Transgenic and histological approaches to investigating the development of the stalk-eyed fly, *Teleopsis dalmanni*

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DECLARATION

I, Ian Andrew Warren, confirm that the work present in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Stalk-eyed flies of the family Diopsidae have their eyes laterally displaced on the end of head extensions called "eye-stalks". Diopsids vary in their degree of sexual dimorphism for eyespan (distance between the eyes). In some dimorphic species it has been well established that females preferentially mate with males possessing exaggerated eyespan. With over 150 members, the family Diopsidae is an ideal model system for analysing the evolution and development of exaggerated sexual traits.

Progress towards understanding the mechanisms underlying the development of exaggerated eyespan has been significantly hampered by the lack of modern molecular genetic technology in stalk-eyed flies. I have developed a transgenic protocol in *Teleopsis dalmanni*, a highly dimorphic diopsid species. I selected and tested embryo microinjection procedures. I used excision assays to compare the activity of three potential transposable element vector systems. *Minos* and *piggyBac* demonstrated suitable activity in *T. dalmanni* embryos but *mariner* did not. Using *Minos* and the transgene construct *Px3-eGFP*, I successfully achieved stable germline transformation in *T. dalmanni*. A number of transgenic lines were created. In one, a single copy of the insertion was seen to segregate with the X chromosome.

I used a histological approach to investigate the relative contributions of cell size and cell number to variation in eyespan. I compared estimates of cell size in the eye-stalks of newly eclosed flies among fully fed and nutritionally stressed individuals. For comparison, I assessed cell size in a non-sexually dimorphic organ, the wing. I found that variation in eyespan was explained, at least in part, by variation in cell size. No inherent difference in eye-stalk cell size was detected between the sexes.

The implications of the cell size findings and of the future studies made possible by transgenic technology are discussed.

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1.1 THE STALK-EYED FLY

1.1.1 Exaggerated novel morphologies

In nature, evolution has produced a vast array of organisms with a bewildering variety of morphologies. Some of the most striking and remarkable features are those of novel and exaggerated traits. Classic examples include the elaborate tail feathers of the male peacock (Ohlsson *et al.* 2002), the brightly coloured dewlaps of the anole lizard (Vanhooydonck *et al.* 2005), the extended incisor of the narwhal (Nweeia *et al.* 2005) and the large antlers seen in many species of deer (Markusson & Folstad 1997). There has been a great deal of interest over the last century in the selective forces underlying the evolution of these traits (Fisher 1930; Müller & Wagner 1991; Andersson 1994). To fully understand the evolutionary mechanisms that produce such extreme phenotypic variation the developmental genetics of the trait must be understood (Emlen & Nijhout 1998).

Insects provide attractive model systems for the study of development in general as they can be reared in large numbers and many species have short generation times. A large array of novel and exaggerated morphologies are found in insects (reviewed in Emlen & Nijhout 2000). For example, the male dung beetles, *Onthophagus taurus*, possess large thoracic horns (Hunt & Simmons 1997), exaggerated forelegs are observed in the harlequin bug, *Acrocinus longimanus* and tree hopper species in the group membracidae exhibit many different elaborations of the pronotum (Zeh & Zeh 1992; Stegmann 1998). Males of the Hawaiian drosophilid species *Drosophila heteroneura* have greatly enlarged heads with their eyes displaced laterally (Grimaldi & Fenster 1989). This expansion of the head capsule is an example of hypercephaly. The form of hypercephaly can vary greatly ranging from gross head enlargement as seen in the ant *Pheidole bicarinata* (Wheeler & Nijhout 1983), to long thin eye-stalks observed in several Dipteran species (Grimaldi & Fenster 1989) with much variation seen inbetween. The degree of displacement of the eye can vary, for example males in *D. heteronuera* have broadened heads with unmodified eyes, whereas in the closely related drosophilid *Zygothrica latipanops*, the eyes in males are displaced anterio-laterally (McAlpine 1979). Hypercephaly is present in many insect orders including the Hymenoptera, Heteropera and Diptera (Emlen & Nijhout 2000).

Diptera, Diopsidae and hypercephaly

One group that provides an excellent example of hypercephaly is the stalk-eyed flies (Diopsidae) which are members of the Diptera order of insects. The Diptera (true flies) are a diverse order, consisting of approximately 150 families which together contain 150,000 species making up 10-15% of all known animal species (Yeates *et al.* 2007). Fossil evidence indicates that the order formed about 250 million years ago (MYA). Within the Diptera are several commonly studied families including the Culicidae (mosquitoes), Tetriphidae (true fruit flies), Muscidae (house flies) and the Drosophilidae, which includes the laboratory favourite *Drosophila melanogaster* (Fig. 1.1).

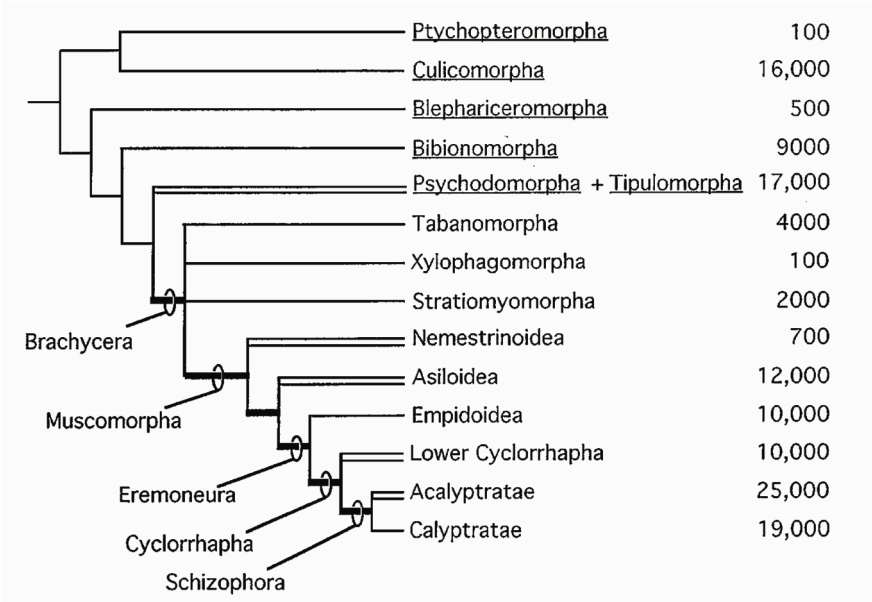

Figure 1.1. Dipteran phylogeny. Estimation of species number for each group is given. Diopsidae reside within the Calyptratae along with the Drosophilidae and Tetripidae. Muscidae (house flies) are found in the Acalyptratae and the Culcidae (mosquitoes) are found in the Culicomorpha. Adapted from Yeates and Wiegmann 1999.

Hypercephaly is present in several dipteran groups, most strikingly in the Diopsidae (stalk-eyed flies) (Shillito 1971; Fig. 1.2). The Diopsidae are members of the Brachycera (or higher diptera). There is much debate as to the exact details of brachyceran phylogeny but it is generally agreed that Cyclorrhapha, which formed over 200 MYA (late Triassic), is a monophyletic group. The Cyclorrhapha are characterised by its members developing from a pupa enclosed in a modified form of the larval cuticle (puparium). The Cyclorrhapha can be sub-divided into the Aschiza and Schizophora,

the latter are thought to be more derived. The Schizophora are, in turn, subdivided into Calyptrata, which includes the muscidae family (including the house fly, *M. domestica*) and the Acalyptrata, which includes Drosophilidae, Tetriphidae and Diopsidae. The Acalyptrata consists of 61 families and hypercephaly is observed in 8 of these (Grimaldi & Fenster 1989; Wilkinson & Dodson 1997). The Acalyptrata and Calyptrata are thought to have diverged 48-86 MYA.

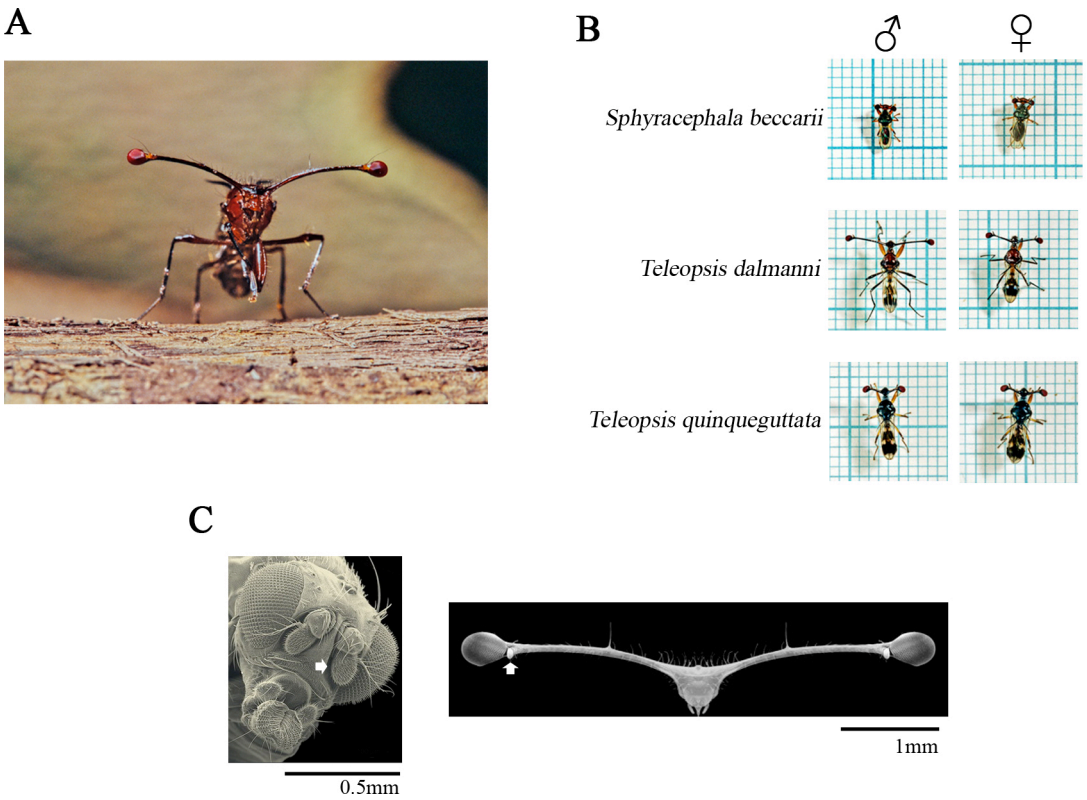

Figure 1.2. Hypercephaly and the stalk-eyed fly. **A.** Photograph of *Teleopsis dalmanni* to show hypercephaly. The eyes are laterally displaced on the ends of large stalks. **B.** Representative species of stalk-eyed fly demonstrating hypercephaly and sexual dimorphism. *Sphyracephala beccarii* exhibit weak hypercephaly. *T. dalmanni* shows extreme hypercephaly and sexual dimorphism for eyespan. *Teleopsis quinqueguttata* is monomorphic for eyespan. Each individual square = 1mm^2. **C.** Reorganisation of head structures in *T. dalmanni* (right), compared with *D. melanogaster* (left). In *T. dalmanni*, the eyes and the antennae (white arrow) are both located at the ends of the eye-stalks. In *D. melanogaster* the antennae (white arrow) are found in the medial anterior portion of the head. (Photographs A & B by Sam Cotton, UCL. C by Imogen Hurley).

Diopsid hypercephaly is unusual in two respects. All species of the family possess the trait in both males and females by contrast with other dipteran families where only a few species show the trait and it is also only observed in one sex, usually males

(Grimaldi & Fenster 1989). Furthermore hypercephaly in Diopsidae involves a major re-arrangement of the head, with both the eyes and antennae along with parts of the brain re-located to the ends of eye-stalks. Other hypercephalic dipteran groups only exhibit lateral displacement of the eyes.

Diversity of eye-stalk size in the Diopsidae

There are over 160 described diopsid species spanning 11 genera and it is estimated that there are over 200 extant species. *Diopsis* (>50 species), *Diasemopsis* (>30 species), *Teleopsis* (>40 species) and *Sphyracephala* (~20 species) are the major genera with the other genera containing only a few species each. (Fig. 1.3) (Meier & Baker 2002; Kotrba 2004; Carr *et al.* 2006; Földvarí *et al.* 2007). The genus *Cyrtodiopsis* was originally given independent status but is now regarded as part of the genus *Teleopsis* (Meier & Baker 2002) and is treated as such in this thesis. There is debate as to whether the centrioncidae are part of the Diopsidae or a separate family and recent opinion favours the former interpretation (Feijen 1983; 1989; Meier & Hilger 2000). Stalk-eyed flies are found predominantly in old-world tropics but are also located in nearctic and palearctic regions (Feijen 1989).

Although both male and female diopsids possess eye-stalks, there is a great deal of both inter- and intra-specific variation in eyespan (distance between the tips of the eyes) (Wilkinson & Dodson 1997; Baker & Wilkinson 2001). Sexual dimorphism for eyespan is present in many stalk-eyed fly species with males having dramatically larger eyespan than females. In addition, many monomorphic species exist with males and females having similar eyespan. In *Sphyracephala beccarii*, part of what is thought to be the oldest diopsid genus, both males and females possess small eyespans that are much smaller than a body length (Fig 1.2; Baker & Wilkinson 2001). In *Teleopsis dalmanni* males have eyespans greater than the length of their bodies with the females possessing comparatively modest eye-stalks (Fig. 1.2). However, eyespan in *T. dalmanni* females, is still significantly greater that that of *S. beccarii* (Fig. 1.2). A large amount of variation is also observed in more closely related species with females of the sexually dimorphic *Teleopsis breviscopum* possessing eye-stalks twice the length of females of the monomorphic *Teleopsis quadriguttata* and *T. breviscopum* males possessing eye-stalks twice the length of *T. breviscopum* females (Wilkinson & Dodson 1997).

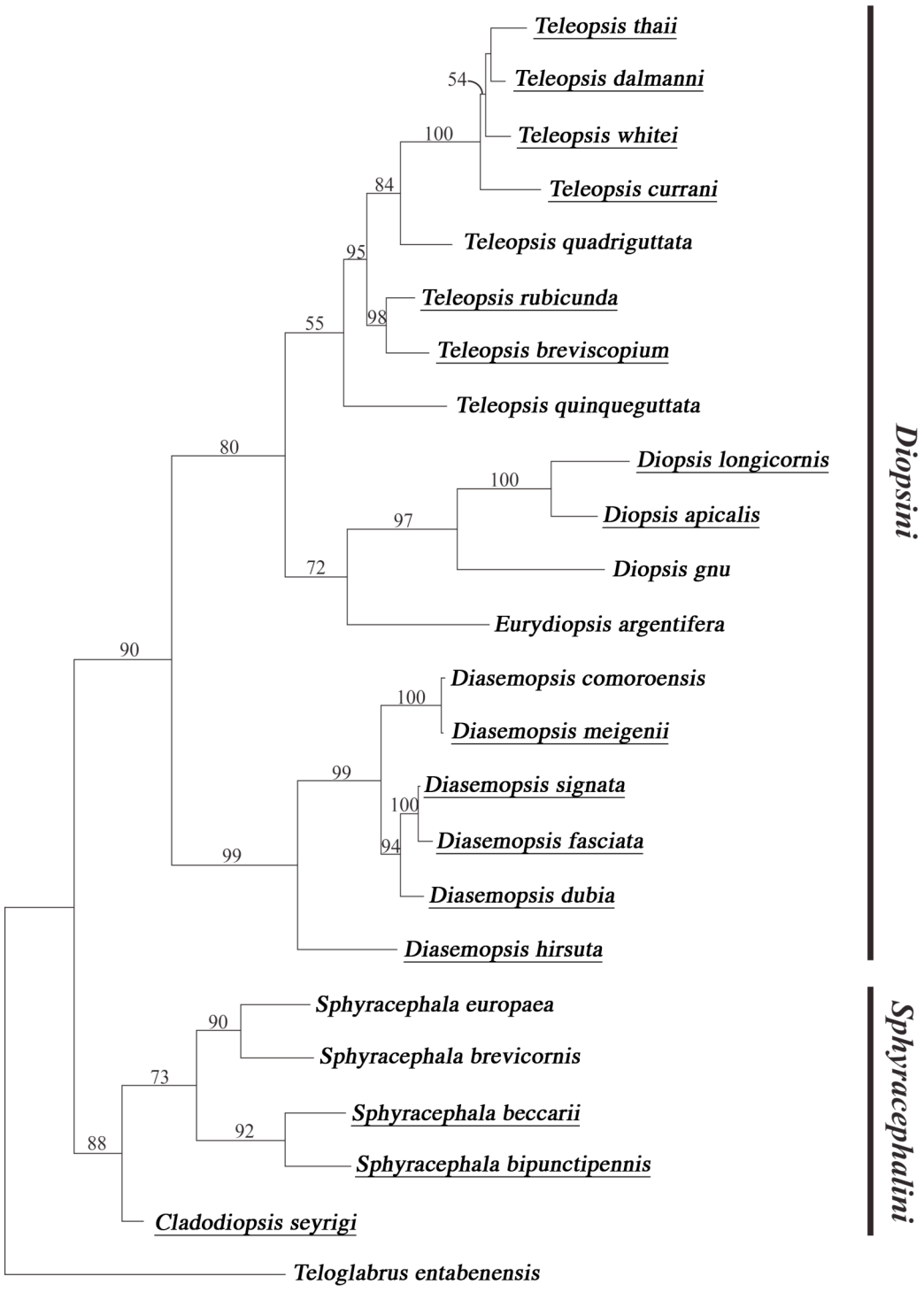

Figure 1.3. A simplified stalk-eyed fly (Diopsidae) phylogeny. Species that are sexually dimorphic for eyespan are underlined. Distance-based neighbour-joining tree, constructed using sequence of *white*. The tree has been rooted with the sequence from the non-hypercephalic species, *T. entabenensis*. Bootstrap values are positioned on the nodes and are percentages taken from 1,000 replicates. Tree adapted from Carr 2008. Information on dimorphism from Baker & Wilkinson 2001; Carr *et al.* 2006; Földvári *et al.* 2007.

Although the ancestral state of the group is unclear, sexual dimorphism appears to have arisen on at least four separate occasions within the group (Baker & Wilkinson 2001). There are several examples of closely related species, such as *Diasemopsis meigenii* and *Diasemopsis comoroensis*, that diverge in relation to eye-stalk dimorphism with the former being sexually dimorphic and the latter monomorphic (Carr *et al.* 2006).

With so many species possessing such variation in the eyespan trait, stalk-eyed flies show great potential for understanding the evolution and development of exaggerated morphologies. I shall review what is known about eye-stalk evolution and development in Diopsidae. I go on to argue the importance of Diopsids as potential model organisms for understanding the development and evolution of novel and exaggerated traits.

1.1.2 Natural selection and eye-stalks

Exaggerated morphologies have evolved for a variety of reasons in different species. In soldier ants enlarged mandibles are used to defend the nest, however in most studied species these traits are involved in sexual selection to enhance a male's chances of reproduction. Exaggerated traits, such as enlarged legs in harlequin bugs, *Acrocinus longimanus* (Zeh & Zeh 1992) and the horns of scarab beetles (Emlen *et al.* 2007). Alternatively exaggerated traits act as as signals to which females respond. This has been the focus of the majority of the work on stalk-eyed flies and will be discussed later.

In stalk-eyed flies both sexes possess eye-stalks and many monomoprhic species exist where eyespan is not used as a signal of quality by males, indicating that there is an advantage conferred to males and females via natural selection. This suggests that ecological factors specific to stalk-eyed flies initially drove natural selection in favour of lateral displacement of the eyes onto stalks. This aspect of eye-stalk evolution is often neglected. Burkhardt and de La Motte (1983) showed evidence for increased visual acuity conferred by the displacement of eyes on stalks. The eyes are a bulbous shape and much wider than the eye-stalks while there are a large number of ommatidia with many facing medially towards the fly itself. This structural arrangement gives stalk-eyed flies a binocular field of vision of 135º. In addition the angle of divergence between separate ommatidia is very small, 1.3º, compared to other dipteran species (Burkhardt & de la Motte 1983). Taken together these data imply high visual acuity in

stalk-eyed flies. Although not experimentally tested, it is possible that this may have given stalk-eyed flies improved all round peripheral vision and aided orientation and flight in their natural habitats such as dense forests.

1.1.3 Sexual selection and eye-stalks

Eye-stalks as ornamental signal traits subject to female preference

In recent years there has been much interest in the signalling value and evolution of exaggerated male sexual ornaments. One focus has been the condition-dependent expression of sexual traits because this is predicted by handicap models of sexual selection and is in line with "good genes" interpretations of their signalling value (Iwasa & Pomiankowski 1994; Iwasa & Pomiankowski 1999; Maynard Smith & Harper 2003). The handicap theory of sexual selection proposes that male sexual ornaments are used as a cue in mate choice by females because such traits are costly and so reflect the quality of their bearer (Zahavi 1975; Andersson 1994; Pomiankowski 1987; Grafen 1990; Iwasa and Pomiankowski 1994; 1999). The logic is that poor quality males are unable to suffer the cost, or enjoy the benefits, of possessing a large sexual trait, and hence only high quality males can afford to signal at high levels (Grafen 1990; Iwasa and Pomiankowski 1994, 1999; Getty 2006). Given this variation in male quality (environmental or genetic), there is the potential for the evolution of female preference for ornamented males, through either direct benefits to the female, and/or indirect benefits to her offspring (Iwasa and Pomiankowski 1999; Kokko *et al.* 2006).

In *T. dalmanni*, there is ample field and laboratory evidence that females have a strong preference for mating with and preferring to roost with large eyespan males (Burkhardt & de la Motte 1988; Wilkinson & Dodson 1997; Hingle *et al*. 2001a, b; Cotton *et al*. 2006; 2009). Several laboratory studies have investigated the signalling function of exaggerated eyespan in *T. dalmanni*. These show that male eyespan is highly condition-dependent, with a heightened response to stress. Male eyespan becomes progressively smaller as phenotypic condition is reduced by larval dietary stress (David *et al.* 1998; Cotton *et al.* 2004a, b), desiccation and heat shock (Bjorksten *et al*. 2001). Quantitative genetic studies have shown that there is a strong genetic component to the response to stress. Under stress, male eyespan becomes more phenotypically and genetically variable and the relative performance of genotypes is maintained across different

environments with the consequence that performance differences between individuals are amplified (David *et al*. 2000; Cotton *et al*. 2004a, b). Overall, these data constitute evidence that male eyespan is a reliable signal of individual condition and are also in line with "good genes" arguments postulating indirect benefits arising from female mate choice for males with exaggerated sexual traits.

There is also recent evidence, in *T. dalmanni*, that direct benefits can accrue from matings to large eyespan males because male eyespan predicts male reproductive quality. The development and maturation of the male reproductive organs (testes and accessory glands) show highly condition-dependent responses to food stress and larger eyespan males cope better with such stress (Baker *et al.* 2003; Rogers *et al*. 2008). These attributes have important direct consequences for females who achieve elevated fertility when kept with large eyespan males (Rogers *et al*. 2008).

In contrast to the wealth of data on the potential benefits to males bearing large ornaments, there is relatively scarce information on the costs of exaggeration of the eyespan trait. Increased eye-stalk size has been shown to impair the flying abilities of stalk-eyed flies (Swallow *et al.* 2000; Ribak & Swallow 2007). In a comparative study, the overall aerial manoeuvrability and vertical velocity of males from a dimorphic species, *T. whitei*, was lower than that of males from a monomorphic sister species, *T. quinqueguttata* (Swallow *et al.* 2000). A related finding was that, within the dimorphic *T. dalmanni*, females can fly faster and make larger turns than males (Ribak & Swallow 2007). One consequence of exaggerated eyespan is that the bearer will experience large inertia-driven rotations of the head while moving. There is recent evidence that in order to compensate for this, heavily ornamented males have developed stronger musculature to regulate the movement of their heads (Ribak *et al.* 2009).

Eye-stalks as signal traits in male-male contests

The dominant explanation for the evolution of the male sexual ornament (eyespan) in stalk-eyed flies is that it acts as a signal of male quality that is used by females in their choice of mates. However there is also evidence that the trait plays a role in male-male competition. The two most commonly studied species of stalk-eyed fly, *T. dalmanni* and *Teleopsis whitei*, live in the rainforests of Malaysia and flies roost overnight on root hairs that hang from the eroded banks of forest streams (Burkhardt & de la Motte 1985,

Wilkinson & Dodson 1997; Cotton *et al.* 2009). Adults form groups ("leks") consisting of 1-4 males and upwards of 10-20 females (Burkhardt & De La Motte 1985; Wilkinson & Dodson 1997; Lorch *et al.* 1993). More than 90% of the matings occur at dawn and are carried out by the males roosting on the leks (Lorch *et al.* 1993). At dusk males will fight for control, and therefore mating rights, of a roosting site. Males with the largest eyespan usually win such male-male contests (Panhuis & Wilkinson 1999). In staged laboratory fights between males in three closely related stalk-eyed fly species that differed in the degree of eyespan dimorphism, it was found that males with larger eyespan won more fights than their smaller eyespan competitors in the sexually dimorphic species, further indicating that larger male eyespan is a signal of greater competitive ability (Panhuis & Wilkinson 1999). In laboratory lines of *T. dalmanni* that had been selected for an increased ratio of eyespan to body size, fight duration reduced as the difference in eyespan between competing males increased and the outcome of male-male contests was positively correlated with male eyespan, independent of body size (Panhuis & Wilkinson 1999).

However, note that the eye-stalks are not used as weapons in fights. This is in sharp contrast to the hammerhead drosophilid, *D. heteroneura*, in which males use their expanded head capsules as weapons against other males (Speith 1981) and males with wider heads win the majority of battles (Boake *et al.* 1997). The horns of the dung beetle, *Onthophagus taurus*, are weapons that are used to settle intra-sexual disputes. Mated females lay eggs within burrows constructed and defended by males. *O. taurus* males with larger horns are more likely to win male-male contests and so gain greater access to females (Emlen & Nijhout 1998). One interpretation of the intra-sexual signalling function of male eyespan in *T. dalmanni* is that eyespan is a condition-dependent trait which is highly correlated with male body size and reflects overall male quality. Consequently, male eyespan reliably signals a male's ability to cope with the demands of intensifying aggressive interactions.

1.1.4 Early eye-stalk development

As I have already stated, understanding the development of a trait is key to understanding its evolution. The study of eye-stalk ontogeny in diopsids can be studied from two perspectives.

The first addresses how the eye-stalk itself arises. It looks at the differences that are present in diopsid head development, compared with non-hypercephalic species from closely related genera such as the Drosophilidae, that give rise to the eye-stalk. This approach treats the eye-stalk as a novel phenotype.

The second perspective addresses why there is such inter- and intra-specific variation in eyespan observed within the Diopsidae and looks at the growth mechanisms underlying the diversity observed. Understanding this variation in growth regulation is critical to understanding the mechanisms of insect organ growth in general, but also in elucidating the mechanisms underlying the evolution of secondary sexually selected traits.

Generation of a novel phenotype

Novel phenotypes are intuitively understood to cover distinctive morphological, physiological or behavioural features, which are characteristic of particular taxonomic groups. Examples include the colour patterns of lepidopteran wings, the bioluminescent organs of fireflies and the horns of scarab beetles. The definition of a novel phenotype has been the source of some controversy (Müller & Wagner 1991). The most restrictive definition is that novel morphological phenotypes are traits that show no homology to any other structure. However, the definition of homology is also contentious and it can be argued that all phenotypes are based on pre-existing developmental machinery present within an organism. Moczek (2008) argued that it is more productive to focus on the evolutionary origins of innovations than their definitive categorisation. How novel phenotypes arise remains an understudied question (Müller & Newman 2005; Bowsher & Nijhout 2007).

Diopsid eye-stalks can be viewed as novel since the eye-stalk structure seen is unique to the Diopsidae and involves a major re-arrangement of the head rather than the simple overgrowth observed in most other hypercephalic species (Shillito 1971). The arista and

antennae, which are medial structures in most dipteran flies, are displaced laterally along with the eyes at the end of the stalks (Fig. 1.2C). This is associated with a relocation of the optic lobe away from the rest of the brain to the end of the eye-stalks, along with the eyes and antennae (Buschbeck & Hoy 1998; Buschbeck *et al.* 2001). It is also rare for such a phenotype to be observed in both sexes. Most examples of hypercephaly and other novel morphologies in insects are only observed in males (Emlen & Nijhout 2000; Bowsher & Nijhout 2007).

Diopsidae are sufficiently related to Drosophilidae, and therefore *D. melanogaster*, that developmental comparisons of the two can be informative and techniques that are well developed in *D. melanogaster* can be applied to stalk-eyed fly species. In diopsids and drosophilids, as in other Dipteran flies, the progenitors of all adult epidermal structures grow as small groups of cells known as imaginal discs. Prior to metamorphosis the discs proliferate and then go through a series of complex folds. During metamorphosis the discs evert to produce the adult structure (Haynie & Bryant 1986). In *D. melanogaster* the head, eyes and antennae are formed from the eye-antennal imaginal disc, which consists of two joined lobes (Haynie & Bryant 1986). A smaller anterior lobe gives rise to the anterior portions of the head including the antennae and mouthparts whilst the larger posterior lobe gives rise to the dorsal part of the head, including the eye . An initial comparison between the eye-antennal discs of *D. melanogaster* and *T. dalmanni* showed them to be of similar structure except that the lobes in *T. dalmanni* are separated by a thin piece of connecting tissue (Hurley *et al.* 2001). Studies comparing head development in different diopsid species and *D. melanogaster* have analysed gene expression patterns, regional specificity and pupal brain development (Hurley *et al.* 2001; 2002; Buschbeck & Hoy 1998; 2005; Buschbeck *et al.* 2001).

Hurley and co-workers (2001) initially used a candidate gene approach to compare expression of genes between *T. dalmanni* and *D. melanogaster*. *Wingless* (*wg*), a gene that in *D. melanogaster* is expressed in the lateral part of the head next to the eye, and *engrailed* (*en*), which expresses in the medial head, were used to compare regional identity between the discs. In addition the expression of *Distal-less* (*Dll*), a gene associated with proximodistal outgrowths of the body, was examined. The expression patterns of all three genes were conserved between *T. dalmanni* and *D. melanogaster* (Hurley *et al.* 2001). A further gene, *defective proventriculus* (*dve*), a gene co-expressed with the homeobox gene *ocellaless* (*oc*) in the medial regions of the disc, was also seen

to have a conserved expression pattern between the two species (Carr *et al.* 2005). These studies implied that the developmental genetics of early head development is conserved between *T. dalmanni* and *D. melanogaster* but failed to identify any differences between the two species that might indicate how eye-stalks develop.

To understand how an organ develops it is important to identify the cells that give rise to it. Dissecting and culturing different regions of the disc and observed patterns of differentiation were used to generate a fate map of the eye-antennal disc in *T. dalmanni* (Hurley *et al.* 2002). Clearly distinguishable structures of the adult head were mapped to different regions of the imaginal disc. The anterior lobe gave rise to the palpus, which is medially positioned on the head and the antennae which are laterally displaced on the eye-stalk. The posterior lobe gave rise to the ocelli, which are located medially on the head, as well as the outer vertical bristle and the eye which are displaced laterally on eye-stalks. The inner vertical bristle, which is located half way along the eye-stalk, originates from the posterior lobe. Therefore both lobes gave rise to a mixture of proximal and distal adult derivatives.

A comparison of pupal brain development brain of stalk-eyed flies with that of *D. melanogaster* was performed by taking sections of the developing brain (Buschbeck & Hoy 1998; 2005; Buschbeck *et al.* 2001). Brain development is remarkably similar for both species except that approximately two-thirds of the way through the pupal stage, the optic lobe, which usually flanks the brain in *D. melanogaster* becomes constricted and separated from the rest of the brain in *T. whitei* (Buschbeck *et al.* 2001). An invagination forms and the eye-stalk develops between the optic lobe and the rest of the brain. In addition the neuropils that connect the optic lobe to the central part of the brain become grouped together into a thin fibre bundle that runs down the stalk. The division between the optic lobe and the rest of the brain increases as development progresses and the eye-stalks form into a rolled up structure within the pupal case. The axons of the neuropil also grow into a coiled structure (Buschbeck & Hoy 2005). Upon eclosion the eye-stalk is rolled up tightly and internal pressure is used for its expansion (Buschbeck & Hoy 1998).

Growth of an exaggerated trait

Early gene expression and regional specificity of the eye-antennal disc in *T. dalmanni* is similar in both sexes and follows the same basic pattern as in the non-hypercephalic *D. melanogaster*. By contrast adult morphology is highly variable. Sexual selection in stalk-eyed flies drives diversity in eyespan in a number of ways (section 1.1.3). In addition to the dramatic differences in male and female eyespan in dimorphic species such as *T. dalmanni*, a great deal of interspecies variation exists for absolute eyespan and for the scaling relationship between eyespan and body size. Understanding the growth differences underlying this variation will shed light on the developmental mechanisms involved in the evolution of sexually selected ornamental traits.

Despite the potential of stalk-eyed flies as model organisms for understanding the growth of ornamental traits relatively few studies have been published. Wright *et al.* (2004) developed microsatellites covering large sections of the *T. dalmanni* genome, which have been used for quantitative trait locus (QTL) mapping. Four polymorphisms associated with variation in eyespan were identified, one of which was specific to males (Johns & Wilkinson unpublished data in Wilkinson *et al.* 2005). In the longer term higher resolution mapping of the regions associated with eyespan variation has the potential to uncover individual genes regulating eye-stalk growth.

The growth of holometabolous insects is regulated by nutrition via the insulin and target of rapamycin (TOR) signalling pathways while transitions between different periods of growth and development are controlled by hormones such as juvenile hormone (JH), ecdysone and prothoracicotropic hormone (PTTH) (Edgar 2006). Fry (2006) conducted a study addressing the effect of JH on eyespan and testis size. Following topical application of a JH analogue (methoprene) during the third larval instar, eyespan and testis size were recorded at sexual maturity. An increase in eyespan, relative to body size, and a decrease in relative testis size was observed. However, attempts to replicate these results proved unsuccessful (Rogers 2005) and there are a number of problems with this paper. The effect of JH treatment on absolute eyespan was not reported and a decrease in body size was observed as well as a high mortality. JH and ecydsone signalling are two interconnected pathways that have a wide range of effects: any conclusions about the interactions between JH and eyespan regulation from the general application of methoprene alone will be naïve. More rigorous studies using

physiological hormone titres and combining such treatments with other forms of manipulation are required (Zera 2007).

1.1.5 Stalk-eyed flies and other model species for studying the evolution and development of an exaggerated sexual trait

Many organisms have been used to study the evolution, and to a lesser extent the development, of sexually selected traits. The classic examples of sexual selection and ornamental traits are often observed in birds and species, such as the zebrafinch *Taeniopygia guttata*, have been important in elucidating many aspects of sexual selection (Bolund *et al.* 2007; Schielzeth *et al.* 2008). The zebrafinch genome is now available (NCBI website) enabling the genetics underlying female preference to be studied.

A restriction of working with birds and mammals is the difficulty in rearing and housing large populations, along with long generation times. Fish and, to a greater extent, insects are attractive models for developmental studies as they can be mass reared easily and possess short generation times. Male swordfish of the genus, *Xiphorus*, possess elongations of the ventral fin-rays of the caudal fin, referred to as a "sword", for which females demonstrate preference (Basolo 1990). The understanding of the development of the sword is expanding. Comparative studies, with zebrafish, have detected novel high expression of *muscle segment homeobox gene* (*msxC*) in developing swords (Zauner *et al.* 2003) and transplants have shown presumptive swords to have autonomous organisers (Eibner *et al.* 2008).

An increasingly important model organism for the development of exaggerated traits is the scarab beetle (Emlen & Nijhout 2000). The males of many species possess large horns and there is much inter-specific diversity in the location, size and shape of the horn. Often small males and females possess either severely reduced horns or no horns at all. Genes involved in horn patterning and hormone regulation are being increasingly well characterised. These include the involvement of *Dll* and *aristaless* (*al*) in horn patterning and the identification of the threshold levels of hormones required to promote growth of large horns (Moczek & Nagy 2005; Emlen *et al.* 2007).

The primary role of horns in beetles is as weapons in intra-sexual selection whereas exaggerated male eyespan in diopsids is primarily involved in inter-sexual selection. As with scarab beetle horns, a wide range of stalk-eyed fly species exist that exhibit varying degrees of inter and intra-specific variation in eyespan. Stalk-eyed flies are amenable to culturing in laboratory conditions with a life cycle of approximately two months, which is an important consideration when selecting a model organism (Hughes & Kaufman 2000a; Jenner & Wills 2007). It is possible to maintain large populations permitting long-term selection experiments and quantitative genetic analysis (Wilkinson *et al.* 2005; Cotton 2004; Bellamy pers. comm.). Much progress has been made into understanding the evolutionary basis of eyespan and initial studies have demonstrated that it is possible to manipulate and observe development at key stages (Buschbeck & Hoy 1998; 2005; Buschbeck *et al.* 2001; Hurley *et al.* 2001; 2002; Carr *et al.* 2005: 2006; Fry 2006). Although these have shed some light on some of the morphological changes involved in eye-stalk formation, fundamental aspects of the underlying genetic and cellular mechanisms that regulate eye-stalk size remain unclear. In the next section I address approaches to studying development in a non-model organism and in the final section discuss how features of the stalk-eyed fly can be used to give a unique perspective on the growth of an exaggerated trait.

1.2 TRANSGENICS AND STALK-EYED FLIES

1.2.1 Studying evolution and development in non-model organisms

Until recently the study of developmental genetics has concentrated on model organisms that readily adapt to the laboratory, possess short lifespans and are capable of generating large numbers of offspring. In insects the classic model organism is *D. melanogaster*. The amount of work and knowledge, which has been built up by more than a century's worth of work, of *D. melanogaster* developmental genetics dwarfs that of any other insect species. *D. melanogaster* is by no means representative of all insect species. Furthermore there are many interesting traits that they do not possess like polyphenism as seen in aphids, social insect structure as seen in many species of hymenoptera and exaggerated traits which, as already discussed, is a common feature across insect orders. In order to understand the diversity of phenotypes and morphologies present in insects many different species need to be, and are being, studied.

Studying development in many non-drosophilid species, including the stalk-eyed fly, has been hampered by the paucity of molecular and genomic tools available. However there are many emerging approaches and technologies that are enabling increasingly detailed developmental studies in non-model species. Here I assess these strategies and discuss their potential application to stalk-eyed flies. These include candidate gene approaches, high throughput expression studies and different methods for assaying gene function and I will focus in particular on transgenics due to its potential in stalk-eyed flies.

Candidate genes

A large number of genes and regulatory pathways have been isolated and characterised in *D. melanogaster*. Inter-species comparisons have shown that gene sequence, expression and function are often conserved across taxa (Carroll *et al.* 1994; Chen *et al.* 1996; Economou & Telford 2009). Investigating genes that have already been identified to be involved in the tissue of interest in a related species has proved to be a successful approach. This is known as the candidate gene method and has been successfully applied many times and examples are discussed below. Sequence data from *D.*

melanogaster can be used to clone potential homologues in non-model organisms and their expression pattern assessed using mRNA *in situ* hybridisation. Alternatively antibodies, that have been shown to work in a range of species, can be used to assess protein expression patterns provided there is sufficient similarity in amino acid sequence present. Comparative studies of candidate genes have been used to investigate eye-stalk development in stalk-eyed flies and identify similar patterns of expression for the developmental regulators, *wg , en, Dll* and *dve* expression in *T. dalmanni* and *D. melanogaster* (Hurley *et al.* 2001; Carr *et al*. 2005; section 1.1.4). Analyses of horn development in beetles and wing vein patterning in butterflies provide further examples of the approach.

The candidate gene approach was used in two examples of scarab beetle, *O. taurus* and *Onthophagus nigriventris* (Moczek & Nagy 2005). *O. taurus* possesses horns derived from the head and thorax while the horns of *O. nigriventris* are derived from the thorax only. In both species large adult males possess the horn, but small males and females do not. Primitive horns are seen in pre-pupal stages in both males and females. Horns develop from novel pieces of tissue that behave like imaginal discs. Therefore genes involved in outgrowth development and regulation in the antennae and legs of *D. melanogaster* were assessed in the imaginal discs of horns in *O. taurus* and *O. nigriventris*. In the legs of *D. melanogaster, dpp* and *wg* encode two diffusible morphogens that form two interacting gradients of expression, which give positional identity to regions of the developing leg imaginal disc and regulate downstream transcription factors. *Dll* is one target of WG/DPP patterning which is found in the centre of the leg disc and eventually gives rise to the distal tip of the outgrowth. Other targets of WG/DPP patterning are the epidermal growth factors (EGFRs), which in turn regulate a further set of transcription factors including *aristaless (al)*. *Dll* and *al* expression were assessed in *O. taurus* and *O. nigriventris*. In *O. taurus* males, but not females, *Dll* was associated with expression in the tip of the pre-pupal horn indicating a similar role as is observed in other insect appendages. Interestingly in *O. nigriventris* both males and females expressed *Dll* in the head primordium that gave rise to the horn. This indicates a species difference in the mechanisms underlying dimorphism. The work further indicated that there were different regulatory patterns in different horns. In *O. taurus* no expression of *al* was detected in head horns. However, in both species *al* expression was observed in thoracic horns. This sets them apart from other arthropod appendages where both *al* and *Dll* expression is observed (Moczek & Nagy 2005).

Butterflies possess coloured wing patterns. Examples of such patterns are found in the intervein regions of the wing and are symmetrical around the mid-point of the surrounding veins. The patterns vary in shape and are seen as eyespots, ellipses and midlines. Conservation of patterning genes involved in wing development between the butterfly species *Precis coenia* and the involvement of *Dll* expression associated with eyespot formation has long been established (Carroll *et al.* 1994). In *D. melanogaster* wings, *Notch* signalling and its inhibitory interactions with its ligand *Delta* define the dorsoventral boundary and intervein tissue identity. *Notch* signalling is also capable of causing ectopic *Dll* expression in imaginal discs. Reed and Serfas (2004) wished to look at the intervein patterns and hypothesised that the *Notch* regulation of *Dll* expression could be involved in the formation of the intervein patterns. Antibody staining assessed expression patterns of *notch* and *Dll* in three butterfly species, *Vanessa cardui*, *Precis coenia* and *Bicyclus anynanna*. It was shown that appropriate timing of midline expression of *notch* and *Dll* were associated with the development of the intervein patterns.

High throughput sequencing and expression methods

Candidate gene selection based on homology is restricted by the traits available for study in model organisms and the extent to which genetic mechanisms are characterised. More recent technology offers the possibility of identifying candidate genes based on their expression in the non-model organisms. In the past 10-20 years sequencing and expression based technology has improved rapidly. As a result mass sequencing and gene expression studies using technical advances such as 454-pyro-sequencing and micro-arrays are becoming increasing economical and widespread and available to any organism of study (Shaffer 2007; Vera *et al.* 2008). This technology enables the harvesting of large amounts of sequence information in any species. Thirty-six insect genomes have now been fully sequenced and information on their expression is available from micro-arrays and EST data (Reumer *et al.* 2008). Candidate genes identified in this way can be studied further by using mRNA *in situ* hybridisation and antibody expression patterns as well as more recently developed techniques such as real-time quantitative PCR (qPCR) (Johnson *et al.* 2005). Large scale sequencing techniques have been applied to stalk-eyed flies and an expressed sequence tag (EST) library for the stalk-eyed fly head has recently been generated with an aim to making

micro-arrays (Baker pers. comm.). The EST library has generated a large amount of sequence data for a large number of genes (~4,000) that could be used in further studies of stalk-eyed fly development.

High throughput sequencing and microarrays have been employed to identify genes involved in sex determination and differentiation in mosquitoes (Hahn & Lanzaro 2005) and in generating polyphenisms in the green peach aphid, *Myzus persicae* (Ghanim *et al.* 2006) and the pea aphid, *Acrthosiphon pisum* (Brisson *et al.* 2007; Cortés *et al.* 2008). The malaria carrying mosquito, *Anopheles gambiae*, is an important vector of disease. Malaria is caused by the protozoan *Plasmodium falciparum* which is carried by female mosquitoes, placing importance on understanding the difference between males and females in this species. The *An. gambiae* genome has been sequenced but is largely unannotated. Using micro-arrays the differences between male and female gene expression was tested (Hahn & Lanzaro 2005). It was found that 10% of the genome had at least a four-fold difference in expression level, 71% of which was female biased. This provides a large number of candidate genes that could be used for controlling the spread of malaria.

Aphids are important plant pest species. Both *M. peresicae* and *A. pisum* have wingless and winged morphs. At high population densities females produce winged morphs that are capable of dispersion but at low population densities wingless morphs are produced. In both *M. peresicae* and *A. pisum*, where no previous sequence data was available, cDNA libraries that covered approximately 10% of the genome were created and used to make micro-array chips to assess the differences in gene expression of winged and wing-less morphs. This generated a range of novel candidate genes for caste determination for further study (Ghanim *et al.* 2006; Brisson *et al.* 2007).

Functional assays

Whether identified through sequence homology to developmental regulators in model systems or directly from their expression in the study organism, candidate genes require further functional assessment in order to determine their biological role. Functional studies involve manipulation of gene expression or activity, and correlating gain or loss of function with phenotypic effects. Gain of function assays typically involve over or misexpression of the gene at the level of transcription. Alternatively the coding

sequence may be mutated to generate, for example, constitutively active receptor proteins. Loss of function may be induced at the DNA level through mutagenesis or gene 'knockout', at the RNA level through antisense or RNA interference (RNAi) mediated gene 'knockdown' or through the expression of dominant negative proteins. Many gain and loss of function approaches require the delivery of exogenous DNA or RNA to cells or tissues. For some purposes this can be achieved by the use of virus-based vectors and in many species effective RNAi mediated gene knockdown can be induced by injection, electroporation or transfection of double stranded RNA (dsRNA). In other cases more stable and heritable expression of exogenous sequences via germline transformation or transgenics is required.

Transient expression systems and RNAi

Viruses are commonly employed in insects to induce transient gene expression (Foy & Olson 2008). The *Sindbis* virus was first successfully used to express the selectable marker chloramphenicol acetyltransferase in *Aedes triseriatus* (Olson *et al.* 1994) and *Aedes aegypti* (Johnson *et al.* 1999). The *Sindbis* based expression system has also been used to drive green fluorescent protein (GFP) expression in another mosquito species *Culex tritaeniorhynchus* and two lepidoteran species, *Manducta sexta* and *Bombyx mori* (Foy *et al.* 2004). A further virus based expression system using *Autographa californica nucleopolyhedrovis* (AcNPV) has been successfully used in *Apis mellifera* (Ando *et al.* 2007), *B. mori* (Shiomi *et al.* 2003), *D. melanogaster* and *Tribolium casteneum* (Oppenheimer *et al.* 1999). There is little restriction on the genes that can be expressed using virus based systems and both mis-expression and knockdown studies have been carried out (Foy & Olson 2008). One of the major drawbacks in working with virus based expression systems is that they are a serious biohazard and special precautions need to be taken when working with them (Jourdan *et al.* 1990; Bossin *et al.* 2007). Virus mediated transgene systems have not been attempted in stalk-eyed flies, but due to the problems of working with virus are not an attractive option.

The use of RNAi to induce loss of function by degradation of gene-specific, endogenous mRNA sequences is a powerful new technology, which exploits conserved cellular defence mechanisms against viral infection. Many viruses generate dsRNA as an essential component of their life cycle. RNAi reduces steady state RNA levels of cytoplasmic and nuclear transcripts with sequence homology to viral dsRNA; it can also

be induced by the introduction of exogenous dsRNA, this was first performed in *Caenorhabditis elegans* (Fire *et al.* 1998).

Although mechanisms vary between species there are two main types of RNAi, transient and systemic (Geley & Müller 2004). Transient RNAi is a cell autonomous response and the effect only lasts for a number of hours, it is sometimes referred to as "embryologic RNAi" since it is widely used in the embryos of *D. melanogaster* (Hannon 2002). In systemic RNAi, the silencing signal, mediated by the *sid-1* gene, spreads through the organism and can remain for days and even weeks (Winston *et al.* 2002) while the silencing effect can be passed to an individual's offspring. Systemic RNAi was first reported in *C. elegans* but appears to be applicable to many insect groups and has worked in such species as the honey bee *Apis mellifera* (Amdam *et al.* 2003), the flour beetle *T. casteneum* (Tomoyasu & Denell 2004), the ladybird *Harmonia axyridis* (Kuwayama *et al.* 2006) and the cockroach *Blattella germanica* (Martín *et al.* 2006). The advantage of systemic RNAi is that injections at larval or adult stages result in a response throughout the body of an organism. Systemic RNAi has been demonstrated to be absent in *D. melanogaster* (Miller *et al.* 2008) and injections of dsRNA in mosquito species and tsetse flies imply that the strong systemic response is not present in dipteran species and therefore unlikely to be found in stalk-eyed flies (Blandin *et al.* 2002; Boisson *et al.* 2006; Walshe *et al.* 2009).

1.2.3 Stable and heritable expression systems (Transgenics)

When exogenous DNA is incorporated into the genome of a host organism, the inserted gene will be passed down through generations allowing the full effects of its expression and activity to be assessed. Incorporation of foreign DNA is often referred to as stable germline transformation and the resulting organism is referred to as a transgenic. After many failed attempts during the 1960s and 1970s to create a stable transgenic insect line (Capari & Nawa 1965; Nawa & Yamada 1968; Fox *et al.* 1970; Nawa *et al.* 1971) transgenesis was first achieved in *D. melanogaster* in the early 1980s (Rubin & Spradling 1982). It was more than ten years before a non-drosophilid insect species, the medfly *Ceratitis capitata*, was successfully transformed (Loukeris *et al.* 1995a). Since then germline transformations have been carried out in 27 insect species (Table 1.1). Transgenics has revolutionised the study of developmental genetics in *D. melanogaster* and is slowly becoming a powerful tool in other insect species (O'Brochta & Handler

2008). Applications range from the analysis of *cis*-regulatory elements to functional assays of hormonal signalling and behaviour. In addition to being used to characterise gene regulation and function, transgenics have also facilitated cell marking for fate mapping or investigating sperm competition. Genome wide screens of regulatory elements and insertional mutations using appropriately designed transgene constructs are another possibility (e.g. Metaxakis *et al.* 2005).

Transgenics and functional assays

Drosophila biarmipes possesses a region of pigmentation on its wing. The wings of the closely related *D. melanogaster* and the more distantly related *Drosophila pseudobscura* do not possess such patterns implying it is not the ancestral state. Antibody staining showed that the gene, *yellow*, was expressed in the wing spot. To investigate the evolution and regulation of this gene a transgenic construct containing the *cis*-regulatory region of the yellow gene from *D. biarmipes* driving the expression of the reporter gene eGFP was introduced into *D. melanogaster*. The resulting transgenic lines expressed eGFP in the same region as the wing spot in *D. biarmipes* showing that the change in expression of *yellow* between different species was due to a change in its *cis*-regulatory regions rather than the gene itself or an upstream regulator (Gompel *et al.* 2005).

The use of transgenics in functional assays to induce ectopic or over-expression can have lethal effects such that it is not possible to create a viable line using a single insert. This issue can be addressed by the use of the binary GAL4/UAS system in which two transgenic lines are created (Fischer *et al.* 1988; Brand and Perrimon 1993; Viktorinová & Wimmer 2007). One contains the gene for the yeast transcriptional activator GAL4 under the control of a promoter. The second contains the upstream activation sequence (UAS) which is a short promoter sequence whose expression is regulated by GAL4 and drives the expression of any downstream genes. Altered expression of the gene of interest will only occur when the lines are crossed. Tan *et al.* (2005) used the GAL4/UAS system to investigate the effect of juvenile hormone and pupation in *B. mori*. Pupation in insects is associated with a drop in juvenile hormone (JH) titre during the final instar. JH is broken down by the enzyme juvenile hormone esterase (JHE).

Order (underlined) Family or Superfamily	***Common Name***	***Species***	***Transposon***	***Reported transformation efficiencies****	***References***
Diptera					
Culicidae	Mosquitoes	*Aedes aegypti*	*Hermes, mariner, piggyBac,*	0.7%-8.6%	Coates *et al*. 2000; Jasinskiene *et al*. 1998; Kokoza *et al*. 2001
		Aedes fluviatilis	*piggyBac*	~27%	Rodrigues *et al*. 2006
		Anopheles albimanus	*piggyBac*	20%-43%	Perera *et al*. 2002
		Anopheles gambiae	*piggyBac*	0.01%	Grossman *et al*. 2001
		Anopheles stephensi	*Minos, piggyBac*	6.7-12%	Catteruccia *et al*. 2000; Nolan *et al*. 2002; Ito *et al*. 2000
		Culex quinquefasciatus	*Hermes*	12%	Allen *et al*. 2001
Tephritidae	Fruitflies	*Anastrepha ludens*	*piggyBac*	4.3%-10%	Condon *et al*. 2007
		Anastrepha suspensa	*piggyBac*	~5%	Handler & Harrell 2001
		Bactrocera dorsalis	*hobo, piggyBac*	2%	Handler & McCombs 2000
		Bactrocera oleae	*Minos*	N.R.	Koukidou *et al*. 2006
		Bactrocera tyroni	*hobo*	N.R.	Wyhard *et al*. unpub. data in O'Brochta & Atkinson 1996
		Ceratitis capitata	*Hermes, Minos, piggyBac*	0.6%-13%	Michel *et al*. 2001; Loukeris *et al*. 1995a; Handler *et al*. 1998; Salvemini *et al*. 2006.
Calliphoridae	Blow flies	*Lucilia cuprina*	*piggyBac*	1%-2%	Heinrich *et al*. 2002
		Cochliomyia hominivorax	*piggyBac*	13%-33%	Allen *et al*. 2004
Muscidae	House flies	*Musca domestica*	*piggyBac*	15.4%	Hediger *et al*. 2001
		Stomoxys calcitrans	*Hermes*	4%	O'Brochta *et al*. 2000

Table continues on the next page.

Drosophilidae	Drosophilids	*Drosophila hawaiiensis*	*P*	N.R.	Brennan *et al.* 1984
		Drosophila melanogaster	*Hermes, hobo, mariner, Minos, P, piggyBac*	1%-50%	Berghammer *et al.* 1999; Blackman *et al.* 1989; Loukeris *et al.* 1995b; Rubin & Spradling 1982
		Drosophila simulans	*P*	NR	Scavarda & Hartl 1984
		Drosophila virilis	*Hobo, mariner, Minos, P*	>0.5%	Lozovskaya *et al.* 1996; Lohe & Hartl 1996; Megna & Cline pers comm. in O'Brochta & Atkinson 1996; Scavarda & Hartl 1984
		Drosophila willistoni	*piggyBac*	66.70%	Finokiet *et al.* 2007
Lepidoptera					
Bombycidae	Silk worm	*Bombyx mori*	*Minos, piggyBac,*	0.9%-40%	Uchino *et al.* 2008; Tamura *et al.* 2000; Dai *et al.* 2008
Nymphalidae	Butterfly	*Bicyclus anyana*	*Hermes, piggyBac*	4.2%-10.2%	Marcus *et al.* 2004
Gelechiidae	Pink bollworm	*Pectinophora gossypiella*	*piggyBac*	3.50%	Peloquin *et al.* 2000
Coleoptera					
Coccinellidae	Ladybird	*Harmonia axyridis*	*piggyBac*	3.70%	Kuwayama *et al.* 2006
Tenebrionidae	Flour beetle	*Tribolium casteneum*	*Hermes, piggyBac, Minos*	1%-60%	Berghammer *et al.* 1999; Pavlopoulos *et al.* 2004
Hymenoptera					
Symphyta	Saw fly	*Athalia rosae*	*piggyBac*	5%	Sumitani *et al.* 2003

Table 1.1. Insect species in which germline transformation has been performed. Transposon vectors used in each species are listed. *Transformation efficiencies are given as percentage of total number of surviving injected individuals that give rise to transgenic offspring. N.R. denotes that transformation efficiencies were not reported.

Over-expression of JHE is lethal so two lines were created, one carrying GAL4 coding sequence under the control of a ubiquitous promoter and one containing the UAS fused upstream of the JHE coding sequence. When the lines were crossed JHE was over-expressed resulting in a drop in JH titre in developing larvae and premature pupation demonstrating the role of JH in pupation.

Transgenesis can be combined with RNAi to deliver dsRNA to specific cells and tissues which are not accessible to direct delivery. A systemic RNAi response is not present in *D. melanogaster* and other dipterans but gene knockdown can be induced in post-embryonic stages if the dsRNA is expressed as a transgene product. Expression of a construct containing two copies of the gene of interest in opposing orientations causes dsRNA hairpins to form, which then induce RNAi in a cell autonomous fashion that can be regulated spatially and temporally (Kennerdall & Carthew 2000). This approach was used to show the role of *Ecdysis triggering hormone* (*ETH*) in the development of *B. mori* (Dai *et al.* 2008). Two transgenic lines were created with one containing the *B. mori* actin3c ubiquitous promoter driving *gal4* gene expression and the other possessing UAS driving a hairpin of dsRNA of *ETH*. When the lines were crossed, the resulting offspring showed reduced levels of *ETH* protein, were deficient in ecdysis and were unable to generate normal second instar larvae. This confirmed the role of *ETH* in normal ecdysis.

As well as morphology and physiology, transgenics have been used to study the genetic and cellular basis of behaviour. In *D. melanogaster,* males perform a courtship "song" using their wings to entice females to mate using uni-lateral vibrations of the wing. This is associated with sexual dimorphism in splicing of transcription factors encoded by the *fruitless* (*fru*) gene (Clyne and Miesenböck 2008). A transgenic line was constructed that expressed light sensitive ion channels in specific neurons within the thoracic-abdominal ganglion. Activation of the channels was associated with movement and sound in both sexes, but a recognisable song was only observed in males and females expressing the male form of the *fru* gene product. This demonstrated the sex-specific differences in the genetic basis of this courtship behaviour.

Transgenics and cell marking

Determining the developmental origins of organs, tissues and cells is of obvious importance in developmental biology but fate mapping procedures in insects are often very invasive, which limits their resolution (Hurley *et al.* 2001; 2002). In *D. melanogaster* and the butterfly *B. anynana* constructs were introduced that possessed eGFP expression driven by the *D. melanogaster* heat sensitive promoter, *hsp70* (Halfon *et al.* 1997 & Ramos *et al.* 2006). *Hsp70* initiates persistent expression when exposed to higher temperatures (>35°C). Modified cell ablation lasers were used to apply heat to individual cells and switch on eGFP expression in a cell autonomous fashion with expression persisting to adult stages. When performed in pupal wings of *B. anynana,* the fate of individual cells in adult wings could be followed (Ramos *et al.* 2006).

In *D. melanogaster* transgenic markers have been used to assess male mating success by looking at sperm competition within the female reproductive tracts (Price *et al.* 1999). Sperm specific expression of an eGFP reporter gene was used to label the sperm produced by experimental males and distinguish them from unlabelled controls (Civetta *et al.* 1999; Price *et al.* 1999). This construct was used to directly assess sperm competition by looking at the spermathecae of multiple-mated females. A similar construct is available in *C. capitata* but has not yet been used to assess sperm competition (Scolari *et al.* 2008). In stalk-eyed flies, models of ornament evolution predict large eyespan males will be at a selective advantage and obtain more matings. The use of transgenic markers could facilitate studies of sperm competition in *T. dalmanni*.

Genomic and post-genomic transgenic research tools

In *D. melanogaster*, and a few other insect species, the technology has been developed for generating and mapping very large numbers of independent transgenic lines. This in turn allows insertions to be used as tools for genome wide screens. Early insertion based screens used transgenes, which could act as easily identifiable genetic markers for insertional mutagenesis (Spradling *et al.* 1999). Hundreds of lines, each carrying a mapped single copy insertion, are maintained at stock centres and available for further characterisation on request (Metaxakis *et al.* 2005). Later screens, instead of simply using insertions to disrupt endogenous sequences, employed transgenes designed to

report on or manipulate the function of genes in the vicinity of the insertion (Rørth 1996; Bellen 1999). Enhancer-trapping is a method for locating *cis*-regulatory regions responsible for driving gene expression in an organ of interest (O'Kane & Gehring 1987; Bellen, 1999). It requires a transgene consisting of a weak or minimal promoter driving expression of a reporter gene. In *D. melanogaster* a vast number of enhancer trap lines have been developed from various sources and these have been collected together as a resource for researchers looking for promoters to drive expression in a tissue of interest. *D. melanogaster* enhancer trap lines were used to screen for novel genes expressed in the head region that may be of interest to studying eye-stalk development in stalk-eyed flies (Carr *et al.* 2006). Enhancer trapping can be combined with the binary GAL4/UAS system to not only identify stage and tissue specific regulatory regions but make them available for misexpression studies (Gustafson & Boulianne 1996; Horn *et al*. 2003). These and other advanced applications of genome-wide technology have been developed in, and until recently restricted to, *D. melanogaster* (reviewed in Venken & Bellen 2007). Comparable approaches are being applied in other insect species and can be combined with increasing amounts of genome sequence data becoming available (Scali *et al.* 2007). Databases are currently being developed in the flour beetle *T. casteneum* where both enhancer-trap lines and genome wide mutation projects are underway (Lorenzen *et al.* 2007).

It is clear that the study of development of any organism is going to be greatly enhanced by possessing transgenics as a tool and that there are many possible uses of transgenics in stalk-eyed flies. For example, the EST library has produced a number of interesting sequences involved in growth pathways: their functional role in eye-stalk development could be tested by using over-expression studies or by knock-out studies using expression of RNAi constructs. Fate mapping would be a valuable asset to ascertaining which cells give rise to the actual eye-stalk.

1.2.4 Creating a transgenic line

Transposons

Transgenesis in insects is mediated by mobile transposable elements (O'Brochta & Handler 2008). Transposons are self-replicating DNA sequences that exist in the genome of all sexually reproducing organisms (Hickey 1982). They are widespread and

constitute 50% of the maize genome, 45% of the human genome and 15% of the *D. melanogaster* genome (Hurst & Werren 2001 and references within). The DNA of all transposons includes a sequence encoding the transposase protein. This mediates the insertion of the transposon into new locations within the host genome (transposition) thus increasing copy number and the probability of the transposon being passed onto the next generation.

There are two nominal classes of transposable elements, class I and class II. Class I elements transpose via an RNA intermediate which is reverse-transcribed into DNA and then inserted into a new location within the genome. Some class I elements are flanked by long terminal inverted repeats (TIRs). Examples of class I elements are the mammalian LINES and SINES elements (Smit & Riggs 1996; Kazazian & Moran 1998). Class II elements do not use an RNA intermediate for transposition. Instead the DNA is directly excised from its location and inserted into a new position in the genome, again mediated by the transpose enzyme. All class II elements are flanked by TIRs. Examples of class II elements are the *P* element in *Drosophila* species (Rubin & Spradling 1982) and the *Ac* element in maize (McClintock 1956).

All the commonly used vectors for transgenesis in insects are based on class II transposable elements. To construct a vector the native element is cloned into a plasmid and the transposase and other coding sequences deleted, often leaving only the TIRs intact. Multiple restriction sites engineered between the TIRs allow transgenes and markers to be inserted between the TIRs. The plasmid carrying the TIR flanked transgene is introduced to an organism along with a source of transposase. This is most commonly achieved by co-introduction of a functional transposase gene on a separate plasmid but transposase function may also be supplied in the form of capped mRNA (Kapetanaki *et al.* 2002) or *in vitro* translated protein (Coates *et al.* 2000). The transposase mediates the integration of the transgene and other sequences flanked by the TIRs but is not itself able to integrate into the genome and is eventually degraded or diluted out by subsequent rounds of cell division over time. In the absence of a cellular source of transposase, the integrated DNA sequence is immobile and persists as a stable insertion (Fig. 1.4). For stable germline transformation, integration of the transgene must occur within the germline of an individual so that the transgene can be passed onto its offspring. The efficacy of a transgenics protocol is assessed by the transformation efficiency which, given as a percentage, is the total number of surviving injected

individuals that give rise to transgenic offspring.

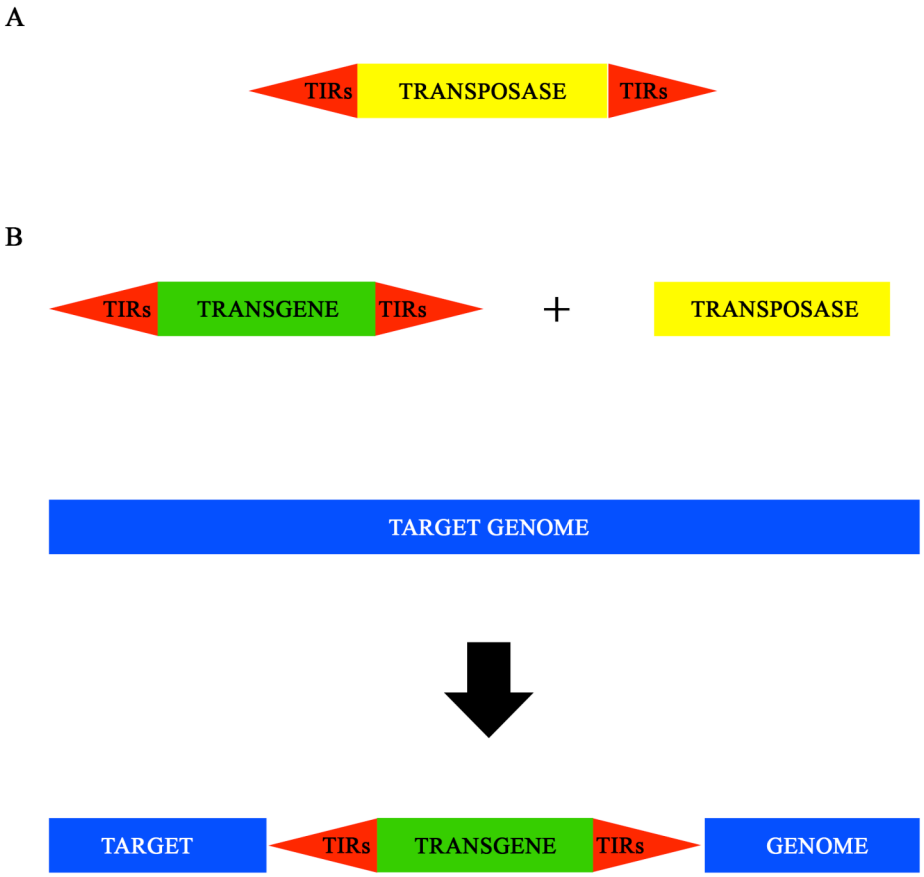

Figure 1.4. Schematic representation of transposons and their use in insect transgenics. **A.** Functional class II transposons minimally consist of the coding and promoter sequences of the transposase gene flanked by terminal inverted repeats (TIRs). **B.** For use in transgenics, the transposase is removed and the transgene of interest placed between the TIRs of the transposon. The transgene and an exogeneous source of transposase are co-injected into an embryo. Transposase mediated integration of the TIR flanked transgene into the target genome follows. The transposase source will eventually be degraded or diluted through cell division. In the absence of transposase, the transgene will remain stably integrated in the target genome.

Transposons used for transgenesis

The first insect transformation was performed using the *P*-element as a vector in *D. melanogaster* (Rubin & Spradling 1982). Unfortunately the activity of the *P*-element is restricted to drosophilid species (O'Brochta & Handler 1988). It was not until the medfly, *C. capitata* was transformed with the *Minos* element that a stable germline transformation was performed in a non-drosophilid insect (Loukeris *et al.* 1995a). Three other transposable elements are now regularly used for insect transgenesis, these are

piggyBac, *Hermes* and the *Mos1/mariner* element. In addition, *hobo* has been used in a limited number of insect species. The range of species in which these elements have been successfully used is summarised in Table 1.2.

Minos was isolated from *Drosophila hydei* (Franz & Savakis 1991; Franz *et al.* 1994) and is in the same family as the *Tc* element from *C. elegans*. Despite being used in the first non-drosophilid insect transformation it has only been used in a limited number of species. Initial attempts resulted in very low transformation efficiencies, 1.5% in *C. capitata* and 2.1% in *D. melanogaster* (Loukeris *et al.* 1995a, b). However, more recent results have indicated that *Minos* will integrate at high efficiencies (eg 32% transformation efficiency in *T. casteneum*) (Pavlopoulos *et al.* 2004) when an mRNA source of transposase is used (Kapetanaki *et al.* 2002; Uchino *et al.* 2007). The element has a low but significant preference for insertion into introns whereas *P*-elements preferentially insert into the 5' region of genes (Metaxakis *et al.* 2005). The complementary preferences have made *Minos* an attractive alternative to the *P*-element for genome-wide insertional mutagenesis programmes. *Minos* has a broad host range and is functional in many non-insect taxa. It shows activity in human cell lines, crustaceans, and flatworms (Klinakis *et al.* 2000a, b; Pavlopoulos & Averof 2005; Sasakura 2007).

Isolated from *Trichoplusia ni* (Cary *et al.* 1989), *piggyBac* is the transposable element that has generated transgenic lines in the highest number of insect species (Table 1.2). The native element is not present in many insect species so resistence to *piggyBac* transposition is unlikely to be present. Observed transformation efficiencies vary from very high in some species, 66.7% in *Drosophila willistoni* (Finokiet *et al.* 2007) and 60% in *T. casteneum* (Berghammer *et al.* 1999) to very low in others, 2% in *Bactrocera dorsalis* (Handler & McCombs 2000) and 4% in *T. casteneum* (Lorenzen *et al.* 2002). It has also been shown to have a broad host range outside of the insect groups with activity in primate cells, zebrafish *Danio rerio* embryos, planarian *Girardia tigrina* and the malaria causing *Plasmodium falciparum* (Lobo *et al.* 2006; González-Estévez *et al.* 2003; Balu *et al.* 2005).

Transposon	*Size*	*Species of origin*	*Number of species transformed*	*Reported transformation efficiencies**	*References*
Hermes	2.7kb	*Musca domestica*	7	0.1%-50%	Jasinskiene *et al.* 1998; Berghammer *et al.* 1999; O'Brochta *et al.* 2000; Michel *et al.* 2001; Allen *et al.* 2001; Marcus *et al.* 2004
Hobo	3kb	*Drosophila melanogaster*	4	0.5%-26.5%	Blackman *et al.* 1989; Lozovskaya *et al.* 1996; Whyard pers. comm. & Whyard *et al.* unpub data in O'Broctha & Atkinson 1996
Minos	1.4kb	*Drosophila hydei*	7	1.5%-32%	Megna & Cline pers. comm. in O'Brochta & Atkinson 1996; Catteruccia *et al.* 1999; Loukeris *et al.* 1995a, b; Pavlopoulos *et al.* 2004; Koukidou *et al.* 2006; Salvemini *et al.* 2006; Uchino *et al.* 2007
Mariner	1.3kb	*Drosophila mauritiana*	3	0.8%-8.6%	Lohe & Hartl 1996; Berghammer *et al.* 1999; Coates *et al.* 2000
P	2.9kb	*Drosophila melanogaster*	4	1%->50%	Rubin & Spradling 1982; Brennan *et al.* 1984; Scavarda & Hartl 1984
piggyBac	2.5kb	*Tricoplusia ni*	19	0.7%-66.7%	Berghammer *et al.* 1999; Handler & McCombs 2000; Peloquin *et al.* 2000; Tamaura *et al.* 2000; Handler & Harrell 2001; Grossman *et al.* 2001; Nolan *et al.* 2002; Perera *et al.* 2002; Sumitani *et al.* 2003; Marcus *et al.* 2004; Adelman *et al.* 2004; Kuwayama *et al.* 2006; Rodrigues *et al.* 2006; Condon *et al.* 2007; Finokiet *et al.* 2007

Table 1.2. Transposable elements used as vectors for insect transgenesis. *Transformation efficiencies are given as percentage of total number of surviving injected individuals that give rise to transgenic offspring

The *Mos1* transposon, part of the widespread *mariner* family of transposons, was originally isolated from *Drosophila mauritiana* (Medhora *et al.* 1988) and is often referred to as *mariner* in the transgenic literature. Compared to *piggyBac*, *mariner* transformation has not been reported in a wide range of insect species. It is unclear whether this is due to failed attempts or because it is not a popular choice. Members of the *mariner* transposon family are widespread and there are negative effects associated with having an element present in a target genome (Sundararajan *et al.* 1999). Reported transformation efficiencies using the *Mos1/mariner* element are relatively low with 8.6% in *M. domestica* being the highest rate of stable integration confirmed (Coates *et al.* 2000). A transformation efficiency of 15.4% was reported in *Ae. aegypti* but the authors were unable to show that integration into the genome had occurred (Yoshiyama *et al.* 2000). *Mariner* has also been used successfully in non-insect species including *D. rerio* (Fadool *et al.* 1998), the chicken (Sherman *et al.* 1998) and *Leishmania major* (Gueiros-Filho & Beverley 1997) although activity was not detected in mammalian cell lines (Wu *et al.* 2006).

The *Hermes* element, isolated from *M. domestica* (O'Brochta *et al.* 1996), appeared promising with a transformation efficiency of 50% recorded in *D. melanogaster*. However, it has had limited success in other species although it is the most popular element for *Ae. aegypti* germline transformations (Adelman *et al.* 2002).

One other potential element for use as a germline transformation vector in insects is *hobo*, which has been successfully used to transform four insect species (Tables 1.1 & 1.2), but no details have been published for the transformations of *Bactrocera tyroni* and *Bactrocera dorsalis*, being reported only as "pers. comm." and "unpublished data" in O'Brochta and Atkinson (1996). In *D. melanogaster* and *Drosophila virilis* reported transformation frequencies are low (Blackman *et al.* 1989; Lozovskaya *et al.* 1996). Finally, the elements *Himar1, Hopper*, *henes* and *Buster* have been touted as possible vectors for insect transgenesis but have not been used (Lampe *et al.* 1996; 1998; Atkinson 2008).

Transgenesis in stalk-eyed flies

To fully understand diopsid eye-stalk development and evolution will require functional assays to complement and enhance findings from studies of gene expression. Virus mediated gene expression is a powerful tool but problematic due to the risks of working with virus making it expensive unless laboratories have pre-existing expertise in the required techniques. RNAi has proved valuable in embryonic studies in *D. melanogaster* and in studying larval and pupal development in non-dipteran insects. Eye-stalk development occurs during the late larval instars and during pupal stages therefore systemic RNAi is required, which is unlikely to be present in stalk-eyed flies. Due to the broad species ranges of transposon vectors such as *piggyBac*, *mariner, Minos* and *Hermes*, germline transformation protocols have been developed in many diverse insect species and are the best options for developing a protocol for assaying gene function in the stalk-eyed fly.

In addition, transgenics enables other applications which would be valuable for diopsid research in development and evolution. For example, the exact location of the eye-stalk progenitor cells within a developing larva is not known. Transgenics has been used in fate-mapping studies in *D. melanogaster* and *B. anynana* using heat shock promoters driving the expression of reporter genes (Ramos *et al.* 2006). Assessing mating success and sperm competition has proved problematic in stalk-eyed flies, especially when attempting to compare success from multiple matings. Transgenic fluorescently labelled sperm allows a simple method for visualisation of sperm within a mated female.

Although transgenic protocols are available in a wide range of organisms, a protocol needs to be developed that is tailored to requirements of the species of study. In particular there are two main elements to address, the mechanism of DNA delivery and the selection of DNA vector. Insects vary dramatically in their life cycles and embryonic morphology. *D. melanogaster* produce many fertile eggs from a single mating that possess a soft chorion which is easily penetrated by a microinjection needle. In contrast, some species of mosquito will only mate in a mass aggregation of individuals and produce embryos whose chorion hardens rapidly making access problematic (O'Brochta & Atkinson 2004).

Although some transposon vectors have broad host ranges it is not guaranteed that they will be appropriate for use in all species. The spread of insect transgenesis was hindered by *P*-element activity being restricted to drosophilid species (Atkinson *et al.* 1993). *hobo* is not a viable vector for *M. domestica* as it is mobilised by the presence of the closely related *Hermes* transposon (Sundararajan *et al.* 1999). Therefore it is important to test potential vector activity within the species of study before embarking on an attempt at a germline transformation. Several plasmid based assays are available for such a purpose.

1.3 GROWTH OF AN EXAGGERATED TRAIT

The eye-stalks of male *T. dalmanni* are larger than those of females due to sexual selection on male but not female eyespan. Male eye-stalk growth is also more sensitive to nutritional stress, than growth of non-sexually selected traits such as the wing (David *et al.* 1998; Cotton *et al.* 2004a, b). Identifying the mechanisms underlying the differential growth and response to nutrition of male eye-stalks, rather than the origin of the novel feature itself, will aid understanding of the development, evolution and maintenance within a population of ornamental sexually selected traits.

The strategies for studying development in non-model systems discussed in section 1.2 can be applied to any species. In this section, I discuss an approach to studying organ growth specific to the stalk-eyed fly by exploiting the structure of the eye-stalk to enable the assessment of the relationships of cell size and cell number with organ size. Trait size is determined by cell size, cell number and, in some instances, extra-cellular mass. Understanding the relationships of these parameters for the eye-stalk will shed light on the underlying mechanisms involved in eyespan regulation. Below, I review the current understanding of growth regulation in holometabolous insects and then address what is known specifically about the relationship of cell size and cell number in insect species.

1.3.1 Determining body size in holometabolous insects

The genetic and hormonal regulation of nutrition-dependent growth in holometabolous insects has been well characterised in model species such as *D. melanogaster* and the sphinx moth, *Manduca sexta*. Holometabolous insects go through several larval stages before pupating and undergoing metamorphosis into the adult form (Truman & Riddiford 1998). All growth is carried out during the larval stages and adult body size is determined by final larval size prior to pupation. Adult epidermal structures develop as imaginal discs, which are discrete clusters of cells set aside in the developing larvae. Imaginal discs commence a period of rapid proliferation during the larval instars that terminates around pupation but timings vary between discs. At this point the discs undergo differentiation, eversion and then a series of complex morphogenetic movements to form the adult organs (Haynie & Bryant 1986).

Most models of growth regulation and its interaction with nutrition have been developed to account for variation in overall body size. Growth variation within and between imaginal discs is not well understood except for a few systems showing discontinuous variation in size, e.g. *O. taurus* where large males possess horns, but small males and females do not (Emlen *et al.* 2007). To understand growth control of individual organs it is beneficial to first look at overall body size regulation.

A basic model for determining body

The final body size (BS) of any organism is a function of the growth rate (GR) and the period of growth (ΔT) (Shingleton *et al.* 2007):

$$BS = GR \times \Delta T$$

An increase in body size will result from an increase in either growth rate or the period spent growing, or both. In holometabolous insects, growth occurs during larval stages, and adult body size is set by larval body size at pupation. In *M. sexta*, when a larva reaches a specific weight ("critical weight"), an irreversible hormone signalling cascade is initiated and the larva can no longer delay pupation (Nijhout 1975; Davidowitz *et al.* 2003). The period between critical weight and pupation is the terminal growth period. Final body size will be determined by critical weight (CW) plus the growth rate (GR) during the terminal growth period (TGP):

$$BS = CW + (GR \times TGP)$$

Attempts to extend this *M. sexta* model to other insect species have met with varying degrees of success. In *D. melanogaster*, critical weight is estimated by assessing the weight at which 50% of larvae will survive to pupation when removed from food. This is the minimal viable weight, or weight that must be achieved for the larva to be able to survive metamorphosis, but critical weight and minimal viable weight are approximately the same in this species (De Moed *et al.* 1999; McBrayer *et al.* 2007). The above equation presents a basic framework for looking at the primary components underlying variation in insect body size and can be modified to fit the life history of a particular species.

Critical weight and the length of the terminal growth period are key parameters in size regulation. *D. melanogaster*, *M. sexta* and *T. dalmanni* at least double their body mass during the terminal growth period. (Davidowitz *et al.* 2003; De Moed *et al.* 1999; Ingraham pers. comm.). Critical weight is determined by both environmental and

genetic factors. In *M. sexta* early larval nutrition modulates critical weight whereas in *D. melanogaster* critical weight is only sensitive to extremely low levels of nutrition (Robertson 1963; De Moed *et al.* 1999; Mirth *et al.* 2005). In flies, variation in critical weight is also seen in response to radiation, which retards the growth of the imaginal discs (Stieper *et al.* 2008). Rearing temperature has no effect on critical weight in *M. sexta* but in *D. melanogaster* critical weight is increased at low temperatures. Significant genetic variation for critical weight has been observed in both species (Davidowitz et al 2003; De Moed *et al.* 1999; Partridge *et al.* 1999).

In a small number of insect species with blood sucking larvae, such as *Dipetalogaster maximus* (Nijhout 1984), larval weight is sensed by abdominal stretch receptors that can be manipulated to alter critical weight. In *D. melanogaster* prothoracicotropic hormone (PTTH) has been shown to play a part in sensing critical weight. PTTH is secreted by neurons innervating the prothoracic gland (part of the ring gland). If these neurons are ablated in *D. melanogaster* critical weight is delayed dramatically, but pupation still eventually occurs (McBrayer *et al.* 2007).

Ecdysone, juvenile hormone and the terminal growth period

Larval development is regulated by several hormones and has been well studied in the lepidoterans *M. sexta* and *B. mori* (Fig. 1.5) (MacWhinnie *et al.* 2005; Flatt *et al.* 2005; reviewed in Edgar 2006). Transition between lepidoteran larval stages are regulated by the interaction of ecdysone, a steroid hormone secreted from the prothoracic gland, and juvenile hormone (JH), a sesquisterpene released by a region of the corpus allata (Nijhout 1994). Evidence from assessing hormone titres in developing larvae has been combined with observations of the effects of manipulating hormone levels to arrive at a detailed model of the interactions between them. For example, over-expression of juvenile hormone esterase (JHE), which degrades JH, causes premature pupation in *B. mori* (Tan *et al.* 2005). The application of a JH analogue prevents metamorphosis in *M. sexta* (Abdell-Aal & Hammock 1986; Tan *et al.* 2005). During early larval stages JH levels are maintained at high levels. Pulses of ecdysone regulate changes between larval stages. After critical weight is attained, JH levels drop. This causes a release of PTTH, which induces an ecdyone pulse. In the absence of JH, an initial ecdysone pulse induces the cessation of feeding and a second pulse causes pupation. This short pulse is followed by a sharp increase in both JH and ecdysone resulting, eventually, in the

cessation of proliferation and the onset of differentiation. At some point after pupation the levels of both hormones drop (Fig. 1.5).

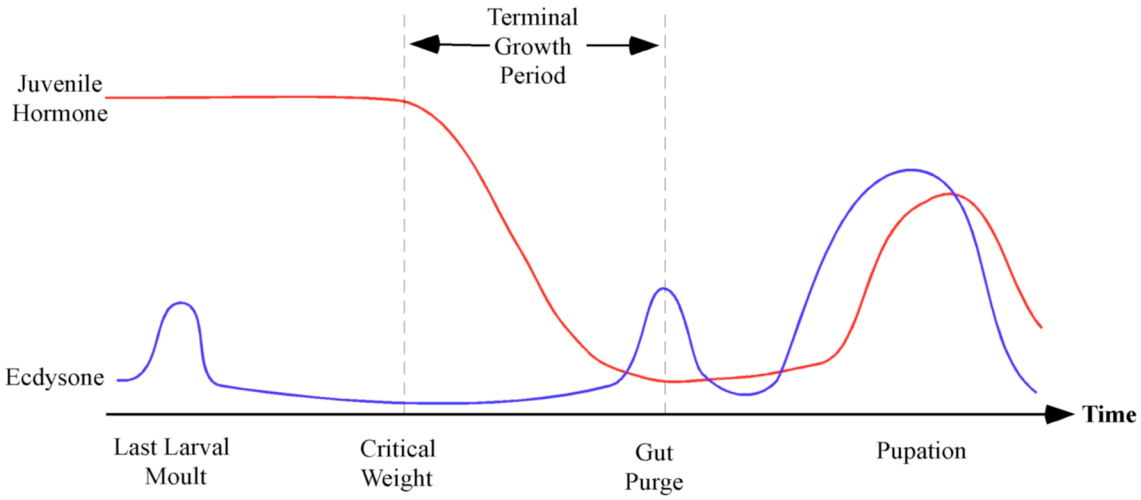

Figure 1.5. Generalised view of levels of juvenile hormone (Red, JH) and Ecdysone (Blue) on transition between developmental stages in holometabolous insects based on work from *D. melanogaster*, *M. sexta* and *B. mori* (Emlen & Allen 2004). Whilst JH levels are high pulses of ecdysone initiate transitions between larval stages. Upon the attainment the attainment of critical weight JH levels drop and cause, via the action of PTTH, a pulse of ecdysone that causes gut purging. A second pulse of ecdysone initiates pupation and metamorphosis. The period between critical weight attainment and pupation is the terminal growth period and the amount of growth during this period is critical in determining final adult body size. After pupation, JH and ecdysone levels drop but the relative timing of these changes is not well established.

The hormone cascade in *D. melanogaster* is less well understood but some deviation from the lepidopteran model has been observed. The application of a JH analogue does not prevent metamorphosis in *D. melanogaster* as in *M. sexta* (Wilson 2004). However, the role played by ecdysone appears similar. Reducing ecdysone levels by decreasing the size of the ring gland caused prolonged over-growth in larvae and the opposite is seen if the size of the ring gland is increased (Colombani *et al.* 2005).

Growth rate and insulin signalling

The insulin signalling pathway is a highly conserved pathway with homologues and common effects seen in mice, fish, fruit flies and nematode worms (Chen *et al.* 1996). It affects many parts of an organism's life cycle including reproduction and lifespan (Wu

& Brown 2006). It is also important in regulation of nutrients and in growth (Chen *et al.* 1996). It is therefore an obvious candidate for regulation of growth rate in insects and has been well studied in *D. melanogaster*. The main components of the insulin signalling pathway are shown in Fig. 1.6. It can be divided into two connected pathways that work in a cell autonomous manner, one pathway initiated by the insulin receptor (InR) and another based around the protein kinase target of rapamaycin (TOR) (Fig. 1.6).

InR detects signals from insulin-like peptides (ILPs) that circulate in the body (Brogiolo *et al.* 2001) and mediates its effect by the insulin receptor substrate, *chico*, starting a signalling cascade (Bohni *et al.* 1999), including PI3K and AKT/PKB, which results in the removal of the growth inhibitor FOXO from the nucleus (Weinkove *et al.* 1999; Puig *et al.* 2003; Jünger *et al.* 2003). The InR pathway is inhibited by the PTEN which prevents PI3K activity (Goberdhan *et al.* 1999; Gao *et al.* 2000).

Target of rapamycin (TOR) responds more directly to intracellular levels of amino acids, ATP and oxygen (Oldham *et al.* 2000; Zhang *et al.* 2000). Increased levels of amino acids and ATP inhibit tumour suppressor complexes 1 and 2 (TSC1, TSC2) (Potter *et al.* 2000) and indirectly increases levels of TOR. This causes a signalling cascade that promotes S6K and TIF-IA which promote growth. The InR and TOR signalling pathways are connected by the inhibitory action of AKT on TSC1 and TSC2 (Scanga *et al.* 2000; Oldham *et al.* 2003) and the competitive action of FOXO and TOR on S6K and 4EBP, an inhibitor of growth.

The role that the InR/TOR pathway plays in regulation of growth in response to nutrition in *D. melanogaster* is well established. Increased nutrition is associated with increased levels of InR and phosphatidylinositol-3 kinase (PI3K) (Britton *et al.* 2002) and food deprivation is associated with decreased levels of ILPs (Ikeya *et al.* 2002). Direct manipulations of the pathways have confirmed the pathway's importance. Over-expression of the main constituents InR, chico and TOR result in increased body size in flies and mutants whilst decreased activity of the same components result in reduced body size (Chen *et al.* 1996; Bohni *et al.* 1999).

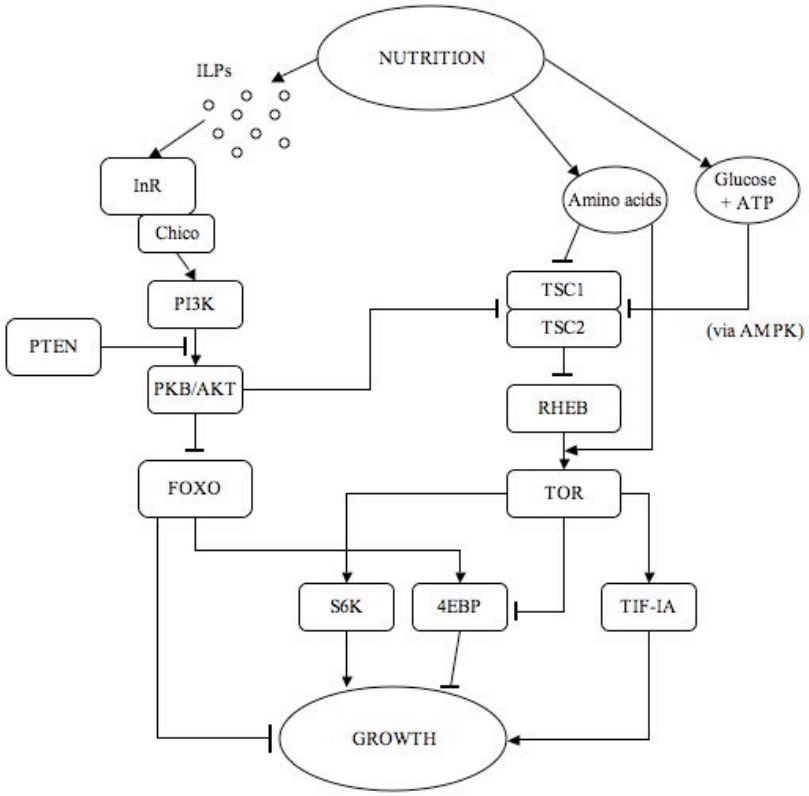

Figure 1.6. Main components of the InR/TOR signalling pathways based on work in *D. melanogaster*. The pathway can be split into two connected pathways, the InR pathway (left) and the TOR pathway (right). In the former, the insulin receptor (InR) is activated by circulating insulin-like peptides (ILPs). This upregulates the insulin receptor subtrate, chico, which in turn activates phosophatidylinositol 3-kinase (PI3K). PI3K phosophorylates PIP2 to PIP3 and this reaction is reversed by PTEN. PIP3 activates protein kinase B/AKT which causes the removal of the FOXO transcription factor from the nucleus. FOXO inhibits cell growth. The TOR (target of rapamycin) signalling pathway responds to intracellular levels of amino acids and ATP. It is suppressed by the actions of tumour suppressor complexes 1 and 2 (TSC1 & TSC2). Removal of this repression enables TOR to upregulate S6 kinase (S6K) and TIF-IA transcription factor induces growth as well as repressing the growth repressor 4EBP. The InR and TOR pathways are connected by PKB/AKT inhibition on TSC1 and TSC2 and the FOXO and TOR upregulation and inhibition of 4EBP. TOR negatively regulates InR signalling by feedback from S6K on InR and upregulates activity by TOR acting on PKB/AKT (not shown). See text for references along with Oldham & Hafen 2003, Edgar 2006 and Shingleton *et al.* 2007 for further review.

Null mutations in InR are lethal and mutants in TOR arrest development completely (Oldham *et al.* 2003). Conversely, over-expression in PTEN, FOXO, TSC1 or TSC2, antagonists of the InR/TOR pathways, result in smaller flies and reduced expression of

these components causes increased body size (Goberdhan *et al.* 1999; Potter *et al.* 2001). The signals that the InR/TOR pathways respond to are less well characterised. Seven ILPs (ILP1-7), which activate InR (Brogiolo *et al.* 2001) exist with differing expression patterns. ILP2 is released from medial neurosecretory cells and ablation of these cells in *D. melanogaster* results in a decrease of ILP2 and a reduction in growth. The wild-type phenotype can be restored with the application of ILP2 (Brogiolo *et al.* 2001).

Clearly variation in any part of the insulin signalling will affect growth rate. Changes in the expression of pathway components are correlated with changes in body size and functional assays confirm this. One study of a wild population of *D. melanogaster* indicated that insulin plays a role in causing natural size variation. Chromosome inversions are often associated with reduced gene expression. An inversion on chromosome two, In(2l)t, spans the *Gpdh* and *Adh* loci, which are involved in the starvation responses and the insulin signalling pathway (Oudman *et al.* 1994). This inversion is correlated with reduced body size in African and European populations of *D. melanogaster* (De Jong & Bochdanovits 2003).

The interactions of hormones and insulin signalling and detection of nutrient levels

Hormones regulate the duration of the TGP and insulin signalling acts in response to the level of nutrients to mediate the growth rate during the TGP. There is also evidence that more complex interactions between hormone and insulin signalling are important in growth regulation in *D. melanogaster* (Colombani *et al.* 2003; Mirth 2005; McBrayer *et al.* 2007). The fat body in flies is a possible nutrient sensor and acts as a site of integration of the two pathways (Colombani *et al.* 2003; Britton *et al.* 2002). Reduction in the level of amino acids in the fat body results in reduced insulin signalling (Colombani *et al.* 2003) and inhibition of InR or PI3K specifically in the fat body causes a reduction in the size of the fat body but also a reduction in larval growth (Britton *et al.* 2002).

There is increasing evidence for interactions between InR/TOR signalling and ecdysone release in the prothoracic gland (Colombani *et al.* 2005; Mirth *et al.* 2005; Layalle *et al.* 2008). Colombani *et al.* (2005) demonstrated that over-expression of PI3K specifically in the ring gland saw an increase in the size of the gland and alterations of

developmental timings, but an overall decrease in body size. The decrease was associated with an increased level in 20-hydroxy-ecdysone (20E), an active form of ecdysone, which caused premature differentiation. Reduction in PI3K levels caused the opposite effect. Insulin signalling was shown to have a specific role, as altering levels of myc and cyclinD/Cdk4, which affect growth independently of insulin, resulted in an alteration of prothoracic gland size but no had effect on overall body size. Similar results were observed when the size of the prothoracic gland was altered by manipulating levels of dPTEN or Dp110 (co-factor of PI3K) (Mirth *et al.* 2005) and by altering TOR signalling in the prothoracic gland (Layalle *et al.* 2008). In the latter experiment it was shown that the effects of altering PI3K and TOR were different from each other. Other circumstantial evidence implies an interaction between insulin signalling and JH. Decreased levels of JH are observed under starvation and in mutants with reduced InR signalling and in adult flies reduced expression of genes involved in JH signalling is associated with starvation (Tatar *et al.* 2001; Terashima & Bownes 2005).

1.3.2 Growth regulation in organs and imaginal discs

To understand eye-stalk variation in stalk-eyed flies, growth regulation within individual organs must be addressed. The control of overall body size by insulin and hormone signalling provides a framework within which growth of imaginal discs occurs.

Imaginal discs vary in their periods of growth and proliferation can occur both prior to the attainment of critical weight and after pupation has occurred (Peel & Milner 1992; Stern & Emlen 1999). Therefore the constraints on the final size of individual organs are different from those on overall body size, which is determined by larval dry weight at pupation. As with overall body size, organ size will be determined by the length of the growth period and by growth rate. In addition, there is evidence for the presence of an autonomous regulation within the disc and interactions between discs.

Imaginal disc growth period and regulation by hormone signalling

Imaginal discs differ in their period of growth. The timing of both initiation and termination varies between species and between discs. In *B. mori* cell proliferation in

the wing discs is first detected in the early larval stages while the discs that give rise to the legs and mandibles do not initiate proliferation until the final instar (Fukuda 1952; Ohtaki *et al.* 1986). In *M. sexta* a similar pattern is seen with the further feature that eye disc growth does not begin until after the attainment of critical weight (Champlin & Truman 1998). In *D. melanogaster,* wing and eye discs begin proliferation early in the first larval instar whereas the leg and genital discs initiate proliferation in the second larval instar (Truman & Riddiford 2002).

There is evidence that the imaginal discs continue nutrient-dependent growth after feeding and larval growth has ceased. Shingleton *et al*. (2005) used a temperature sensitive variant of the *D. melanogaster* insulin receptor to perform timed suppression of insulin signalling. When insulin signalling was removed after pupariation, no effect on overall body size was observed but adult wing size was reduced. Termination points may also vary between discs. The wing discs continue proliferating 24 hours after pupariation whereas the eye discs initiate differentiation prior to pupation (Truman & Riddiford 1999; Shingleton *et al.* 2005).

Hormones play a large part in the timing of disc proliferation and the onset of proliferation, with key changes in levels of JH and ecdysone taking place after the attainment of critical weight (Truman & Riddiford 1998). In *M. sexta* it has been shown that proliferation within the eye disc only occurs between two levels of ecdysone titre (Champlin & Truman 1998) and similar responses are seen in *D. melanogaster* cell lines derived from imaginal discs (Peel & Milner 1992). It is proposed that sensitivity to hormone levels determines the growth period of a disc (Truman & Riddiford 1998). There is evidence for variation in sensitivity to ecdysone levels within discs. Different regions of the eye discs of *M. sexta* differentiate at different time points and this is associated with regional variation in the level of expression of the ecdysone receptor in the discs (Fujiwara *et al.* 1995; Jindra *et al.* 1996). Similar evidence is observed in the leg discs of *D. melanogaster* where different regions of the disc vary in their response to the application of the active form of ecdysone, 20-hydroxyecdysone (20E) (Mirth 2005). However, there is no direct evidence of variation in hormone responsiveness of the same disc between individuals of differing body and organ size.

Regulation of disc growth by the InR/TOR pathway

The effects of the insulin signalling pathway are cell autonomous and therefore proposed to regulate growth rate within discs (Gao *et al.* 2000). For example, clonal expression of PTEN, an inhibitor of growth, causes reduced growth only in the affected clone and if members of the InR/TOR pathways are either over-expressed or knocked-out in individual discs, the response is restricted to those discs (Leevers *et al.* 1996; Huang *et al.* 1999; Weinkove *et al.* 1999; Goberdhan & Wilson 2002). There is experimental evidence for variation in the expression of insulin signalling components in different discs in *D. melanogaster* (Shingleton *et al.* 2005). When flies carrying a temperature sensitive variant of the InR receptor were reared at restrictive temperatures, derivatives of the wing and eye-antennal discs were reduced in size while the genital arches, which are a product of the genital disc, remained the same size.

Target size and disc growth

D. melanogaster imaginal discs will grow to approximately the same size when cultured in permissive medium as they would attain in a fully fed larva (Johnston & Gallant 2002). This suggests that each disc has an inherent maximum size (target size) which is independent of nutritional and hormonal factors. It is well established that wing growth is sensitive to the opposing gradients of the morphogens *decapentaplegic* (*dpp*) (Martin-Castellanos & Edgar 2002) and *wingless* (*wg*) (Neumann & Cohen 1997). Mutations of either *dpp* or *wg* result in reduced wing size or complete wing loss while manipulation of the *notch* and *wnt* signalling pathways, which regulate *dpp* and *wg* signalling, results in changes in wing size. Similar effects of manipulating morphogen expression on organ size have been observed in the antennae, legs and eyes (Johnston & Gallant 2002). A model has been proposed whereby cells grow until specific gradients of morphogens are generated (Johnston & Gallant 2002). One of the major gaps in our understanding of how organ size is determined is to establish how this level of regulation by morphogen signalling interacts with nutrition-sensitive and hormonal control of growth (Shingleton *et al.* 2007).

Coordination of organ growth by interactions between imaginal discs

In a small number of species, direct manipulation of an imaginal disc can result in alterations to the growth of other discs. In the hymenopteran *Precis coenia,* the removal of one wing disc results in an increase in size of the adjacent wing (Emlen & Nijhout 1998). The removal of the horn generating disc in *O. taurus* is correlated with an increase in size in the nearby eye (Emlen & Nijhout 1998) and ablation of the genitals results in an increase in horn size (Moczek & Nijhout 2004). Contrary to this, over-expression of the insulin signalling pathway in *D. melanogaster* eyes results in a dramatic increase in eye size but compensation by other discs is only observed when systemic levels of *chico* are also reduced (Goberdhan & Wilson 2002). Some models propose that signalling between discs may be mediated by a group of secreted proteins known as imaginal disc growth factors (IDGFs) which were originally isolated from cultures of imaginal disc cells and found to promote growth (Kawamura *et al.* 1999). The effect of IDGFs on cell growth in culture was enhanced by application of insulin although the addition of insulin alone was not enough to promote extra growth. IDGFs are often also presented as a mechanism for the regulation of disc growth via insulin signalling (Edgar 2006; Shingleton *et al.* 2007). More studies are required to fully understand the role of IDGFs in organ growth.

Possible mechanisms for regulation of eye-stalk size

To summarise, factors involved in the regulation of organ size include nutritional levels, hormone signalling and intrinsic disc growth (Fig. 1.7). There are also varying degrees of evidence for interactions between these factors. Any of these could be involved in driving the dramatic diversity in eyespan in stalk-eyed flies. Variation in responsiveness to hormone signalling between discs may affect the period of eye-stalk growth. Different levels of insulin signalling present in different discs may be responsible by altering growth rate in response to nutrition. Additionally, eye-stalk size may be set by autonomous disc signalling, which in turn could be affected by insulin signalling. There is evidence for all of these mechanisms being present in other organisms, but currently little is known in stalk-eyed flies. Progress is hindered by the current paucity of sequence data available in stalk-eyed flies. Therefore in order to study eye-stalk growth different approaches must be taken such as assessing the relationships of cell size, cell number and trait size.

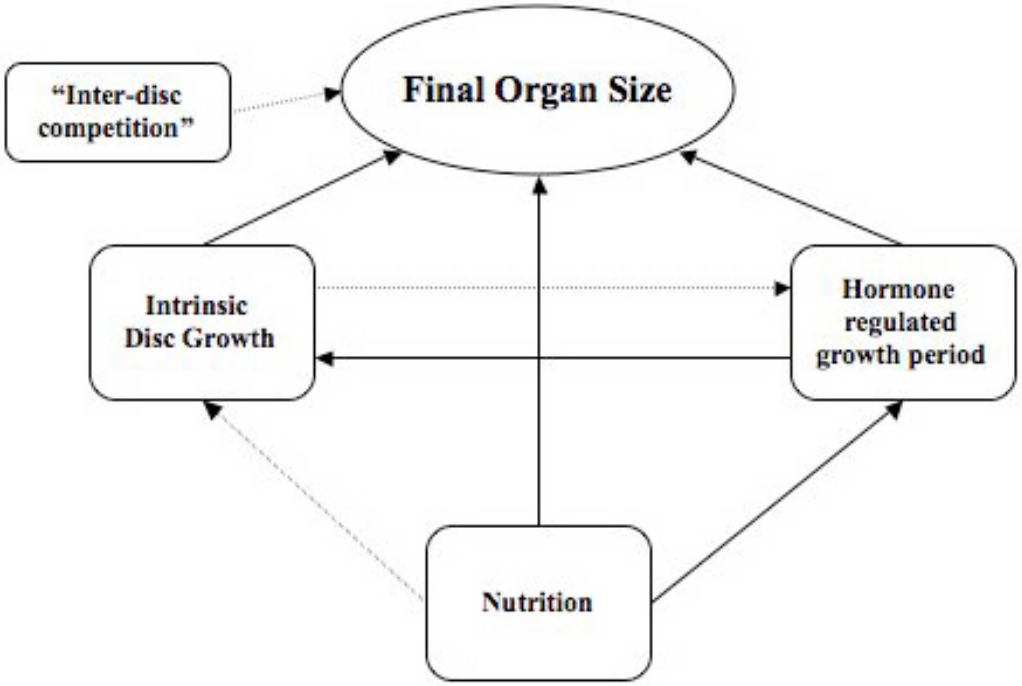

Figure 1.7. Schematic representation of factors that affect organ growth in holometabolous insects. Solid lines indicate interactions that have been experimentally tested. Dotted lines denote interactions that have not been experimentally tested or are contentious (eg inter-disc competition).

1.3.3 Cell size, cell number and organ size

The relative contributions of cell size and cell number are important for understanding both the developmental regulation of growth and the evolution of body/organ size (Shingleton *et al.* 2005; Arendt 2007; Stevenson *et al.* 1995). Developmental manipulation of the insulin signalling pathway and the consequent effects on size are associated with changes in cell size, cell number or both. These relationships are summarised in Table 1.3. Some older reviews suggest that changes in the InR pathway result in changes in cell number whereas changes in the TOR pathway are involved in changes in cell size, but this is an over-simplification. The current understanding is that some components (e.g. *chico* and PTEN) affect both cell size and cell number. FOXO, the growth inhibitor and final target of the InR pathway results in changes purely in cell number (Jünger *et al.* 2003; Puig *et al.* 2003) whereas alterations in Tsc1 and Tsc2 appear to result in changes in mainly cell size (Potter *et al.* 2001).

Gene	Effect observed on cell size (CS) and/or on cell number (CN)	References
InR	CS & CN	Chen *et al.* 1996; Tatar *et al.*2001; Brogiolo *et al.* 2001
chico	CS & CN	Bohni *et al.* 1999; Clancy *et al.* 2001
Dp110/p60	CS & CN	Weinkove *et al.* 1999; Verdu *et al.* 1999; Scanga *et al.* 2000
AKT/PKB	CS only	Verdu *et al.* 1999; Scanga *et al.* 2000
S6K	CS only	Montagne *et al.* 1999
TOR	CS & CN	Oldham *et al.* 2000; Zhang *et al.* 2000
PTEN	CS & CN	Goberdhan *et al.* 1999; Scanga *et al.* 2000.
FOXO	CN only	Junger *et al.* 2003

Table 1.3. Effect of manipulations of InR/TOR pathway component function on cell size and cell number in *D. melanogaster*. See Fig. 1.6 for abbreviations. Dp110 and p60 are catalytic and adaptor subunits of phosophatidylinositol 3-kinase (PI3K), respectively.

There have been a number of studies addressing cell size and cell number variation in response to developmental conditions and to selection (both artificial and natural). These have mainly been based on cell size and cell number estimates in the *D. melanogaster* wing due to the relatively straightforward method of estimation in this organ (Robertson 1959). In *D. melanogaster,* nutritional stress during the larval stages results in a decrease in adult body size while reduced temperature during the same period causes an increase in adult body size. The developmental response to nutritional stress involved changes in both cell size and cell number (Robertson 1959). The response to temperature differed between the sexes. Early experiments indicated that the increase in body size seen at lower temperatures were mainly due to increases in cell size in females and to changes in both cell size and cell number in males (Robertson 1959). Later experiments demonstrated an interaction between the effects of food and temperature (De Moed *et al.* 1997a). At high temperatures (above 20°C), response to nutritional stress was mediated solely by cell number, but at lower temperatures responses to nutritional stress involved a reduction in both cell size and cell number. The plastic response of cell size and cell number to temperature was also shown to differ between different selection lines and with wing size (De Moed *et al.* 1997b; Noach *et al.* 1997). In small-winged individuals, variation in size was associated with cell number but in larger-winged individuals cell size had a more significant role. Outside of *D. melanogaster*, response of cell size and cell number to temperature has been studied in the yellow dung fly *Scathophaga stercoraria*. In this species, an increase in rearing temperature was associated with a decrease in organ size mediated by both cell size and cell number in eyes and wings in females. In males cell size and cell number varied in the eye, but only cell size was affected in the wing (Blanckenhorn & Llaurens 2005). This appears to be contrary to what was observed in *D. melanogaster* wings, however in *S. stercoraria* males are the larger sex whereas in *D. melanogaster* females are the larger sex, therefore the larger sex in each species does respond in the same fashion.

Evolutionary changes in cellular patterns have been evaluated by monitoring responses in experimentally evolved or artificially selected lines in a variety of contexts. For example, lines of *D. melanogaster* kept at different temperatures mimicked the developmental responses documented above for phenotypic studies. At low temperatures, body size and cell size increased in females and cell size and cell number changed in males although the relative contributions of response were not consistent

between lines (Partridge *et al.* 1994; Noach *et al.*1997). When exposed to variation in humidity, cell number was seen to predominantly mediate both the increase in wing size, associated with decreased humidity, and the decrease in wing size, observed with increased humidity (Kennington *et al.* 2003). When selection on body size itself was carried out, increased size was predominantly mediated by cell number, in contrast to the trend in temperature selection, and decreased size was associated with a decrease predominantly in cell size (Partridge *et al.* 1999).

Patterns of cellular responses have also proved useful in documenting the mechanisms underlying variation in size between natural populations. Insects adapt very rapidly to temperature variation. Some *D. melanogaster* populations have been shown to largely conform to Bergmann's rule (Blackburn *et al.* 1999) in that flies at higher latitudinal clines, and therefore lower temperatures, have larger body sizes (James *et al.* 1995). In clines from two continents (South America and Australia), increases in wing and body size with increased latitude are accompanied by increases in cell number and cell size with the relative contributions of cell size and cell number differing between clines (James *et al.* 1995; 1997; Zwaan *et al.* 2000) This contrasts with the response to general trends observed in laboratory populations, in which cell size makes a larger contribution to increased size (Noach *et al.* 1997; Partridge *et al.* 1994).

Cell size and cell number are final factors which the genetic pathways that regulate growth act on to determine organ size. The case studies described above demonstrate that assessment of variation in cell number and cell size can provide sensitive measures of a range of developmental and evolutionary responses. The relationships between cell size and number are seen to vary with species and experimental manipulation or selection regime. In stalk-eyed flies, a comparison of cell size and cell number in male and female eye-stalks will be an immensely valuable step towards identifying the basic parameters underlying differential growth in these sexually dimorphic organs.

Most of the work on cell size and cell number in insects has focused on the wing due to its simple structure and because every cell is easily identified since each produces a single trichome. Clearly, no single organ will be representative of growth mechanisms present in all the structures within an organism therefore it is important to sample as many organs as possible. Most organs are complex structures surrounded by an opaque cuticle and so cell size and cell number assessments are not straightforward. Buschbeck

et al. (2001) examined eyestalk structure in *Teleopsis whitei* and suggested that a single mono-layered epidermis lay beneath the cuticle. After eclosion, stalk-eyed flies spend a significant amount of time expanding their eye-stalks prior to melanisation of the cuticle. During this period the cells underneath are visible. These two features make cell size readily assessable in the eye-stalk.

Analysis of the relationship between cell size and eye-stalk size will not only give insight into the mechanisms underlying sexual selection but also into growth regulation of insect organs in general. Comparisons can be made between sexes, within sexes and even between species to assess the contributions of cell size and cell number to this exaggerated sexually selected trait.

1.4 THESIS STRUCTURE

The work in this thesis was funded by the Biotechnology and Biological Sciences Research Council and was performed under the supervision of Kevin Fowler and Hazel Smith. The execution of all the experiments was by the author. Additional instruction and assistance was provided for sequencing reactions (S. Martin, University College London), and for electron microscopy and sectioning (M. Turmaine, University College London). Plasmids used in Chapters Two and Three were kindly provided by E. Wimmer (George August Universität, Göttingen, Germany) and C. Savakis (Univeristy of Crete, Greece). Technical advice on microinjection techniques was provided by N. Gompel (University of Marseille, France) and A. Pavlopoulos (University of Cambridge, UK).

Chapter Two describes the development of two aspects of a transgenic protocol. An embryo microinjection protocol for physical delivery of foreign DNA to the stalk-eyed fly *Teleopsis dalmanni* was designed and tested. In addition, three transposable element vectors, *mariner*, *Minos* and *piggyBac* were assayed for their activity in *T. dalmanni* embryos. *Mariner*, *Minos* and *piggyBac* were selected because they had been shown to be capable of activity in a wide range of insect species. A robust microinjection protocol was successfully developed. *Minos* and *piggyBac*, but not *mariner,* were shown to be active in stalk-eyed fly embryos and therefore were potential vectors for germline transformation.

Chapter Three describes how the findings from Chapter Two were exploited to achieve the first stable germline transformation in *T. dalmanni*. The *Minos* element was used as a vector and the *px3-eGFP* transgenic construct as a marker gene. At least three separate insertion events were observed and the transgenic line created has immediate practical use.

Chapter Four investigates the relative contribution of cell size and cell number to variation in eye-stalk and wing size in *T. dalmanni*, which shows sexual dimorphism for eyespan. A protocol for estimating cell size in eye-stalks was developed and used to compare cell size and its relationship to cell number in unstressed and nutritionally stressed males and females. Cell number was shown to be the main contributor to variation in eye-stalk size with no difference observed in the relationship of cell size,

cell number and eyespan between the sexes.

Chapter Five summarises and discusses the findings of the experimental chapters and relates them to the overall objective of investigating the evolution and development of an exaggerated ornamental trait in the stalk-eyed fly.

Some of the material from Chapter One was used to contribute towards a published review written in collaboration with Hazel Smith (Warren & Smith 2008).

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Chapter Two: Development of a transgenic protocol in the stalk-eyed fly, *Teleopsis dalmanni*

2.1 INTRODUCTION

Background

In Chapter One I established the importance of developing transgenics as a tool for assaying gene function in stalk-eyed flies. This chapter examines the development and evaluation of two key aspects of the necessary protocol for stable germline transformation (transgenesis) of the stalk-eyed fly *Teleopsis dalmanni*.

A fundamental requirement of insect transgenesis is that the DNA vector containing the transgene is physically delivered to prospective germline cells (pole cells). If incorporation of the DNA into the genome of these cells is achieved then the resulting transgene insert will be inherited by at least a proportion of the offspring of the original embryo. Here I describe key aspects of procedures developed in various insect species for the effective delivery of transgene DNA to pole cell nuclei that also maximise the survival of injected embryos. I discuss the features relevant to germline transformation in stalk-eyed flies before designing and testing a microinjection protocol for *T. dalmanni*.

In insects, transposon based vector systems are used to facilitate germline transformations. It is important to assay for transposon integration and stability before devoting significant effort to making transgenic lines. Using a vector that is incapable of integration or shows unstable integration in the species of interest constitutes a waste of both temporal and financial resources. Six transposons have been used for insect transgenesis. *P*-elements are only active in drosophilid species and *hobo* and *Hermes* have each been shown to be inappropriate vectors for at least one insect species (Atkinson *et al.* 1993; Sundararajan *et al.* 1999). In this chapter I describe and evaluate methods for assessing the species-specific activity of transposon vector systems and then apply one of the methods to test the suitability of three potential systems for the germline transformation of *T. dalmanni.*

2.1.1 Transgene delivery

Several different methods of DNA delivery have been used for transgenesis. Some have proved more applicable in species other than insects. Electroporation employs a large electric impulse to disrupt cell membranes and allow polar DNA molecules to pass into cells (Kamdar *et al.* 1992). It has been used in *Drosophila melanogaster* and honeybees but only to enable transient DNA expression (Kamdar *et al.* 1992; Leopold *et al.* 1996; Kunieda & Kubo 2004). The small size and delicate nature of the embryos together with the impermeable cuticle of the adults make electroporation a non-viable method for germline transformation in insects. In organisms where such problems are not present, such as the planarian *Ciona intestinalis* (Sasakura 2007) and *Girardia tigrina* (González-Estévez *et al.* 2003), the technique has been applied to produce stable transgenic lines. Biolistics or particle bombardment involves coating gold or tungsten microbeads with DNA and bombarding recipient tissue so that the beads pass through the cells while leaving the DNA inside (Klein *et al.*1987). It has been used in *D. melanogaster* and *Anopheles gambiae* with limited success (Baldarelli & Lengyel 1990; Miahle & Miller 1994) but is a routinely employed technique in plants (Klein *et al.* 1987; Sussex 2008).

In all successfully transformed insect species, and indeed in the majority of animal taxa, the most common method of DNA delivery is microinjection during the early stages of embryonic development (Handler 2000; O'Brochta & Atkinson 2004). A variety of procedures have been developed in *D. melanogaster* and other insects to maximise embryo survival following microinjection. Although these protocols share many common features any microinjection technique has to be tailored to the needs of the individual study species.

Insect embryology and timing of microinjections

Embryonic microinjection presents a number of challenges. Access to a plentiful supply of eggs for manipulation is ideal as is the ability to rear injected eggs to adulthood under laboratory conditions. In some species there are empirical constraints on egg availability. The tsetse fly, *Glossina palpalis*, is viviparous rendering embryos inaccessible (Geiger *et al.* 2005). Most transgene expression systems in *Apis mellifera* are transient: this is due to the nature of the honeybee's life cycle and the social

structure of a hive which make egg manipulation and post-injection rearing problematic (Robinson *et al.* 2000). However in stalk-eyed flies, although direct manipulations of embryos have not been attempted, previous studies have shown that newly fertilised eggs can be collected in large numbers and reared to adulthood in the laboratory without significant difficulty (David *et al.* 1998; Cotton *et al.* 2004; Author's personal observations).

For incorporation of DNA into the germline it is most efficient to deliver it before the prospective germ cells begin to proliferate and differentiate. In organisms that develop from a single cell embryo (e.g. vertebrates and crustaceans) microinjections are performed at the single or two-cell phase (Fadool *et al.* 1998; Pavlopoulos & Averof 2005). Stalk-eyed flies, being dipterans, undergo long-germ band embryonic development. This mode of development has been characterised in great detail in *D. melanogaster* (Campos-Ortega & Hartenstein 1985). Starting with a single nucleus, several rounds of nuclear division occur in the absence of cytokinesis forming a syncytium (several nuclei within one cell membrane). The nuclei migrate to the periphery of the embryo to create a syncytial blastoderm. Pole cell nuclei, which are the progenitors of the germline, are located at the posterior pole of the syncytial blastoderm stage embryo. DNA delivery to the posterior pole of these embryos therefore increases the probability of its incorporation into the gametes (Handler 2000; O'Brochta & Atkinson 2004). The pole cells are the first cells to develop individual plasma membranes. Once cellularisation is complete the newly-formed membranes present an additional barrier to DNA entry into the nucleus. It is critical that microinjections be carried out before the pole cells begin to cellularise. In *D. melanogaster* pole cell formation occurs about 1.5 hours after fertilisation. The timing of this event is unknown in *T. dalmanni* or any stalk-eyed fly species and needs to be determined.

Microinjection protocols

Microinjection at early embryonic stages potentially constitutes a major insult to an organism's development. Consequently it is critical to minimise the impact of microinjection and so increase survival rates. Techniques that were pioneered and developed in *D. melangaster* have formed the template for transformation protocols in most insect species (Loukeris *et al.* 1995a; Lobo *et al.* 2006a; Condon *et al.* 2007; O'Brochta & Handler 2008). These protocols address several specific issues including

the preparation of eggs prior to injection, design of injection needle to deliver the injection mix, damage due to desiccation and/or cytoplasmic leakage, and post-injection rearing of injected embryos.

Insect embryos are surrounded by a shell-like chorion, which varies in structure, hardness and opacity between species. This is relevant to decisions about how to prepare eggs for microinjection because hard chorions can prevent penetration of the needle into the cytoplasm. Moreover, a completely opaque chorion will prevent visual confirmation of needle penetration of the embryo and successful fluid delivery. In *D. melanogaster* the chorion is frequently removed by physical or chemical (bleach) treatment (Bachmann & Knust 2008). In many mosquito species, eg. *Aedes aegypti* and *Culex quinquefasciatus*, survival is not possible without the chorion so embryos are injected without dechorionation (Lobo *et al.* 2006a; Allen *et al.* 2001). In some mosquito transformation protocols, dextran sulphate is applied to inhibit the hardening of the chorion that occurs following oviposition (Morris 1997). Alternatively, injections can be timed so that they occur before the chorion hardens but after the point at which the action of microinjection will cause fatal damage to a soft embryo (Lobo *et al.* 2006a). In stalk-eyed flies the chorions are opaque and certainly harder than *D. melanogaster* chorions, but not as hard as those seen in mosquito species. It would therefore be prudent to explore microinjection of dechorionated embryos.

The nature of the chorion and whether embryos can survive without it will affect the needles required for microinjection. For the microinjection of embryos that have soft chorions or have been dechorionated, glass needles with gradual tapers are used which cause minimal damage upon injection (Fig. 2.1). For injection of embryos surrounded by a hard chorion, needles made from thicker glass with more severe tapers are required to give them the required strength to pierce the chorion and vitelline membrane without frequently breaking (Fig. 2.1; Miller *et al.* 2002). Larger needles will cause more damage to an embryo increasing the risk of mortality (O'Brochta & Atkinson 2004). Microinjection needles are generated by shaping glass capillary tubes using a heat-based glass pulling device (Miller *et al.* 2002). The most common type of capillary used are borosilicate glass needles as they are inexpensive and easy to shape. Aluminosilicate glass capillary tubes are less delicate but harder to shape. For mosquito injections the use of quartz needles is becoming increasingly popular because they are stronger, although they require less commonly available laser based pulling machines (Lobo *et al.*

2006a; O'Brochta & Atkinson 2004). In order to reduce mortality, a fine needle designed with a gradual taper resembling those typically used in transformation studies of *D. melanogaster* would be the best choice for dechorionated stalk-eyed fly embryos.

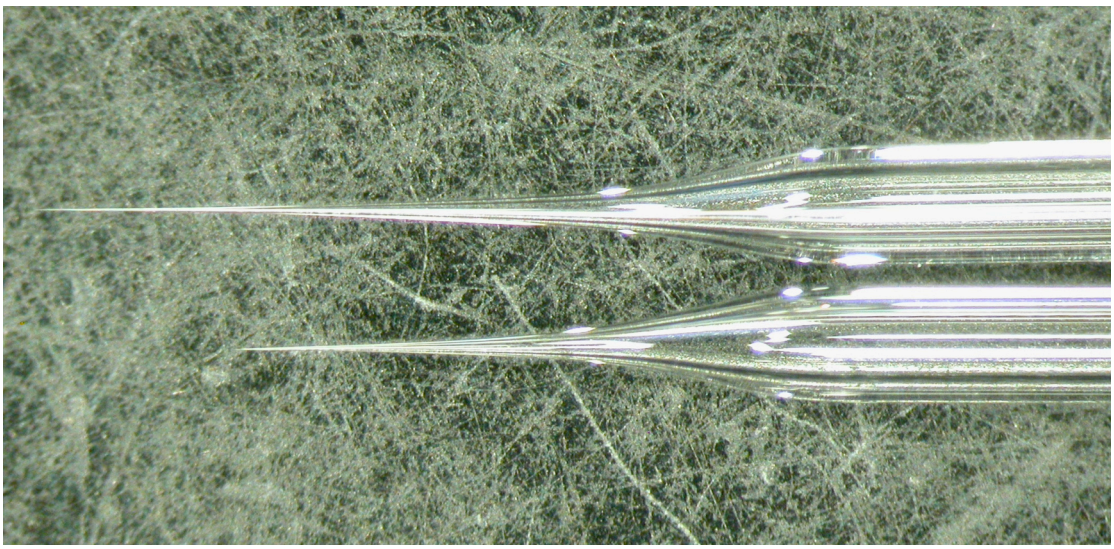

Figure 2.1. Different designs of microinjection needle. The upper needle (needle A) has a gradual taper and a longer tip than the lower needle (needle B), which results in a more delicate structure. Needle A would be used for the microinjection of dechorionated embryos of *D. melanogaster*. Needle B has a steep taper and short tip to give it more strength and is designed for injection of embryos with harder chorions from species such as *Ae. aegypti*.

Minimising damage associated with microinjection is a key concern in protocol design. The action of piercing the vitelline membrane and increasing the internal pressure of an embryo by inserting fluid can cause excessive leakage of cytoplasm. This can be reduced by a number of approaches. A brief period of desiccation prior to injection has been used to reduce the volume of liquid within the embryo thus enabling it to readily accommodate extra volume without a dramatic increase in internal pressure (Handler 2000; O'Brochta & Atkinson 2004). Alternatively, some experimenters perform two injections (O'Brochta & Atkinson 2004). The first injection is away from the posterior pole during which no fluid is delivered. This causes cytoplasmic loss but appears to cause less damage than the leakage from the posterior pole. The second injection delivers the injection mix to the posterior pole of the embryo. Note also that injection technique varies between experimenters and may have a large affect on survivorship. Variation due to the skill of individual experimenters may be controlled for by using an automated injection system such as that developed by Peloquin *et al.* (2000) for the pink bollworm, *Pectinophora gossypiella* but this is a not a widespread approach.

In many protocols, embryos are covered with halocarbon oil 700 during injection. This has two potential benefits. Firstly the high viscosity of the oil creates a pressure gradient which prevents excessive cytoplasmic leakage. Secondly the oil covering the embryos reduces desiccation during the injection procedure. However, halocarbon oil 700 has also been shown to have detrimental effects on larval survival in *Tribolium confusum* (Chang & Wade 1994) and *Musca domestica* where it is thought that the viscosity of oil reduces larval movement (O'Brochta & Atkinson 2004). In *M. domestica* a mixture of halocarbon oil 700 and halocarbon oil 27 is used to reduce viscosity and results in increased survivorship. Although the use of halocarbon oil is standard in most insect microinjection protocols, in some species, such as *C. quinquefasciatus* and all lepidopteran species, its application has been shown to be unnecessary (Allen *et al.* 2001; O'Brochta & Atkinson 2004; Marcus *et al.* 2004).

After microinjections, embryos are reared under optimal rearing and nutritional conditions, according to the particular requirements of the specific study species. When manipulating Drosophila and mosquitoes, a key issue is rapid transfer to defined food medium for development under sufficiently humid conditions. In species with relatively large embryos such as *M. domestica*, *Ceratitis capitata* and *Stomoxys calcitrans*, increased survivorship is seen when embryos are reared under conditions of elevated oxygen tension (O'Brochta & Atkinson 2004). Stalk-eyed fly embryos are not abnormally large (750μm in length) and there are well established laboratory procedures for the successful rearing of embryos to pupation and eclosion which should suffice (David *et al.* 1998; Cotton *et al.* 2004).

2.1.2 Vector selection

In insects, by far the most successful vectors used for stable transgene integration are class II DNA transposable elements (Atkinson *et al.* 2001). Individual transposable elements differ in their mechanisms of transposition and in their host range (Atkinson *et al.* 2001; Handler 2001). Transposable element activity can be tested using transposition and/or excision assays carried out in embryos or cell lines. Such assays were vital in demonstrating the limited species range of the *P*-element (O'Brochta & Handler 1988; Handler *et al.* 1993) and in developing transposable element vectors with activity in a wider range of hosts such as *mariner* (Coates *et al.* 1995), *piggyBac* (Thibault *et al.*

1999), *Hermes* (Sarkar *et al.* 1997a, b), *Minos* (Klinakis *et al.* 2000) and *hobo* (Handler & Gomez 1996).

Successful germline transformations also require stable integration of the transgene. Transposons of the same family as the transgene vector system can act as endogenous source of transposase and be capable of mobilising an integrated transgene rendering it unstable (Lobo *et al.* 1999; Atkinson *et al.* 1993; Handler & Gomez 1995). Such effects can be tested for by performing a transposition or excision assay without an exogenous source of transposase. Activity without an exogenous source of transposase indicates the presence of an endogenous source of transposase in the genome. For example *hobo* based vectors show activity in *M. domestica* due to the presence of the closely related *Hermes* elements (Atkinson *et al.* 1993). *Hobo* is therefore not considered a viable vector for transgenesis in *M. domestica*.

A range of plasmid based assays are available to test the activity of a transposon within embryos of the organ of study. These can be divided into transposition and excision assays. A third factor influencing the choice of vector system is the availability of an effective source of exogenous transposase.

Transposition assays

In transposition assays two plasmids, the donor and the target plasmid, are co-injected into embryos (or introduced to available cell lines) with and without a source of transposase (Fig. 2.2A). The donor plasmid contains a set of selectable markers flanked by the terminal inverted repeats (TIRs) of the transposon. The target plasmid contains a different set of selection properties from the donor plasmid. After a period of time, DNA, including the plasmids, is extracted from the embryos or cell lines and used to transform *Escherichia coli* cells. If the transposon has excised from the donor plasmid and then inserted into the target plasmid, then the resulting plasmid will possess a unique set of selection properties that can be observed in the transformed *E. coli* cells.

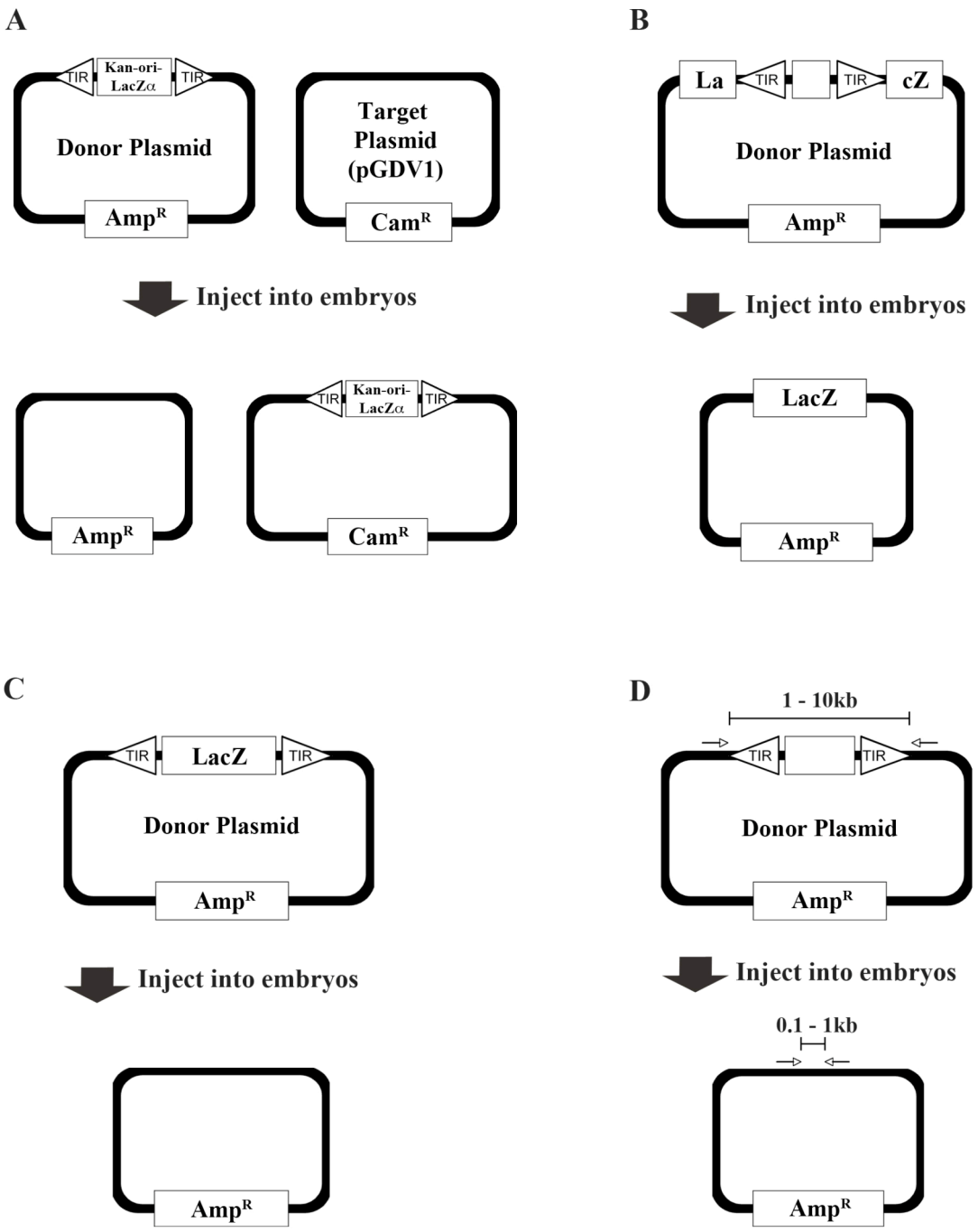

Figure 2.2. Transposition and excision assays. In all cases the donor plasmid is injected into embryos with an exogenous source of transposase. The plasmid DNA is then extracted and used to transform *E.coli* or used as a template for PCR. TIR = transposon terminal inverted repeats, AMP^R = Ampicillin resistance gene, CAM^R = Chloramphenicol resistance gene, LacZ = *lacZ* gene. **A**. Transposition assay (Lobo *et al.* 1999): the target plasmid, pGDV1, is injected along with the donor plasmid. The donor plasmid contains the *lacZ* gene, a kanamycin resistance gene and an *E.coli* origin of replication flanked by the transposon terminal inverted repeats. If the transposition into pGDV1 occurs, *E.coli* transformed with the resulting plasmid will be resistant to chloramphenicol and kanamycin as well as producing blue colonies in the presence of X-gal. **B**. Excision assay – *lacZ* restoration (Rio *et al.* 1986): the donor plasmid contains the transposon of interest

interrupting the *lacZ* gene. Excision will result in the *lacZ* no longer being interrupted. *E.coli* cells transformed with the excised plasmid will produce blue colonies in the presence of X-gal. **C**. Excision assay – *lacZ* removal (O'Brochta *et al.* 1991): TIRs flank the *lacZ* coding sequence. Excision results in the removal of the *lacZ* gene from the donor plasmid and *E. coli* cells transformed with the excised plasmid will produce white colonies in the presence of X-gal. **D**. PCR based excision Assay (Klinakis *et al.* 2000): PCR primers (arrows) are designed that flank the transposon. With the transposon present on the donor plasmid the primers are too far apart to produce a PCR product under standard PCR conditions. Upon excision the primers will be located closer to one and other and capable of producing a PCR product under standard PCR conditions.

A common transformation assay used by Lobo *et al.* (1999) to test *piggyBac* activity in *D. melanogaster, Ae. aegypti* and *Trichoplusia ni* illustrates this technique (Fig. 2.2A). The donor plasmid contained an ampicillin resistance gene, and the *piggyBac* terminal inverted repeats flanking a *LacZ* gene, an *E. coli* origin of replication and a kanamycin resistance gene. The target plasmid, pGDV1, contained a chloramphenicol resistance gene, lacked an *E. coli* origin of replication but was capable of replication in *Bacillus subtilis*. If the transposon inserted into the target plasmid, it was detected due to its ability to support the growth of *E. coli* transformants on media containing chloramphenicol and kanamycin as well as producing blue colonies in the presence of X-gal. This assay has been used in a wide range of species to test a variety of elements with slight variations of the selectable markers flanked by the transposon (Lobo *et al.* 1999; Coates *et al.* 1997; Coates *et al.* 1998; Horn & Wimmer 2000; Thibault *et al.* 1999; Ren *et al.* 2006; Lobo *et al.* 2006b; Sarkar *et al.* 1997a, b; Pledger *et al.* 2004; Pledger & Coates 2005).

Klinakis *et al.* (2000) produced a variation based on similar principles that has been used to test *Minos* in a wide range of species (Catteruccia *et al.* 2000; Shimizu *et al.* 2000; Zhang *et al.* 2002; Pavlopoulos & Averof 2005). The donor plasmid possesses a tetracycline resistance gene flanked by TIRs of the *Minos* transposon. The target plasmid possesses a chloramphenical resistance gene as well as the *SacRB* gene, which inhibits *E.coli* growth on sucrose media. Transposition is only detected when insertion into the *SacRB* gene has occurred thus disrupting the gene. Both donor and target plasmid possess an *E. coli* origin of replication, which means that culturing *B. subtilis*, a non-standard procedure in most laboratories, is not necessary.

Excision assays

Transposition events are rare and therefore transposition assays can prove time consuming due to the high number of bacterial transformations required and subsequent testing of transformed bacteria on selective media to fully demonstrate a transposition event has occurred. Excision assays are simpler and more sensitive than transposition assays as they only evaluate the ability of the transposase to excise TIR flanked sequences. A single donor plasmid is injected into embryos with a source of transposase, plasmid DNA extracted from injected embryos is used to transform bacteria and the transformants analysed for the expression of various marker genes. Donor plasmids are similar in design to the donor plasmids of a transposition assay. TIR flanked sequences may be present as an insert in a visible marker gene such as *lacZ* (Fig. 2.2B; Rio *et al.*1986) or alternatively selectable or visible markers may be flanked by the TIRs of the transposon (Fig. 2.2C; O'Brochta *et al.* 1991; Handler *et al*. 1993; Handler & Gomez 1995, 1996). The latter method was used to show that the range of *P*-element activity is restricted to *Drosophilid* species and that *mariner* is active in a wide range of insect species including *Lucilia cuprina* and *Bactrocera tyroni* (Coates *et al.* 1995). The exact nature of the excision event can be ascertained by sequencing excised plasmids. An alternative to using marker gene expression/non-expression to detect excision is to insert a unique restriction site between the TIRs in the test plasmid (Ren *et al.* 2006; Elick *et al.* 1997; Thibault *et al.* 1999; Catterrucia *et al.* 2000). After injection and recovery, the plasmid DNA is treated with restriction enzyme. Unexcised plasmids will be cut at the restriction site but excised plasmids which lack the site will be protected from digestion and remain capable of transforming *E. coli* cells.

Excision is a rare event and detection can require the screening of anywhere between 1,000 and 10,000,000 bacterial colonies in order to detect one excision event, therefore such assays need to be performed on a large scale if they are to be reliable (Ren *et al.* 2006; Takahashi *et al.* 2008). Klinakis *et al.* (2000) developed a potentially more sensitive PCR-based assay for detection of excision in *Minos* based vectors (Fig. 2.2D). Primers were designed that flanked the transposon so that excision events would bring them closer together and significantly reduce the size of the predicted PCR product. The assay has been successfully applied in *Gryllus bimaculatus* (Zhang *et al.* 2002), in *Parahayle hawaiiensis* (Pavlopoulos & Averof 2005), *D. melanogaster* and *C. capitata* (Kapetanaki *et al.* 2002). PCR based excision assays are fast, extremely sensitive and

simple to run requiring only a single PCR reaction to be performed on the DNA extracted from test plasmid injected embryos, so are an attractive choice for testing transposon activity.

Source of transposase

Transposition events are mediated by transposase enzymes the source of which has a large affect on transformation efficiency. The most common source of transposase in transformation experiments is a co-injected helper plasmid containing the transposase gene under the control of a constitutive promoter (O'Brochta & Atkinson 2004). The gene is transcribed and translated in the embryo. Both transcription and translation of the transposase needs to be reliable and efficient when using DNA sources of transposase and the promoter driving the expression of the exogenous source of transposase will have an effect on transformation efficiency (Li *et al.* 2001; Heinrich *et al.* 2002). The use of a capped mRNA source of transposase has been observed to increase transposition frequency, possibly because translation and transposition can occur earlier in development as transcription of the gene is not required (Kapetenaki *et al.* 2002; Heinrich *et al.* 2002). In addition it removes variation due to inter-species differences in promoter efficiency.

Using an mRNA source of transposase for *Minos* saw increases in transformation efficiency compared to previous studies in *D. melanogaster* (2.1% vs 22.7% Loukeris *et al.* 1995b; Kapetanaki *et al.* 2002) and in *C. capitata* (1% vs 6%, Loukeris *et al.* 1995a; Kapetanaki *et al.* 2002) although this is comparing different experiments. In germline transformations of *Tribolium castaneum* (Pavlopoulos *et al.* 2004) and *B. mori* (Uchino *et al.* 2007) dramatic increases in transformation efficiencies are seen with the use of an mRNA source of transposase when directly compared to a helper plasmid source of transposase. The availability of an mRNA source of *Minos* transposase is therefore an additional factor to consider when selecting the vector system for transgenesis in stalk-eyed flies.

2.1.3 Experimental outline

In this chapter I describe the development of a robust microinjection protocol for *T. dalmanni* embryos. Initial observations identified the location and timing of pole cell

formation in stalk-eyed fly embryos and hence the optimal time frame for effective microinjections. Two embryo preparation methods were evaluated for their effects on survival to pupation of uninjected *T. dalmanni* embryos. These methods were based on protocols previously used in successful transformation studies in *D. melanogaster*. The best performing embryo preparation method was adopted for trial microinjections during which modifications to the protocol, including chemically treating embryos and variation in needle design, were evaluated. Survival of microinjections ranged from 12%-18%.

PCR based excision assays were used to test the activity of three potential transposable element vectors *Minos, mariner and piggyBac*, which have all been shown to work in a wide range of species. A previously developed assay for *Minos* was available (Klinakis *et al.* 2000) and new primers were designed for use with *mariner* and *piggyBac* donor plasmids (Handler & Harrell 1999; Coates *et al.* 1998). *Mariner* showed no detectable activity but both *piggyBac* and *Minos* underwent excision in *T. dalmanni* embryos and are therefore candidate vectors for transgenesis in this species. *Minos* was shown to excise in the presence of either an mRNA or a DNA source of transposase and this feature has the potential to increase the efficiency of germline transformation in stalk-eyed flies.

2.2 MATERIALS AND METHODS

Stock population, experimental flies and egg collection

A laboratory-adapted population of *Teleopsis dalmanni* founded from flies collected from Ulu Gombak, Peninsular Malaysia in 1993 was used for these experiments. Laboratory flies have been maintained in cage culture at 25ºC, in a 12h:12h light:dark light cycle regime and fed pureed sweetcorn twice weekly. Population size has been kept high (> 200 individuals) in an effort to minimise inbreeding. In order to obtain a sufficient supply of eggs for experiments, groups of 9-15 sexually mature adult flies (with a ratio of 2:1 females:males) were kept in inverted 1.5L plastic containers with bases comprising damp cotton wool and filter paper together with sweetcorn which was replaced three times weekly. The cotton wool bases were lined with dark blue paper which allowed eggs to be seen easily. Two populations of 40-50 groups were maintained to provide a sufficient supply of eggs. In order to obtain synchronised batches of eggs, the blue paper discs were replaced and the flies allowed to lay eggs for between 1 and 1.5 hours. At the end of the oviposition period, eggs from all groups were transferred using a paintbrush to a moist paper towel and pooled together prior to being placed on "egg-lays". These consisted of a Petri dish containing a moist cotton wool base, filter paper and pureed sweetcorn. The composition of the egg-lays enabled normal development of fertilised eggs and larvae to proceed. Fertilised *T. dalmanni* eggs typically hatch 2-3 days after being laid (Baker *et al.* 2001).

2.2.1 Transgene delivery

Embryology

To estimate the timing of pole cell formation, embryos were observed at hourly intervals between 3 and 8 hours post laying (hpl). Taking note of the orientation of the chorion throughout, eggs were placed on double-sided sticky tape and left to desiccate for 3-5 minutes. Gentle lateral pressure was applied with forceps to the embryos in order to break the chorion. The dechorionated eggs were then placed, using forceps, in halocarbon oil 700 in a depression slide, observed under a light compound microscope and photographed with a Nikon P5100 digital camera.

Embryo preparation protocols and post-operative care

Two methods of embryo preparation were trialled. One involved the preparation of dechorionated embryos and was based on an established method for *D. melanogaster* (Rubin & Spradling 1982; Handler 2000). Eggs were collected after a 1.5 hour laying period and then dechorionated on double-sided sticky tape using gentle lateral pressure applied by forceps. 10-25 dechorionated embryos were arranged side by side (not touching) in a line on a square of moist agar. A heptane based glue (G. Vinti pers. comm.) was spread in a thin line along the centre of a glass slide. The glass slide was inverted and then briefly and gently laid on top of the embryos so that they would stick to the line of glue on the slide. The embryos were left for five minutes to desiccate before being covered in halocarbon oil 700. A square was drawn around the halocarbon oil using a wax crayon to prevent the halocarbon oil running off the slide. The slide was placed on an egg-lay and the embryos left to develop at 25ºC. After 3 days any first instar larvae remaining on the slide were transferred to the egg-lay with a paintbrush.

The second method involved the preparation of embryos in their chorions and was based on prior protocols used in many insect species (Lobo *et al.* 1999; O'Brochta & Atkinson 2004; Gompel pers comm.). Eggs were collected after a 1.5 hour laying period and pooled on a damp paper towel. 10-30 embryos were arranged side by side on a coverslip laid on a slide and left for 5 minutes to desiccate and attach (to the coverslip). The embryos were then covered with halocarbon oil 700. After one hour as much of the halocarbon oil was removed as possible and the slide placed at an angle on an egg-lay and kept at 25ºC.

In a later modification of the protocol, embryos were treated with bleach prior to being transferred to the slide to evaluate the consequences for survival of softening of the chorions prior to microinjection. The damp paper towel containing the embryos was placed on cotton wool saturated with 50% bleach for 2 minutes. The bleach was removed by placing embryos on three successive water-saturated cotton wool pads.

For both protocols control embryos were directly transferred to egg-lays (10-25 per egg-lay) after collection and maintained at 25ºC. Survivorship of experimental and control embryos was recorded at hatching and pupation.

Microinjection needles

Needles for microinjection were made from borosilicate glass capillaries (Harvard apparatus, UK) and pulled using a P-97 needle puller (Sutter Instruments, USA). Two designs of needle were used for microinjections. Needle A was made from a glass capillary with length of 100mm, outer diameter of 1.0mm, inner diameter of 0.78mm, and shaped using the programme: heat = 200, pull = 445, velocity = 80, time = 0, delay = 90, based on a design for *D. melanogaster* microinjections (G. Vinti pers. comm.). Needle B was designed with reference to Miller *et al.* 2002 and made from a glass capillary with length of 100mm, outer diameter of 1.0mm, inner diameter of 0.58mm, and shaped using the programme: heat = 489, pull = 0, velocity = 90, time = 0, delay = 200.

Microinjection

To minimise dust, which could block needles, injection buffer (15μm NaH_2PO_4, 850μm Na_2HPO_4, 5mM KCl at pH7.6-7.8, 0.05% phenol red (Sigma)) was passed through a 2μm Acrodisc syringe filter (Pall Corporation, UK). Prior to use, the injection mix was centrifuged on a bench top centrifuge for 15-30 minutes at 14,000rpm. The supernatant was transferred to a fresh tube and used to load needles. Needles were filled by placing a drop of injection buffer at the blunt end of the needle and allowing the buffer to flow to the tip of the needle via capillary action.

Microinjections were performed on an inverted microscope (IDOC, Zeiss, Ukraine) by attaching the needle to a micromanipulator (MN-153, Narishige, UK), an electric microinjector (IM-30, Narishige, UK) and an oil air compressor (Jun-air, Norgren, Denmark). Embryos were arranged in a line and attached to a coverslip while still in their chorions. The coverslip was then placed on the microscope stage with the posterior ends of the embryos facing the needle. The needle was positioned at a shallow angle (10 º to 25º) so that the tip was at the same height as the embryos. The needle tip was opened by gentle agitation with a pair of forceps or against the chorion of an embryo. Embryos were injected by laterally moving them onto the microinjection needle via manipulation of the microscope stage. A short burst of pressure was applied to deliver the injection mix. After injections, virtually all of the halocarbon oil was removed and the coverslip containing the embryos transferred to an egg-lay and maintained at 25ºC.

2.2.2 DNA vector selection

Excision assay plasmids and mRNA

The donor plasmids used for each assay were: *piggyBac* - pBac[3xP3-EGFPafm] (Berghammer *et al.*1999), *mariner* - pMos[3xP3-EGFPafm] (Horn *et al.* 2000), *Minos* - pMiLRTetR(L) (both assays; Klinakis *et al.* 2000). The helper plasmids for each assay were: *piggyBac* - phsp-pBac (Handler and Harrell 1999), *mariner* - pKhsp82MOS (Coates *et al.* 1998) and *Minos* - pHSS6hsILmi20 (Klinakis *et al.*2000). *piggyBac* and *mariner* plasmids were kindly donated by E. Wimmer (George August Universität, Göttingen, Germany) and *Minos* plasmids by C. Savakis (University of Crete, Greece).

To generate necessary amounts of donor and helper plasmid DNA for microinjection, Sub-Cloning Efficiency^{TM} DH5α^{TM} *E.coli* cells (Sigma, UK) were transformed, cultured and plasmid DNA purified using the Qiagen Endofree Plasmid Maxi Kit^{TM} (Qiagen, UK). The DNA pellet was dissolved in 50µl TE buffer and its concentration measured using an Eppendorf Biophotometer (Eppendorf, UK). *Minos* transposase mRNA was synthesised using the MEGAscript® T7 Kit (Ambion Inc., UK) and pBlueSKMimRNA linearised with *NotI* as the template (Pavlopoulos *et al.* 2004).

Excision assay primers

All primer sequences are given in Table 1. Primers were designed using the programs primer3 (http://frodo.wi.mit.edu/) and Amplify 3x (Bill Engels, University of Wisconsin, USA) and ordered from MWG (Europe) They are listed in table 2.1. For the *piggyBac* and *mariner* assays, a pair of primers flanking the transposon were designed to detect excision (PbacFor1 & PbacRev1; MosRev1 & MosFor1). For the *Minos* excision assay, primers from Klinakis *et al.* 2000 were used (MiR-hydei & MiL-Lorist). A pair of primers based on the donor plasmid backbone and therefore outside of the TIR flanked transposon was designed to test for efficient extraction of the donor plasmid (PbacConf1 & PbacConf1 for *piggyBac*, MosConf1 & MosConr1 for *mariner* and Lorist6conl1 & Lorist6conr1 for *Minos*, see table 2.1). *piggyBac* primers were designed based on the available sequence of the donor plasmid pBac[3xP3-EGFPafm]. The published sequences for the *mariner* donor plasmid pMos[3xP3-EGFPafm] were

incomplete. The relevant regions were sequenced and primers designed based on the sequences obtained.

Assay	*Test*	*Primer Name*	*Sequence (5'-3')*	*Expected band size with excised donor plasmid (bp)*	*Expected band size with control plasmid (bp)*
piggyBac	Excision	PbacRev1	GGTCGAGTAAAGCGCAAATC	269	361
		PbacFor1	TACGCACCATATACGCATCG		
	Plasmid extraction	PbacConf1	AGGAGAAAATACCGCATCAG	450	450
		PbacConr1	CACAGATTTAAAGAACCAAAA		
mariner	Excision	MosRev1	GCGTAAGAACGGGGACCTA	402	510
		MosFor1	ATTAGGCACCCCAGGCTTTA		
	Plasmid extraction	MosConf1	AACGATCAAGGCGAGTTACA	246	246
		MosConr1	GTATTGACGCCGGGCAAG		
Minos	Excision	MiR-hydei	TGCATTCTCTATGCT	211	501
		MiL-Lorist	CCAGCTGGCTTATCGAAA		
	Plasmid extraction	Lorist6conl1	GGCTGAACACACCGTTGAT	465	465
		Lorist6conr1	GGGACAGCAGAAGACCTGAC		

Table 2.1. Primers used in excision assays. Primers were designed to either test for excision of the transposon or for successful extraction of the donor plasmid from the injected embryo. MiR-hydei and MiL-Lorist were designed by Klinakis *et al.* (2000).

Excision primers were tested using modified donor plasmids as templates for PCR. In the unmodified donor plasmids, primers were too far apart to produce a reliable amplifiable product under standard PCR conditions. To generate derivatives in which part of the sequence between the two primer sites had been deleted, each donor plasmid was digested with an appropriate restriction enzyme: (pBac[3xP3-EGFPafm] with *EcoRV*; pMos[3xP3-EGFPafm] with *HindIII*; pMiLRTetR(L) with *NotI*). Digested plasmid fragments were treated with T4 DNA ligase (Invitrogen, UK) and the mix used to transform Sub-Cloning Efficiency^{TM} DH5α^{TM} *E.coli* cells (Sigma, UK). DNA was prepared from transformant colonies using the Qiagen Miniprep Kit (Qiagen, UK). Deletion-carrying derivatives of the original donor plasmids were identified by restriction analysis.

Excision assay

50-100 eggs were injected with 250µg/ml of both helper and donor plasmid in injection buffer for the *piggyBac* and *mariner* excision assays. For the *Minos* assays, embryos were injected with 300µg/ml of donor plasmid and 150µg/ml helper plasmid or 500µg/ml donor plasmid and 300µg/ml transposase mRNA. 50-100 embryos were also injected with the donor plasmid for each assay without an exogenous source of transposase.

DNA was extracted following a 48 hour incubation period. Eggs were placed in 10µl HM buffer (0.1M NaCl, 0.2M Sucrose, 0.1M tris-HCl pH9.1, 0.05M EDTA, 0.5% SDS) and homogenised using a flame blunted pipette tip. 90µl of HM buffer was added and left to stand for 20 minutes at room temperature. 25µl of 5M NaCl was slowly added and then left at 4°C for 8-16 hours. The mix was then centrifuged at 14,000rpm at 4°C for 30 minutes. The supernatant was carefully removed and placed in a new eppendorf tube and the precipitate discarded. An equal amount of a 1:1 phenol/chloroform (1:1) was added and centrifuged at 10,000rpm for 5 minutes at room temperature. The aqueous (upper) layer was removed and placed in a fresh eppendorf tube and the lower layer discarded. An equal volume of chloroform/isoamyl alcohol (24:1) was added. The mix was centrifuged at 10,000rpm and the aqueous layer removed and placed in a fresh eppendorf tube. To precipitate the DNA, 0.1 volumes of 3M sodium acetate ($NaCH_3COO$, pH5.2) along with 2-2.5 volumes of chilled 100% ethanol was added. The mix was incubated on ice for 20 minutes. The mixture was centrifuged for 10-15 minutes at 10,000rpm, the supernatant was discarded and 1ml of 70% ethanol was added. After 10-15 minutes of centrifuging at 10,000rpm the supernatant was removed and discarded. The pellet was air-dried for 5-10 minutes before being dissolved in 20µl TE buffer (Tris 10mM, EDTA disodium 1mM, pH 8).

50-500ng of the DNA was used as a template for PCR (GoTaq PCR, Promega, UK): 95°C for 10 minutes; 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute; 72°C for 10 minutes. PCR reactions were run on either a Master Cycler Gradient thermocycler (Eppendorf, UK) or a GeneAMP® PCR System 2700 thermocycler (Applied Biosystems, UK) and the products analysed on a 1.7% agarose gel.

Mariner sequencing

The flanking sequences of mariner transposon on pMos[3xP3-EGFPafm] were sequenced using primers given in Table 2.2. The primers MseqLL and MseqLR were used to obtain genomic DNA flanking the left arm of the transposon. The primers MseqRR and MseqRL were used to sequence the genomic DNA flanking the right arm of the transposon.

Primer Name	*Sequence (5' - 3')*	*Position of primer ‡*
MseqLL	TAGCTAGCGACGGCAAATAC	Plasmid, left arm
MseqLR	CATGATTACGCCAAGCTC	Transgene, left arm
MseqRR	GTGGTTCGACAGTCAAGGT	Plasmid, right arm
MseqRL	GCTGCAAGGCGATTAAGT	Transgene, right arm

Table 2.2. Primers used for sequencing the flanking regions of the insert in the plasmid pMos[px3-eGFPafm] (Horn *et al.* 2000). ‡ For the plasmid, pMos[px3-EGFPafm] the DNA sequence flanking the terminal inverted repeats of transgene were unknown. Sequencing primers were designed either side of the unknown DNA sequence on known sequences of the plasmid backbone and the transgene for each arm of the transgene.

A sequencing reaction mix was made with the following: 4µl of Big Dye® Terminator v1.1 pre-mix (Applied Biosystems, UK), 2µl of 1.6µM primer, 150-500ng plasmid DNA made up to 10µl with ddH_20 and run on a Master Cycler Gradient (Eppendorf, UK): 96°C for 3 minutes, 25 cycles of 96°C for 3 minutes, 50°C for 15 minutes, 60°C for 4 minutes. The products were purified by adding 10µl ddH_20, 2µl 3M sodium acetate ($NaCH_3COO$, pH5.2) and 50µl 100% ethanol. After a 10 minute incubation on ice, samples were centrifuged for 15 minutes at 12,000rpm at 4°C on a bench top centrifuge. The supernatant was removed and discarded. 60µl of 70% ethanol was added and the mixture spun at 12,000rpm for 5 minutes at room temperature. The supernatant was removed and discarded and the pellet air-dried for 10 minutes before being dissolved in 10µl TE buffer and being analysed on an automated capillary DNA sequencer (ABI Applied Biosystems 3100-Avant Genetic Analyzer) by Stuart Martin (UCL).

2.3 RESULTS

2.3.1 Transgene Delivery

Embryology

Despite lacking obvious anterior or posterior markers, the two longitudinal poles of the *T. dalmanni* egg are distinct (Fig. 2.3A). One end possesses a gradual taper leading to a blunt edge, whereas the other end has a more severe taper leading to a pointed edge. In older embryos viewed under halocarbon oil to reveal embryo morphology the posterior pole of the embryo is located at the pointed end of the egg.

To calculate the timing of pole cell formation, embryos were observed under a light microscope from 3 to 8 hpl (Fig. 2.3B). There was no evidence of cells forming before 4 hpl but cellularisation had clearly occurred by 6 hpl.

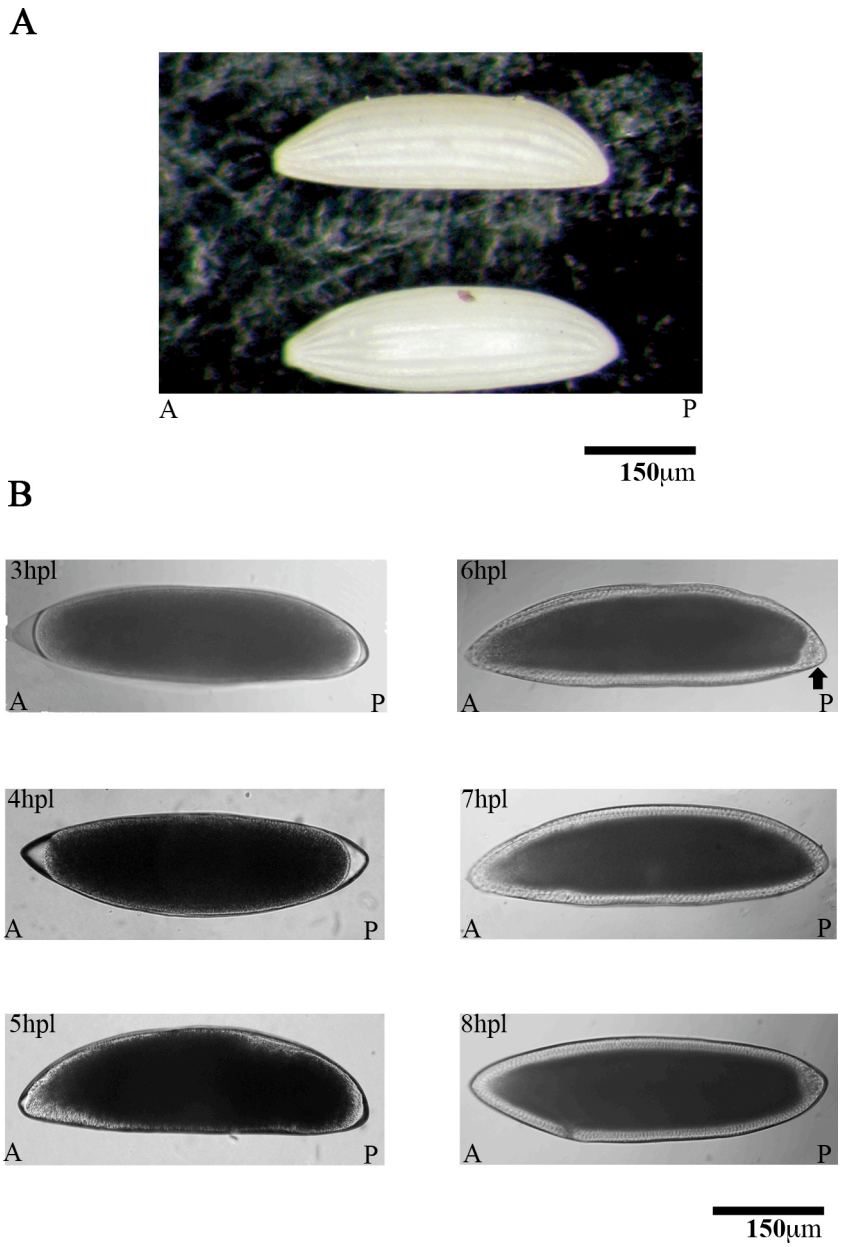

Figure 2.3. Stalk-eyed fly embryo morphology and development. Anterior (A) is to the left and posterior (P) is to the right in all panels. **A.** Light microscope photographs of a pair of *T. dalmanni* embryos with their chorions attached and demonstrating the distinct morphology of the poles. A more severe taper is seen at the posterior pole, which is pointed while the anterior pole appears blunt. **B.** Light microscope photographs of embryos of *T. dalmanni* from 3 hours post laying (hpl) to 8hpl. Pole cells (arrow) are evident from 6 hpl. Photographs show typical embryos for each time point. 10-20 embryos were observed for each time point.

Embryo preparation for microinjection

Prior to commencing microinjections, trials of two embryo preparation protocols were carried out. The first protocol was based on standard methods using in *D. melanogaster* transformations and involved dechorionation of the embryos (Table 2.3; Rubin & Spradling 1982). Although the proportion of dechorionated eggs giving rise to first instar larvae was high (65%), dechorionation had a significant deleterious effect on survival to pupation (dechorionation treatment 7.5%, control 48.7%; $\chi^2 = 68.1$, $df = 1$, $p < 0.0001$).

Method	*Treatment Group*	*Total number of eggs*	*First instar larvae (%)*	*Pupae (%)*
Dechorionation	Experimental	160	104 (65)	12 (7.5)
	Control	166	Not recorded	81 (48.7)
Non-dechorionation (trial 1)	Experimental	143	82 (57.3)	55(38.4)
	Control	143	55 (38.5)	44 (30.8)
Non-dechorionation (trial 2)	Experimental	435	332 (80.5)	263 (60.5)
	Control	220	144 (65.5)	117 (53.2)

Table 2.3. Survival following 'dechorionation' and 'non-dechorionation' protocols for embryo preparation. Control embryos were placed directly onto egg-lays with a minimum of handling/manipulation. Number of individuals produced at each developmental stage is given. Number of individuals as a percentage of the number of eggs manipulated is given in brackets. Trial 1 and trial 2 of the non-dechorionation protocol used eggs that had been generated by different independent samples of flies.

In an attempt to improve survivorship, a second protocol was tested that used intact embryos, which were fixed to a coverslip by desiccation (Table 2.3) (Gompel pers. comm.). In two replicate trials, survival to pupation of non-dechorionated embryos was similar to that of the untreated control embryos (trial 1, $\chi^2 = 1.87$, $df = 1$, $p = 0.17$; trial 2, $\chi^2 = 3.18$, $df = 1$, $p = 0.07$). The survival rates were significantly greater in trial 2 than in trial 1 for experimental treatments and controls (treatment: $\chi^2 = 42.01$, $df = 1$, $p < 0.0001$; controls, $\chi^2 = 17.63$, $df = 1$, $p < 0.0001$). The replicate trials were conducted at different times and a likely explanation of their differences in survivorship is that there was temporal variation in the fertility of the females that produced the eggs for each trial. In both trials, survival to pupation was significantly better than the procedure using dechorionated embryos (Trial 1: $\chi^2 = 131.9$, $df = 1$, $p < 0.0001$; trial 2: $\chi^2 = 42.03$,

df = 1, $p < 0.0001$). As a result the intact method of embryo preparation was used for future microinjections.

Microinjection

The presence of hard chorions may hinder microinjections and cause needles to break. Modifications to the injection protocol designed to counteract the chorion effect were trialled and their effects on survival evaluated. One trial assessed the effect on survival of using bleach to soften the chorions prior to injection (Table 2.4). Experimental groups (microinjection with and without bleach) did not differ in their survival to pupation (bleach 12.0%, no bleach 13.7%, $\chi^2 = 0.32$, *df* = 1, $p = 0.57$). Both experimental treatments suffered significant reduction in survival to pupation relative to the control (non-injected) group (bleach treatment, $\chi^2 = 58.38$, *df* = 1, $p < 0.0001$; no bleach treatment, $\chi^2 = 58.22$, *df* = 1, $p < 0.0001$). As expected microinjection significantly impaired survival. Bleach treatment had no significant effect and involved additional effort so was not used in subsequent microinjection procedures.

Treatment	***Number of eggs microinjected***	***First instar larvae (%)***	***Pupae (%)***
Bleach	225	28 (12.4)	27 (12.0)
No bleach	270	67 (24.8)	37 (13.7)
Control	294	208 (70.7)	126 (49.6)

Table 2.4. Survival following microinjection of eggs treated with and without bleach treatment. Number of individuals produced at each developmental stage is given. Survival as a percentage of embryos microinjected is given in brackets. Control embryos were placed directly onto egg-lays with a minimum of handling/manipulation.

The needles used for evaluation of the bleach treatment were based on a design developed for injection of dechorionated *D. melanogaster* embryos (denoted needle A, Fig. 2.1). Needle A broke frequently when injecting stalk-eyed fly embryos. A needle with a thicker circumference and a more severe taper was designed (needle B). These attributes would give the needle more strength resulting in less frequent breaking but the increased external diameter might cause more damage. Hence a further trial compared the effects of needle A and needle B on survival (Table 2.5). Microinjections using the two types of needle did not differ significantly in survival to pupation (needle A 15.6%, needle B 18.1%, $\chi^2 = 0.21$, *df* = 1, $p = 0.65$). Both experimental treatments suffered

significant reduction in survival to pupation relative to the control (non-injected) group (needle A, χ^2 =23.63, df = 1, p < 0.0001; needle B, χ^2 = 20.32, df = 1, p < 0.0001).

Treatment	***Number of eggs injected***	***Pupae (%)***
Needle A	90	14 (15.6)
Needle B	94	17 (18.1)
Control	122	58 (47.5)

Table 2.5. Survival following microinjection of eggs using two different designs of injection needle. Total number of pupae produced is given for each treatment and in brackets as a percentage of total number of eggs injected. Control embryos were placed directly onto egg-lays with a minimum of handling/manipulation.

2.3.2 Vector selection

Excision assays

PCR-based excision assays were performed for *piggyBac, mariner* and *Minos* with and without a DNA source of transposase and additionally for *Minos* with and without an mRNA source of transposase. In all cases plasmids were successfully delivered to, and extracted from, the embryos as shown by positive results for PCR primers situated on the donor plasmid backbone (Fig. 2.4A-D). DNA extracted from uninjected embryos was used as a negative control (data not shown).

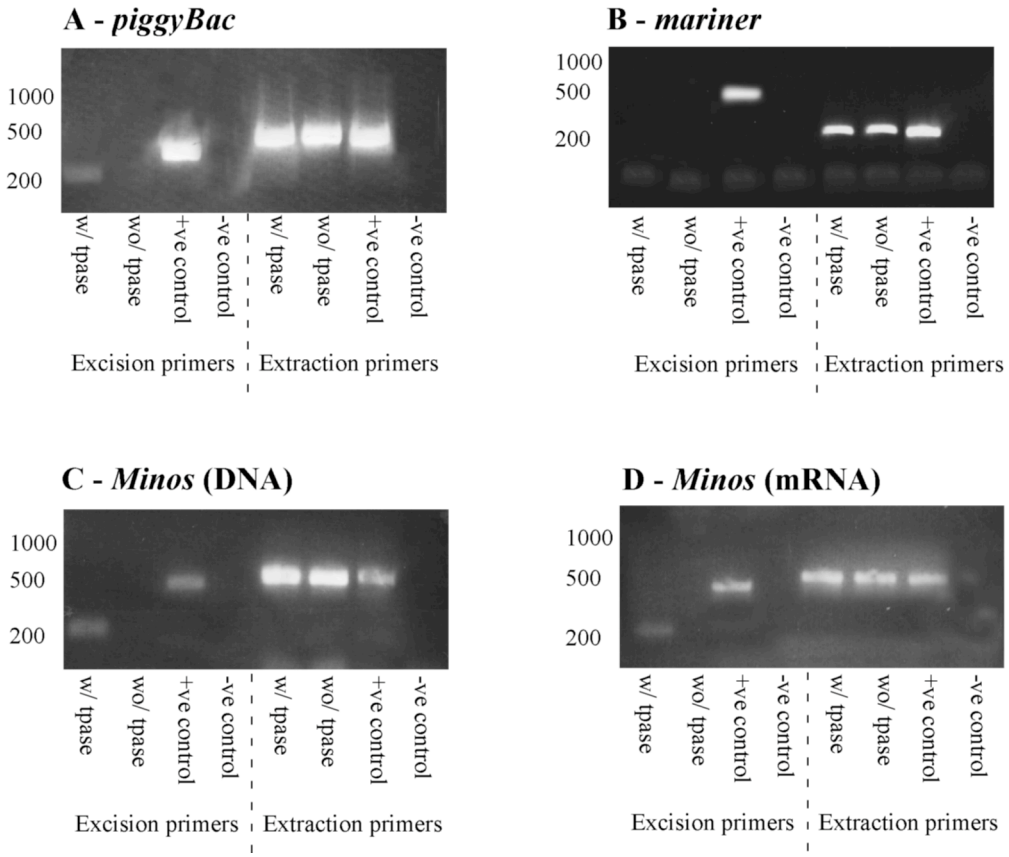

Figure 2.4. Results for PCR-based excision assays for the transposable elements: *piggyBac* (**A**), *mariner* (**B**) and *Minos* (**C**&**D**). Templates for PCR were as follows: DNA extracted from embryos injected with donor plasmid and a source of transposase ("w/ tpase"); DNA extracted from embryos with donor plasmid without a source of transposase ("wo/ tpase"); Donor plasmids with the transposase removed, referred to as control plasmids in Materials and Methods ("+ve control"); Water (H_2O). PCR reactions either used "excision primers" to test for excision of the plasmid, or "extraction primers" to test for successful extraction of the plasmids from injected embryos (see table 2.1). For the *Minos* assays both a DNA source of transposase (**C**) and an mRNA source of

transposase were tested (**D**). Excision was detected for *piggyBac* and both *Minos* assays when the donor plasmid was injected with a source of transposase but not in the *mariner* assay. In all cases no excision was detected when donor plasmids were injected without a source of transposase. In all cases donor plasmids were successfully extracted from embryos.

2.4 DISCUSSION

A robust protocol has been developed for embryonic microinjection of the stalk-eyed fly, *T. dalmanni.* Survival of injected eggs to pupation was between 12% and 18% which is comparable to survival rates observed in other dipteran species (Atkinson *et al.* 2001; O'Brochta & Handler 2008). Three transposable elements, *Minos*, *piggyBac* and *mariner*, were tested for their suitability as vectors in *T. dalmanni*. *Minos* and *piggyBac* were shown to be active in excision assays indicating that either could be used as a vector for transgenesis in stalk-eyed flies.

2.4.1 Transgene delivery

Embryology

Anterior-posterior asymmetries in external morphology proved sufficient for simple identification of the posterior pole of a stalk-eyed fly egg. Timed observations of early embryo development indicated that cellularisation of the posterior pole cells, which give rise to the germline, occurs between 4-6 hours post laying. The ability to orient eggs so that DNA will be delivered to future germline nuclei will increase the likelihood of germline transformation. Microinjection prior to cellularisation is also readily achievable as eggs can be collected after a 1.5h laying period and injected within 1-2 hours after collection.

Microinjection protocol

Between 12% and 18% of microinjected eggs survived to pupation when embryos were left in their chorions throughout the procedure (Tables 2.4 and 2.5). Dechorionation and associated procedures caused increased mortality during larval stages. The exact reason for this is unclear. It may be a side-effect of immersion in halocarbon oil 700, as seen in *M. domestica* (O'Brochta & Atkinson 2004). The heptane-based glue may also be a problem. Therefore injecting with the chorion intact is advantageous, as glue is unnecessary and since halocarbon oil is not required to prevent desiccation most of it can be removed post-injection. Modifications to needle design were found to have little impact on embryo survival. Needles with a steep taper and a short tip were preferred because they broke less frequently and so were more convenient.

The survival of other insect species following microinjection varies considerably. The lowest reported rates are 2.5% in *M. domestica* and 10% in *L. cuprina* (Hediger *et al.* 2001; Heinrich *et al.* 2002), while rates of 25-30% have been described in *B. mori* (Uchino *et al.* 2007) and *D. virilis* (Lozovskaya *et al.* 1996) and of up to 50% in *D. melanogaster* (Atkinson *et al.* 2001). The stalk-eyed fly survival rates seen here are at the lower end of this range. In part this may be due to the low fertility of *T. dalmanni* since only 30-50% of untreated eggs gave rise to larvae (Tables 2.3-2.5). This was not unexpected because similarly low fertilities have been documented for other laboratory and field populations of *T. dalmanni* (Cotton *et al.* 2009). Fertilised and unfertilised eggs cannot be distinguished during the injection procedure so that low fertility levels cause effort to be wasted in collection and injection of unfertilised eggs.

The physical acts of piercing the embryos and injecting fluid appear to be the main causes of mortality since non-injected individuals survive the preparation protocol with a similar mortality rate to that of control embryos. While developing the protocol a stronger needle was designed to penetrate the hard chorion. The needle could be further improved by bevelling the tip of the needle as a sharper tip would enable smoother and possibly less damaging injection. Bevelled needles are employed in other insect species where the embryo remains in the chorion for injection. In mosquitoes, which have particularly hard chorions, an improved survival rate is obtained by using aluminosilicate needles which are strong but expensive and hard to shape (O'Brochta & Atkinson 2004; Lobo *et al.* 2006a). With the available microinjection set-up it was not possible to tightly regulate the volume of fluid injected: switching to an automated unit (Picospritzer® III, Intracel, UK) would alleviate this problem.

Taking into account the number of *T. dalmanni* eggs which can be collected and processed, the microinjection survival rates observed here were sufficient to make transgenesis feasible. The number of microinjected eggs required to produce a transformant depends on the product of the survival rate and the transformation efficiency (the proportion of surviving embryos giving rise to transgenic offspring). In insects, transformation efficiencies vary from 1% up to 60% depending on the species and vector. The highest transformation efficiencies are reported in *T. castaneum* and *Drosophila* species (Berghammer *et al.* 1999; Finokiet *et al.* 2007) but in most non-drosophilid dipteran flies transformation efficiencies are between 5% and 15% (Hediger

et al. 2001; Kokoza *et al*. 2001; Handler *et al.* 1998). The populations of *T. dalmanni* females used here were capable of laying up to 150 eggs in a 1.5 hour oviposition period and up to three oviposition periods could be processed in a day. Given the observed survival rates of approximately 15% (Table 2.5), even with transformation efficiencies at the low (5%) end of the range typical for dipteran flies, 4-5 transformants would be expected for every 600 flies injected.

2.4.2 DNA vector selection

Three candidate transposon vector systems for mediating germline transformation of *T. dalmanni* were evaluated using a PCR-based excision assay. No excision was detected using the *mariner* element *Mos,* with or without an exogenous source of transposase (Fig 2.4B). PCR is a very sensitive technique capable of detecting small amounts of DNA. If *mariner* is excising at such a low rate that it cannot be detected by PCR then it should not be considered as a potential vector. Recent studies have shown that potentially active *mariner* elements from the vertumnana subfamily are present in *T. dalmanni* and other stalk-eyed fly species (Carr 2007). The fact that no excision of the donor plasmid is detected indicates that these elements are either inactive or incapable of cross-mobilisation of the *Mos1* element. Often host genomes evolve mechanisms for inhibition of transposon activity, this being well demonstrated by hybrid dysgenesis in *D. melanogaster* (Black *et al.* 1987). Several mechanisms of self-inhibition are suggested specifically for *mariner* elements with some supporting evidence (Hartl *et al.* 1997a, b). These include overproduction inhibition which implicates a negative feedback on transposase activity. Competitive inhibition and transposase titration occur when excess transposase is present and the proteins either interact with each other directly or occupy potential binding sites.

PiggyBac and *Minos* mediated excision was detected in *T. dalmanni* embryos supplied with an exogenous source of transposase (Fig. 2.4A, C and D). No excision was detected in assays when donor plasmid alone was injected, indicating that there is no endogenous source of transposase for either element in the *T. dalmanni* genome. A reason for preferring *Minos* over *piggyBac* is that an mRNA source of *Minos* transposase is available and was confirmed to mediate excision of the transposon (Fig. 2.4D). Increases in transformation efficiency have been seen when using an mRNA source of transposase compared to a DNA source of transposase in four species

(Pavlopoulos *et al.* 2004; Kapetanaki *et al.* 2002; Uchino *et al.* 2007).

2.4.3 Conclusions

This chapter has described the necessary preparatory work that has been carried out in order to go on to perform a germline transformation in *T. dalmanni*. A robust method of microinjection for DNA delivery has been established. Survival is sufficient for the protocol to be used as part of a viable transformation protocol even if low transformation efficiencies are present.

Two potential transposable element vectors, *Minos* and *piggyBac* have been used as vectors for transgenesis in a wide range of species and often reported with high associated transformation efficiencies. Both have been shown to be active in *T. dalmanni*. Thus either vector would be a potentially sensible choice for use in trials of germline transformation in *T. dalmanni*. Additionally, excision of *Minos* was observed when using an mRNA source of transposase. Given that increases in transformation efficiency have been observed in other species with an mRNA source of transposase it was decided to use *Minos* as a promising candidate vector for transgenesis in *T. dalmanni*.

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Chapter Three: Stable germline transformation of the stalk-eyed fly, *Teleopsis dalmanni*

3.1 INTRODUCTION

Background

Diopsid stalk-eyed flies provide a promising model organism for investigation of the evolution and development of novel and exaggerated phenotypes. Developmental studies in stalk-eyed flies have been restricted by the paucity of available genetic and molecular tools. Expression studies of candidate stalk-eyed fly developmental regulator genes have generated some insights (Hurley *et al.* 2001; Carr *et al.* 2005). However, studies of gene expression need to be complemented by studies of gene function to test for causal relationships between genotype and phenotype. Transgenics provide the most promising strategy for fulfilling this role. Not only are transgenic organisms versatile tools for assaying gene function, but they also have diverse applications in the contexts of, for example, fate-mapping (Halfon *et al.* 1997; Ramos *et al.* 2006) and analysing sperm competition (Price *et al.* 1999).

The preceding chapter gave details of the development of a method for delivering transgene DNA to germline nuclei in stalk-eyed fly embryos and selection of an appropriate transposon vector, *Minos*. In this chapter I provide a description of the first successful germline transformation of the stalk-eyed fly, *Teleopsis dalmanni*. Transgenic individuals are initially identified by their expression of marker phenotypes. Once a transgene insertion(s) has been detected, its stability and heritability are established by backcrossing to the untransformed base stock and analysing segregation and linkage of the transgenic phenotype. Southern blot and PCR-based analysis are used to confirm transgene integration into the host genome and to determine the number, location and underlying mechanism of the insertion event.

3.1.1 Detection of the insertion event

In insect transgenesis, insertion events are detected by the observation of a phenotype (usually visual) encoded by the transgene (Handler 2000). For germline transformation the transgene must be detected in the offspring of the injected individual. Transgene expression has been reported in injected individuals but this can be due to either somatic integration events of the persistence of the donor plasmid containing the transgene (Nolan *et al.* 2002; Catteruccia *et al.* 2000). In long germband insects, including

Diptera, the germline is derived from multiple pole cells. Often transgene incorporation only occurs in one of these cells and therefore only a small percentage of gametes will possess the transgene. If integration occurs late in development, after several rounds of mitosis, the proportion of gametes possessing the transgene will be reduced even further. As a result the rates of transmission from injected individuals to offspring can be low (eg 0.9% in Jasinskiene *et al.* 1998; 0.2%-1.5% in Coates *et al.* 2000; 0.75% in Koukidou *et al.* 2006; 3.7% in Kuwayama *et al.* 2006).

Low transmission frequencies from injected individuals to the next generation, combined with low transformation efficiencies, require a large number of offspring to be screened in order to detect an insertion event. Screening is facilitated by the incorporation of a reporter gene within the transgenic insertion. Activity of the reporter confers a visible phenotype through expression of different marker proteins. Reporter genes consist of the regulatory sequences and promoters that drive the expression of a marker protein. Care should be taken when selecting the appropriate promoter sequences and marker protein coding sequences in order to maximise the chances of detection of the insert.

Regulatory sequences and promoters

Effective species-specific promoters have been developed including *BmA3* in *Bombyx mori* (Tamura *et al.* 2000) and *polyubiquitin* (*pUb*) in *Tribolium castaneum* (Lorenzen *et al.* 2002). However, in most non-model species, including *T. dalmanni*, well characterised promoter regions are not available. Although not designed to be pan-specific, two ubiquitous promoters from *Drosophila melanogaster* have driven reporter gene expression in other dipteran species. The *polyubiquitin* (*pUb*) promoter has been used to drive expression in *Anastrepha suspensa* (Handler & Harrell 2001a), *Cochliomyia hominivorax* (Allen *et al.* 2004), *Anopheles albimanus* (Perera *et al*. 2002) and *Lucilia cuprina* (Heinrich *et al.* 2002). The *actin5C* promoter has been used in *Anopheles stephensi* (Catteruccia *et al*. 2000), *Culex quinquefasciatus* (Allen *et al.* 2001), *Stomoxys calcitrans* (O'Brochta *et al*. 2000) and *Aedes aegypti* (Pinkerton *et al.* 2000).

A self-activating derivative of the tetracycline-responsive promoter (*tTA*) provides another potential option for driving transgene expression in diverse insect species

(Koukidou *et al.* 2006). It has been shown to be functional in *Ceratitis capitata*, *D. melanogaster*, *Bactrocera oleae* and *Caenorhabditis elegans* as well as in tobacco leaves, *Nicotiana tabacum* and *N. bentamiana* (Koukidou *et al.* 2006).

The pan-specific *3xP3* promoter is by far the most successful transgene promoter construct available (Czerny & Busslinger 1995; Berghammer *et al.* 1999; Horn & Wimmer 2000). It consists of three minimal *pax6* binding sites and an *hsp70* promoter that drives localised expression in the adult and developing eye (Horn *et al.* 2000). This enables simple detection in larvae and adults with non-pigmented eyes. In addition, expression can be seen in the parts of the brain, digestive system and anal pads. Since it does not require co-factors for expression, *3xP3* has been successfully used in a wide range of species including: *Ae. aegypti* (Kokoza *et al.* 2001; Adelman *et al.* 2008), *Ae. fluviatilis* (Rodrigues *et al.* 2006), *An. stephensi* (Ito *et al.* 2002), *Bicyclus anynana* (Marcus *et al.* 2004), *D. melanogaster* (Berghammer *et al.* 1999), *Drosophila willistoni* (Finokiet *et al.* 2007), the planarian *Girardia tigrina* (González-Estévez *et al.* 2003), *Harmonia axyridis* (Kuwayama *et al.* 2006), *T. casteneum* (Berghammer *et al.* 1999), *B. mori* (Thomas *et al.* 2002), *Musca domestica* (Hediger *et al.* 2001) and the crustacean *Parahyale hawaiiensis* (Pavlopoulos & Averof 2005).

Marker proteins

In some species it has been possible to use marker coding sequences that can rescue eye colour phenotypes such as *rosy* in *D. melanogaster* (Rubin & Spradling 1982)*, white+* in *D. melanogaster* (Klemenz *et al.* 1987) and *C. capitata* (Loukeris *et al.* 1995; Handler *et al.* 1998; Salvemini *et al.* 2006) and *cinnabar* in *Ae. aegypti* (Coates *et al.* 1998). This approach requires both the availability of loss of function mutant strains and molecular characterisation of the gene in question.

A more widely applicable strategy exploits coding sequences for fluorescent protein derivatives of the jellyfish, *Aequorea victoria*, Green Fluorescent Protein (GFP) or the more recent derivative enhanced-GFP (eGFP) (Cormack *et al.* 1996; Horn *et al.* 2002). The expression of eGFP does not have major effects on viability of an organism and acts as a dominant marker trait (Irvin *et al.* 2004). As the larval cuticle of most insects is to some extent transparent, detection simply requires observation of (live) larvae under fluorescent light of the appropriate wavelength. All insect species for which a successful

germline transformation protocol has been developed have been shown to be capable of expressing eGFP (Horn *et al.* 2002; O'Brochta & Handler 2008).

In some species autofluorescence is observed at certain wavelengths of light. Autofluorescence is defined as the fluorescence of other substances than the fluorophore of interest. If present in an organism being screened then it may produce a background fluorescence which obscures the signal from the reporter gene. As a result an alternative protein, *Dicsosoma sp.* Red (dsRed), is becoming increasingly popular because less autofluorescence is observed at its low wavelength of fluorescence activity than is typical for eGFP (Matz *et al.* 1999; Handler & Harrell 2001b). A dsRed based reporter gene was used for transgenesis in the crustacean *P. hawaiiensis* since autofluoresence inhibited clear visualisation in eGFP expression in this species (Pavlopoulos & Averof 2005). A further benefit of having more than one reporter gene available for a given species is that it enables the insertion of multiple transgenes to be detected. For instance, in the GAL4/UAS systems different reporter constructs containing different reporter genes are used to differentiate between lines carrying the GAL4 driver transgene and the UAS reporter transgene (Imamura *et al.* 2003; Dai *et al.* 2008; Ito *et al.* 1997). eGFP and dsRed are the most commonly used reporter genes, but others are used such as yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), CopGreen and J-Red but their usage is not as widespread (Horn & Wimmer 2000; Sarkar *et al.* 2006; Condon *et al.* 2007a; Shagin *et al.* 2004).

3.1.2 Confirmation and characterisation of the insertion event

To generate a stable transgenic line the initial identification of potentially transgenic individuals is followed by tests to determine whether the transgene(s) is stably integrated and inherited. Further molecular characterisation is also required to determine the number of insertions present and the nature of the insertion events. Molecular characterisation requires extraction of genomic DNA and consequently sacrifice of transgenic individuals. Prior to this, transgenic lines need to be amplified to generate sufficient numbers so that the loss of a few individuals will not risk loss of the line. Analysing segregation patterns during the establishment and amplification of a transgenic line can be informative about the nature of the original insertion event(s) in addition to demonstrating heritability and stability.

Rearing and segregation analysis of transgenic lines

Random transposon mediated integration can occur almost anywhere within the genome and can occur more than once within a cell or in more than one different germline cell. This can affect segregation and expression pattern of the transgene in the offspring of a transformant fly. In the event of a single integration event within an injected individual, its offspring will be heterozygous carriers of the transgene. When crossed with a wild-type individual, 50% of offspring will express the transgene phenotype and subsequent crosses would be expected to follow standard Mendelian ratios of inheritance. Many deviations from this pattern are possible depending on the number and location of integration events present.

Multiple insertion events can occur within a pole cell and different insertion events can occur in different pole cells within an individual. Both types of event are commonly reported (Tamura *et al.* 2000; Peloquin *et al.* 2000; Kuwayama *et al.* 2006). The former (multiple insertions in one pole cell) is detected when carrier offspring of the individual are crossed with wild-types and more than 50% of the progeny express the transgene. The latter (insertion occurring in multiple pole cells) can result in differences between the observed segregation patterns in lines derived from different offspring of an injected individual.

Departures from Mendelian ratios can be due to classical effects such as sex-linkage or recessive lethality. Insertions in sex chromosomes are often observed (eg Allen *et al.* 2004; Condon *et al.* 2007b). This results in exclusively female expressing or male expressing offspring in specific test crosses. In the case of *C. capitata*, transgene insertion into the Y-chromosome resulted in male only carriers (Condon *et al.* 2007b). This proved beneficial as it provided a mechanism for larval sex determination. Recessive lethality was observed in lines created by a *piggyBac* transformation of *Cochliomyia hominivorax*, crosses between heterozygous carriers of the transgene resulted in less than the expected 75% of offspring carrying the transgene (Allen *et al.* 2004). It was discovered that this was due to the insertion causing a lethal mutation so that homozygous carriers of the transgene were not viable.

Copy number of the transgene in the genome has been associated with increased level for fluorescence of reporter genes. However, this can be confounded by the effects of

promoters local to the insertion site and as a result is not a reliable guide to copy number (Hediger *et al.* 2001; Handler & Harrell 2001a).

It is well established that the expression pattern of transgenic inserts can be influenced by the particular locus at which the insertion occurred (Levis *et al.* 1985; Wilson *et al.* 1990; Hazelrigg *et al.* 1984). These can be separated into epigenetic effects and *cis*-regulatory effects (Hediger *et al.* 2001; Ito *et al.* 1997). If either type of effect results in complete silencing of transgene expression the insertion will not be detected and investigated. As a result such effects may be occurring more frequently in transgenic lines than is reported.

By far the most commonly studied case of epigenetic effect is position effect variegation (PEV). This is often seen with transgenes rescuing the *white* mutant phenotype in *D. melanogaster*. In PEV, expression is silenced by the spread of heterochromatin into euchromatic regions (Wilson *et al.* 1990). *White+* expression is cell autonomous so mosaic expression of the phenotype can result and in extreme cases the rescue phenotype is not observed at all. Similar telomeric position effects (TPE) are associated with repeats of genes found distal to the telomere (Dorer & Henikoff 1994; Roche & Rio 1998; Kurenova *et al.* 1998; Ronsseray *et al.* 2001). PEV is often erroneously used as a general term to refer to any transgene silencing where in fact many mechanisms appear to be present although they are not as well characterised as PEV (Adelman *et al.* 2004). In transgenic insects, PEV in *D. melanogaster* is the only form of epigenetic silencing that has been clearly identified due to the easily detectable mosaic phenotype, although general epigenetic effects have been suggested in a number of cases without exact mechanisms being described (Lorenzen *et al.* 2007; Koukidou *et al.* 2006).

The expression pattern of inserted transgenes will be affected by enhancer or suppressor regulatory regions in the vicinity of the site of integration. Such *cis*-regulatory effects form the basis of enhancer trapping techniques (Imamura *et al.* 2003; Ito *et al.* 1997) and are commonly reported in transgenic insect lines (Hediger *et al.* 2001; Lorenzen *et al.* 2003). For example, an insertion of the *3xP3-eGFP* construct into *M. domestica* resulted in variation between lines in the expression pattern (Hediger *et al.* 2001).

Loss of expression can be due to insert instability rather than silencing. Transgene loss was reported in a study of a *piggyBac* transformation of *Ae. aegypti* (Adelman *et al.* 2004). In a transformed line a low transmission rate of GFP expression across generations was observed. Crosses between individuals with the GFP-positive phenotype produced progeny with a lower than expected proportion of progeny showing transgene expression. Southern blot analysis and in situ hybridisation showed multiple tightly linked copies of the transgene, described as "plaques". Further analysis showed that regions of the donor plasmid not flanked by the terminal inverted repeats of the transposon were also present in the genome, indicating a recombination rather than transposase mediated mechanism of incorporation into the genome. Loss of transgene expression was associated with loss of the plaques. Similar plaque formation of different transposable elements has been reported on a number of occasions and their insertion sites are associated with chromosomal break points (Perucho *et al.* 1980; Barth *et al.* 1998).

Complete silencing of expression or instability of the insertion event can result in an under-representation of the expression pattern in crosses (Coates *et al.* 1998; Jasinskiene *et al.* 1998). When testing a germline transformation technique in a species, at least one stable integration event free from positional silencing effects would be the minimal desired outcome.

Southern blot confirmation and molecular characterisation

Simple initial genetic confirmation of an insertion event into the genome can be performed using polymerase chain reaction (PCR) based assays (Pavlopoulos & Averof 2005; Yoshiyama *et al.* 2000; Marcus *et al.* 2004; Rodrigues *et al.* 2006). However, PCR is highly sensitive to contamination of the template sequence making PCR alone an unreliable method for detection (O'Brochta & Atkinson 2004; Handler 2000). In addition, PCR only tests for presence of the transgene and does not give any information about copy number.

Almost all reports of stable transgene integration events include confirmation of the presence of the transgene by Southern blot hybridisation. The advantages of this method are that it is less prone to giving false positives than PCR and can also give information as to the nature of the insertion event, such as copy number and location of the

insertions. Insertions at different genomic locations will generate fragments of different sizes and the number and relative intensities of bands will indicate the number of copies present within the genome.

Insertion mechanism and location of insertion events

In insects transposase mediated integration is used to insert DNA into the genome, however other mechanisms of integration have been reported to occur during such attempts. It is important to confirm that the insertion mechanism is transposon mediated as non-transposon mediated integrations are associated with instability of the transgene (Adelman *et al.* 2004). Recombination of regions of the donor plasmid outside of the terminal inverted repeats (TIRs) of the transposon has been reported frequently in transposition assays (O'Brochta & Atkinson 1996) but also in genomic integration events (Adelman *et al.* 2004). This can be tested for by sequencing the flanking regions of the insert, which will show whether any of the donor plasmid has been incorporated into the genome. Transposon mediated mechanisms can be further confirmed as many transposons, including *Minos* and *piggyBac*, possess specific insertion sequences which are duplicated upon integration (Arca *et al.* 1997; Elick *et al.* 1997). As a result, a characteristic duplication of the insertion site can be seen flanking the TIRs when transposon mediated integration has occurred. The integration site for *Minos* is TA (Arca *et al.* 1997).

Characterization of the endogenous sequences adjacent to an insert may also be informative with respect to the genomic location of the insert. In species for which genomic or EST data is available, sequences that flank an insert can be compared with previously identified sequences in the databases for the organism. To date, relatively little sequence information is available for stalk-eyed flies. Some small scale EST projects (covering about 4,000 genes) have been carried out in *T. dalmanni* and microsatellite markers covering the genome are also available (Baker pers. comm.; Wright *et al*. 2004).

Inverse PCR

The standard method used to characterise the flanking sequence of an inserted gene is inverse-PCR (iPCR) (described in Fig. 3.1) (Sambrook & Russel 2001). In iPCR two

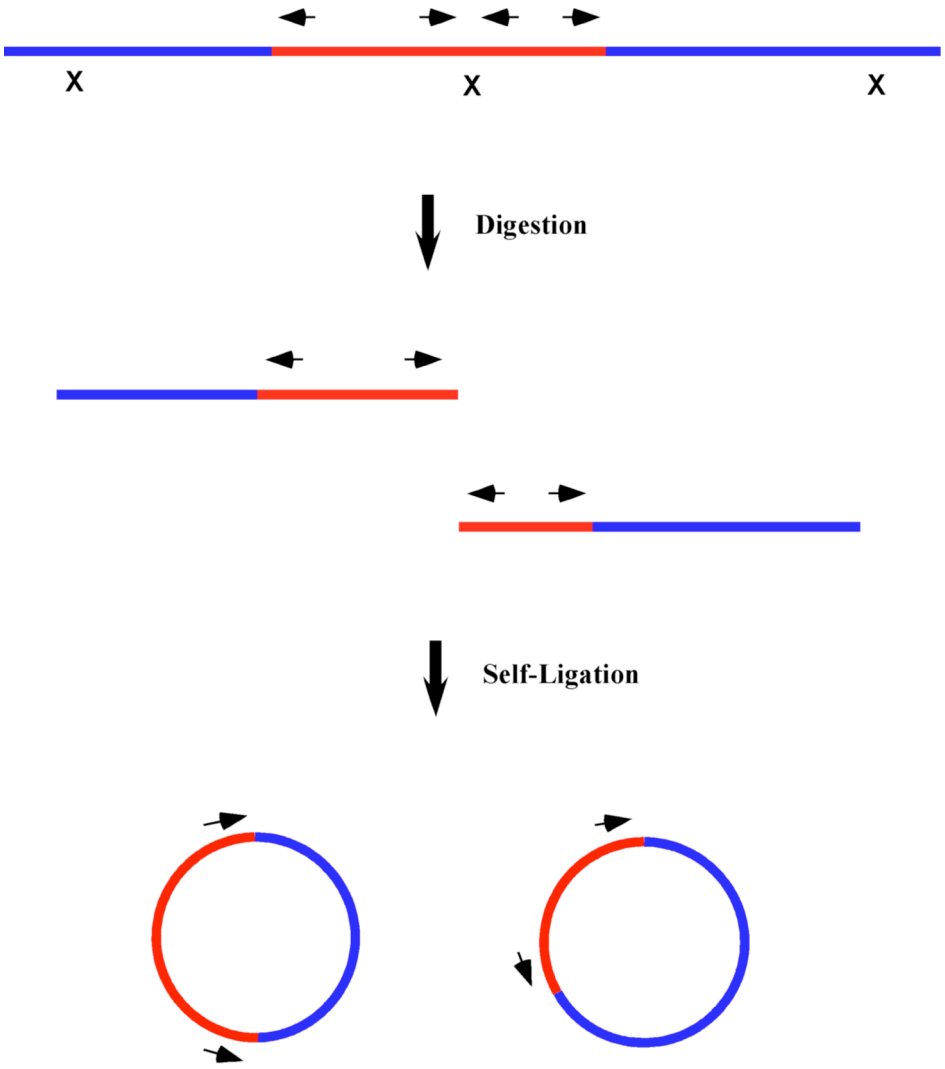

Figure 3.1. Inverse PCR. Solid lines = dsDNA. Arrows = primers. Red line = transposon sequence, blue line = flanking genomic sequence. X = restriction enzyme cutting site. Two sets of primers each facing away from each other are designed based on the transgene sequence. The pairs of primers are separated by a restriction enzyme site (X). The DNA is then cut with the restriction enzyme, which will also cut at sites in the flanking genomic DNA. The resulting fragments of DNA are then self-ligated. As a result the primers within each pair now face each other such that the resulting PCR product will include a small part of the transposon DNA along with some flanking DNA. PCR products are then cloned and sequenced.

pairs of terminal insertion-specific primers are designed each consisting of one outward facing (towards the flanking sequence) and one inward facing (towards the insert) primer. Genomic DNA extracted from transgenic individuals is digested with a

restriction enzyme that cuts at a site within the inserted transgene and the resulting DNA fragments are subjected to ligation conditions that favour circularisation (self-ligation) of fragments. In those self-ligated fragments that include the insert sequences, the primer pairs will now face each other and be capable of producing a PCR product that includes flanking genomic sequence. Problems with iPCR are often reported and it is common for publications of germline transformations of insect species not to give the flanking sequences of all insertion events (Marcus *et al.* 2004; O'Brochta *et al.* 2000; Thomas *et al.* 2002; Uchino *et al.* 2007).

Two-step gene walking

Due to the problems reported in using iPCR, other methods for finding the sequence of unknown flanking regions of DNA have been developed. Pilhofer *et al.* (2007) reported a technique called two-step gene walking (Fig. 3.2). A single primer located within the known DNA and facing the unknown DNA, is used throughout the two-step procedure. Multiple rounds of high stringency PCR are first carried out on a genomic DNA template to produce single stranded copies of DNA (ssDNA) covering the region of interest. A single round of low stringency PCR is then carried out. DNA synthesis initiated from non-specific binding of the primer to the ssDNA product of previous rounds will produce complementary ssDNA covering the region of interest. The resulting dsDNA copies of the region of interest will be flanked by primer sites. Several rounds of high-specificity PCR will then produce multiple copies of the region of interest.

The advantages of this method are that it avoids the digestion and self-ligation steps of iPCR, eliminates the need for DNA extractions between each step and requires only a small amount of template DNA. Pilhofer *et al.* 2007 reported recovery of more than 80% of sequences attempted and similar success rates were seen in a repeat of the process (Pilhofer *et al.* 2008). It has been suggested that the approach would be applicable to isolating the regions flanking transgene insertions in many organisms (Philhofer *et al.* 2007).

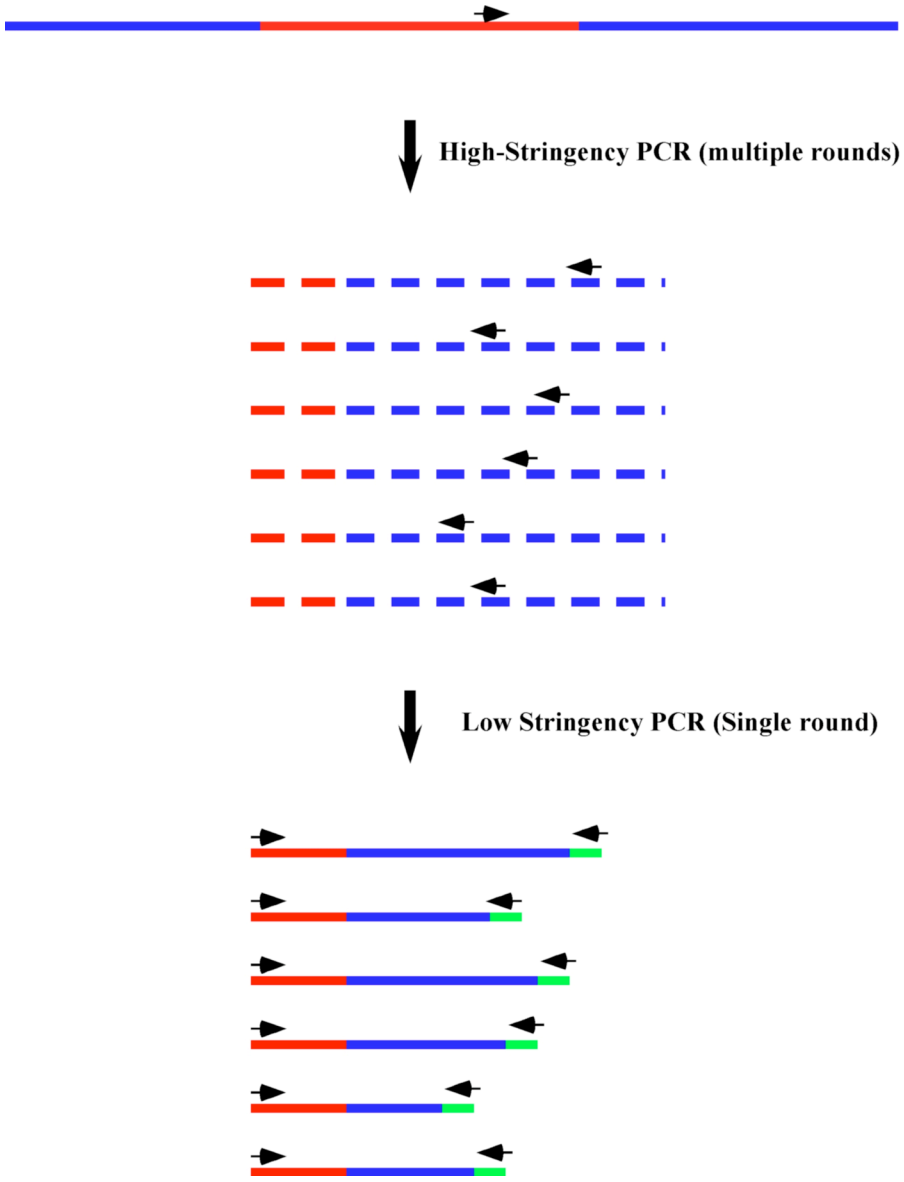

Figure 3.2. Two-step gene walking. Solid lines = dsDNA, dashed lines = ssDNA. Red lines = transposon sequence, blue lines = flanking genomic sequence, green line = inserted primer binding site. Arrow = primer. A single primer is designed within the known sequence facing the genomic sequence. Several rounds (~30) of high stringency PCR produce multiple ssDNA copies of the sequence extending from the primer. A single round of low stringency PCR is carried out and the primer will bind to random sites in the genomic sequence, including the ssDNA facing towards the transposon DNA. This will produce dsDNA copies of the sequence of interest with primer binding sites at both ends, which then acts as the template for a standard PCR reaction. The products of this reaction will be of differing lengths but can be used directly for sequencing reactions.

3.1.3 Experimental outline

Here I describe germline transformation of the stalk-eyed fly *T. dalmanni* with a vector based on the *Minos* transposable element. The vector selected, pMi[3xP3-eGFP], carries eGFP coding sequences driven by the 3xP3 synthetic promoter, which drives expression in the larval and adult eye, the brain and anal pads. The *3xP3-eGFP* reporter gene was chosen because the 3xP3 promoter does not require host factors and has been shown to work reliably in a far wider range of organisms than any other promoter. In addition, its restricted expression means that detection is made easier in the presence of autofluorescence than would be the case for a promoter with ubiquitous expression. Since a small degree of autofluorescence had been observed in the digestive tracts of stalk-eyed fly larvae under both dsRed and eGFP filters (Author's personal observations), eGFP was chosen as the marker. This has been used successfully in a far wider range of organisms than dsRed.

Embryos were microinjected with donor plasmids carrying the *3xP3-eGFP* construct flanked by the TIRs of *Minos* along with a capped mRNA source of *Minos* transposase using protocols developed previously (see Chapter Two). The offspring of injected individuals were screened for eGFP expression. Expression of eGFP was restricted to the anal pads and not detected in the eyes or brain. A transformation efficiency of 4% was observed, which is at the low end of transformation efficiencies observed in other insect species.

In order to evaluate linkage and stability of the transgenes, individuals showing eGFP expression in the anal pads were backcrossed with wild-type individuals and the resulting progeny scored for eGFP. The segregation of the eGFP phenotype in subsequent crosses indicated that at least three insertion events had occurred. Two were X-linked and the other was autosomal. The insertions were shown to persist in inbred populations over several generations. Integration and copy number of the transgene insertions were confirmed by Southern blot analysis. One line carried a single copy X-linked insertion and the two other lines contained multiple insertions. iPCR and two-step gene walking were used to obtain flanking sequences. A TA duplication characteristic of *Minos* insertions provided confirmatory evidence that integration was transposase mediated.

3.2 MATERIALS AND METHODS

Experimental flies and generation of embryos for microinjection

A laboratory-adapted population of *Teleopsis dalmanni* founded from flies collected from Ulu Gombak, Peninsular Malaysia in 1993 was used for these experiments. Laboratory flies have been maintained in cage culture at 25ºC, fed pureed sweetcorn twice weekly, and kept on a 12h:12h light:dark light cycle. In order to minimise inbreeding, population size has been kept high (> 200 individuals). For these experiments, eggs were obtained from sexually mature adult flies kept at a ratio of 2:1 female:male in groups of 9-15 individuals. Two sets, each consisting of 40-50 groups, were maintained to provide a sufficient supply of eggs for experiments. Each group was housed in an inverted 1.5L plastic container with a base containing damp cotton wool, moistened filter paper and sweetcorn replaced thrice weekly. Bases were lined with dark blue paper discs so that freshly laid eggs could be readily identified. In order to collect freshly laid eggs for microinjection, blue paper towels were replaced and females were left for a period of 1.5h. At the end of the laying period, fertilised eggs from all groups were transferred using paintbrushes to be pooled on moistened paper towel discs prior to microinjection.

Microinjections

Groups of 10-30 embryos were arranged side by side on a coverslip, attached to a slide, and left for 5 minutes to desiccate and attach to the coverslip. The embryos were then covered with halocarbon oil 700 and placed on an inverted microscope (IDOC, Zeiss, Ukraine) and microinjections performed using a needle attached to a micro-manipulator (MN-153, Narishige, UK), an electric microinjector (IM-30, Narishige, UK) and an oil air compressor (Jun-air, Norgren, Denmark). Needles for microinjection were made from borosilicate glass capillaries and shaped using a P-97 needle puller. Detailed specifications of the needles ('needle B') are given above in section 2.2.1.

Needles were filled by placing a drop of injection mix at the blunt end of the needle and allowing the buffer to flow to the tip of the needle via capillary action. Injection mix consisted of 500µg/ml donor plasmid (pMi[3xP3-EGFP], Pavlopoulos *et al.* 2004), and 300µg/ml *Minos* transposase mRNA in injection buffer (15µm NaH_2PO_4, 850µm

Na_2HPO_4, 5mM KCl at pH7.6-7.8) with 0.05% of the inert dye phenol red (Sigma) added to facilitate visual monitoring of the process. To minimise dust, which could block needles, injection buffer was passed through a 2μm Acrodisc syringe filter (Pall Corporation, UK). Prior to use, the injection mix was centrifuged on a bench top centrifuge for 15-30 minutes at 14,000rpm. The supernatant was carefully transferred to a fresh tube and then used to load needles.

The needle was positioned at a shallow angle (10^{o} to 25^{o}) with the tip at the same height as the embryos. The needle tip was opened by gentle agitation against the chorion of an embryo. Embryos were injected at their posterior end. Needle penetration of the embryos was achieved by moving the embryos onto the needle via lateral manipulation of the microscope stage. A short burst of pressure was applied to deliver the injection mix. After injections, virtually all of the halocarbon oil was removed and the coverslip containing the embryos transferred to an 'egg-lay' (described in section 2.2) and maintained at 25ºC.

Three series of injections were carried out. In each series, experimental and control treatments were carried out. In the experimental treatment, embryos were injected with a solution made up of a mixture of *Minos* transposase mRNA and donor plasmid DNA in injection buffer. Two types of control were used. In the first two series of injections, as a sham control for the effect of mRNA and DNA on survival, a proportion of embryos were injected with injection buffer alone. In all three series, as a control for the effect of microinjections, a proportion of collected embryos were transferred directly to egg-lays, without being subjected to microinjection and maintained at 25ºC. After injection treatment, the development of embryos was monitored and survivorship was recorded at hatching, pupation and eclosion.

Plasmids and mRNA

The donor plasmid used was pMi[3xP3-eGFP] kindly provided by C. Savakis (University of Crete, Greece). In vitro synthesis of *Minos* transposase mRNA was performed using pBlueSKMimRNA, which contains the tranposase coding sequence under the control of the T7 promoter (Pavlopoulos *et al*. 2004). To generate necessary amounts of plasmid for injection or synthesis, Sub-Cloning Efficiency^{TM} DH5α^{TM} *E.coli* cells (Sigma, UK) were transformed with plasmid DNA and purified using the

Qiagen Endofree Plasmid Maxi Kit™ (Qiagen, UK). DNA was dissolved in 50µl TE buffer (10mM Tris, 1mM disodium EDTA, pH 8) to generate a stock solution.

For the majority of the injections, pMi[3xP3-eGFP] DNA was added to injection buffer directly from the stock solution. In the final series of injections, a further purification step was added in an attempt to reduce the deleterious effects of the plasmid DNA on embryo survival. For purification, 50µl stock plasmid solution was made up to 400µl with H_2O. 1ml (2.5 x vol) EtOH and 40µl (0.1 x vol) 3M sodium acetate ($NaCH_3COO$, pH5.2) in an eppendorf tube and gently mixed. After a period of 15 minutes at -80ºC the contents of the tube were centrifuged at 13,200rpm for 30 minutes at 4ºC using a bench top centrifuge. The supernatant was removed and discarded and 70% EtOH was added to the pellet. The mixture was centrifuged at 13,200rpm for 15 minutes at 4ºC. The supernatant was removed and discarded. The pellet was air-dried for 5-10 minutes before being dissolved in 50µl TE buffer to generate a purified stock.

Transposase mRNA was prepared by fully digesting pBlueSKMimRNA plasmid with *NotI*. The linearised plasmid acted as a template to make mRNA using the MEGAscript® T7 Kit (Ambion Inc., UK). Concentrations and purity of stock DNA and mRNA solutions were measured using a Biophotometer (Eppendorf, UK).

Screening of injected *T. dalmanni* offspring

Sexually mature adults derived from injected embryos (G0) were crossed with 1-3 wild-type virgin individuals of the opposite sex to generate offspring (G1). All eggs produced were collected and transferred to egg-lays. Third instar larvae were screened for eGFP expression using a UV-dissecting microscope (Leica MZII, Leica, UK). Pictures were taken using a Nikon 5100 digital camera or a Nikon 4900 digital camera.

Not all G1 larvae produced could be screened before reaching pupation. An additional screening process was carried out to maximise the number of transformants which could be recovered. Individual intercrosses between unscreened G1 offspring of the same G0 founder were set up and the resulting offspring (G2) screened for eGFP expression as larvae. If more than 10 offspring showed no detectable expression, both parents were discarded. If expressing larvae were produced, the male and female parents were separated and mated with wild type flies to identify the transgene carrier.

Genetic characterisation, breeding and maintenance of insertions

All surviving G1 individuals which had shown eGFP expression as larvae or been identified as transgene carriers were initially crossed with 1-3 virgin wild-type individuals of the opposite sex to generate male and female expressing offspring (G2) for further analysis.

To determine whether the insert(s) in a particular G1 individual was sex-linked or autosomal, individual crosses of a carrier male and a wild-type virgin female or of a carrier female and a wild-type male were set up. Offspring were screened for eGFP expression as larvae. Expressing and non-expressing larvae were reared to adulthood and their sex determined. An insert was inferred to be X-linked if, in the progeny from the carrier male cross only females expressed eGFP, and in the progeny from a carrier female cross, both males and females expressed. For a Y-linked insert only male progeny of a carrier male would express the transgene.

Transgene stability and susceptibility to silencing was assessed by recording the ratio of expressing to non-expressing offspring of carrier individuals over at least three generations. For the X-linked lines, to create stocks in which all individuals were homozygous/hemizygous for a stable insertion, ten pairwise crosses were set up between eGFP-expressing males and females. 25 offspring of each cross were screened for eGFP expression. Pairs producing 100% eGFP expressing offspring were identified and used to set up a stock population. For autosomal lines, homozygosity was increased by mass crosses of expressing individuals in each generation.

DNA extractions

For the extraction of good quality genomic DNA for Southern analysis, the head, wings and abdomen were removed from 20-30 flies. The remaining tissue was placed in an eppendorf and immersed in liquid nitrogen for 1 minute. The flies were homogenized using a flame-blunted pipette tip. 300µl HM buffer (0.1M NaCl, 0.2M Sucrose, 0.1M tris-HCl pH9.1, 0.05M EDTA, 0.5% SDS) was added to the homogenate followed by 5µl of 20mg/ml proteinase K. The contents of the tube were mixed before being left at 55°C for 2-3 hours with occasional agitation. 85µl of 5M NaCl was gently added and

left at 4°C for 20 minutes before the mixture was centrifuged on a bench top centrifuge at 14,000rpm for 20 minutes at 4°C. The supernatant was carefully removed and placed in a fresh eppendorf tube. If any precipitate was left in the supernatant it was centrifuged again to remove the precipitate. This was repeated until the supernatant was completely free of precipitate. 1ml of cold EtOH was added to the supernatant and the DNA spooled at the interface. A flame-modified glass pipette with the tip blunted and slightly rounded was used to transfer the precipitated DNA to an eppendorf containing 70% EtOH. The DNA was then transferred to 30µl TE buffer.

For DNA extraction from individual flies for PCR-based analysis, the same protocol was used with minor modifications: 100µl of HM buffer and 1.6µl of proteinase K was added to the homogenate, 30µl of NaCl was added after incubation and 350µl of cold EtOH was used to precipitate the DNA. To separate the precipitated DNA from the supernatant the mixture was centrifuged at 14,000rpm for 20-30 minutes at 4°C on a bench top centrifuge. The supernatant was discarded and cold 70% EtOH was added. The mixture was centrifuged at 14,000 rpm for 15 minutes at 4°C on a bench top centrifuge. The supernatant was discarded and the pellet air-dried for 5-10 minutes before being dissolved in 20µl TE buffer.

Southern analysis

DNA was extracted from 20-30 female flies. DNA concentration and quality was assayed using an Eppendorf Biophotometer (Eppendorf, UK) and 10µg plasmid pMi[3xP3-EGFP] (Fig. 3.3) was digested with *EcoRI* and separated on a 0.5% agarose gel. The gel was then washed twice for 45 minutes in 10 gel volumes of denaturation buffer (1.5M NaCl, 0.5M NaOH) with gentle shaking. The gel was rinsed in ddH_2O followed by two 15 minutes washes in neutralisation buffer (1.5M NaCl, 0.5M Tris-HCl pH 7-8). A 20x stock solution of SSC was made and then diluted with ddH_2O to required concentrations (20xSSC = 1.5M NaCl, 0.15M $Na_3C_6H_5O_7$ (sodium citrate)).

DNA was transferred to nylon membranes (Amersham Biosciences, UK) by capillary action. This was achieved by placing the gel in contact with a reservoir of transfer buffer (10x SSC) supported by a filter-paper wick. The membrane was positioned on the agarose gel and blotting pads consisting of paper towels were placed on top of the membrane. A weight (200-300g) was placed on top of the assembly, which was then

left for 8-16 hours for the transfer to take place. The blotting apparatus was carefully dismantled and the membrane washed twice for five minutes in 6x SSC. The membrane was then dried for 30 minutes on Whatman paper before being placed in an 80°C oven for 2 hours between two pieces of Whatman paper. The membrane was marked and stored at room temperature until required.

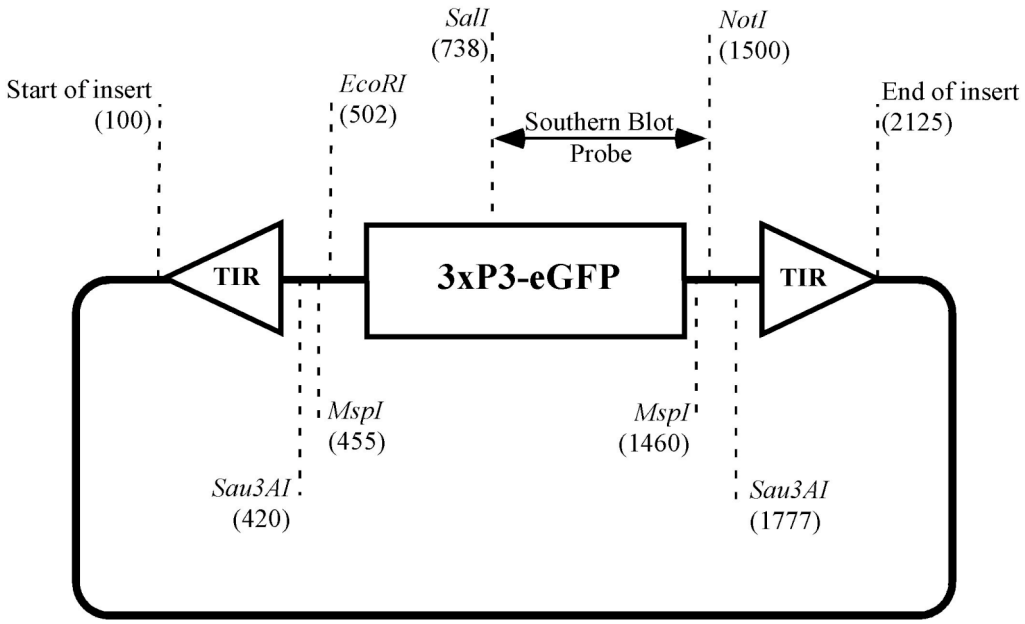

Figure 3.3. Schematic representation of the donor plasmid, pMi[3xP3-eGFP], used to transform *T. dalmanni.* The diagram is based on a plasmid map and sequence data donated by A. Pavlopoulos. Position on the plasmid is shown in brackets. TIR denotes terminal inverted repeats. The insert runs from positions 100 to 2125 of the plasmid. The plasmid is 5041bp long in total. The *Sau3AI* and *MspI* restriction sites were used for inverse PCR. *NotI* and *SalI* restriction sites were used to make the probe for the Southern blot analysis. *EcoRI* was used to digest genomic DNA of transgenic flies for Southern blot analysis.

A 762bp SalI/NotI fragment of pMi[3xP3-eGFP] with *SalI* and *NotI* was isolated using a Qiagen Gel Purification Kit (Qiagen, UK). The fragment was radioactively labelled (Prime-It II Random Primer Labelling Kit, Stratagene UK) to use as a probe (Fig. 3.3). 10µl random oligonucleotide primers were added to a 23µl solution containing 25ng of the purified DNA fragment in ddH_2O. The mixture was heated in a boiling water bath for 5 minutes. After brief centrifugation 10µl 5x reaction buffer was added along with 5µl dCTPs radioactively labelled with P^{32} (PerkinElmer, UK) and 1µl Klenow enzyme. The mixture was incubated at 37°C for 10 minutes then transferred to a glass test tube containing 5ml pre-hybridisation solution (6x SSC, 0.5% SDS, 5x Denhardt's solution,

0.1mg/ml denatured salmon sperm DNA, 50% formamide) and placed in a boiling water bath for 10 minutes before being cooled for 5 minutes on ice. The mixture was added to the membrane that had been soaked for 2 hours in pre-hybridisation solution and left overnight at 42°C with constant shaking.

The membrane was then washed twice in low stringency wash solution (2x SSC, 0.1% SDS) for 20 minutes at room temperature with shaking, followed by at least two 20 minute washes at 65°C with shaking in high-stringency wash solution (0.1x SSC, 0.1% SDS). The last step was repeated to remove residual radioactivity. The membrane was dried, briefly wrapped in saran wrap and placed, along with a sheet of Kodak MXB blue sensitive X-ray film, in a cassette containing an intensifier screen film. Following storage at -80°C for at least 16 hours, the film was developed in an automated film developer.

Inverse PCR

1µg of DNA extracted from individual female transgenic flies was digested with either *Sau3A* or *MspI*. On completion of the digest the reaction was stopped by phenol:chloroform extraction and ethanol precipitation. The precipitate was centrifuged for 10 minutes at 14,000rpm and the pellet air-dried for 5-10 minutes before being dissolved in 10µl TE buffer. DNA concentration was measured using an Eppendorf Biophotometer (Eppendorf, UK).

For each digestion, ligations were performed using T4 DNA ligase (Invitrogen, UK) at three different concentrations of DNA, 0.1ng/µl, 0.5ng/µl and 1ng/µl. Following phenol: chloroform extraction and ethanol precipitation 50-500ng of the ligated DNA was used as a template for inverse PCR. In addition to the primers described in Klinakis *et al.* (2000) several additional "inward facing" primers were designed using the primer3 website (http://frodo.wi.mit.edu/) and Amplify 3x (Bill Engels, University of Wisconsin). All primers and annealing temperatures used are listed in Table 3.1. PCR reactions (GoTaq PCR, Promega, UK) were run on either a Master Cycler Gradient thermocycler (Eppendorf, UK) or a GeneAMP® PCR System 2700 thermocycler (Applied Biosystems, UK). The conditions used were: 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 55°C to 50°C (depending on melting temperature of the primers) for 30 seconds and 72°C for 1 minute.

Primer name	*Sequence (5'-3')*	*Position on pMi[3xP3-eGFP]*	*Position on Insert (Direction)*
IMio1	AAG AGA ATA AAA TTC TCT TTG AGA CG	1991-2016	Left & right arms (Outwards)
IMio2	GAT AAT ATA GTG TGT TAA ACA TTG CGC	2051-2077	Left & right arms (Outwards)
IMii1	CAA AAA TAT GAG TAA TTT ATT CAA ACG G	274-301	Left & right arms (Inwards)
IMii2	GCT TAA GAG ATA AGA AAA AAG TGA CC	305-330	Left & right arms (Inwards)
MiL1	TTA AGA GAT AAG AAA AAA GTG ACC	307-330	Left arm (Inwards)
MiL2	AAT CAT TAT CTA GTT ATG ATC TGC	404-427	Left arm (Inwards)
MiL3	CTG CAA ATA ATG CTG CAG AGC TGG	424-447	Left arm (Inwards)
MiL4	TAA TGC TGC AGA GCT GGG GGA TCC	431-454	Left arm (Inwards)
MiR1	AGT GGA TCC CCC CCT AGG CGC GCC	1762-1785	Right arm (Inwards)
MiR2	CCG CTC TAG AAC TAG TGG ATC CCC	1775-1798	Right arm (Inwards)
MiR3	TAT TAG AAT TGT GTA ACG TCC CGC	1807-1830	Right arm (Inwards)
MiR4	TAA TGT GAT TTA CTA TCA TAC TTA	1849-1872	Right arm (Inwards)
MiR5	GAA CGC GGC GTA ATG TGA TTT ACT	1859-1882	Right arm (Inwards)

Table 3.1. Primers used for inverse PCR. *IMio1*, *IMio2*, *IMii1* and *IMii2* were designed by Klinakis *et al.* (2000). Position refers to the location on the original donor plasmid, pMi[3xP3-eGFP] (Fig. 3.3). The 2.025kb insert runs from position 100 to 2125 on the plasmid. Direction refers to whether the primer faces away from the centre of the insert or towards it.

Two-step gene walking

DNA from individual female transgenic flies was extracted and 50-500ng used as the template for the two-step PCR reactions (Philhofer *et al*. 2007). Three primers were designed for each end of the insert (see Table 3.2). Conditions were identical for all primers: 94°C for 10 minutes; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 3 minutes of 72°C; 1 cycle of 30 seconds at 94°C, 30 seconds at 40°C, 3 minutes at

72°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 3 minutes at 72°C; and a final stage of 10 minutes at 72°C. PCR products were analysed by agarose gel electrophoresis, DNA was isolated from those reactions that gave products (PCR purification kit, Qiagen, UK) and sequenced by Macrogen (Korea) using the sequencing primers listed in Table 3.3.

Primer name	***Sequence (5'-3')***	***Position on pMi[3xP3-eGFP]***	***Position on Insert***
2stepfor_A	CTG CAT TCT AGT TGT GGT TTG TCC	1698-1721	Right arm
2stepfor_B	GTA TGA TAG TAA ATC ACA TTA CG CC G	1852-1877	Right arm
2stepfor_C	CAC ACC TCC CCC TGA ACC	1570-1587	Right arm
2steprev_A	ATC AAG CTT ATC GAT ACC GTC G	477-498	Left arm
2steprev_B	GAA CTT CAG GGT CAG CTT GC	899-918	Left arm
2steprev_C	AGC TTA TCG ATA CCG TCG ACC TC	472-494	Left arm

Table 3.2. Primers used for two-step gene walking. Position refers to location on the original donor plasmid, pMi[3xP3-eGFP] (Fig. 3.3). The 2.025kb insert runs from position 100 to 2125 on the plasmid.

Primer name	***Sequence (5'-3')***	***Position on pMi[3xP3-eGFP]***	***Position on Insert***
seq_a	CCG CGT TCG AAT TAA TAG TGG	329-349; 1875-1895	Both arms
seq_b	TTT TGT TGT TGT TGG AAA TAG AGC	255-278; 1946-1969	Both arms
seq_k	TAA GCT TAC GCC GCG TTC	342-359	Left arm
seq_g	GCA TTC TAG TTG TGG TTT GTC C	1700-1721	Right arm
seq_j	TCA CAT TAC GCC GCG TTC	1865-1882	Right arm
2stepfor_B	GTA TGA TAG TAA ATC ACA TTA CGC CG	1852-1877	Right arm
2steprev_C	AGC TTA TCG ATA CCG TCG ACC TC	472-494	Left arm

Table 3.3. Primers used for sequencing products of two-step gene walking. Position refers to location on the original donor plasmid, pMi[3xP3-eGFP] (Fig. 3.3). The 2.025kb insert runs from position 100 to 2125 on the plasmid.

3.3 RESULTS

3.3.1 Microinjection and detection of the transgene

Microinjection

The ability of *Minos* based transgene vectors to transpose into the germline of stalk-eyed flies was tested by co-injecting *T. dalmanni* embryos with a donor plasmid containing the sequence for *3xP3-eGFP* flanked by the *Minos* TIRs and capped *Minos* transposase-encoding mRNA. Three series of microinjections were carried out.

In the first series, the majority of collected eggs (135) were injected with DNA/mRNA mix, 36 were injected with buffer alone as a sham control for the effect of the addition of DNA and mRNA to the injection buffer. In addition 38 eggs were transferred directly to egg-lays to test for the effect of microinjection. Survivorship for all groups was recorded at larval, pupal and adult stages (Table 3.4). The addition of DNA/mRNA had a weakly detrimental effect on survival to adulthood relative to the sham control (injection of DNA/mRNA = 4.4% survival, injection of buffer = 13.9% survival, χ^2 = 4.211, df = 1, $p < 0.04$). Survivorship of uninjected eggs (31.6%) was significantly higher than of those injected with DNA/mRNA (χ^2 = 23.421, df = 1, $p < 0.0001$) but also indicates that the fertility of the egg-laying adults was low.

Injection treatment	***Number of eggs manipulated***	***First instar larvae (%)***	***Pupae (%)***	***Adults (%)***
DNA/mRNA & injection buffer	135	9 (6.7)	6 (4.4)	6 (4.4)
Injection buffer alone	36	6 (16.7)	5 (13.9)	5 (13.9)
Non-injected control	38	17 (44.7)	14 (36.8)	12 (31.6)

Table 3.4. Survival following the first series of injections. Eggs were injected with a mix of plasmid DNA, mRNA and injection buffer, or injection buffer alone, or were not injected. Number of individuals produced at each developmental stage are shown. This number is also given, in brackets, as a percentage of the number of eggs manipulated per treatment.

To further evaluate the effect of including DNA/mRNA in the injection mix, a second and larger scale series of microinjections was carried out. Equal numbers of eggs were allocated to injection with either DNA/mRNA or injection buffer alone, although not all eggs were successfully injected. This increased the proportion of eggs in the sham control group and also enabled the injections to be performed double blind thus removing experimental bias. 123 eggs were injected with DNA/mRNA, 131 with buffer alone and a further 42 were used as non-injected controls (Table 3.5). The injection of DNA/mRNA had a significantly detrimental effect on survival to adulthood relative to the buffer control (injection of DNA/mRNA = 4.1% survival, injection of buffer = 16.0% survival, $\chi^2 = 9.885$, $df = 1$, $p < 0.02$). and also relative to the non-injected controls ($\chi^2 = 20.346$, $df = 1$, $p < 0.0001$). As in the first round of injections, approximately 30% of eggs in the untreated control group produced viable adults.

Injection treatment	***Number of eggs manipulated***	***First instar larvae (%)***	***Pupae (%)***	***Adults (%)***
DNA/mRNA & injection buffer	123	16 (13.0)	9 (7.3)	5 (4.1)
Injection buffer alone	131	41 (31.3)	27 (20.6)	21 (16.0)
Non-injected control	42	18 (42.9)	12 (28.6)	12 (28.6)

Table 3.5. Survival following the second series of injections. Eggs were injected with a mix of plasmid DNA, mRNA and injection buffer, or injection buffer alone, or were not injected. Number of individuals produced at each developmental stage are shown. This number is also given, in brackets, as a percentage of the number of eggs manipulated per treatment.

The difference in survival between buffer injected and non-injected controls was marginally non-significant ($\chi^2 = 3.240$, $df = 1$, $p = 0.072$). In a third and final series of injections, a modified DNA extraction protocol was tested to determine whether the deleterious effects of DNA on embryo survival could be reduced by using DNA with increased purity. Again eggs were evenly distributed between two groups. In one group 222 eggs were injected with DNA which had been purified by the standard protocol using only a Qiagen plasmid purification kit, and in a second group 219 eggs were injected with DNA that had been purified using a Qiagen plasmid purification kit

followed by an additional ethanol precipitation. Both groups were co-injected with *Minos* transposase encoding mRNA, as in the previous two series. A control group of 68 eggs were transferred directly to egg-lays (Table 3.6). The variation in DNA purification protocol did not significantly affect survival to adulthood (standard protocol = 12.6%, standard protocol plus ethanol precipitation = 16.0%, $\chi^2 = 1.021$, $df = 1$, $p > 0.05$). Survivorship of uninjected eggs (47.1%) was significantly higher than injected eggs for both DNA extraction treatments (vs. standard protocol, $\chi^2 = 37.641$, $df = 1$, $p < 0.0001$; vs. ethanol protocol, $\chi^2 = 28.004$, $df = 1$, $p < 0.0001$).

Injection treament	***Number of eggs manipulated***	***First instar larvae (%)***	***Pupae (%)***	***Adults (%)***
DNA/mRNA (1ppt)	222	32 (14.4)	29 (13.1)	28 (12.6)
DNA/mRNA (2ppt)	219	41 (18.7)	41 (18.7)	35 (16.0)
Non-injected control	68	40 (58.8)	35 (51.5)	32 (47.1)

Table 3.6. Survival following the third series of injections. Eggs were injected with plasmid DNA that had been purified using a Qiagen maxiprep kit, mRNA and injection buffer (1ppt) or with plasmid DNA that had been purified with a Qiagen maxiprep kit followed by a second ethanol precipitation, mRNA and injection buffer (2ppt). As a control, non-injected eggs were directly transferred to egg-lays. The number of individuals produced at each developmental stage is shown. This figure is also given, in brackets, as a percentage of the number of eggs manipulated for each group in brackets.

Phenotypic detection of transformation events

Transposition and integration of transgene DNA into the host germline was assayed by screening for eGFP expression in the offspring of the injected embryos. Germline transformation is a rare event and likely to occur in only a small proportion of injected embryos. In these embryos, typically only a small proportion of germline nuclei are transformed and the transgene may be transmitted to only 1-2% of their offspring. As a result, detection of a transformation event in this species requires that as large a number as possible of the offspring of each injected individual be screened.

For all three series, injected (G0) embryos that survived to sexual maturity were crossed with wild-type individuals to generate independent family lines. The majority of first generation (G1) offspring were screened for eGFP expression as larvae. A small proportion of G1 adults that had pupariated before they could be screened were intercrossed so that their offspring could be tested and transgene carriers identified.

In total, over 5000 G1 individuals were screened (Table 3.7). Expression was only detected in offspring derived from two G0 individuals, labelled 17 and 34. Fly 17 was a product of the first series of injections and fly 34 of the third series. In the lineage derived from fly 17, 93 G1 individuals were screened as larvae and 33 screened via their G2 offspring. Expression was detected in three G1 larvae (2.4%). In the lineage derived from fly 34, 241 individuals were screened as G1 larvae and 42 were screened via their G2 offspring. Expression was seen in 13 larvae and in the offspring of two G1 individuals (5.3%).

Injection series	***Group***	***Number of eggs injected***	***Number of G0 adults***	***Number of fertile G0 adults***	***Number of G1 individuals screened***	***Number of G0 transformants***	***Transformation efficiency***
1	-	135	6	2	290 (33)	1	50%
2	-	123	5	3	32 (2)	0	0
3	1ppt	222	28	20	2350 (42)	1	5%
3	2ppt	219	35	25	2479 (0)	0	0
3	Total	441	63	45	4829 (42)	1	2%
TOTAL	-	699	74	50	5151 (77)	2	4%

Table 3.7. Summary of transformation results of all injection series. Transformation efficiency is given as the percentage of fertile G0 individuals giving rise to transformed G1 offspring. The majority of G1 individuals were screened as G1 larvae. For three lines (17, 34, 44) a proportion of G1 individuals were screened by crossing with other G1 individuals from the same line and then screening the resulting G2 progeny as larvae. The number of individuals screened in this manner is given in brackets. Results from the third series of injections are given separately by DNA preparation method and subsequently combined (see Table 3.6).

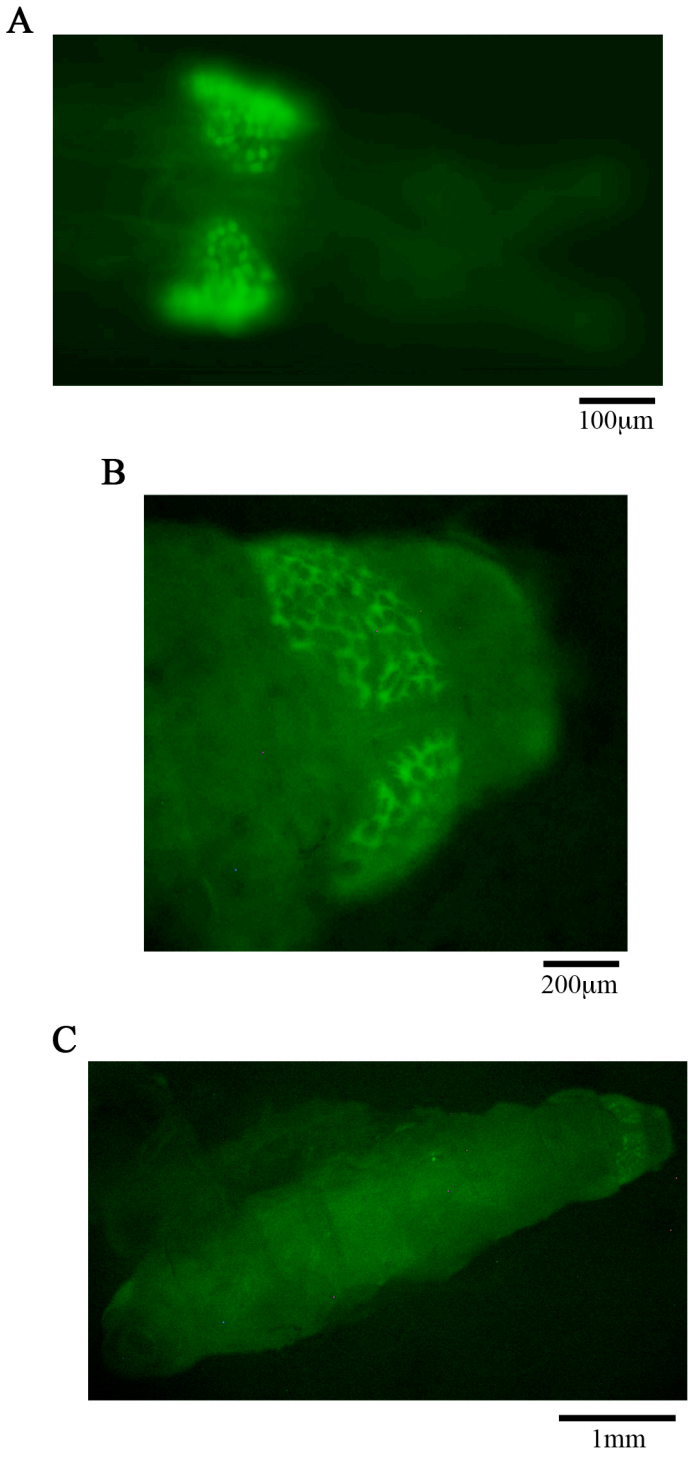

Figure 3.4. eGFP expression in the anal pads of transgenic larvae. Posterior is to the right in all panels. **A**. Posterior end of 3-5 day old larva (first instar) showing expression in discrete round cells within the anal pad. **B**. Posterior end of a third instar larva. Boundaries between expressing cells are not clear. **C**. Full length third instar larva. eGFP expression is seen in the posterior end. A degree of autofluorescence is visible in the gut.

eGFP expression pattern

For both *3xP3-eGFP* lines, eGFP expression was restricted to the larval anal pads (Fig. 3.4). Expression was detected in all three larval instars. No expression was visible during embryonic stages of development and a dissection of pupae and adult flies revealed no detectable expression in the eyes or any other structure at either stage.

The eGFP protein localises to the cytoplasm making it possible to identify the shape of those cells in which it was expressed. Anal pad cell shape appeared to vary between individuals. In some larvae, eGFP expressing cells were round in shape and clear boundaries between cells could be seen (Fig. 3.4A). In other larvae, cells were long and thin and the boundaries between them were relatively indistinct (Fig. 3.4B). Often the latter expression pattern was associated with low levels of fluorescence.

3.3.2 Confirmation and characterisation of the insertion event

Transgene insertions can occur at any location within the genome. Location may affect the segregation and stability of transgene expression. Transgene segregation, transmission and expression over several generations was followed in order to gain more information about the nature of the insertion events in the two founder flies 17 and 34. In addition, Southern blots were carried out to confirm genomic integration and estimate transgene copy number. Finally, inverse PCR (iPCR) and two-step gene walking were used to sequence the flanking regions of the insertions. Lines derived from flies 17 and 34 produced insertions with different genetic and molecular characteristics and will be discussed them separately.

Line 17: Transgene transmission in G1 crosses

Three G1 individuals showed expression of the transgene (Table 3.8). Of these only two (denoted 17.1 and 17.2) survived to sexual maturity. Both were female and were crossed separately to wild-type males. For 17.1 the proportions of eGFP-expressors and non-expressors were consistent with a 1:1 ratio ($\chi^2 = 0.0077$, $df = 1$, $p = 0.933$). In 17.2 there was a significant deficit of eGFP-expressors ($\chi^2 = 5.476$, $df = 1$, $p = 0.019$). The lower than 1:1 ratio of expressing:non-expressing larvae could be due to weak expression in some individuals resulting in false negatives. For both sublines the

observed proportions of expressing offspring are consistent with there being a single insert with variable expressivity inherited from the founder (fly 17).

Fly	***Sex***	***G2 larvae***		***Sex of eGFP expressing G2 larvae***	
		Expressing	*Non-expressing*	*Male*	*Female*
17.1	♀	128	130	38	46
17.2	♀	133	174*	44	47

Table 3.8. Segregation analysis for transgenic line 17, G1 crosses. Surviving eGFP expressing G1 progeny of G0 individual 17 were individually crossed with wild-type virgin flies of the opposite sex to generate G2 offspring. Larvae were checked for eGFP expression and sex was recorded when individuals eclosed. Not all individuals were reared to adulthood. * In line 17.2 there was a significant deficit of eGFP-expressors

Line 17: Sex-linkage in G2 crosses

T. dalmanni possess a chromosomal mechanism for sex determination (Wright *et al.* 2004) whereby females are the homozygous sex (XX) and males are the heterozygous sex (XY). X-linked insertions will therefore only be passed on to female offspring of a carrier male as his male offspring will inherit the Y chromosome and therefore not the transgene-carrying X. To test for sex linkage of the 17.1 and 17.2 insertion(s), a pair of G2 crosses were set up for each subline (17.1 and 17.2; Table 3.9). Carrier males were individually crossed with virgin wild-type females and virgin carrier females were individually crossed with wild-type males. In both sublines the segregation of the transgene with sex was consistent with X-linkage. For crosses involving a carrier male and a wild-type female all male offspring were non-expressing (from at least 24 individuals assessed in each subline; Table 3.9). By contrast for crosses involving a carrier female, progeny that expressed eGFP were equally distributed between the sexes (17.1: $\chi^2 = 0.0370$, $df = 1$, $p = 0.85$; 17.2: $\chi^2 = 0.0238$, $df = 1$, $p = 0.89$; Table 3.9). These results demonstrate that the insert is not sex-limited but can be expressed equally in males and females.

Subline	*Cross*	*G3 larvae*		*Sex of surviving G3 offspring*			
		Expressing	*Non-expressing*	*Expressing*		*Non-expressing*	
				Male	*Female*	*Male*	*Female*
17.1	$eGFP^+$ ♂ × wt ♀	98	111	0^+	60	101	2
17.1	$eGFP^+$ ♀ × wt ♂	81	79	28	26	29	30
17.2	$eGFP^+$ ♂ × wt ♀	27	38	0^+	24	19	3
17.2	$eGFP^+$ ♀ × wt ♂	32	46	11	10	11	14

Table 3.9. Segregation of the insert with sex in line 17, G2 crosses. G2 individuals that had expressed eGFP as larvae ($eGFP^+$) were individually crossed with wild-type (wt) virgin flies of the opposite sex. Results are given as total output of five crosses. Fly 17 produced a third expressing larva that died prior to adulthood, so is not included.
$^+$ Crosses involving $eGFP^+$ male flies did not produce any male offspring positive for eGFP expression in both sublines..

Line 17: Genetic confirmation and characterisation

Stock populations were set up for both sublines using eGFP-expressing (hemizygous) males and homozygous carrier females. Transgene expression was detected in all individuals sampled in eight successive generations confirming that the insertion(s) is stable. Integration of the *Minos* element into the host genome was confirmed by Southern analysis (Fig. 3.5). Genomic DNA was isolated from a pool of 20-30 homozygous transgenic females from the stock populations for each subline. A single 6500bp fragment was detected in sublines 17.1 and 17.2 indicating that both sublines are derived from a single insertion event in the G0 parent fly 17.

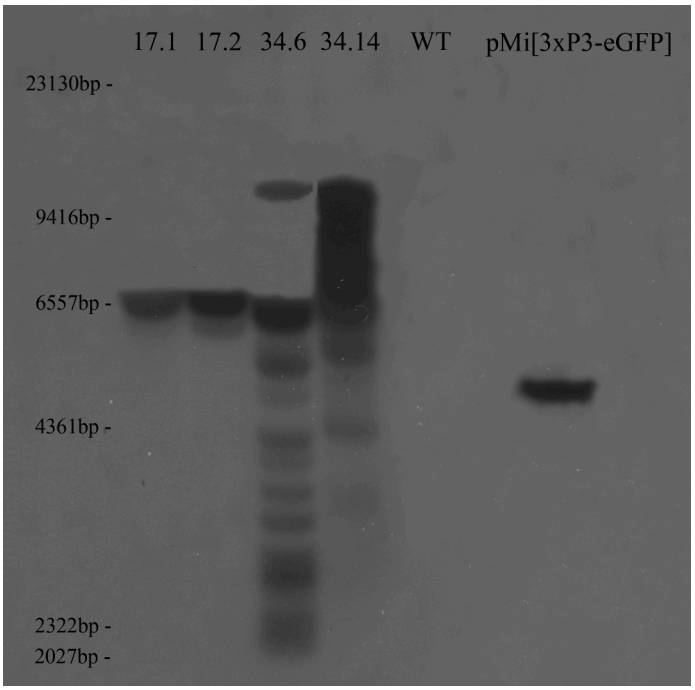

Figure 3.5. Southern analysis to confirm transgene integration and estimate insert number. DNA was extracted from individuals from four sublines carrying the transgene (17.1, 17.2, 34.6 & 34.14) and digested with *EcoRI* (see Fig. 3.3). The probe was generated from the NotI/SalI fragment of pMi[3xP3-eGFP] (Fig. 3.3). DNA from wild-type (WT) individuals and the pMi[3xP3-eGFP] plasmid were included as controls. Single bands of the same size (approximately 6500bp) are seen for sublines 17.1 and 17.2. For sublines 34.6 and 34.14 multiple bands are seen indicating multiple inserts at different locations in the genome. No bands are seen in wild-type DNA. *EcoRI* cuts pMi[3xP3-eGFP] once resulting in a single 5041bp band.

Both iPCR and two-step gene walking approaches were used in an attempt to obtain sequence data from the genomic regions flanking the transgene. iPCR was performed with a range of concentrations of genomic DNA but no PCR products were generated. Two-step gene walking using three primers for each arm of the insert initially appeared more successful. For both sublines, positive PCR products were produced for the flanking DNA of each arm of the insert from at least one of the primers. These products were sent away for sequencing but no positive sequences were returned.

Line 34: Transgene transmission and sex-linkage in G1 crosses

15 G1 offspring showed expression of the transgene and 11 of these individuals (5 females and 6 males) survived to sexual maturity. Individual matings were carried out with wild-type flies and the G2 offspring from each subline scored for eGFP expression

(Table 3.10, where relevant statistics also can be found for these crosses). The expression of the sublines was heterogeneous. In 4 sublines (34.3, 34.4, 34.6 & 34.9) there was no significant deviation from the expected Mendelian proportion (50%) of G2 larvae showing eGFP expression (Table 3.10). However note that in two cases the sample size was small (line 34.3: n = 32; line 34.9: n = 18) and in the other cases the deviation from expectation failed only very narrowly to reach the lowest level of significance (34.4: $p = 0.05001$; 34.6: $p = 0.06$). In the other seven sublines (34.5, 34.7, 34.8, 34.10, 34.11, 34.14 & 34.15) there was significant deviation from the expected proportion of 50% expression among the G2 individuals and in each case there was a deficit of expressors (Table 3.10). This outcome indicates that the inserted transgene is subject either to instability and loss or to epigenetic silencing effects.

Fly	**Sex**	**G2 larvae**		**Sex of surviving G2 offspring**			
		Expressing	*Non-expressing*	*Expressing*		*Non-expressing*	
				Male	*Female*	*Male*	*Female*
34.3	♀	12	20	3	3	1	2
34.4	♀	52	74†	22	20	16	15
34.5	♂	12	33*	1	6	10	10
34.6	♂	50	71†	13	17	70	38
34.7	♂	65	172*	0^{+}	32	70	38
34.8	♀	24	47*	7	13	8	15
34.9	♀	7	11	5	2	1	2
34.10	♀	15	103*	4	6	26	35
34.11	♂	30	100*	0^{+}	14	27	6
34.14	♂	96	215*	0^{+}	72	41	17
34.15	♂	10	43*	0^{+}	5	5	6

Table 3.10. Segregation analysis for transgenic line 34, G1 crosses. Surviving eGFP expressing G1 progeny of the G0 fly 34 were individually crossed with wild-type virgin flies of the opposite sex to generate G2 offspring. G2 offspring were screened for eGFP expression during the third larval instar and sex was recorded when individuals eclosed. Not all individuals were reared to adulthood. Four expressing offspring of fly 34 (34.1, 34.2, 34.12, 34.13) did not develop into fertile adults and are not listed.

[*] A significant deviation from the 50:50 expressing:non-expressing ratio expected with a single insertion event was observed in the offspring of seven flies and in each case there was a deficit of expressors (34.5: $\chi^2 = 9.8$, $df = 1$, $p = 0.002$; 34.7: $\chi^2 = 48.308$, $df = 1$, $p < 0.0001$; 34.8: $\chi^2 = 7.451$, $df = 1$, $p = 0.006$; 34.10: $\chi^2 = 65.627$, $df = 1$, $p < 0.0001$; 34.11: $\chi^2 = 37.692$, $df = 1$, $p < 0.0001$; 34.14: $\chi^2 = 45.534$, $df = 1$, $p < 0.0001$; 34.15: $\chi^2 = 20.547$, $df = 1$, $p < 0.0001$).

[†] In the offspring produced from flies 34.4 and 34.6 the deviation from the expected 50:50 ratio was only marginally non-significant (34.4: $\chi^2 = 3.841$, $df = 1$, $p = 0.05001$; 34.6: $\chi^2 = 3.645$, $df = 1$, $p = 0.05625$).

[+] A significant deviation from a 50:50 male:female ratio was observed amongst expressing offspring in four sublines where male expressors were absent (34.7: $\chi^2 = 32$, $df = 1$, $p < 0.0001$; 34.11: $\chi^2 = 14$, $df = 1$, $p = 0.0002$; 34.14: $\chi^2 = 72$, $df = 1$, $p < 0.0001$; 34.15: $\chi^2 = 5$, $df = 1$, $p = 0.025$).

Six of the G1 founders were male. The segregation of the transgene with respect to sex in their G2 offspring was analysed for evidence of sex-linkage (Table 3.10). Two of the sublines derived from G1 males (34.5 & 34.6) produced expressing progeny of both sexes. In the four remaining sublines derived from G1 males (34.7, 34.11, 34.14 & 34.15), eGFP expression was only seen in female offspring. This pattern is consistent with X-linkage of the insert. The fact that expression is restricted to female offspring in some, but not all, sublines indicates that at least two inserts are present among these sublines, one of which is X-linked and the other autosomal. One X-linked subline (34.14) and one autosomal subline (34.6) were selected for further analysis of the instability/silencing phenomenon observed in lineages derived from founder 34.

Line 34: Silencing effects in G2 crosses

Since the insert in the male G1 fly 34.14 appeared to be X-linked, all female G2 progeny would be expected to have inherited the transgene. eGFP expression was not seen in all female progeny. Crosses were set up to test whether this was due to transgene loss or epigenetic silencing. 10 G2 expressing females and 10 G2 non-expressing females were individually crossed with wild-type males. Both types of cross gave rise to expressing G3 offspring (Table 3.11). This indicates that in the non-expressing females the transgene was present but silenced rather than lost from the genome due to instability. Had the transgene simply been lost then expression could not have reappeared in subsequent generations. In both types of cross, significantly fewer than

the expected proportion of 50% of offspring expressed the transgene (χ^2 = 227.8 & 97.2, df = 1, p < 0.001 for both). The proportion of offspring expressing the transgene was significantly lower in crosses involving non-expressing females than expressing females (33% vs. 10%, χ^2 = 31.51, *df* = 1, *p* < 0.0001).

Subline	***Cross***	***G3 larvae***		***Sex of surviving G3 offspring***			
		Expressing	*Non-expressing*	*Expressing*		*Non-expressing*	
				Male	*Female*	*Male*	*Female*
34.14	$eGFP^+$ ♀ × wt ♂	166	335*	11	10	44	60
34.14	$eGFP^-$ ♀ × wt ♂	15	137*	NR	NR	NR	NR

Table 3.11. Transgene expression in offspring of expressing and non-expressing individuals in subline 34.14, G2 crosses. Female flies that showed expression as G2 larvae ($eGFP^+$) were crossed with wild-type virgin males. Female flies that did not show expression as G2 larvae ($eGFP^-$) were crossed with wild-type virgin males. G3 offspring were screened for eGFP expression during the third larval instar and sex was recorded when individuals eclosed. NR = data not recorded. * Both crosses produced less than the expected proportion of 50% expressors (statistics in text).

In the autosomal subline 34.6 the ratio of expressing to non-expressing G2 larvae was only marginally not significantly different from 50:50, which raises the possibility that silencing effects were occurring (Table 3.10). Two further replicate G2 crosses were carried out to investigate this. Each cross mated three expressing males to three expressing females (Table 3.12). In both replicates, there were significantly fewer transgene expressors than the proportion of 75% predicted from such a cross between heterozygotes (replicate 1: χ^2 = 1783.1, *df* = 1, *p* < 0.0001; replicate 2: χ^2 = 9.6, *df* = 1, *p* < 0.002). This confirms that silencing effects were present in the subline.

Subline	*Cross*	*G3 larvae*		*Sex of surviving G3 offspring*			
		Expressing	*Non-expressing*	*Expressing*		*Non-expressing*	
				Male	*Female*	*Male*	*Female*
34.6	$eGFP^+$ ♂ × $eGFP^+$ ♀	57	50*	NR	NR	NR	NR
34.6	$eGFP^+$ ♂ × $eGFP^+$ ♀	46	31*	NR	NR	NR	NR

Table 3.12. Segregation of the insert in subline 34.6, G2 crosses. The two replicate crosses consisted of 3 male G2 flies that showed eGFP expression ($eGFP^+$) as larvae with 3 female $eGFP^+$ flies. G3 offspring were screened for eGFP expression during the third larval instar and sex was recorded when individuals eclosed. NR = data not recorded.
* In both replicates, the proportion of G3 expressors was significantly lower than the expected 75:25 ratio (statistics in text).

Line 34: Genetic confirmation and characterisation

Within sublines 34.14 and 34.6, mass crosses, using only expressing individuals, were performed to yield an increased population size for genomic DNA extractions and subsequent maintenance in the laboratory. Although the mass crosses yielded fewer than the expected numbers of expressing individuals, expression persisted in the population for six generations. Both sublines were culled in the seventh generation.

Genomic DNA from sublines 34.14 and 34.6 were analysed for presence and copy number of the transgene using a Southern blot (Fig. 3.5). Both sublines appeared to possess many copies of the transgene as multiple bands were present. No bands were less than the minimum expected fragment size (1339bp) for the insertion digested with *EcoRI*. Both iPCR and two-step gene walking were used to obtain flanking sequence for the insertions. No iPCR products were observed, but with the two-step gene walking approach at least one PCR product was produced for each end of the insert in each subline. The PCR products were sent away for sequencing and sequence data for DNA flanking the right arm of an insert in subline 34.6 was returned (Fig. 3.6). The characteristic TA duplication associated with *Minos* mediated insertion was observed indicating a transposase mediated insertion event. The sequence of the flanking region showed no homology with any known genes for stalk-eyed flies or for other species.

5’AATTATGATTAAGCTTATGATCGCGTACGGCGCGCCTAGGGGGGGATCCACTAGT
TCTAGAGCGGCCGCCACCGCGGGACGTTACACAATTCTAATATTAATTAAATTATT
GTTTTAAGTATGATAGTAAATCACATTACGCCGCGTTCGAATTAATAGTGGTCACTT
TTTTCTTATCTCTTAAGCAAACCGTTTGAATAAATTACTCATATTTTTGTTGTTGTTG
GAAATAGAGCAAAACTTTTTTTTTCGTCGTGAAGAGAATAAAATTCTCTTTGAGAC
GAAATGCATTGGTATGTGTTATCTTTAGTAGTATTGATAATATAGTGTGTTAAACAT
TGCGCACTGCAAAAAAAACATGCTGTTCGAATTAATAGTGGTTGGGGCTCGTAtatatt
ctaagtttagcttcgtaggtacgccgataaaaaatatacaaaaaaatttaaacaacaaattgtttatatattagtttacatatatacatatataaata
aacatatatttttatttgttttttttttttaaaaaatttttggttgggcgaagccccttgatctctttaaaaaaaaccagggtataaaggaaaacccc
gcatttaagtctttaaaaatttcccaaattttcccggaaaatttttatatttttttttggggccg 3’

Figure 3.6. Flanking sequence of right arm of an insert present in subline 34.6. pMi[3xP3-eGFP] sequence is shown in capital letters. Flanking genomic sequence is shown in lower case letters. TA duplication, characteristic of a *Minos* mediated insertion event, is underlined.

3.4 DISCUSSION

This chapter describes the first successful germline transformation of the stalk-eyed fly, *Teleopsis dalmanni*. At least three insertion events occurred, one of which involved a single insert on the X chromosome which was stable for at least eight generations. The presence of the inserts was confirmed by Southern blot analysis and flanking sequence from one insert was recovered and shown to include the TA duplication, which is characteristic of a *Minos* mediated insertion event.

3.4.1 Analysis of insertion events

Expression pattern

As expected the *3xP3-eGFP* construct drives detectable expression in stalk-eyed flies. eGFP expression was seen solely in the anal pads of developing larvae in all three larval instars. No expression in the developing or adult eye was seen but note that expression may be obscured in adult stages by eye pigment.

The eye-localised activity is one of the attractive properties of the *3xP3* promoter for transgenics. In insects transformed with *3xP3-eGFP*, eGFP expression is commonly seen in the eye or the developing eye primordia such as in *Ae. aegypti* (Adelman *et al.* 2008; Kokoza *et al.* 2001), *Ae. fluviatilis* (Rodrigues *et al.* 2006), *B. mori* (Tan *et al.* 2005), *B. anynana* (Marcus *et al.* 2004), *D. melanogaster* (Berghammer *et al.* 1999 & Horn *et al.* 2000), *T. casteneum* (Berghammer *et al.* 1999 & Lorenzen *et al.* 2003), *Harmonia axyridis* (Kuwayama *et al.* 2006) and *M. domestica* (Hediger *et al.* 2001). Developmental expression has been noted elsewhere including other regions of the central nervous system, the digestive tract and anal pads (Horn *et al.* 2000; Kokoza *et al*. 2001; Rodrigues *et al.* 2006; Hediger *et al.* 2001). In *M. domestica,* two different larval patterns of eGFP expression were reported with one having expression restricted to the anal pads (Hediger *et al.* 2001).

Variation in the location of transgene expression is common between insect transgenic lines due to *cis*-regulatory effects. The restriction of expression of *3xP3-eGFP* to the anal pads in all sublines indicates that either *3xP3-eGFP* is incapable of driving expression in the eyes of stalk-eyed flies or that the expression is being restricted due to

the local genomic environment of the insert. With only three independent insertions available for analysis, it is not possible to decide between these alternatives.

The structure and function of the anal pads has been well studied in species with aquatic larvae, such as *Ae. aegypti*, where they are referred to as anal papillae (Clark *et al.* 2007). In *D. melanogaster* the anal pads are not an extensively studied organ but have been shown to be involved in larval osmoregulation (Keyser *et al*. 2007). They lie either side of the anus, are one cell thick and secrete a thin cuticle. The cells of the pad swell and deflate depending on the salinity of the medium (Jarial 1987) and are capable of ion transport (Pielage *et al.* 2002). The anal pads of stalk-eyed flies are larger than those of *D. melanogaster* and occupy both the dorsal and ventral sides of the larva while *D. melanogaster* pads are restricted to the ventral side. In stalk-eyed fly larvae, initial observations indicate that the volume of fluid contained in the anal pads and their cellular structure varies between individuals, which may reflect variation in the developmental stage of the larva when screened. Without knowing more about the structure and role of the anal pads in *T. dalmanni* larvae it is not possible to say what might be underlying these variations. One practical consequence was that at certain stages of development the GFP expression was weak and difficult to score. This problem was compounded when the anal pads were swollen.

Different insertion events and transgene silencing/loss

Segregation analysis indicated that at least three different insertion events had occurred and each insertion event demonstrated different characteristics. Line 17 possessed a single X-linked insert which was inherited in the expected Mendelian ratios except for one subline cross where the transgene was slightly under-represented. The proportion of individuals inheriting the transgene was never greater than predicted, indicative of either a degree of transgene silencing or the occurrence of false-negatives due to the weak expression associated with some of the different cell structures observed in the anal pad.

The insertions in sublines derived from fly 34 showed characteristics previously associated with insert instability (Adelman *et al.* 2004). Multiple copies of the insert were present and there was under-representation of GFP expression in crosses involving carriers of the transgene. However, the flanking sequence of one arm of the insert from

subline 34.6 showed the characteristic TA duplication associated with *Minos* integration events indicating that a transposase mediated and typically stable insertion event had occurred. Additional direct evidence that loss of expression was reversible, and therefore due to epigenetic silencing rather than insert loss was obtained in subline 34.14. In addition, in subline 34.14 non-expressing carrier females produced a smaller proportion of expressing offspring than expressing carrier females. This is consistent with there being a heritable component underlying the silencing effect, as has been well established for epigenetic mechanisms (eg TPE/PEV) of gene silencing (Singh *et al.* 2008). Epigenetic silencing has been observed in a number of insect germline transformations although the exact mechanism is rarely known (Coates *et al.* 1998; Handler *et al.* 1998; Kurenova *et al.* 1998; Jasinskiene *et al.* 1998; Nolan *et al.* 2002; González-Estévez *et al.* 2003; Kokoza *et al.* 2001; Lorenzen *et al.* 2007; Koukidou *et al.* 2006).

Inverse PCR and two-step gene walking

Knowledge of the flanking sequences confirms the mechanism of the insertion event and also can give information as to the location of the insert within the genome. Only one flanking sequence of one arm of one of the inserts was recovered even though two approaches, iPCR and two-step gene walking, were taken. iPCR was unsuccessful: problems with this technique are frequently reported in the literature, for example it is common for studies of germline transformation to fail to obtain all of the flanking sequences for all insertions (Marcus *et al.* 2004; O'Brochta *et al.* 2000; Thomas *et al.* 2002). The two-step gene walking method has been proposed to be a more effective and reliable alternative to iPCR but did not prove entirely successful here. Many of the two-step PCR reactions generated products but sequencing reactions using these products were only successful in one case.

Difficulties with primer design may have affected the efficacy of the two-step procedure. Reliable PCR/sequencing primers should be 18-24bp in length, have a melting temperature of 55ºC-62ºC and avoid inverted repeats. In the *Minos* vector the presence of the TIR and multiple cloning sites in the critical regions for two-step primer design severely restrict the sequences available. A second confounding factor is the presence of multiple inserts in sublines 34.6 and 34.14. If these were due to multiple integration events in different locations the two-step PCR procedure would generate

more than one type of product, which would need to be separated before they could be sequenced. From the data reported here, two-step gene walking is not as reliable as claimed (Pilhofer *et al.* 2007; Pilhofer *et al.* 2008). However, it did prove to be a more effective protocol than iPCR as produced one piece of sequence data and was less labour intensive.

3.4.2 Assessment of and potential improvements to the transgenic protocol

The present study demonstrates that transposon mediated germline transformation is possible in stalk-eyed flies. The transformation efficiency achieved (4%) was comparable to other insect transgenic systems, but was at the lower end of the spectrum of reported transformation efficiencies. Caution should be taken when interpreting transformation efficiencies. Transformation efficiencies reported are often overestimates since failed attempts are rarely reported (O'Brochta & Handler 2008; O'Brochta & Atkinson 2004). On the other hand the method for estimating transformation efficiency does not take into account whether all insertion events are suitable for further study. Only insertion events that sustain stable and reliable expression through multiple generations will be useful. In this experiment one line met the criteria of reliable and stable expression.

Although the transformation efficiency reported is low, the fact that germline transformation has occurred in *T. dalmanni* should be seen as a very positive result and a starting point for the development of transgenic tools in stalk-eyed flies. There are several insect species for which attempts to create transgenic lines have failed, for instance *Spodoptera frugiperda*, *Phthorimaea operculella, Gryllus bimaculatus* and *Heliothis virescens* (Mohammed & Coates 2004; Shinmyo *et al.* 2004; O'Brochta & Handler 2008).

The efficiency of transgenic protocols can be limited at a number of stages. Mortality due to microinjection is high in many species. For example, in *M. domestica* (Hediger *et al.* 2001) only 41 fertile G0 adults survived from 1668 injected embryos and this was exacerbated by a low fertility rate. A transformation rate of 17% meant that seven insertion events were recovered. If high mortality rates are combined with low transformation rates then a transgenic protocol will require a large workload. Certain applications of transgenics such as promoter and *cis*-regulatory element characterisation

require high transformation efficiencies, however many other applications do not require this (discussed in Chapter Five). Reported transformation rates in *C. capitata* are similarly low (~5%, Handler *et al.* 1998) but recent work by Condon *et al.* (2007b) has involved experiments that require a high number of transformation events to occur. Furthermore there are opportunities for further optimisation of the protocol in stalk-eyed flies, along with general improvements to transgenic protocols that will arise with increased understanding of the mechanism.

Improving transformation efficiency in stalk-eyed flies

Only one transposable element has been used in this study. Evidence described in Chapter Two indicates that *piggyBac* is another viable vector for stalk-eyed flies. Transposable elements vary considerably in their efficiency depending on species, *Hermes* in *T. casteneum* resulted in a transformation efficiency of 1% whereas *piggyBac* in the same experiment produced a transformation efficiency of 60% (Berghammer *et al.* 1999). In the same study the reverse was seen in *D. melanogaster* where the transformation rates for *Hermes* and *piggyBac* were 50% and 4% respectively. As previously stated, transformation rates are very variable so it is unclear whether these are trends or isolated results.

Transgenes are very susceptible to silencing effects and consequently a significant proportion of insertions may not be detected in initial phenotype based screens. In both insertion events in line 34, silencing of the transgene was observed. Elements such as scs-scs', *gypsy* transposon and chicken *β-globin 5' HS4* element have been found to prevent transgene silencing (Cai & Levine 1995; Gerasimova & Corces 1996; Bell *et al.* 2001; Sarkar *et al.* 2006). This is commonly mediated by preventing the spread of heterochromatin through the recruitment of histone modifying enzymes. Sarkar *et al.* (2006) used the scs-scs' element to flank reporter constructs which included 3xP3 driving the expression of one of three fluorescent reporter genes. Very little variation in intensity of fluorescence was observed within and between lines. Compared to other studies no dramatic increase in transformation rate was observed, although this may be due to random variation of transformation rates across experiments. Reduced variability in reporter gene expression will result in fewer G1 individuals needing to be processed before a transformation event is detected.

The phage ϕ31integrase, a site-specific recombinase, has recently emerged as an attractive prospect for insect transgenesis. Insertion requires the presence of *attP* sites, which can be already present in or be readily introduced to the genome. Its use is well established in mammalian cell lines (Thyagarajan *et al.* 2001). ϕ31integrase has been used to create transgenic lines in *D.melanogaster* (Groth *et al.* 2004) and *Ae.aegypti* (Nimmo *et al.* 2006) with high transformation efficiencies being associated. Furthermore transposon vectors are, in general, limited in efficiency by the size of the insert being carried but this effect is not seen with ϕ31 integrations (Lorenzen *et al.* 2003; Geurts *et al.* 2003; Nimmo *et al.* 2006). Since no genome sequence is available for stalk-eyed flies it is not known whether *attP* sites are present in the genome. However, they could be introduced via a transposon mediated transformation event.

In some transformation systems in *D. melanogaster* (Cooley *et al.* 1988; Häcker *et al.* 2003), *T. castaneum* (Lorenzen *et al.* 2007) and *Ae. aegypt* (Scali *et al.* 2007) an endogenous source of transposase is used for microinjections. This is introduced by a prior insertion of a transgene containing the relevant transposase sequence. The transgene is either rendered stable by removal of its TIRs, or is introduced using a different transposon vector to the transposase it is carrying thus maintaining stability. The endogenous source of transposase has been seen to cause an increase in efficiency of the transformation protocol compared to an injected source of transposase (Häcker *et al.* 2003; Lorenzen *et al.* 2007). Previous experiments (Chapter Two) showed that both *Minos* and *piggyBac* were active in stalk-eyed fly embryos, therefore a *Minos* based insertion could be used to supply a *piggyBac* source of transposase and vice-versa.

Optimisation of transposase and transposon activity

The mechanisms of transposition are not fully understood. When using *piggyBac*, *mariner* and *Minos,* the transposon vector and the transposase used in transgenics are derived directly from the originally isolated "wild" transposon (Atkinson 2008). Little attempt has been made to improve the efficacy of the enzyme with a view to improving transformation efficiency. In nature, transposons with high amounts of activity are selected against (Hurst & Werren 2001; Tosi & Beverley 2000). Alteration of the TIRs of the *Tn5* transposon resulted in a 1000 fold increase of *in vitro* activity (Goryshin & Reznikoff 1998). Recently the crystal structures of the *Hermes* (Zhou *et al.* 2004) and *Mos1/mariner* (Hickman *et al* 2005; Richardson *et al.* 2006) transposase proteins were

assessed giving information on their DNA binding properties. Directed mutation of the *Himar1* transposase protein has resulted in two hypersensitive mutants that caused increased activity (Butler & Chakraborty 2006) and variation in transposition has been detected using different mutants of the *Himar1* and *Mos1* transposases in *Ae. aegypti* (Pledger *et al.* 2004; Pledger & Coates 2005). In *piggyBac,* nuclear localizing signals on the transposase protein have been identified and tested (Keith *et al.* 2008). Increased understanding of the mechanisms involved in transposition could be utilized to improve transformation efficiencies in transgenic protocols.

Recently the regulation of transposon activity by micro-RNAs (miRNAs) has been proposed (Brennecke *et al.* 2007). A large number of miRNAs associated with transposases were found when purifying proteins that make up the RNA induced silencing complex (RISC-complex). This implies that the miRNAs are involved in transposase silencing. Furthermore *piwi* interacting RNA (piRNA) is associated with the *flamenco* locus which regulates activity of the *gypsy* transposon. Further understanding of the activity of miRNA interaction may improve mechanisms and selection of transposable element vectors (Atkinson 2008). If miRNAs are present in a genome that inhibit the action of specific transposon they will also inhibit activity of a transposon that shares sequences in common. This could explain why *mariner* was seen to be inactive in *T. dalmanni* embryos. *Minos* is a member of the *Tc1/Mariner* super-family and distantly related to the *mariner* type elements found in the stalk-eyed fly genome (Carr 2008) and this may reduce *Minos* activity in this organism.

3.4.3 Conclusions and future work

The results described in this chapter provide proof of principle that stable germline transformation of the stalk-eyed fly, *T. dalmanni,* is possible. The observed transformation efficiency of 4% is at the low end of those reported in insect species but could potentially be increased by using a vector or reporter gene system better suited to stalk-eyed flies.

One immediately accessible application of transgenic stalk-eyed flies exploits the X-linkage of the eGFP transgene in line 17 to enable a simple sex determination method for live larvae. If a carrier male from this line is crossed with a wild-type female then only female larval offspring will express eGFP. One of the key features of *T. dalmanni*

is the extreme sexual dimorphism for eyespan (Wilkinson & Dodson 1997). To fully understand the developmental and genetic basis of the difference between male and female eyespan it will be necessary to identify differences between the sexes at larval and pupal stages. Previously it has only been possible to sex larvae and pupae based on the analysis of microsatellite markers or genital disc morphology (Carr *et al.* 2006). Both methods are time consuming and require the larvae/pupae to be sacrificed. Female-specific transgene expression will allow living female and male larvae to be identified and processed separately.

With minor modifications to currently available constructs, transgenics in *T. dalmanni* could be combined with developmental gene expression studies to further understand the evolution and development of novel and exaggerated traits. Mis-expression constructs for candidate genes or transgene mediated RNAi could be introduced into the genome to manipulate function. Reporter constructs can be also used to assay potential *cis*-regulatory regions and further extend the range of manipulations possible. In the longer term, constructs for enhancer trapping and insertional mutagenesis may be used to identify new candidate genes on the basis of stage and tissue specific expression or phenotype.

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Chapter Four: Cell size and cell number in the eye-stalk

4.1 INTRODUCTION

Background

Sexual selection has driven the evolution of an array of bizarre and exaggerated morphologies in nature, classic examples include the elaborate tail feathers of the peacock and the bright plumage seen in birds of paradise. In these species, the exaggerated, or ornamental, trait is used by males to attract females, with possessors of the most elaborate traits experiencing increased mating success. The processes underlying the evolution of such ornamental traits have fascinated biologists for many years (Fisher 1930, Andersson 1994).

Hypercephaly in stalk-eyed flies (Diopsidae) provides a well-studied example of an exaggerated ornamental trait, in both sexes the eyes are laterally displaced from the head on long eye-stalks (Wilkinson & Dodson 1997; Chapman *et al*. 2005). Many species of stalk-eyed fly exhibit sexual dimorphism for eyespan (distance between the eyes) with males possessing eyespans of up to twice those of females. Models of sexual selection predict that for these exaggerated (and often costly) traits to be maintained within a population, a female preference for the trait must be present that confers an advantage to choosy females. It has been demonstrated on a number of occasions that females show a marked preference for males with large eyespans in dimorphic stalk-eyed fly species (Hingle *et al.* 2001a, b; Cotton *et al.* 2006; 2009). In addition, eyespan has been shown to be heritable (Wilkinson & Reillo 1994; David *et al.* 2000), therefore females will benefit from mating with a male with increased eyespan as their attractive male offspring will sire a larger proportion of the next generation. It has also been shown that eyespan is an honest signal of male quality as the trait is highly sensitive to larval nutritional stress in males (David *et al.* 1998, 2000; Cotton *et al.* 2004a, b). A similar scenario is observed in females but to a far lesser extent (Cotton et al. 2004a). Furthermore response to nutrition has been shown to have a genetic component (David *et al.* 2000; Cotton 2004), as a result the offspring of females that demonstrate preference may inherit higher quality genes (Iwasa & Pomiankowski 1994).

Although the selective pressures driving the evolution of exaggerated eyespan have been well studied, little is known about the underlying developmental mechanisms regulating variation in this sexually selected trait. The observed dimorphism observed is

purely a difference in size, and therefore growth, rather than structure as seen in horned beetles, for example (Emlen *et al.* 2007). Information can be obtained on the ontological processes shaping the evolution of this trait by investigating how eye-stalk growth, that causes variation in eyespan, differs between and within sexes, and in response to environmental factors.

4.1.1 Organ growth in holometabolous insects

Stalk-eyed flies are holometabolous insects, which hatch from eggs and go through three larval instars before pupariating and undergoing metamorphosis to generate the adult form. Final body size is determined by larval weight at pupariation. A significant body of research on growth control in animals has focused on holometabolous insects, in particular, *Manduca sexta*, *Drosophila melanogaster* and *Bombyx mori* (Emlen & Nijhout 2000). In such species all adult epidermal structures arise from clusters of cells called imaginal discs. These discs proliferate during the larval stages before everting and undergoing a complex process of morphogenesis and differentiation to form the adult organs during metamorphosis. The final size of each organ will be determined by the product of the duration of its growth period and the growth rate during this period (Shingleton *et al.* 2007; Edgar 2006; Emlen & Nijhout 2000; Chapter 1.3.2). In addition, there is evidence that intrinsic patterning signals in imaginal disc development set a limit or target size for the adult organ(s) (Johnston & Gallant 2002). Variation in any of these parameters will result in a change in organ size.

Cell size, cell number and organ growth

Alterations in the growth of an organ by any mechanism will ultimately be mediated by alterations in cell size and cell number. The relative contributions of cell size and cell number have never been studied in a sexually selected ornamental trait. Comparing cell size and cell number in males and females or between individuals exposed to different environments will give clues as to the mechanisms driving differential organ growth.

Cell size and cell number are differentially affected by changes in the duration and rate of organ growth. Increased eyespan might be due to an increased period of proliferation in eye-stalk progenitor cells. Differences in growth period of imaginal discs are commonly reported (Shingleton *et al.* 2005) and it has been suggested that this reflects

different sensitivities to circulating hormones such as ecdysone and juvenile hormone (Reddy *et al.* 1980; Mirth 2005). Manipulations that alter the level of ecdysone expression and hence the duration of the growth period primarily affect cell number rather than cell size (McBrayer *et al.* 2007; Colombani *et al.* 2005). Differences in eyespan may be mediated by the rate of growth as well as its duration. The rate of nutrition sensitive growth is regulated by the InR/TOR signalling pathway (Chen *et al.* 1996; Oldham *et al.* 2000), manipulations of which have disc autonomous effects on cell size and cell number (Goberdhan & Wilson 2002). Different components vary in their effects. For instance, altering FOXO expression or activity causes changes in cell number and not cell size (Puig *et al.* 2003; Jünger *et al.* 2003), while altered expression of TSC1 or TSC2 only affects cell size (Potter *et al.* 2001).

The duration and rate of growth are proximate developmental factors that determine organ size. Evolutionary relationships between organ size, cell size and cell number vary between species, sexes and populations. Changes in cell size and cell number can be very sensitive indicators of differences between responses to experimental manipulation or selection regime (Robertson 1959a, b; Stevenson *et al.* 1995; Arendt 2006; Chapter 1.3.3). For example, in *D. melanogaster* and the dung fly, *Scathophaga stercoraria*, males and females show significantly different responses in wing size, cell size and cell number to varied rearing conditions (Blanckenhorn & Llaurens 2005). If the relationship between eye-stalk length, cell size and cell number were to vary between the sexes in *Teleopsis dalmanni* this would imply different mechanisms of size regulation in males and females: for example, that factors other than hormone sensitivity or InR/TOR signaling may be involved in causing exaggerated male eyespan in dimorphic species.

4.1.2 Experimental measurement of cell size in insects

Estimation of variation in cell size and cell number in most insect organs is problematic as adults are surrounded by melanised cuticle and the underlying tissue is not easily visible. Cell size and number can be assessed in imaginal discs by disseminating the tissue and fluorescence activated cell sorting (FACs) (e.g. Weinkove *et al.* 1999). Counting the ommatidia present in the insect eye is another approach used to assess cell number and cell size (e.g. Stevenson *et al.* 1995). Most commonly in insects, cell size and cell number estimations are carried out in the wing. There is a body of literature

looking at cell size and cell number variation with regard to developmental plasticity and in response to selection in both wild and laboratory based populations using insect wings (Partridge *et al.* 1999; Zwaan *et al.* 2000). Wing cell counts are also used when assessing the effects of mutations of the InR/TOR signalling pathways (Shingleton *et al.* 2005). In *D. melanogaster* wings, each cell secretes a single trichome (Robertson 1959a; Partridge *et al.* 1994). This feature, which appears to be conserved across insect groups (Parachem *et al.* 2007; Blanckenhorn & Llaurens 2005), enables simple estimation of cell size and cell number and assessment of their relative changes in relation to organ size. Caution should be exercised when using wing hair counts for two reasons. First, cells are three dimensional entities but wing cell size is estimated in two dimensions and the effects of cell flattening are not always taken into consideration (Kuo & Larson 1987). Second, the relationship of cell size and cell number with organ size will vary between organs and therefore generalisations made from sampling one organ are suspect (Stevenson *et al.* 1995; Arendt 2006). Despite these limitations, wing hair counts are a very widely used tool for cell size and cell number estimates (Partridge *et al.* 1994; Robertson 1959a, b; De Moed *et al.* 1997; James *et al.* 1995; 1997; Zwaan *et al.* 2000; Chapter 1.3.3).

The eye-stalk structure lends itself to simple assessment of cell size and cell number and a number of histological techniques could be used to visualize cells. One possible approach would be to take sections through the eye-stalk. Buschbeck and co-workers used a serial sectioning approach to study the development of the adult brain during the pupal stages (Buschbeck & Hoy 1998; 2005; Buschbeck *et al.* 2001). Although their studies focused on neural development they indicated that eye-stalk structure was relatively simple: a cylindrical outer integument was composed of a mono-layered epidermis beneath the overlying cuticle and a single bundle of nerve fibres ran down the middle of the stalk.

The melanised cuticle of adult insects creates problems for visualizing cells in whole mount preparations. As with all cyclorrhaphan flies, stalk-eyed flies pupate within the sclerotized cuticle of the third instar larva (Fraenkel & Bhaskaran, 1973). The eye-stalks develop as coiled structures within the barrel shaped pupal case (Buschbeck & Hoy 2005). Upon eclosion from the pupal case, the eye-stalks are unfolded by an internal pressure caused by a pumping action. Once all external organs, including the eye-stalks, are fully inflated then the cuticle hardens and darkens. The eye-stalks achieve their adult

size around the time of melanisation of the cuticle. If fixed at this point, cuticle hardening is arrested and hence it is possible to count the epidermal cells within whole mount preparations.

4.1.3 Experimental outline

In this study I examine the relative contributions of cell size and cell number to variation in eyespan in the strongly dimorphic stalk-eyed fly, *Teleopsis dalmanni*. I used transmission electron microscopy to evaluate eye-stalk structure and trialed several approaches to assessing cell size and cell number in eye-stalks. Eye-stalks sustained significant damage during sectioning and due to the hard resins required were resistant to staining. Fuschin and 4',6-diamidino-2-phenylindole (DAPI) based nuclear staining protocols of whole mount eye-stalk preparations were evaluated as alternative approaches to assessing cell size in eye-stalks. Fuschin produced limited staining and the associated protocol was found to cause large amounts of damage to the eye-stalk. The staining procedure for the fluorescent dye DAPI was less damaging and staining was reliably achieved, for at least some part of the stalk in the majority of flies.

I used DAPI staining to test for differences in cell size between eye-stalks from male and female individuals that had been grown in two environments that differed in their degree of nutritional stress. In flies reared under low stress conditions, male eye-stalk length was almost twice that of females. There was a slight but significant difference in cell size between the sexes. Individuals exposed to high nutritional stress were smaller than low stress flies with much greater variation in eye-stalk length and overlap between males and females. Cell size was correlated with eye-stalk length but cell number made a larger contribution to variation in eye-stalk length. Furthermore, although the scaling relationship between eyespan and body size varied between the sexes there was no difference in the relationship between cell size and organ size between the sexes.

Finally, cell size estimates were made in a non-sexually selected control trait, the wing. As in other insects, wing cell size could be measured very simply by counting trichomes in an area of defined size. However, unlike in *D. melanogaster*, cell size was not uniform across the wing in *T. dalmanni*. The pattern was complex with variation between different locations on the wing. In nutritionally stressed individuals, cell number made a greater contribution to variation in wing size.

4.2 MATERIALS AND METHODS

4.2.1 Fly stocks, and generation of low stress and high stress flies

A laboratory-adapted population of *Teleopsis dalmanni* founded from flies collected from Ulu Gombak, Peninsular Malaysia in 1993 was used for this experiment. Laboratory flies have been maintained in cage culture at 25ºC, in a 12h-light and 12h-dark light cycle regime and fed pureed sweetcorn twice weekly. In order to minimise inbreeding, population size has been kept high (> 200 individuals). Flies for experiments were obtained from 8-10 population cages each containing 30-50 adult flies.

To generate 'low stress' flies, eggs and larvae were reared on 'egg-lays' containing excess food. Eggs were collected by placing egg-lays in the base of a population cage for 24 hours. Egg-lays consisted of moist cotton wool in a Petri dish with pureed sweetcorn covered by a circle of damp blue paper. Subsequently, pupae were transferred with forceps to moist cotton wool and kept in a population cage until eclosion. To generate 'high stress' flies, collected eggs were transferred to fresh egg-lays containing 0.369g of pureed sweetcorn (*sensu* Cotton *et al.* 2004a). Approximately 15 eggs were placed on each fresh egg-lay. Pupae were processed as for low stress flies.

4.2.2 Embedding and sectioning for electron and light microscopy

Eye-stalks were removed from adult flies and fixed in 2% paraformaldehyde (PFA), 1.5% glutaraldehyde in 0.1M phosphate buffered solution (PBS) for 1.5 hours at room temperature. After three 10 minute washes in 0.1M PBS, the fixed tissue was transferred to 1% osmium oxide (OsO_4) for 45 minutes, followed by three 10 minute washes in double distilled H_2O (ddH_2O) and then to 2% uranyl acetate ($UO_2(CH_3COO)2{\cdot}2H_2O$) for 5 minutes, followed by three 10 minutes washes in ddH_2O. The eye-stalks were then dehydrated through an ethanol (EtOH) series (5 minutes at 25% EtOH, 5 minutes at 50% EtOH, 5 minutes at 75% EtOH, 5 minutes at 100% EtOH). This was followed by four 10 minute washes in 100% EtOH and three in propylene oxide (C_3H_6O).

The eye-stalks were then embedded in the epoxy resin, Agar 100® (Agar Scientific, UK) in a stepwise manner, being transferred to 2 parts propylene oxide: 1 part Agar 100® resin for 1.5 hours and then 1 part propylene oxide: 2 parts Agar 100® resin for 1.5 hours. The samples were left in 100% Agar 100® for 8-16 hours at room temperature before the Agar 100® was replaced and the samples placed in resin in moulding blocks at 42ºC, to harden overnight.

80nm wet sections were taken using a microtome and treated with lead citrate for 5 minutes followed by two 10 minute washes in ddH_2O. Sections were placed on a copper grid and observed under a transmission electron microscope and photographs taken with the aid of M. Turmaine (UCL). For viewing under a light microscope (Leica DMLB), dissected adult eye-stalks were embedded as above except that the uranyl acetate step was omitted. 1μm wet sections were taken, stained with toluidine blue and placed on glass slides to be viewed.

For embedding in JB-4 resin, eye-stalks from adult eye-stalks were fixed in 2% PFA, 1.5% glutaraldehyde in 0.1M PBS for 1.5 hours at room temperature then dehydrated using an alcohol series as above. The JB-4 Embedding Kit® was used. Infiltration solution (100ml JB-4 solution A with 1.25mg benzoyl peroxide) was added in a stepwise manner. 50% ethanol:50% infiltration solution, followed by 25% ethanol:75% infiltration solution, 10% ethanol: 90% infiltration solution followed by three washes of 100% infiltration solution. Specimens were placed in blocks and embedding solution added (25ml infiltration solution, 1ml JB-4 solution B). Blocks were left for 2-3 hours at room temperature under anaerobic conditions to harden. Once hard, 1μm sections were taken using a microtome and observed under a light microscope.

Whole mount eye-stalk preparation and staining

For fuschin staining (Smith 1989), freshly emerged flies (imagos) were allowed to extend their eye-stalks (the process takes 15-30 minutes) before being placed at 20ºC for at least 20 minutes. The eye-stalks were removed and fixed for 1 hour at room temperature in 2% formalin dissolved in 95% ethanol and 10% glacial acetic acid. After one 10 minute wash in 70% ethanol, specimens were heated for 10 minutes at 60°C in 2M hydrochloric acid (HCl). Specimens were washed in ddH_2O for 10 minutes. Specimens were then left for 1 minute in fuschin staining solution (1% (w/v) basic

fuschin in 2.5% acetic acid), followed by 1 minute in 5% acetic acid. A 10 minute wash in ddH_2O preceded a stepwise transfer to 100% ethanol (via 25% ethanol, 50% ethanol and 75% ethanol). Specimens were cleared in xylene and then mounted in DPX (TCS Biosciences ltd., UK).

For DAPI staining, eye-stalks were removed from imago heads as described for the fuschin staining and fixed in 4% paraformaldehyde for 20 minutes. This was followed by three rinses in PBS (phosphate buffered solution) and three 10 minute washes in PBS. Eye-stalks were then placed in DAPI (1μg/ml) for 10 minutes. Following three rinses in PBS and three 10 minute washes in PBS, eye-stalks were transferred to 75% glycerol in a stepwise manner (e.g. via 25% glycerol and 50% glycerol) and then mounted in 75% glycerol.

Staining was observed using a compound microscope (Leica DMLB) with a UV lamp and DAPI filter attached. Photographs were taken with a Nikon Coolpix P4500 digital camera.

4.2.3 Morphological measurements

Flies killed soon after eclosion, when they had extended their eye-stalks but prior to full melanisation of the cuticle, are referred to as "imagos." Flies allowed to complete melanisation and become at least one day old are referred to as "adults". Imagos and adults were collected and sacrificed by placing them at -20°C for at least 20 minutes. Low stress imagos were sacrificed while the cuticle was still transparent. However, it was shown that flies were not fully expanding their eye-stalks and therefore high stress imagos were sacrificed when the cuticle had started to darken. Photographs of the thorax were taken and the sex of the fly was recorded. The head was removed and photographed. Eye-stalks were removed from the head and the eyes removed from the eye-stalk by cutting distally to the arista (Fig. 4.1). Eye-stalks were then stained with DAPI and successful stainings were photographed. The wings were removed and placed in isopropanol before being mounted in aquamount (Lerner Laboratories, USA) on glass slides, under a coverslip and photographed. Thoraxes were kept at -20°C. All photographs were taken using either a Nikon Coolpix 4500 or a Nikon Coolpix 5100 digital camera mounted on a microscope. Measurements were carried out using ImageJ 1.40g (National Institute of Health, Bethesda, MD, USA).

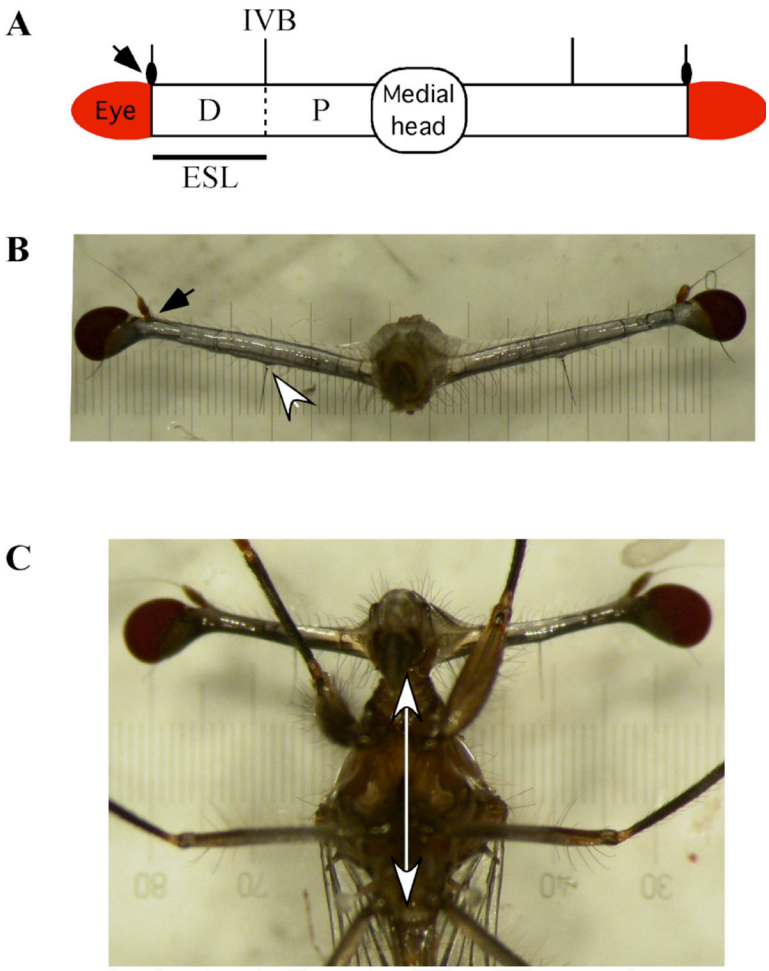

Figure 4.1. Eye-stalk and thorax measurements. **A**. Schematic representation of the eye-stalk showing the different regions of the stalk. The distal region (D) runs from the arista (black arrow) to the inner vertical bristle (IVB). The proximal region (P) is from the IVB to the medial part of the head. Cell areas where any part of the area lay directly beneath the IVB were labeled as IVB. Eye-stalk length (ESL) is measured as the distance between arista (black arrow) and the IVB. A small number of specimens could not be assigned to any region and were designated as unknown. **B**. Head of an imago fly showing the arista (black arrow) and IVB (white arrowhead). **C**. Thorax length is measured as the distance from the junction of the head and the thorax and the most posterior part of the thorax (shown by white arrows).

Eye-stalk length (ESL) was estimated as the length between the inner vertical bristle and the base of the arista (Fig. 4.1) and used as a proxy (*sensu* David *et al*. 1998) for eyespan (the distance between the tips of each eye). It was not possible to measure eyespan reliably in imagos since the eye-stalk cuticle was still soft and loss of internal pressure during the dissection process altered the orientation of the eye-stalks with respect to each other and the head. In adult flies both ESL and eyespan were recorded. Thorax length was measured from the posterior limit of the thorax to the junction of the

head and thorax (Fig. 4.1). Wing area was estimated by tracing around the edge of the wing using a line connecting the edges of the wing and the bases of the R_{2+3} and R_{4+5} veins as the base (Fig. 4.2). Two wing dimensions (corresponding to length and width) were estimated as distances between pairs of easily identified landmarks (Fig. 4.2) (David *et al.* 1998).

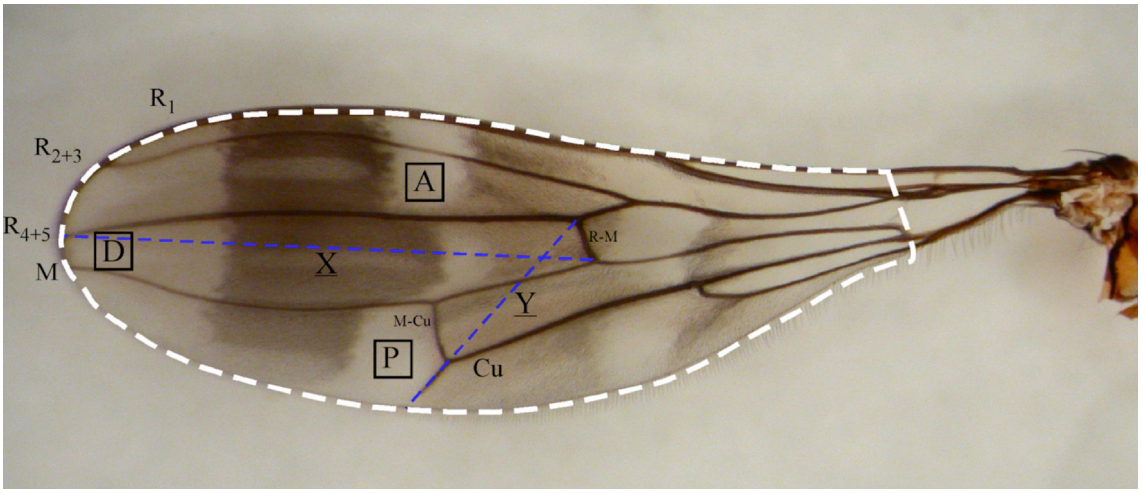

Figure 4.2. Wing measurements and locations of cell areas sampled. Wing veins are labeled using standard nomenclature (Papp & Darvas 2000). Wing area (shown by white dashed line) was estimated by tracing round the outside of the wing using as a start point a line connecting the intersection points of the medial (M) and cubital (Cu) veins and radial vein 1 (R_1) with radial veins 2+3 and 4+5 (R_{2+3} & R_{4+5}). Blue dashed lines show internal measurements of the wing. Wing width (Y) was estimated as the distance from the junction of the radial-medial intervein (R-M) and R_{4+5} to the edge of the wing via the junction of the cubital (Cu) vein and the medial-cubital inter-vein (M-Cu). Wing length (X) was estimated as the distance between the junction of the medial vein (M) and R-M and the most distal point of R_{4+5}. Cell size estimates were taken from three locations, shown by squares. The posterior location (P) was positioned distally to the junction of Cu and M-Cu. The distal location (D) lay between R_{4+5} and M in line with the distal tip of R_{2+3}. The anterior location (A) lay between R_{2+3} and R_{4+5} in line with M-Cu.

Cell size estimation

Images of successfully stained eye-stalks were processed using ImageJ 1.40g (NIH, USA). Free hand traces of stained areas were taken. The area was measured and the number of cells counted. The location of the area with respect to the proximo-distal axis of the eye-stalk was noted (see Fig. 4.1). To give an estimation of eye-stalk cell size for an individual, the total area measured was divided by the total number of cells counted,

across both eye-stalks. Areas were not included if local tissue was clearly damaged or if all the cells were not in the same plane of focus. Areas of less than 1500μm^2 were excluded.

The one-to-one relationship of trichome and cell number in wings was confirmed in six day old DAPI-stained pupal wings viewed using a compound light microscope with Nomarski optics. Cell size estimations were made at three different wing locations (posterior, distal and anterior) using readily identified landmarks (Fig. 4.2). Areas were photographed using a compound light microscope and a Nikon Coolpix 5100 digital camera. Using ImageJ 1.40g, the number of hairs in a 15,000μm^2 rectangle at each wing location was counted. Cell size for each area was estimated by dividing the area by the number of hairs. In each individual, for each location, the average of the cell size from both wings was used.

4.2.4 Statistical analysis

All statistics were performed using JMP 5.0.1a (SAS Institute Inc., USA).

Eye-stalks

The repeatability of the pair of ESL values for each individual was tested using one-way ANOVA (Lessels & Boag 1987). A regression analysis of ESL and eyespan in adults was performed to test whether the ESL was a suitable proxy for eyespan. Residuals of all models were tested for normality (Shapiro-Wilks tests). *t*-tests were used to assess differences between the sexes for ESL and thorax length for imagos, and between ESL, eyespan, and thorax length for adults. Differences between trait size in adult and imago datasets were assessed using *t*-tests. *t*-tests assuming unequal variances were used if required (Ruxton 2006).

The repeatability of eye-stalk cell size estimations was assessed using one-way ANOVA to test for differences between individuals for whom more than one area had been measured (Lessells & Boag 1987). To test for regional differences between cell size estimates, average cell sizes of each individual, classified by region, were subjected to one-way ANOVA. Where there was a significant difference between regions, then *a posteriori* Tukey Kramer honestly significant difference (HSD) tests were performed to

identify the regions responsible.

Sequential general linear modeling (GLM) was used to analyse the relationships between multiple traits in high stress flies. GLMs were constructed using main effects, their interactions, a constant, and an error term. Each term's significance was determined by an *F*-test of the change in explained variance when sequentially added to the model. Residuals of the models were tested for normality (Shapiro-Wilks).

To demonstrate that the scaling relationship of eyespan and body size differed between the sexes, a general linear model was created with *ESL* as the dependent variable and *thorax*, *sex* and *thorax* × *sex* as the effect terms.

A GLM was constructed to test the relationship of cell size with trait size and sex. Cell size (area) is a two-dimensional measure and therefore the square root of cell size ($\sqrt{C_S}$) was used in all GLMs to enable appropriate comparison with other one-dimensional effect terms (*ESL* and *thorax length*). The interaction term of *sex* × *ESL* was added to test whether the relationship of ESL and the square root of cell size differed with sex. To test whether cell size was correlated to overall body size, to actual organ size or an interaction of the two, a further GLM was designed in which the effect *thorax length* was added first and therefore treated as a covariate before adding *ESL* to the model. The interaction term of *ESL* × *thorax length* was used to test whether the relationship between cell size and trait size varied with body size.

Estimations of the relationship of cell size, cell number and organ size

The relative contributions of cell size and cell number to organ size was estimated by examining the covariation of log-transformed organ size and cell size (Zwaan *et al*. 2000). The interaction of organ size (O_S) is related to cell number (C_N) and cell size (C_S) by:

$$O_S = C_S \times C_N \quad (1)$$

Log transformations produce:

$$\log O_S = \log C_S + \log C_N \quad (2)$$

The following regression models can be fitted:

$$\log C_S = \log a + b.\log O_S \tag{3}$$

$$\log C_N = \log c + d.\log O_S \tag{4}$$

The regression slopes are given by (*Cov* = covariance, *Var* = variance):

$$b = \frac{Cov\ (\log C_S, \log O_S)}{Var\ (\log O_S)} \tag{5}$$

$$d = \frac{Cov\ (\log C_N, \log O_S)}{Var\ (\log O_S)} \tag{6}$$

From these formulae and equation (2), it can be shown that:

$$Cov\ (\log C_S, \log O_S) + Cov\ (\log C_N, \log O_S) = Var\ (\log O_S) \tag{7}$$

and:

$$b + d = 1 \tag{8}$$

From these formulae, the proportion of the variance in organ size explained by cell size is given by b (the slope of the regression of log C_S against log O_S). If $b = 1$, then any change in organ size is explained completely by cell size. If $b = 0$, there is no relationship between cell size and organ size. If b lies between 0.5 and 1, cell size is increasing more rapidly than cell number, if between 0.5 and 0 cell number is increasing more rapidly. In examining cell number in the eye-stalk, one dimensional measures of organ size and cell size were used for comparison. Accordingly, ESL and the square root of cell size values were used as the biologically relevant measures. Where log-transformation was performed, natural logarithms were used.

Wings

Repeatability of measurements for wing area, wing width, wing length and cell size for each of the three sampling locations was tested by one-way ANOVAs (Lessells & Boag 1987). Datasets were tested for normality (Shapiro-Wilks $p > 0.05$) before applying *t*-

tests to evaluate differences in wing area between imagos and adults and between males and females. Differences in wing shape between *sex*, *life-stage* (imago or adult) and *thorax length* were assessed by generating GLMs with either *wing width* or *wing length* (Fig. 4.2) as the dependent variable and adding the effect term *square root of wing area* ($\sqrt{WA}$) first, therefore treating it as a covariate. The effects *thorax length*, *life-stage* and *sex* were added to the model along with higher interactions. Each term was sequentially added to the model and the significance of the effect was tested using an F-test. Residuals of all models were tested for normality (Shapiro-Wilks, $p > 0.05$).

In low stress adults a one-way ANOVA was used to test for cell size differences between locations. To test the relationship of cell size with wing area in high stress adults, GLMs were used with the *square root of cell size* ($\sqrt{C_S}$) as the dependent variable: the effects *sex* and *thorax length* were added first and therefore treated as covariates to control for sex specific and body size related differences in wing shape. As for the eye-stalks, the logarithmic transformation/regression based approach was (Zwaan *et al.* 2000) used to assess the relationships of cell size and cell number with organ size in wings from high stress adult flies. Since both cell (C_S) and organ (O_S) measures were areas, there was no need to use the square root of cell size in this analysis. Log C_S was plotted against log O_S to determine the slope (b).

4.3 RESULTS

4.3.1 Eye-stalk structure, and histology

Electron microscopy confirmed that *T. dalmanni* eye-stalks were structurally similar to those of *Teleopsis whitei* and possessed a structure amenable to cell size estimations (Buschbeck *et al.* 2001; Fig. 4.3A). A simple mono-layered epidermis lies beneath the cuticle, which enables simple estimation of cell area in whole mount preparations. In longitudinal sections (running from the head to the eye) eye-stalk curvature proved too marked for it to be possible to cut a single section containing an entire cell and hence to use such sections to measure cell length.

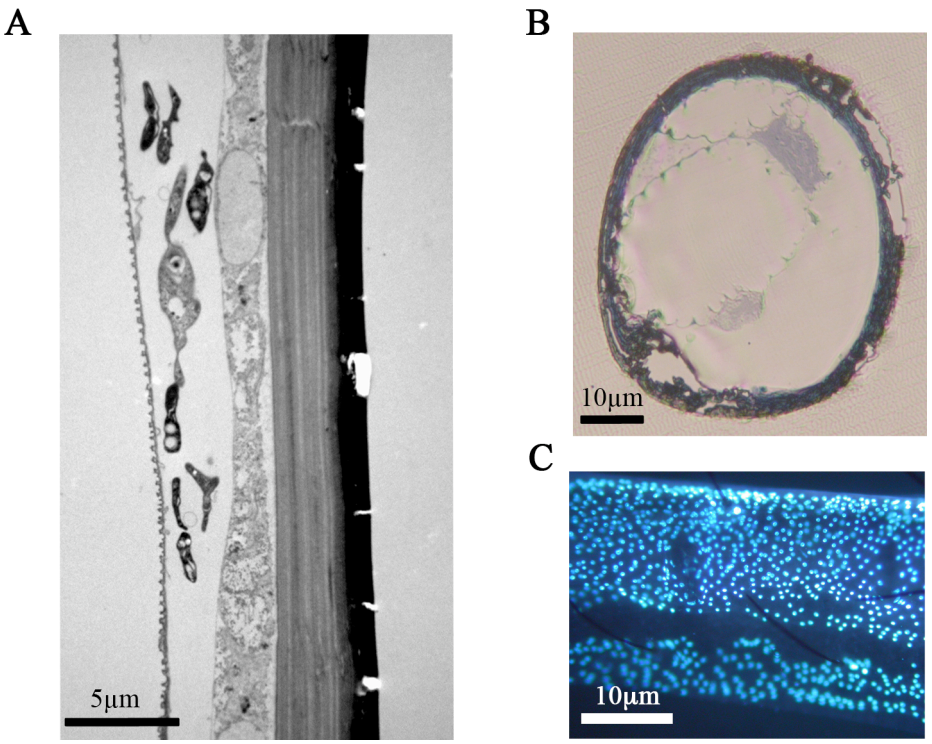

Figure 4.3. Eye-stalk structure and histology. **A.** Electron micrograph of 80µm longitudinal eye-stalk section. A single layer of epidermal cells is apposed to the cuticle (right). Medial to the epidermis is the outer surface of the airbags used to inflate the eye-stalks upon eclosion. **B.** 1µm cross-section of an eye-stalk stained with toluidine blue. The stalk is hollow except for two nerve fibres. Note the connective tissue linking the nerves to the epidermis. The cuticle has sustained damage during the sectioning process, most clearly visible in the top right of the picture. **C.** DAPI-stained whole mount preparation of an imago eye-stalk.

Electron microscopy is impractical for comparisons of cell size in large samples. Light microscopy, which tolerates thicker sections, is less labour intensive. Eye-stalks are delicate and brittle hence cutting thick sections caused significant damage but it was still possible to take sections although identification of cell boundaries was problematic (Fig. 4.3B). The general stain toluidine blue, improved visualisation of the epidermis but cell structure was not sufficiently resolved for cell dimensions to be estimated. The hard embedding resin (Agar 100®) required for electron microscopy is incompatible with more specific stains or immunohistological procedures but a softer aqueous resin (JB-4) was unable to support the eye-stalk during the embedding process.

Stained whole mount preparations of newly expanded eye-stalks provided a potential alternative for visualising epidermal cells. The nuclear dye, fuschin, produced limited staining and a large amount of damage to the tissue. DAPI, a fluorescent nuclear stain, has a much shorter and less invasive protocol than fuschin. Although damage was still present, large sections of the stalk epidermis were successfully stained in about one third of preparations (Fig. 4.3C). Therefore, DAPI stained whole mount preparations were used to compare cell size in low and high stress *T. dalmanni* eye-stalks.

4.3.2 Eye-stalks and cell size in low stress flies

The whole mount DAPI staining protocol was used to test for a difference in cell size between full sized 'low stress' males and females, which were generated by allowing individuals access to excess food during larval stages. 210 imago flies were stained and ESL, thorax length and cell size estimates were obtained from 81 individuals (44 males and 37 females). To determine whether imagos had reached full size, morphological measurements were also made for 80 adult flies (40 males and 40 females). Significant repeatability was seen for ESL measurements (imago ESL: $r^2 = 0.999$, $F_{71,72} = 828$, $p < 0.0001$; adult ESL: $r^2 = 0.997$, $F_{77,78} = 359$, $p < 0.0001$). Imago and adult males were significantly larger than females for ESL (imagos: $t_{79} = -30.2$, $p < 0.0001$; adults: $t_{78} = -33.261$, $p < 0.0001$; Fig. 4.4A) and thorax length (imagos: $t_{79} = -2.83$, $p = 0.0058$; adults: $t_{77} = -4.850$, $p < 0.0001$; Fig. 4.4B). Adult ESL values were significantly correlated with eyespan ($r^2 = 0.984$, $F_{1,78} = 5062.7$, $p < 0.0001$) confirming that ESL is a suitable proxy measure for eyespan. Adult males had significantly larger ESL than imago males ($t_{82} = 2.53$, $p = 0.0269$; Fig 4.4A) while adult and imago females did not differ in ESL ($t_{75} = 0.060$, $p = 0.952$). Thorax length did not differ between adults and

imagos in either sex (males: t_{82} = 0.064, p = 0.949; females: t_{74} = -0.662, p = 0.510; Fig. 4.4B). These data suggest that the male imagos had not been given sufficient time to fully extend their eye-stalks so that cell size measurements in imago male eye-stalks are likely to be underestimates, however the difference in mean ESL (mm±S.E.) between imago (1.46±0.018) and adult (1.52±0.019) males was small.

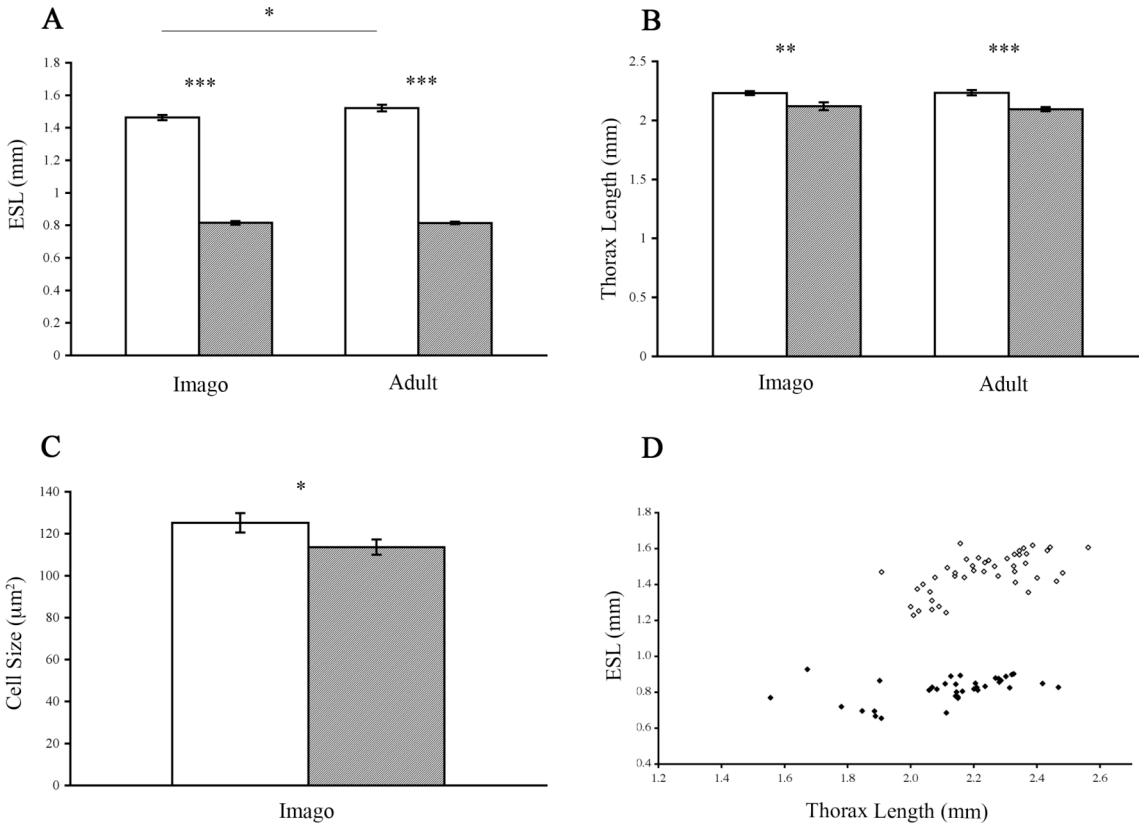

Figure 4.4. Trait size (imagos and adults) and cell size (imagos) in low stress flies. **A-C.** Non-shaded = male, shaded = female. Error bars denote standard errors. **D.** Scatter plot for ESL against thorax length for imago males (open diamonds) and females (closed circles). Asterisks indicate significant differences between paired categories. ***p < 0.0001, **p < 0.01, *p < 0.05. ESL = eye-stalk length.

Cell size estimates in the stained imagos were derived from between 1 and 5 areas taken from both left and right eye-stalks (mean of 2.23±1.19 areas per individual). Repeatability for cell size between individuals was significant (r^2 = 0.490, $F_{56,101}$ = 1.73, p = 0.0038). There was no significant relationship between proximodistal location and cell size ($F_{3,108}$ = 0.196, p = 0.09; Table 4.1). Cell size was significantly larger in male eye-stalks than in female eye-stalks (t_{79} = -2.00, p = 0.0487; Fig. 4.4C).

	Proximal	*Distal*	*IVB*	*Unknown*
Cell size	119.5 ± 25.1 (57)	121.7 ± 31.7 (45)	128.5 ± 39.0 (6)	120.9 ± 18.8 (4)

Table 4.1. Mean cell size estimation per individual by region of the eye-stalk in low stress flies. Mean cell size is given in μm^2 ± standard deviation. Sample size is given in brackets. Refer to Fig.4.1 for definitions of regions of the stalk. In six cell area estimations it was not possible to identify which area they came from.

4.3.3 Eye-stalks and cell size in high stress flies

In low stress flies, little variation in eyespan is observed between flies of one sex (Fig 4.4A, D and see Cotton *et al.* 2004b) and so it was not possible to observe how cell size and cell number varied with eyespan. The observed difference in cell size in the low stress flies may have been due to an inherent difference in cell size between the sexes. Alternatively, cell size could be a function of organ size, in which case the difference would reflect the difference between male and female eyespan. To distinguish between these possibilities, flies were generated that had experienced restricted access to food during larval stages to generate males and females with a wide range of eyespan values (Fig. 4.4D & 4.5D) (David *et al.* 1998; Cotton *et al.* 2004b).

300 imagos were stained and ESL, thorax length and cell size estimates were obtained from 178 individuals (100 males and 78 females). Morphological measurements were made for 130 adult flies (65 males and 65 females). ESL values showed significant repeatability (imagos: $r^2 = 0.996$, $F_{159,160} = 234.1$, $p < 0.0001$; adults: $r^2 = 0.983$, $F_{122,123} = 57.9$, $p < 0.0001$). Imago and adult males had significantly larger eye-stalks than females (imagos, $t_{133} = 11.7$, $p < 0.0001$; adults: $t_{76.2} = 9.07$, $p < 0.0001$) but the distributions overlapped (Fig. 4.5A, D). Thorax length did not differ significantly between the sexes (imagos, $t_{176} = 0.543$, $p = 0.588$: adults: $t_{128} = 0.859$, $p = 0.392$). Adult ESL measures were again significantly correlated with eyespan ($r^2 = 0.940$, $F_{1,127} = 1980$, $p < 0.0001$). Neither ESL nor thorax length differed significantly between imago and adult datasets (male eye-stalk: $t_{162} = 0.554$, $p = 0.580$; female eye-stalk: $t_{142} = 1.65$, $p = 0.102$; male thorax length: $t_{162} = 0.188$, $p = 0.851$; female thorax length: $t_{142} = 0.684$, $p = 0.495$; Fig. 4.5B).

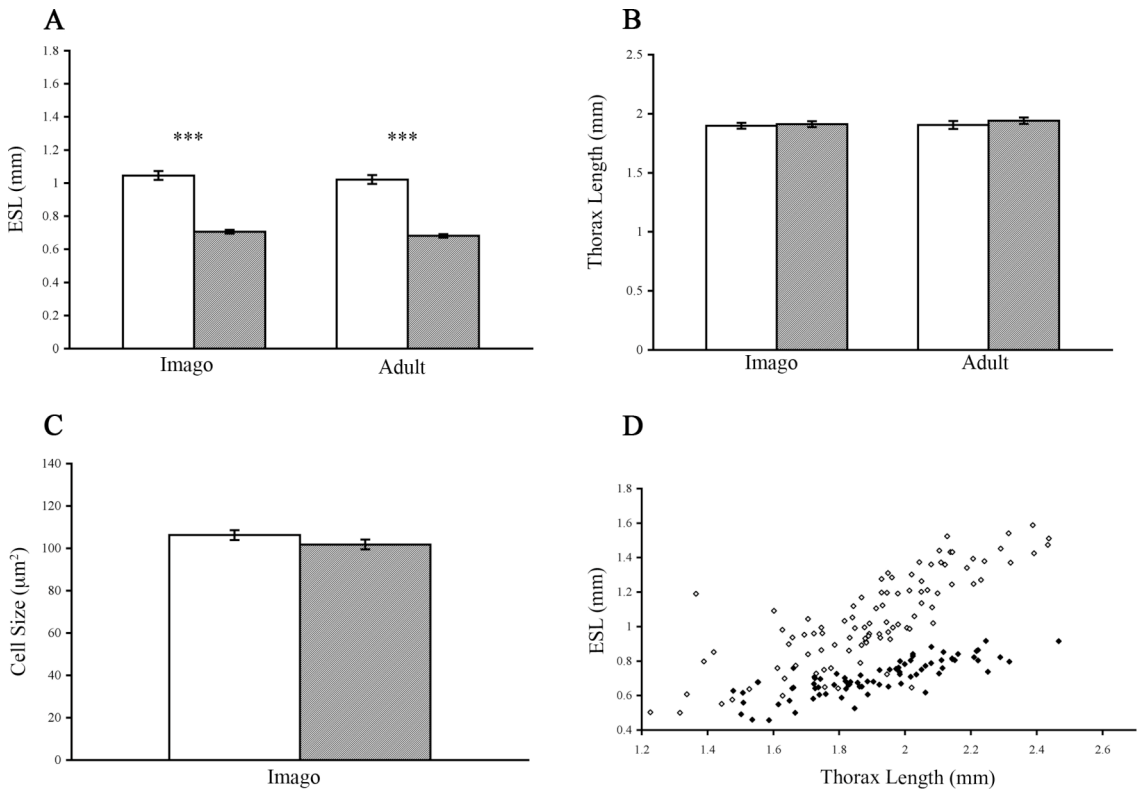

Figure 4.5. Trait size (imagos and adults) and cell size (imagos) in high stress flies. **A-C.** Non-shaded = male, shaded = female. Error bars denote standard errors. **D.** Scatter plot for ESL against thorax length for imago males (open diamonds) and females (closed circles). Asterisks indicate significant differences between paired categories. ***$p <$ 0.0001, **$p < 0.01$, *$p < 0.05$. ESL = eye-stalk length.

It has been shown on a number of occasions that the relationship of eyespan and body size is different for each sex (Baker & Wilkinson 2001; Cotton *et al.* 2004a). A GLM was used to show this to be true for the imago dataset. Details of the results are given in Table 4.2. The interaction term *ESL × thorax length* was significant confirming a different relationship of eyespan and body size between the sexes.

	Source of variation	***df***	***MS***	***F Ratio***	***p-value***
ESL	*Thorax length*	1,173	4.258	279	< 0.0001
	Sex	1,172	5.220	342	< 0.0001
	Thorax length × Sex	1,171	0.499	32.8	< 0.0001

Table 4.2. Analysis of covariance of *ESL* with *thorax length* and *sex* for high stress flies. Significance of adding each term to the model was tested using an *F*-test. MS = mean squares.

Cell size estimates were derived from between 1 and 13 areas taken from the left and right eye-stalks (mean = 2.61±1.71 counts per individual). Repeatability of cell size estimates was significant (r^2 = 0.480, $F_{124,294}$ = 2.19, p < 0.0001). Cell size varied according to location within the eye-stalk ($F_{2,262}$ = 6.94, p = 0.0012; Table 4.3). IVB measures were significantly different from those of the proximal and distal regions (Tukey-Kramer HSD) and were removed from further analyses. In total, 15 (out of 472) areas and 3 (out of 100) male individuals were discounted. Repeatability of cell size estimates in the new dataset remained highly significant (r^2 = 0.490, $F_{123,276}$ = 2.16, p < 0.0001).

	Proximal	***Distal***	***IVB***
Cell size	106.9 ± 22.56 (138)	103.6 ± 27.5 (112)	129.6 ± 34.9 (15)

Table 4.3. Mean cell size estimation per individual by region of the eye-stalk in high stress flies. Mean cell size is given in μm^2 ± standard deviation. Sample size is given in brackets. Refer to Fig.4.1 for definitions of regions of the stalk.

Results for the general linear model assessing the relationship of *ESL* and cell size ($\sqrt{C_S}$) are summarised in Table 4.4. The relationship of *ESL* and $\sqrt{C_S}$ was significant. No significant relationship were seen between $\sqrt{C_S}$ and *sex*, and between $\sqrt{C_S}$ and *ESL* × *sex* demonstrating that there is no inherent cell size difference between males and females and that the relationship of eyespan and cell size did not differ between the sexes.

Character	***Source of variation***	***df***	***MS***	***F Ratio***	***p-value***
$\sqrt{C_s}$ *(Eye-stalk)*	*ESL*	1,173	15.77	14.7	0.0002
	Sex	1,172	1.947	1.81	0.181
	Sex × ESL	1,171	0.199	0.184	0.668
	Thorax length	1,173	14.66	13.7	0.0003
	ESL	1,172	4.596	4.30	0.0396
	Thorax length × ESL	1,171	0.442	0.396	0.530

Table 4.4. Analyses of covariance of *eye-stalk cell size* (C_s) with *ESL* and *sex* in high stress flies. Significance of adding each term to the model was tested using an *F*-test. MS = mean squares.

To test whether the variation in cell size was correlated to *ESL* and was not just a body size effect, a second GLM was performed and results are fully summarised in Table 4.4. Even with *thorax length* added first, and therefore treated as a covariate, a significant relationship between $\sqrt{C_S}$ and *ESL* was observed. The relationship of ESL and $\sqrt{C_S}$ did not significantly vary with *thorax length*. These results show that variation of cell size in the eye-stalk is only related to variation in ESL and independent of effects of either sex or body size. This is despite there being a significant difference in the scaling relationship of eyespan and thorax length between the sexes (Table 4.3).

The relative contributions of cell size and cell number to eye-stalk size were estimated by regressing log $\sqrt{C_S}$ against log *ESL* (Zwaan *et al*. 2000). Since the relationship of cell size and organ size did not differ between males and females, cell size and cell number values for the sexes were pooled. The regression plot produced a significant slope (±SE) of 0.108 (± 0.027) ($r^2 = 0.0841$, $F_{1,173} = 15.9$, $p < 0.0001$) showing that cell number plays a larger role in eye-stalk size variation than cell size (Fig. 4.6).

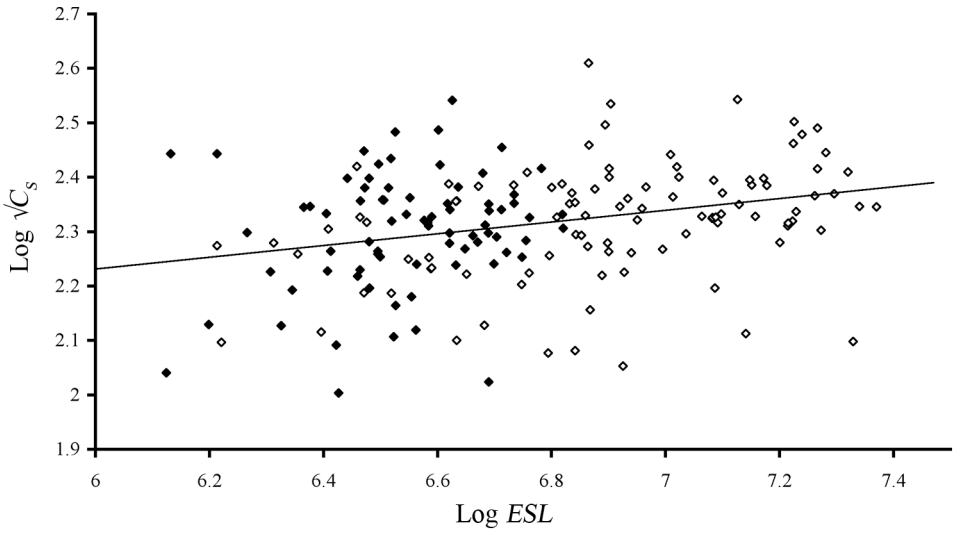

Figure 4.6. Relative contribution of cell size and cell number to organ size (ESL, eye-stalk length) plotted as Log $\sqrt{C_S}$ vs. Log ESL (Zwaan *et al.* 2000). Open diamonds denote males, filled diamonds denote females.

4.3.4 Wings and cell size in low stress flies

For comparison, the relationship between cell size and organ size was analysed in a non-sexually selected control trait, the wing. Wings were collected from the low stress imago and adult populations that had been used for eye-stalk measurements. Repeatability of wing size measurements was significant in both imago and adult

datasets (r^2 = 0.953-0.993, F = 16.6-149, p < 0.0001 for all). Wing area was significantly smaller in females in both datasets (imagos: t_{38} = 2.11, p = 0.0417; adults: t_{78} = -6.26, p < 0.0001; Fig. 4.7A) and smaller in imagos than adults for both sexes (males: t_{60} = 4.76, p < 0.0001; females: t_{56} = 2.48, p = 0.0161; Fig. 4.7A). To test for changes in wing shape between sexes and datasets, variation in wing length and width, with wing area as a covariate, was assessed using a GLM. Significant variation in wing shape was associated with changes in *thorax length*, *life-stage* and *sex* (fully summarized in Table 4.5). The effect of body size may be due to differences in sexes since males had larger thoraxes than females (Fig. 4.4B).

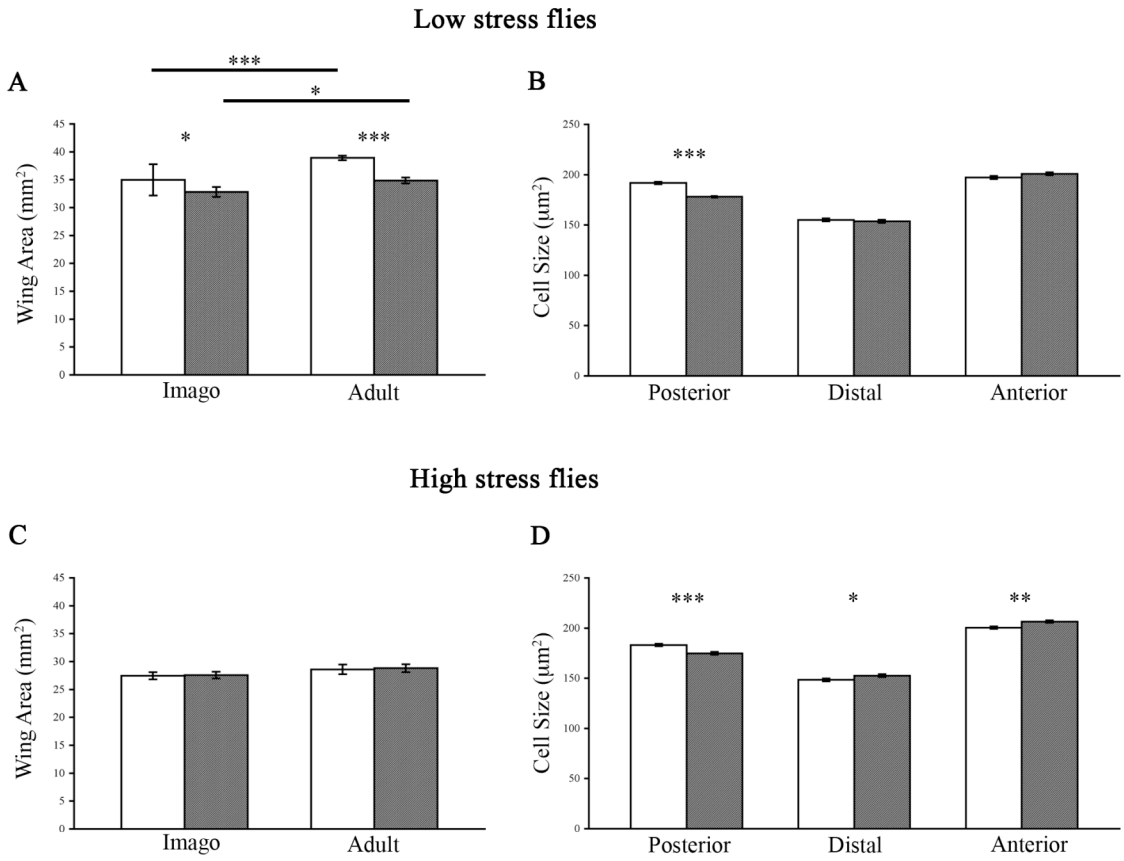

Figure 4.7. Wing area (imagos and adults) and cell size for posterior, distal and anterior locations of the wing in low stress (**A**&**B**) and high stress (**C**&**D**) flies. Non-shaded = male, shaded = female. Error bars denote standard errors. Asterisks indicate significant differences between paired categories. ***p < 0.0001, **p < 0.01, *p < 0.05**.**

The results indicate that in the low stress flies, wings (similar to male eye-stalks) had not reached their final size in the imagos. Wing shape also differed significantly between imagos and adults. The adult data would be expected to show less variation as all individuals had reached their full size and were used for cell size assessment.

	Source of Variation	*df*	*MS*	*F Ratio*	*p-value*
Wing length	*√Wing area*	1,117	1453751	1840	< 0.0001
	Thorax length	1,116	12098	15.3	0.0002
	Life-stage	1,115	37071	47.0	< 0.0001
	Sex	1,114	19699	25.0	< 0.0001
	Sex × Thorax length	1,113	216.5	0.275	0.601
Wing width	*√Wing area*	1,117	297215	492	< 0.0001
	Thorax length	1,116	3314	5.49	0.0209
	Life-stage	1,115	12519	20.7	< 0.0001
	Sex	1,114	61464	102	< 0.0001
	Sex × Thorax length	1,113	1095	1.82	0.181

Table 4.5. Analyses of wing shape in low stress flies. Analyses of covariances for *wing length* and *wing width* were carried out using *wing area* as a covariate. Significance of adding each term to each the model was tested using an *F*-test. Higher interaction terms were non-significant and are not reported. MS = mean squares.

Adult cell size was estimated at three distinct locations within the wing (posterior, distal and anterior; see Fig. 2). Repeatability between for cell size estimates were significant (all locations r^2 = 0.64-0.79, F = 1.85–3.77, $p < 0.004$). Cell size differed significantly between locations ($F_{2,237}$ = 432 $p < 0.0001$; Fig. 4.7B). In the distal and anterior locations no difference in cell size was observed between males and females (distal: t_{78} = 0.641, p = 0.524; anterior: t_{78} = 1.73, p=0.0878). However, for the posterior location males had significantly larger cells (t_{78} = -7.96, $p < 0.0001$).

4.3.5 Wings and cell size in high stress flies

In high stress flies repeatability for wing size measurements was highly significant (r^2 = 0.991-0.999, F = 131-541, $p < 0.0001$ for all). Wing area was not significantly different in males and females (imagos: t_{148} = 0.131, p = 0.896; adults: t_{115} = 0.186, p = 0.8527; Fig. 4.7C) or between imagos and adults (males: t_{139} = 1.10, p = 0.273; females: t_{124} = 1.34, p = 0.184; Fig. 4.7C). As in the low stress flies, to test for changes in wing shape, variation in wing length and width, with wing area as a covariate, was evaluated using a GLM. Significant variation in wing shape was associated with *life-stage*, *sex* and *thorax length* (Table 4.6). Therefore again only adults were assessed for cell size in wings.

	Source of Variation	*df*	*MS*	*F Ratio*	*p-value*
Wing length	*√Wing area*	1,262	10875442	9449	< 0.0001
	Thorax length	1,261	18600	16.16	< 0.0001
	Life-stage	1,260	24132	20.98	< 0.0001
	Sex	1,259	83.0	0.0718	0.789
	Sex × Thorax length	1,258	177	0.154	0.695
Wing width	*√Wing area*	1,262	2314504.5	4641	< 0.0001
	Thorax length	1,261	956.3	1.917	0.167
	Life-stage	1,260	3094.6	6.205	0.0134
	Sex	1,259	76050.6	152.5	< 0.0001
	Sex × Thorax length	1,258	2650.4	5.314	0.0219

Table 4.6. Analyses of wing shape in high stress flies. Analyses of covariances for *wing length* and *wing width* were carried out using *wing area* as a covariate. Significance of adding each term to each the model was tested using an *F*-test. Higher interaction terms were non-significant and are not reported. MS = mean squares.

In adult wings, repeatability for cell size measurements was highly significant (r^2 = 0.74–0.80, F = 2.89–3.96, $p < 0.0001$ for all). As in the low stress adults, cell size estimates differed significantly between locations ($F_{2,347} = 737$, $p < 0.0001$; Fig. 4.7). A GLM was constructed to assess the relationship between cell size and organ size (summarized in Table 4.7). In order to account for effects resulting from sex differences in shape, *sex* was added to the model first so that it could be treated as a covariate. *Thorax length* was also added as a covariate to control for body size effects. In the posterior location, males had larger cells while in the distal location, cells were larger in females. At both posterior and distal locations significant relationships between *cell size* ($\sqrt{C_S}$) and *thorax length*, and between *cell size* and *wing area* ($\sqrt{WA}$) were present. In the anterior location, females had larger cells but no relationship to *cell size* was observed with either *thorax length* or *wing area* (Table 4.7; Fig. 4.7).

Since the three locations sampled for wing cell size had differing relationships with organ size and sex, each area and sex was treated separately when estimating the relationship between cell size and cell number. The results are summarised in Table 4.8. For the posterior and distal locations in both sexes, the slope produced when plotting log C_S against log O_S was shallow indicating a larger contribution of cell number to variation in wing area. For the anterior location, no relationship between log C_S and log

O_S was observed as would be expected given the lack of interaction between cell size and organ size detected in the GLM (Table 4.7).

	Source of Variation	***df***	***MS***	***F Ratio***	***p-value***
$\sqrt{C_S}$: *Posterior*	*Sex*	1,154	2.861	24.9	< 0.0001
	Thorax length	1,153	1.509	13.1	0.0004
	√Wing area	1,152	1.047	9.11	0.0032
	Thorax length × √Wing area	1,151	0.126	1.09	0.298
	Sex × √Wing area	1,150	0.096	0.834	0.363
$\sqrt{C_S}$: *Distal*	*Sex*	1,154	0.893	5.72	0.0186
	Thorax length	1,153	3.472	22.2	< 0.0001
	√Wing area	1,152	1.062	6.80	0.0104
	Thorax length × √Wing area	1,151	0.470	3.01	0.0857
	Sex × √Wing area	1,150	0.176	1.13	0.291
$\sqrt{C_S}$: *Anterior*	*Sex*	1,154	1.307	12.3	0.0007
	Thorax length	1,153	0.239	2.24	0.138
	√Wing area	1,152	0.281	2.65	0.107
	Thorax length × √Wing area	1,151	0.273	2.56	0.112
	Sex × √Wing area	1,150	0.011	0.103	0.749

Table 4.7. Analyses of cell size and trait size in the wing for high stress flies. Analyses of covariance for cell size (C_S) in posterior, distal and anterior locations of the wing were carried out. Significance of adding each term to each the model was tested using an *F*-test. Higher interaction terms were non-significant and are not reported. *MS* = mean squares.

Wing location	*Sex*	*Regression slope from log C_S vs. log WA ± SE*	*Regression statistics*	*Relative contributions of C_N and C_S on wing area*
Posterior	♂	0.079 ± 0.028	$r^2 = 0.127$, $F_{1,57} = 8.29$, $p = 0.0056$	$C_N > C_S$
	♀	0.116 ± 0.036	$r^2 = 0.157$, $F_{1,55} = 10.3$, p = 0.0023	$C_N > C_S$
Distal	♂	0.127 ± 0.039	$r^2 = 0.160$, $F_{1,57} = 10.9$, $p = 0.0017$	$C_N > C_S$
	♀	0.173 ± 0.040	$r^2 = 0.250$, $F_{1,56} = 18.6$, $p < 0.0001$	$C_N > C_S$
Anterior	♂	NS	$r^2 = 0.00030$, $F_{1,56} = 0.0167$, $p = 0.898$	C_N only
	♀	NS	$r^2 = 0.00516$, $F_{1,55} = 0.285$, $p = 0.595$	C_N only

Table 4.8. Relationship of cell size (C_S), cell number (C_N) and wing area (WA) in high stress flies. Regression slopes ± standard error from the regression of log C_S against log WA for each wing location sampled (Zwaan *et al.* 2000). NS = non-significant.

4.4 DISCUSSION

Here I report the first investigation into cell size and cell number variation in a sexually selected ornamental trait, eyespan, in the stalk-eyed fly. I evaluated epidermal structure in the eye-stalks and investigated methods for estimating cell size in sectioned and whole mounted material. Comparisons of cell size in males and females reared in low and high stress environments were made. Low nutritional stress generates males with eye-stalks almost twice the size of those of females and there is a small but significant difference in cell size with males having larger cells. Little variation in trait size is observed within the sexes when reared under these conditions. High stress generates a much wider range of eyespans in both males and females. Cell size varies with eye-stalk size, but cell number makes a greater contribution to eye-stalk variation than cell size. Both cell size and cell number are positively correlated with trait size but no difference in the relationship of cell size and organ size was observed between the sexes. As a control, I assayed cell size, cell number and organ size in a non-sexually selected trait, the wing. Cell size and its relationship with sex and organ size varied significantly between locations in the wing. At two sampling locations, cell number contributed more to wing variation than cell size but at a third location there was no significant correlation between cell size/number and trait size.

4.4.1 Cell size and cell number in eye-stalks

Given the extreme sexual dimorphism observed for eyespan (Figs. 4.4 and 4.5) and the significant effect of sex on the relationship between eye-stalk size and body size (Table 4.3), it is surprising that no difference was seen in the relationship of cell size and eye-stalk length between the sexes. In other flies, the effects of nutritional stress have only been studied in females (Robertson 1959; De Moed *et al.* 1997). However, sex differences in response to temperature effects, with the larger sex responding to lower temperatures via increases in cell size and the smaller sex showing changes in both cell size and cell number, are well established (Partridge *et al.* 1994; Blanckenhorn & Llaurens 2005). In *T. dalmanni,* cell size was uniform across the eye-stalk except in a very narrowly defined region directly below the inner vertical bristle and varied with organ rather than body size. This suggests that cell size reflects the growth history of the specific organ involved rather than any more global factors such as body size or sex. The developmental response to nutrition may function differently from that to

temperature.

Increased eyespan requires a prolonged growth period or increased growth rate. Experimental manipulations that increase the growth period appear to act through increases in cell number (Colombani *et al.* 2005; McBrayer *et al.* 2007). In this study, a linear regression model indicated that cell number made a significantly greater contribution to eye-stalk variation than cell size (Fig. 4.6). This could be verified by comparing the duration and rate of cell proliferation in male and female eye-stalk primordia using the incorporation of immunohistochemically detectable nucleotide analogues such as Bromodeoxyuridine (BrDU) or mitosis-specific antibody markers to compare cell division rates. Current models of organ growth within holometabolous insects suggest that variation in growth period can be mediated by variation in responsiveness to hormone signalling, while growth rate is regulated by insulin signalling. *T. dalmanni* sequence is available for the ecdysone receptor target E75 and the insulin receptor. It would be interesting and informative to see whether the expression levels of either of these genes correlate with cell proliferation in eye-stalk primordia.

4.4.2 Cell size and cell number in the wing

Cell size, cell number and organ size were assessed in a non-sexually selected trait, the wing, as a comparison. Estimates of cell size in *D. melanogaster* have largely been performed in the wing. In this species cell size is essentially uniform across the wing blade (Partridge *et al.* 1994). In stalk-eyed flies this is not the case. The relationship of cell size and overall wing area varied significantly between three sampling locations. Wing shape also differed between the sexes and varied with overall body size. In stalk-eyed flies, the wings have not been previously studied except as a control organ when looking at scaling relationships of eyespan and body size (David *et al.* 1998, 2000; Cotton *et al.* 2004a, b). To fully understand how cell size and number vary with wing size would require morphometric analyses of shape and size changes in the organ and in independent sampling regions (Klingenberg 2002). If the wings of stalk-eyed flies are to be used as a control for cell size and cell number comparisons in further studies, their biological role also needs clarification. Stalk-eyed fly wings are primarily flight organs but they clearly have other roles, which might cause regional variation in cell size. Careful inspection of *T. dalmanni* wings reveals that not only are there patterns of

striated pigmentation but also trichome size clearly varies between regions (Fig. 4.2). Wings have secondary roles in many insect species, including for example those of camouflage and signalling observed in many Lepidopterans. Within Diptera, wingspots are used as part of mating rituals in *Drosophila bimarpes* (Gompel *et al.* 2006).

4.4.3 Conclusions

In this chapter I have made the first assessment of the relative contributions of cell size and cell number to variation in an ornamental sexually selected trait. It was possible to assess cell size and cell number within the eye-stalk using a simple methodology which could be applied to large samples. The results provided some insight as to the underlying mechanisms regulating eye-stalk growth and suggested further avenues of investigation. The pronounced differences in eyespan observed within and between the sexes facilitate detection of growth differences. The study of eye-stalk growth will potentially aid our understanding of the general issue of organ growth regulation and shed light on the development and evolution of ornamental traits.

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Chapter Five: General discussion

5.1 Summary

Stalk-eyed flies have provided an excellent study organism for the evolution of sexually selected ornamental traits. There are over 150 species of stalk-eyed flies, which demonstrate varying degrees of sexual dimorphism for eyespan. In dimorphic species, males typically possess much larger eyespans than females and females demonstrate a marked mating preference for increased male eyespan. Stalk-eyed flies are amenable to laboratory culture with a relatively short lifespan but studies of the development of the eye-stalk have been hampered by the lack of available protocols. In this thesis I describe the successful application of two novel approaches to the study of development in the stalk-eyed fly.

First, I successfully developed a transgenic protocol for the stalk-eyed fly, *Teleopsis dalmanni*. Transgenics is a versatile technique that has revolutionised the study of developmental genetics in *Drosophila melanogaster* and is having a similar impact on other non-model organisms. With a transgenic protocol present in stalk-eyed flies a vast array of experiments are now possible.

Second, I used a histological approach to assess cell size and cell number within the eye-stalk using the nuclear stain, DAPI, in eye-stalks from freshly emerged flies. I showed that cell number made a larger contribution to variation in eyespan than cell size. It will be possible to build upon this result in a number of ways to further understanding of eye-stalk growth regulation, including approaches that exploit the transgenic protocol.

5.2 Transgenics in stalk-eyed flies

Transgenesis in *T. dalmanni* required the development of an embryonic microinjection protocol and the identification of a suitable transposon vector system. I assayed the activity of three transposons in stalk-eyed fly embryos (Chapter Two). Germline transformation was achieved using the *Minos* transposon and I obtained a transformation efficiency of 4% (Chapter Three). The observed transformation rate is towards the low end of reported transformation efficiencies in other insect species (O'Brochta & Handler 2008), but not so low as to present obstacles to future use.

The protocol can be further developed to improve survivorship of the microinjection procedure and increase transformation efficiency (as discussed in Chapters Two and Three). The physical act of piercing the vitelline membrane of an embryo and the insertion of a volume of fluid were the main causes of mortality. Improvement in needle design that confers strength, but that also minimises damage during penetration by retaining a sleek shape, could be achieved by using aluminosilicate rather than the borosilicate needles used in this work. Such a change has resulted in increased efficiency in mosquito transformation protocols (Lobo *et al.* 2006). An introduction of excess fluid causing an increase in internal pressure within an embryo will increase mortality. Improved regulation of the fluid injected could be achieved with automated micro-manipulators. Finally, current research into the basis of the relatively low fertility of *T. dalmanni* adults is likely to provide valuable insights into how to increase the productivity of laying populations.

A number of options for improving transformation efficiency are available. These include modifying or changing the vector, increasing the probability of integration events and altering the source of transposase. Variation in transformation efficiency is observed between transposable elements, and transposon vectors other than *Minos* have proved successful in mediating germline transformation in other insect species. In Chapter Two, *piggyBac* was shown to be capable of excision in stalk-eyed fly embryos and high transformation efficiencies have been reported with this vector (Berghammer *et al.* 1999; Finokiet *et al.* 2007). *Minos* or *piggyBac* could be used to introduce *attP* sites into the *T. dalmanni* genome to facilitate phage ϕ31 integrase mediated germline transformation. This approach has recently been used in *Drosophila melanogaster* and *Aedes aegypti* and is associated with high transformation efficiencies (Groth *et al.* 2004;

Nimmo *et al.* 2006). ϕ31 integrase has two other advantages over transposon based vectors. First, insertion occurs into the same site so that variation in transgene expression due to local enhancer effects is controlled for. Second, ϕ31 integrase activity appears not to be limited by the size of the insert, which is a problem reported in transposon vectors (Lorenzen *et al.* 2003).

Insect transformation protocols in general would benefit from increased understanding and testing of the transposition mechanism and investigations of how transposon and transposase sequences can be altered to increase efficiency. The *piggyBac*, *Minos* and *mariner* vector systems are all based on the unaltered sequence of the 'wild' transposon (Tosi & Beverley 2000). In nature, high transposition activity is selected against (Hurst & Werren 2001). Manipulations of transposon sequences have been shown to increase transformation efficiencies (Butler & Chakraborty 2006) and such alterations could be adapted to manipulate transformation efficiency in insect trangenesis (Atkinson 2008).

In two out of the three insertion events reported in Chapter Three, expression of the transgene was hindered by epigenetic silencing effects although the insertions remained stable. Such silencing effects are common amongst insect transgenic lines with the most well studied example being position effect variegation (Wilson *et al.* 1990). Constructs are available in which the transgenes are flanked by insulator elements such as scs-scs', *gypsy* transposon and chicken β-globin 5' HS4 element, which prevent the spread of euchromatin and have been shown to reduce variability in transgene expression between individuals (Sarkar *et al.* 2006). Such insulator elements will not increase the number of transgene insertions but may cause an effective increase in transformation efficiency since more insertion events will be detected.

Expanding the transgenic protocol

The availability of a transgenic protocol in stalk-eyed flies enables a wide range of experiments on the development and evolution of eye-stalks. There are many experiments that can be carried out, using the transgenic line I have created as well as by the insertion of single, readily available, constructs, which are discussed below. However, it would also be beneficial to develop the protocol further by testing constructs based on heat shock inducible promoters or the binary GAL4/UAS system. These would enable more precise regulation of transgene expression and a more varied range of potential experiments.

The primary purpose of developing the protocol was to test gene function in stalk-eyed flies. A number of candidate genes for involvement in eye-stalk development have been identified from EST and microarray studies (Baker *et al.* 2009), and from QTL mapping based on microsatellites developed for for *T. dalmanni* (Johns pers. comm. in Wilkinson *et al.* 2005). Transgenic analysis of the function of these genes would include over-expression or RNAi-mediated knockdown of the genes and observation of the resulting mutant phenotypes. However any of these types of transgene has the potential to produce a non-viable phenotype that would make creating a transformant impossible. For example many mutant phenotypes of the insulin signalling pathway are embryonic lethal (Chen *et al.* 1996). It would therefore be prudent to use the binary GAL4/UAS system to drive their expression (Fischer *et al.* 1988). The necessary constructs are readily available (Imamura *et al.* 2003).

Many different promoters are available, or can be developed, to regulate transgene expression. Given their widespread activity amongst all other dipteran flies, ubiquitous promoters from *D. melanogaster* are likely to work in stalk-eyed flies (Catteruccia *et al.* 2000; Pinkerton *et al.* 2000). Such promoters would result in high levels of transgene expression though development. However, it would be beneficial to develop methods for more refined temporal and spatial regulation of transgene expression in stalk-eyed flies. Temporal regulation can be mediated by heat shock promoters (eg *Hsp70*) which up-regulate expression in response to high temperatures. Hsp70 promoters from *D. melanogaster* may work in *T. dalmanni* but such promoter regions have been successfully cloned in other species (Pavlopoulos & Averof 2005).

One way of generating tissue- and stage-specific promoters for GAL4 expression would be to clone the upstream regulatory regions of candidate genes that show primordium-specific expression during eye-stalk development. A number of genes expressed in the head have been isolated by Baker *et al.* (2009) and could be used to sequence and test such regulatory elements. In *D. melanogaster,* spatial regulation is also achieved by exploiting enhancer trap lines. Lines are created with a transgene containing the GAL4 coding sequence downstream of a minimal promoter. GAL4 expression will therefore be regulated by the local genomic environment. The expression pattern can be visualised by crossing with a line containing UAS upstream of a marker gene such as eGFP. The GAL4 insert can potentially be mobilised and re-inserted into new genomic locations by crossing with a jumpstarter line carrying a stable source of transposase. Jumpstarter lines are generated by introducing the transposase for one vector system (eg. *Minos*) in a different vector (e.g. *piggyBac*).

Extensive libraries of enhancer trap lines have been generated in *D. melanogaster* to provide a vital resource in functional genomics and are being developed in *Tribolium castaneum* and *Ae. aegypti* (Lorenzen *et al.* 2007; Scali *et al.* 2007). Vector systems for enhancer trapping based on *piggyBac* and *Minos* and are equally applicable to *T. dalmanni.* Such systems can also be used for mutagenesis and, by sequencing the DNA the insert is located in, for identifying genes expressed in the tissues of interest.

Future experiments

As discussed in Chapter One, transgenics can facilitate a varied range of experiments. Below I outline a few specific experiments to demonstrate the diversity possible. These include direct applications for the line created in Chapter Three, the testing of data from sequencing projects, using the technique to build upon and improve results from Chapter Four, physiological studies and the potential for uses other than the study of eye-stalk development.

The insert in line 17 (described in Chapter Three) was X-linked hence a cross between a carrier male and a wild-type female will result in all female expressers and all male non-expressers. The expression pattern is observed during larval stages therefore this line enables the sexing of live larvae. Assessing the differences in development between males and females will be key to understanding the developmental basis of sexual

dimorphism for eyespan. All previous methods of larval sex determination require sacrifice of the individual for either morphological analysis of the genital discs, or microsatellite based approaches (Carr *et al.* 2006b). As discussed in Chapter One, critical weight is an important stage in larval development in insects as it initiates the terminal growth period. The sexual dimorphism for eyespan (and body size) seen in many stalk-eyed fly species, including *T. dalmanni*, may be due to an increased terminal growth period and the results from Chapter Four support this. Successful attempts have been made to determine this point in *T. dalmanni* development (Ingraham pers. comm.). However, any differences in critical weight between the sexes could not be tested for because it was not possible to retrieve enough tissue for morphological or microsatellite based techniques from larvae that had starved to death. By using line 17, larval sex can be determined prior to treatment, which elegantly eliminates this problem.

Transgenics enables functional assays that complement gene expression studies. The generation of EST libraries combined with microarrays in *T. dalmanni* has produced a number of candidate genes involved in eye-stalk development (Baker *et al.* 2009). Microarrays identified genes that differed in expression in the developing eye-antennal disc between selection lines for large and small eyespan. Genes identified of particular interest were *crooked leg* (*crol*) and *cdc2*, which are involved in cell cycle progression regulation. Furthermore, *crol* is regulated by ecydsone and mutant phenotypes affect appendage elongation and head eversion phenotypes (D'Avino & Thummel 1998). Direct insertion of constructs that either over-express these genes using a constitutive promoter or that promote RNAi induced knock-out, can test for direct involvement of these genes in eyespan regulation. As described above it may be prudent to use a GAL4/UAS system to more tightly regulate the expression of these genes and avoid problems of creating a transgenic line if the mutant phenotypes are non-viable.

Exonic insertions where variation in glutamine repeats are correlated with variation in eyespan have been identified in *T. dalmanni* using QTL mapping based on microsatellites developed for the species (Johns pers. comm. in Wilkinson *et al.* 2005). These were again identified in selection lines for high and low eyespan. By using the transgenic protocol to express copies of the gene with varying numbers of repeats, it will be possible to identify whether these repeats are just correlated with the selection lines, or if they are directly involved in eyespan regulation. If the latter is true then transgenics can be used to directly test their role further. A similar approach was used to

test mechanisms of meiotic drive in *D. melanogaster* where different numbers of repeats in certain genes affected sperm formation and cause segregation distortion in gametes (Kusano *et al.* 2002).

In the long term, if it were possible to generate a transgenic construct that caused increased eyespan in *T. dalmanni*, a most apposite experiment would be to introduce individuals with genetically increased eyespan into a standard laboratory population. The spread of the transgene could then be monitored providing empirical tests of some of the central assumptions of the theoretical models of the evolution of ornamental traits. To date comparable tests of the components of sexual selection theory have only been described in yeast (Rogers and Greig 2009). Since female preference for large eyespan would be expected to be present (Burkhardt & de la Motte 1988; Wilkinson & Dodson 1997), one would predict that males with genetically increased eyespan would initially spread through the population.

The transgenic protocol can be used to enhance the approach used in Chapter Four to assess cell size and number. A limitation of the technique used is that it was not possible to stain an entire stalk, and therefore cell size estimates were made from subsections of the stalk. This was due to limited physical penetration of the stain into the stalk. By inserting a construct that expressed nuclear localised GFP under the control of a ubiquitous promoter, every cell in the eye-stalk of a freshly emerged imago can be observed using three dimensional imaging techniques, such as confocal microscopy. This would provide a more accurate measure of both cell size and cell number.

One of the major restrictions in studying eye-stalk development is that the exact location of the eye-stalk progenitor cells is unknown. Attempts were made by Hurley *et al.* (2002) to create a fate map, but the method involved could only compare between three large regions of the disc. Halfon *et al.* (1997) developed a technique whereby the use of a transgenic construct containing a reporter gene, such as eGFP, under the control of a heat shock protein could be controlled at a cellular level. Expression was induced by using a laser to heat individual cells. GFP expression could then be observed in adult tissue and used to generate a fate map. This technique has also been used successfully in the butterfly *Bicyclus anynana* (Ramos *et al.* 2006). It could be used to identify regions that give rise to the eye-stalk in developing eye-antennal discs, in either whole stalk-

eyed fly larva or in cultured discs.

All the experiments so far described are intended for studying eye-stalk development. However, transgenics could also facilitate the study of many other aspects of stalk-eyed fly biology. For example, it is important to test the selective advantage of possessing increased eyespan. One such advantage is increased paternity success when in direct competition with small eyespan individuals. This can be assessed by recording the proportion of sperm within a female's spermathecae. When looking within an individual spermathecae it is not possible to differentiate between the sperm from different males. In *D. melanogaster* a construct that expressed eGFP in sperm was used to assess sperm competition between transgenic and wild-type males (Price *et al.* 1999). A similar construct could be used to assess sperm competition in stalk-eyed flies. Different males each possessing a promoter that drives the expression of different reporter genes in sperm could be used to assess direct competition for matings with single or multiple females.

A similar yet simpler approach could test the benefits of eyespan in competition for matings from a slightly different angle. Instead of sacrificing the females to observe sperm numbers in their spermathecae, their offspring could be scored for expression of the different reporter genes, in order to identify the proportion of the next generation that each male had sired. Paternity is most commonly scored using micro-satellite analysis which can be labour intensive and costly if carried out on large number of individuals since it requires large numbers of DNA extractions and modified sequencing reactions. Using a transgenic approach to determine paternity provides a cheaper and less labour intensive alternative since, once the lines were set-up, it would only require the scoring of larvae under a dissecting microscope fitted with a UV lamp. Furthermore the offspring would not be sacrificed and measures of their fitness could be assessed, such as eyespan and fertility, giving more information on the benefits of female preference for large eyespan.

5.3 Cell size and cell number in eye-stalks

I investigated the differences in growth associated with increased eyespan by assessing cell size and cell number (Chapter Four). My findings show that cell number contributes more than cell size to variation in eyespan. No difference in the relationship of eye-stalk and cell size (and therefore cell number) was observed between the sexes. This is an important result. It is the first time that cell size and cell number have been assessed in a sexually selected trait and provides a solid starting point for further studies of eyespan regulation. Eye-stalks are of interest in the context of sexual selection but their growth is also relevant to studies of organ growth. In *Manduca sexta* and *D. melanogaster* different periods of growth and variation in levels of insulin signalling are observed between the discs of different organs and within individual discs (reviewed in Edgar 2006; Shingleton *et al.* 2007). However, there is a paucity of evidence for variation between individuals in discs for the same organ. In stalk-eyed flies inter- and intra-sexual variation in eyespan is high, with full-sized *T. dalmanni* males possessing eyespan almost twice that of full sized females and nutritionally stressed males. Such dramatic differences may increase the probability of detection of variation in the mechanisms underlying growth.

Future experiments

Since cell number was the main contributor towards increased eyespan, variation in eyespan may be due to changes in proliferation period or rate between males and females. I have outlined above how the transgenic line 17 can be used to compare the terminal growth period in *T. dalmanni* between males and females and how a transgenic approach could be used to improve cell size and number assays. There are many more methods to assess differences in mitotic rate and period between the sexes and the possible mechanisms underlying any differences observed. As stated in Chapter Four, comparisons between male and female eye-stalk primordia could be performed using the incorporation of immunohistochemically detectable nucleotide analogues such as bromodeoxyuridine (BrDU) or mitosis-specific antibody markers to compare cell division rates.

The growth period of discs are determined by the responsiveness to hormones such as juvenile hormone and ecdysone, and the rate of growth by the insulin signalling

pathway (Champlin & Truman 1998; Shingleton *et al*. 2005). EST studies have generated sequences for many members of the ecdysone signalling pathway in *T. dalmanni* that could be used to test the activity of these pathways in developing eye-antennal discs (Baker *et al.* 2009). It has been shown that in *M. sexta,* eye discs are only responsive to ecdysone between two concentrations of the hormone and a similar mechanism may be present in developing eye-stalks. Variation in levels of responsiveness to ecdysone between developing discs could be a mechanism for sexual dimorphism. Quantitative PCR (qPCR) could be used to assess the activity of E75B, a downstream target of the ecydsone receptor, as the disc develops and test for sex specific differences. The activity of this, and related, genes can be compared with larval and pupal ecdysone levels in males and females to give a fuller understanding of the process.

Another method for assessing the growth period of discs is to compare the length of time that the discs are responsive to insulin signalling. This was shown to differ between discs in *D. melanogaster* (Shingleton *et al.* 2005). The *T. dalmanni* insulin receptor has recently been sequenced (Smith pers. comm.). Again using qPCR, the period of insulin receptor expression could be assessed in developing eye-antennal discs and compared between the sexes. Moreover, if the level of insulin receptor expression, also detectable by qPCR, in the developing disc differed between the sexes that would indicate differences in the degrees of male and female responsiveness to nutrition in addition to any differences in the duration of the growth period. Sequences from other members of the insulin signalling pathway, such as PTEN, are available and can be tested as well.

Sexual dimorphism for eyespan has arisen in the Diopsidae on at least four separate occasions (Baker & Wilkinson 2001) and both monomorphism and dimorphism are found in each of the major diopsid genera (Fig. 1.3). It is unclear whether the same developmental mechanism has driven dimorphism each time that it has arisen but comparisons of how cell size varies with sex and organ size in species spanning the Diopsid clade would be informative. If the nature of the relationship between cell size, cell number and eyespan varied between the representative species, it would show that the developmental mechanism underlying the evolution of dimorphism varies within the group. All stalk-eyed species will be amenable to the protocol used in Chapter Four. The UCL stalk-eyed fly group currently maintains diopsids from three different genera

demonstrating dramatic intra- and inter-specific variation in eyespan. In addition to *T. dalmanni*, populations of *Teleopsis thaii*, *Diasemopsis meigenii, Diasemopsis comoroensis, Sphyracephala beccarii* and *Diopsis apicalis* are or have been maintained and proved amenable to mass rearing and experimentation (Cotton *et al*. 2004; Hurley *et al.* 2001; Cotton *et al.* 2006a; Small pers. comm.). *T. thaii* males possess eyespans almost twice the size of *T. dalmanni* males (Földvári *et al.* 2007), and in *D. meigenii* the degree of dimorphism is similar to that of *T. dalmanni* while the sister species *D. comoroensis* is monomorphic (Carr *et al.* 2006a). Comparisons between species should be both straightforward and potentially informative.

5.4 Conclusions

With the development of a germline transformation protocol in *T. dalmanni,* several immediate applications and experiments are possible and further refinement and development of the technique will undoubtedly enhance the range and power of possible future studies. The finding that cell size and cell number vary with eyespan value rather than sex demonstrates that although eyespan is sexually dimorphic the mechanism of growth within developing eye-stalk cells does not appear to be so. This is an important finding that can be exploited in further studies of the development and evolution of exaggerated sexual traits and of organ growth.

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