy, where in most cases, the larger individual is victorious.

This remains true in *T. dalmanni*, where the larger opponent predominantly excludes smaller rivals from resources (Burkhardt and de la Motte, 1987; Wilkinson et al., 1998). One way to understand how a proximate neurobiological mechanism may be acted upon evolutionarily to determine behavioral phenotypes, would be to show that intrinsic factors such as elevated neural 5-HT can influence fight outcomes that would otherwise be decided by morphological discrepancies, and that differences in 5-HT activity are primarily involved in modulation of aggression rather than with factors that may otherwise indirectly influence reproductive success. To investigate how changing 5-HT concentrations could alter a morphologically biased fight, we designed a “David vs. Goliath” experiment where the smaller “Davids” had pharmacologically elevated 5-HT. Although the fight outcome did not significantly change, with larger untreated opponents still winning the majority of the fights, the behaviors of both participants were altered (Swallow, unpublished results). The treated “Davids” performed more high-intensity aggressive behaviors, and retreated less, compared to untreated controls. Interestingly, the “Goliaths” facing treated “Davids” altered their behavior by more quickly escalating to higher intensities, as well as initiating more high-intensity aggressive behaviors. This increased frequency in initiations and decreased latency to high-intensity behavior by the larger, untreated opponents suggests an assessment of their rivals, with the larger male changing behavioral expression to match the increased aggressiveness of the smaller, treated opponent. In what normally presents as a lopsided fight in favor of the larger participant, altering intrinsic neurobiological factors, such as 5-HT in the smaller opponent, creates a contest closely resembling morphologically size-matched opponents.

Applying the stalk-eyed fly as a model system to study aggression is becoming an increasingly attractive opportunity. The species diversity with respect to mono- and dimorphic phenotypes allows researchers to investigate behavioral differences associated with morphological characteristics. Furthermore, the reliable non-invasive ability to manipulate endogenous 5-HT and other neurochemicals from a single animal can be correlated with the well-described and easily quantifiable aggressive behavioral expression by the same individual. Overall, the stalk-eyed fly creates an exciting model system to investigate how central nervous system chemicals modulate behavior and may play an intricate role in shaping overall morphology.

5 Perspectives

To obtain a more complete understanding of aggression, it is imperative to incorporate neurobiological, morphological, and behavioral information. However, obtaining this information, particularly the physiological and neurological data, can prove difficult in organisms with complex central nervous systems and behavioral responses, such as vertebrates. Many of the molecular and physiological processes involved in generating complex behaviors in both vertebrates and invertebrates are highly conserved. Therefore, focusing on invertebrates, with their relatively simple nervous system and often well-characterized aggressive behavioral patterns, could prove beneficial (Bubak et al., 2014). Insects are extremely well suited to aggression models, with significant behavioral (e.g., aggressive and nonaggressive) and morphological (e.g., weapon possessing and non-possessing) diversity providing a rich resource to test larger evolutionary questions. Understanding how the neural circuitries and underlying genetic factors mediate the behavioral processes and development of specific distinct morphological features in individual insect species will help elucidate the evolutionary and ecological connections between morphology, behavior, and physiology, and may provide insights into these same processes in vertebrates. Currently, much of this work being conducted in insects focuses heavily on just a few species such as crickets and *Drosophila*. Obviously, in depth investigations of the physiological and genetic underpinnings of aggression in these species is invaluable. However, it is also vital to apply these findings across a wider range of taxa, with distinctive ornaments, mating systems, and behavioral repertoires, to achieve a more comprehensive understanding of this widely expressed behavior.

A specific advantage to using the stalk-eyed fly as a model system for studying aggression, as mentioned earlier, is the utilization of the vast morphological and behavioral differences we see among species. For instance, sexual dimorphism in eyestalk length is a trait that has arisen and been lost multiple times in the Diopsid family (Baker and Wilkinson, 2001), and appears to correlate with differences in the frequency and components of aggressive behavior. A recent molecular phylogenetic analysis of over 30 (Baker et al., 2009) of the estimated 150+ species in the family (Steyskal, 1972; Feijen, 1983; Feijen, 1989) provides a robust framework for mapping differences in neurochemical modulation of aggression according to species relatedness. In

turn, this may illustrate how selection can shift from emphasis on static morphological signals to favoring more plastic physiological mechanisms, and hence explain continued emergence of dimorphism versus monomorphism in this family. The differences in behavior and morphology among stalk-eyed flies also lends well to large comparative studies. This strategy has been successfully used to study a variety of evolutionary questions at the organismal level, including speciation in closely related populations of *T. dalmanni* (Swallow et al., 2005; Christianson et al., 2005) and the co-evolution of ornaments with morphology and locomotor performance (Husak et al., 2011a; Husak et al., 2011b; Ribak et al., 2009b). Similarly, a comparative genomic approach has been used to investigate genes underlying the development of the sexually selected and sexually dimorphic ornaments, eye span, that define stalk-eyed flies (Baker et al., 2009; Wilkinson et al., 2013). It will be interesting to identify genes responsible for aggressive behavior that have been gained and lost, along with eye span length, throughout evolution of the stalk-eyed fly family. Furthermore, the application of genomic techniques in the field, becoming increasingly more accessible by nucleic acid preservation methods, provides an exciting opportunity to tease out specific proximate mechanisms of aggressive displays by studying gene expression differences in geographically separated wild populations, which also show differences in fighting repertoires.

As research into proximate mechanisms responsible for mediating aggression progresses, it will be interesting to determine the level of conservation between invertebrates, such as insects, and vertebrates. Tools such as next-generation sequencing are invaluable for understanding the degree of conservation at the gene level (see Toth and Robinson 2007 for a review on using “genetic toolkits” for elucidating mechanistic conservation underlying behavioral expression), while the ability to manipulate and measure neurochemical activity within individual insects offers a powerful means of examining why variation exists in aggressive behavior, signal use, and contest outcome. Identifying and comparing such proximate mechanisms among behaviorally and morphologically distinct insect species could provide valuable insight into the evolution of the extensive, often bizarre, variety of morphological features and behavioral repertoires used during aggressive competition.

Acknowledgements We thank Dr. Rebecca Safran and Dr. Maren Vitousek for the invitation to contribute to this special

column of Current Zoology. We also thank the reviewers for their helpful critiques. This work was supported by NSF grant IOS 1256898 (to JGS).

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