Development of handling and transport protocols for *Eldana saccharina* (Lepidoptera: Pyralidae) sterile insect technique (SIT) programme

by

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Summary

Eldana saccharina Walker (Lepidoptera: Pyralidae), is indigenous to sub-Saharan Africa and is a serious economical pest of sugarcane in South Africa. Recent area-wide integrated pest management (AW-IPM) efforts have proven to be effective in lowering infestations and better predicting population growth and spread into new areas. The sterile insect technique (SIT) promises to provide great benefit as a component of the AW-IPM strategy.

This study aimed to establish parameters for collection, packaging, and transport of recently emerged *E. saccharina* adult males, within a maintained cold chain conjointly with methodologies for the routine assessment of the performance and competitiveness of sterilized insects. These treatments should not impact the fitness nor mating ability of the adults exposed to them.

Several major outcomes were realized, summarized as follows: Parental male and female pairs exposed to 100 Gy irradiation prior to mating provided an 83.3 % male biased first filial generation. Thus, a male biased, semi sterile generation could be reared for collection and release using this technique; difference between average male (0.0700 g) and female (0.133 g) pupal weights (as an indication of adult weight) were identified as a morphological trait that could be exploited to obtain a high degree of sex separation; and the plenum collection box was effective in collecting male and female adult *E. saccharina* (a mean of 80.67 % ± 4.56 % adults were collected when placing 200 adult moths (male : female = 1:1) into the prototype collection system overnight for 12 hours replicated three times). Furthermore, the effect of exposure of virgin *E. saccharina* males to 5 ℃ for 24, 48 and 72 hours on male mating frequency and longevity was measured to determine the impact of prolonged periods of cold exposure to male fitness. The 72 hour treatment showed a significant decrease in male mating frequency (average of 4.4 females mated at 48 h versus 2.7 females mated at 72 hours). It is therefore possible to hold *E. saccharina* at 5 ℃ for 48-hours without impacting on moth fitness in terms of mating frequency. Bran was identified as a good temperature insulating material to use as a packaging substrate for adult males, as it buffered temperature fluctuations over time inside a transportable freezer set at 5 ℃ (1.5 ℃ to 5.5 ℃) when fully stocked with bran, versus being empty (0 ℃ to 6 ℃). Bran's effect as a packaging substrate on male fitness during high density packaging and exposure to 5 ℃ for 24, 48 and 72 hours showed no significant decrease in male mating frequency (mean mating frequency of 2.8889 females mated). Males did have a decreased mating frequency (1.4667 females mated) after 72 hours for the no bran packaged adults. A visual rating system was tested to measure the difference in loose scale cover - on the eyes, dorsal surface, ventral surface, as well as scale loss from the pronotum - between bran and no bran packaged moths. There was a clear improved visual quality with bran packaged adults compared to no bran packaged adults with less scale loss from the pronotum and less

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loose scales covering the eyes and ventral and dorsal surfaces, indicating that this visual rating system could be valuable as a quality control check at the point of release.

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Opsomming

Eldana saccharina Walker (Lepidoptera: Pyralidae), is inheems aan Suider Afrika en is 'n ernstige ekonomiese plaag op suikerriet in Suid-Afrika. Onlangse pogings tot area wye geïntegreerde plaag bestuur was effektief. Die steriele insek tegniek (SIT) beloof om 'n groot voordeel te lewer as komponent van die area wye strategie tot beheer.

Die produksie van *E. saccharina* kan voortdurende produksie volumes van mededingende motte verseker. 'n Stralingsdosis van 200 Gy het geen invloed op die paringsgedrag of die algemene fiksheid van volwasse motte nie en laat mannetjies gedeeltelik steriel vir gebruik in ‘n SIT program. Hierdie studie poog om parameters vas te stel vir versameling, verpakking en vervoer binne 'n onderhoude koue ketting, tesame met metodieke vir die roetine-assessering van die mededingendheid van gesteriliseerde insekte. Die blootstelling van mannetjie en wyfie ouer-pare aan bestraling van 100Gy voor paring het 'n 83,3% mannetjie opeenvolhende generasie gegee. Die meerderheid mannetjie, semi-steriele generasie kan dus groot gemaak word vir versameling en vrylating.

Verskil tussen mannetjie en vroulike mot gewigte is geïdentifiseer as 'n morfologiese eienskap wat gebruik kan word om 'n sterk mate van skeiding te verkry. Die plenum-versamelingskis is effektief vir die versameling van mannetjie en wyfie *E. saccharina*.

Deur motte bloot te stel aan koue temperature vir hoë digtheid verpakking vir bestraling, vervoer tot met vrylating verhoed dit dat liggaamlike aktiwiteit mot fiksheid sal beskadig. 'n Immobiliseer- en houtemperatuur van 5 ℃ is gebruik wat bo die motte se kritieke minimum temperatuur van 4,4 ℃ is. Die effek van blootstelling van maagdelike *E. saccharina* mannetjies aan 5 ℃ vir 24, 48 en 72 uur op mannetjie parings frekwensie en langlewendheid is gemeet om die impak van langdurige periodes van koue blootstelling aan mannetjie fiksheid te ondersoek. Die 72 uur behandeling het 'n beduidende afname in die parings frekwensie vir mannetjies getoon in vergelyking met alle ander behandelings. Dit is dus moontlik om *E. saccharina* by 5 ℃ te hou vir tot 48 uur sonder om die motfiksheid in terme van parings frekwensie te beïnvloed.

Semels is geïdentifiseer as 'n goeie temperatuur isolerende buffer om as verpakkingssubstraat te gebruik, en het dus ook getoon dat temperatuurswisseling verminder as die temperatuur met verloop van tyd binne die vervoerbare vrieskas gemeet word wanneer dit vol met semels is teenoor om leeg te wees.

Semels se effek as verpakkingssubstraat op mannetjie fiksheid tydens hoë digtheid verpakking en blootstelling aan 5 ℃ gedurende 24, 48 en 72 uur het geen beduidende afname in mannetjieparings frekwensie getoon nie. Mannetjies het wel na 72 uur 'n verlaagde parings frekwensie gehad vir die nie-semels verpakte motte.

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'n Visuele waarderingstelsel is getoets om die verskil in losskaal bedekking aan die oë, rugkant, ventrale kant sowel as skaal verlies te meet aan die pronotum tussen semels en geen semels verpakte motte. Daar was 'n duidelike verbeterde visuele kwaliteit met semels verpakte motte in vergelyking met geen semels verpakte motte , wat daarop dui dat hierdie visuele waarderingstelsel waardevol kan wees as 'n gehaltebeheer op die punt van vrylating.

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This thesis is dedicated to my wife, Leani, who has been a constant source of support and encouragement during the challenges of work and study that has taken up so much of our time. I hope to also always be such a source of optimism to her as she is to me.

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Biographical Sketch

Adriaan Serfontein earned his BSc (Agric) Horticulture and Entomology degree at Stellenbosch University in 2015. His Master of Science degree in Entomology focusses on the development of a cold chain for *Eldana saccharina* to ensure the release of competitive sterile moths for the implementation of the sterile insect technique in the South African sugar cane production areas.

His interests include the implementation of augmented biological control within a broader integrated pest management system with the aim of achieving more environmentally and economically sustainable crop production.

He lives in Nelspruit, South Africa where he works with citrus producers as a junior crop protection consultant in Mpumalanga, Limpopo and Western Cape production areas

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the journal of the Entomological Society of South Africa (*African Entomology*)

Chapter 1 **Literature review**

Chapter 2 Investigation of possible sex separation and collection methods

Chapter 3 Effect of cold exposure, packaging and transport on *Eldana saccharina* male fitness

Chapter 4 **General discussion and recommendations**

Keywords:
Sterile Insect Technique (SIT); Irradiation; Area-wide Integrated Pest Management (AW-IPM); Collection Box; Handling; Transport; Cold Chain; Temperature curves; Packaging; Insulating; Packaging substrate; Bran; Protandry; Quality Control (QC); Male Mating Frequency; Male Longevity; Visual Rating System

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4. CHAPTER 4: GENERAL DISCUSSION AND RECOMMENDATIONS

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CHAPTER 1: LITERATURE REVIEW

1.1. BIOLOGY AND ECOLOGY

Eldana saccharina Walker (Lepidoptera: Pyralidae) was first described from sugarcane, *Saccharum officinarum* L. (Poales: Poaceae), in Sierra Leone, West Africa by Walker (1865) (Atkinson, 1980). Common names include African stalk borer, eldana borer, bottom borer and broca-dacana—de-acucar (Conlong and Way, 2015). It is indigenous to Africa and widely distributed throughout sub-Saharan Africa (Conlong, 1994). *Eldana saccharina* prefers different natural host plant families in different parts of Africa, with different parasitoid complexes attacking these. It was thus suggested that various biotypes of this species might exist (Conlong, 2001). Further analysis by King *et al.* (2002) confirmed that South African populations are genetically distinct from those present in Uganda, Cameroon and Benin. Assefa *et al.* (2006) further found significant genetic diversity between populations from West Africa, Ethiopia and Southern Africa.

In South Africa *E. saccharina* is present in areas beyond the sugar belt. Extensive surveys conducted on potential indigenous host plants in the proximity of maize, sorghum and sugarcane revealed that populations extend from Thohoyandou in Limpopo province (Northern limit) to Mkambati Nature Reserve in the Eastern Cape (southern limit) to Boskop dam in the North Western Province (Western limit) (Assefa, 2008). Across *E. saccharina's* geographical range in South Africa, subpopulation's critical thermal minimum temperature (CT_{min}) varied, with the average CT_{min} estimates of 2.8, 3.9, 6.9 and 7.2 °C for the Midlands South, Midlands North, Umfolozi and Malelane populations respectively. CT_{min} significantly correlated positively with the climatic mean minimum temperature (Kleynhans, 2014).

Eldana saccharina is polyphagous, and in addition to sugarcane, is found on a wide variety of host plants in four families, namely Cyperaceae, Poaceae, Typhaceae, Juncaceae (Girling, 1972; Atkinson, 1979; Conlong, 2001; Mazodze and Conlong 2001). The specific host plant has been proven to affect *E. saccharina*'s CT_{min} and critical maximum temperatures (CT_{max}). Moths collected from sugarcane have lower CT_{min} compared to those from *Cyperus papyrus* (CT_{min} = 2.8 ± 0.4 vs. 3.9 ± 0.4 °C; CT_{max} = 44.6 ± 0.1 vs. 44.9 ± 0.2 °C) (Kleynhans, 2014). It is capable of year-round development in the presence of suitable host plants where temperatures stay within the specific population's critical thermal limits (Way, 1995). The immature stages' development is highly dependent on temperature and can vary between 33 to 173 days under field temperatures of between 30°C and 15°C, respectively. Up to six generations may develop in one year (Way, 1995). Temperature dependent developmental information is summarized in a population-dynamics model (Horton *et al.*, 2000) that simulates population growth and damage. Most recently, an agent-based simulation model (ABM) by Van Vuuren *et al.* (2015) has been developed. ABM involves the computational study of social agents that interact autonomously as evolving

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systems, allowing for the study of complex adaptive systems and makes possible exploration into how macro-phenomena develop from micro-level behaviors among heterogeneous sets of interacting agents (Janssen, 2005). The aim of the model was to accurately simulate *E. saccharina*'s general biology and population dynamics and spread in a specific area, by incorporating the knowledge of the pest's biological behavior as well as natural climatic variation within an ecological system. The development of a reliable prediction model will provide valuable insight when optimizing integrated pest management (IPM) strategies (Horton *et al.,* 2000).

Atkinson (1981) described the mating behavior and activity patterns of *E. saccharina*. He found that eclosion usually occurred after sunset. Males tended to emerge before females. After emergence, adult *E. saccharina* climbed a short distance up a nearby vertical surface where their wings would be held perpendicularly while they proceeded to expand and set. Wings would set after 10-15 minutes and then fold back to their resting position. Adult males started to display for a potential mate around 30 minutes after wings had set. He also observed that males could lek singly, but more often were found lekking in groups of three to six males perched in close proximity on the canopy of the same host plant. During this lekking behavior a male faces downward while perched on the top section of a host plant, rapidly fluttering his wings in short bursts, curving his abdomen, and protruding his pheromone emitting pencil hairs at the end of the abdomen, into a full round brush. Male pheromones are also emitted from wing glands (Atkinson, 1982). It was estimated that male calling behavior could last 15 to 20 minutes at a time. In the absence of females, male mating displays still initiate and continue up until dawn (Atkinson, 1982). This lekking behavior is unique among lepidopteran species targeted with the Sterile Insect Technique (SIT). However, lekking behavior has been observed in *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and other tropical and subtropical Tephritid flies (eg *Bactrocera*, *Anastrepha* spp.) (Prokopy, 1980). When dealing with this complex mating system, increased overflooding ratios to compensate for reduced sterile male competitiveness is less effective compared to species exhibiting simpler mating systems (Hendrichs *et al*., 2002). This is because females exert mate choice and may favor the less abundant wild males that attract the females better, due to their greater ability to entice females using timely release of pheromones (Heath *et al*., 1994) and performing the proper visual, sound and tactile courtship behaviors (Eberhard, 2000). Mudavanhu *et al.* (2016) proved that wild *E. saccharina* females did not discriminate between irradiated or laboratory-reared males during mating competitiveness trials, suggesting that there are no negative effects on acceptability for mating due to laboratory rearing or radiation treatment. It is nonetheless of utmost importance to focus on delivering consistent, superior sterile male *E. saccharina* adults to establish a successful SIT suppression program.

Conlong (1994) found that females start depositing eggs about 24 hours after mating. Females are equipped with a prehensile ovipositor, allowing for oviposition in cryptic areas such as under leaf sheaths and in the stalk region between the stem and soil, as well as in clods of soil and trash material. Egg masses usually occur in batches of

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about 50-100 eggs with a single female producing up to 450 eggs, according to Walton and Conlong, 2016. Sensitive sensilla on various sides of the ovipositor tip were found to be stimulated for the female to lay eggs when in position between two narrow surfaces and with the tip touching the third egg laying surface (Wallade, 1982). Adult females display preference for oviposition in proximity to indigenous sedge host plants above that of sugarcane and least toward indigenous grass hosts (Conlong *et al*., 2007). However, it was found that females oviposit on any cryptic surface in proximity to hosts, and neonate larvae then forage towards the preferred host plant. *Eldana saccharina* neonate larvae show a clear host preference towards sedges compared to sugarcane and least towards indigenous grass hosts (Conlong *et al*., 2007). After about 8 – 10 days, neonate larvae hatched from eggs and immediately started feeding on surrounding cane leaf sheaths before entering the stem (Leslie, 1993). After development of the third larval instar, the larvae usually bore into host stalks where they feed extensively and develop further. Female *E. saccharina* has 6-7 larval instars compared to 5-6 for males (Walton and Conlong, 2016). It was found that the duration of the larval stages lasts approximately 20 days during summer months, and 60 days during winter months. Before pupation, larvae bore a moth exit hole through the rind of the stalk, which is usually covered with frass, and spin themselves into a protective silk cocoon to pupate. Duration of the pupal stage is 7-14 days (Horton *et al.*, 2000).

1.2. PEST STATUS

Eldana saccharina first assumed pest status in South Africa in September 1939, when an outbreak occurred in two-year-old sugarcane on the Umfolozi Flats, KwaZulu-Natal, South Africa (Dick, 1945). Before specimens were discovered in other areas of the sugarcane belt, *E. saccharina* was thought to be introduced, and there was great fear of it widening its distribution (Dick, 1945). Infestations persisted, confined to the Umfolozi flats until 1953, whereafter infestations ceased in conjunction with the increased planting of the more tolerant sugarcane variety NCo 376, due to the severe impact *E. saccharina* had on the less tolerant variety POJ 2725 (Carnegie, 1974). A second outbreak on NCo 376 was recorded in Hluhluwe, KwaZulu-Natal, South Africa in 1970. Since then, *E. saccharina* has established in sugarcane growing regions of Kwa-Zulu Natal, eSwatini and the Mpumalanga Lowveld (Atkinson, 1981; Carnegie *et al.*, 1976). The spread of *E. saccharina* on sugarcane is attributed to the switch in host plants from surrounding natural hosts (Nuss *et al.*, 1986). The poor dispersal ability of *E. saccharina* can limit gene flow within the metapopulation, increasing the rate of natural selection in specific populations and so increasing the possibility of host shifts (Rutherford, 2015). *Eldana saccharina'*s relatively short lifecycle compared to that of its host plants can cause further strong selective pressures imposed by specific host plant defences. Disturbances of the natural habitat in Umfolozi since the 1930's, whereby wetlands containing natural hosts have been drained and wet soils planted with sugarcane is thought to have caused *E. saccharina'*s host shift to sugarcane. Sugarcane planted in these unsuitable wet soils are stressed and had lowered defence responses against *E. saccharina* (Nuss *et al.*, 1986; Rutherford, 2015). *Eldana saccharina*

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still continues to expand as a pest on sugarcane in previously un-infested regions such as higher altitude areas in the KwaZulu-Natal Midlands and eSwatini (Assefa *et al.*, 2008).

It is the larval stages that account for most damage caused by *E. saccharina*. This infestation lowers sugarcane biomass and quality (Goebel and Way, 2003). Secondary infection of the boring tunnel by opportunistic *Fusarium* spp Link (Nectriaceae: Hypocreales) assemblages causes *Fusarium* stalk rot, metabolizing sucrose into glucose, further decreasing the extractable sugar (Mahlanza, 2015). Thus heavily infested sugarcane has reduced brix, pol, purity and increased fiber (Goebel and Way, 2003). Annual losses to the South African sugar industry due to *E. saccharina* damage are estimated to be in the region of ZAR 344 million (Rutherford 2015).

In order to maintain *E. saccharina* infestations below the economic threshold, a shortened crop cycle of 10-13 months compared to an optimal 15-18 months, is the industry standard (Rutherford, 2015). If not threatened by infestation, a longer crop cycle will have limited input costs and estimated yields can increase considerably from a cutting mass of 5.76 tonnes RV (Recoverable Value)/ha for 12 months, to 10.8 tonnes RV/ha for a 18 month growing period (Rutherford, 2015).

Knowledge of *E. saccharina*'s life history and ecology is important for the formulation of control strategies in the knowledge-based area-wide integrated pest management (AW-IPM) program now advocated (Conlong and Rutherford, 2010). Various aspects of *E. saccharina*'s life cycle give rise to challenges in the implementation of control methods. These include: the cryptic oviposition of egg batches by females, limiting the effectiveness of egg parasitoids for biological control (Conlong, 1994). The damaging larval stages are also cryptic, residing inside sugarcane stalks, making biological control possible with only specialized natural enemies (NE's) and rendering control with contact insecticides ineffective (Conlong, 1994). Additionally *E. saccharina* is a multivoltine pest, usually with several life stages present in a population at a specific time, thus complicating the timing of control methods aimed at specific life stages (Conlong, 1994).

1.3. MANAGEMENT

Conventional control methods for *E. saccharina* include varietal resistance, cultural control and shortened crop cycles (Keeping, 2006; Conlong and Rutherford, 2009). Extensive research has also shown that damage can be minimized by application of insecticides timed to coincide with adult moth peaks during April/May and September/October, targeting newly hatched foraging and dispersing neonate larvae, before boring into host stems occurs (Leslie, 1997). Using monitoring data and predictive modelling, timely application of insecticides can be conducted if all other IPM practices (discussed below) were ineffective in keeping infestation under economic thresholds (Rutherford, 2015). Application of insecticides on a scheduled calendar basis puts pressure on the

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environment and can lead to a loss of diversity (e.g., ants, spiders, wasps) in the agro-ecosystem, which in turn can result in a rise in pest infestations due to decreased competition, predation and parasitism (Roubos *et al.*, 2014). Selection pressure caused by continual insecticide application can also result in resistant pest populations (Helps *et al.*, 2017). It is thus important to focus on the sustainable, conservative use of pesticides within the larger AW-IPM system, and to follow an insecticide resistance management program with different insecticide groups being alternated as well as continually improving application methods/technologies (Kennedy, 2008).

Cultural control practices

A keystone component for the management of *E. saccharina* is the implementation of good cultural control practices to reduce the rate of infestation by lowering egg and pupae numbers in sugarcane fields, ultimately lowering peak population numbers (Girling, 1980). Prescribed measures include: Cutting sugarcane as low to the ground as possible when ratoon cropping, so as to prevent left over larvae and pupae in sugarcane-stubble from carrying over to the ratoon crop (Carnegie, 1974); removal of all left over cut stalks after harvest (Carnegie, 1974); removing trash from ripening sugarcane (pre-trashing) to simultaneously remove eggs, and expose left over egg batches to environmental and biological risks (Rutherford, 2015); seedcane may be contaminated with eggs and larvae, and a hot water treatment at 50 °C for 30 minutes is prescribed to kill any eggs or other life stages prior to planting (Webster *et al*., 2005). Pre-trashing and trash left over after harvest should be left as a mulch to increase soil health and so improve the health and resistance of following crops (Rutherford, 2015). Fertilizers should also be applied according to the specific soils' nutrient requirements, as stress due to toxicity/deficiency increases susceptibility (Keeping *et al.*, 2014). The over application of nitrogen increases sugarcane susceptibility due to the increased production of soft tissue when water availability is sufficient (Keeping *et al.*, 2014). Supplementation of vigorously growing sugarcane with silicon fertilizer can increase resistance against *E. saccharina* infestations (Keeping *et al.*, 2014).

Resistance Breeding

Resistance breeding for *E. saccharina* has been a major focus of SASRI's breeding program since 1980 (Leslie and Keeping, 1996; Keeping, 1999; Keeping and Govender, 2002). Ovipositional antixenosis (resistance a host plant has against ovipositing females) has not been observed for *E. saccharina.* (Nuss and Atkinson, 1983; Mabulu and Keeping, 1999). When various resistant and susceptible sugarcane varieties were placed in cages with mated females, oviposition was observed to be non-preferential. This indicates that resistance is mostly based on the reaction of larval stages to physiological and chemical factors both on the exterior and interior of stalks (Keeping and Rutherford, 2004). It is thus evident that a major role in varietal resistance mechanisms against *E. saccharina* is larval antixenosis and early-stage antibiosis (Keeping and Rutherford, 2004). The extended dispersal period before stalk penetration on resistant varieties increases neonate larval mortality due to the

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prolonged exposure to insecticide applications, natural enemies (ants, spiders) and other natural causes (Girling, 1978; Leslie,1988; 1993). Although internode rind hardness and increased fiber % improves resistance, these two characteristics decrease recoverable sucrose and are therefore undesirable selection traits. More recent resistant varieties are high in recoverable sucrose with only moderate fiber content, suggesting that these varieties are less influenced by physical resistance mechanisms (rind thickness and fiber content) (antixenosis) and rather possess other chemical traits and physiological reactions to damage (antibiosis), for increased resistance (Keeping and Rutherford, 2004). The addition of a successful SIT program for the suppression of *E. saccharina* could allow the focus of cane breeding to be more on increased recoverable sucrose whilst maintaining an adequate level of resistance, albeit lower than that of high fiber and thick rind varieties.

Area-wide Integrated Pest Management (AW-IPM)

AW-IPM is a coordinated, preventative approach to the management of whole pest populations throughout ecosystems at all spatial scales within the larger landscape (Mudavanhu *et al*., 2011). The integration of various management practices must be linked to a comprehensive knowledge resource of the pests' biology and ecology (Mudavanhu *et al*., 2011). Dealing with challenges presented by pests on an empirical basis leads to the development of conventional, localized management strategies that only treat a subpopulation within the greater, usually highly interconnected metapopulation (Webster *et al*., 2005). These conventional management practices are applied independently by separate stakeholders with little coordination. Because pest populations are influenced by many factors e.g. weather patterns, geography, vegetative conditions, biodiversity, etc. reliance on conventional control strategies that focus only on a stakeholder's area within the larger land mosaic, provide limited control (Webster *et al*., 2005). Poorly managed fields containing the host plants of a certain pest will significantly affect surrounding, well managed fields (Webster *et al*., 2005). It is thus much more effective to practice a lower degree of control over the total pest population in an area as opposed to practicing a more intensive control strategy on only a fraction of the total population (Webster *et al*., 2005). This is because the untreated fraction of a pest population will reproduce without control and so easily produce more offspring than what would be the case when the whole population is treated (Knipling, 1972), in addition to providing a source of re-infestation of the control area.

Area-wide efforts for the management of *E. saccharina* include habitat management, cultural control (e.g. trashing), biological control, resistance breeding and chemical control (Rutherford, 2015). Due to the complex chemical ecology used by parasitoids to locate hosts on their natural host plants, successful biological control on sugarcane is yet to be developed (Conlong, 1997), as key chemical cues are missing for host location by parasitoids in sugarcane (Smith *et al*., 2006). Within its natural habitat, *E. saccharina* populations more easily stay below economic threshold levels and are more susceptible to attack by natural enemies (Girling, 1980; Conlong, 1990). Furthermore, *E. saccharina*'s host preference is towards its natural host plant varieties due to a

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more attractive volatile profile, compared to that of cultivated sugarcane varieties (Smith *et al*., 2006). Therefore, it is beneficial for South African sugarcane producers to implement habitat management, controlling wetland and riverine invasive weeds on their land and re-establishing natural wetland sedges (e.g. *Cyperus papyrus* and *Cyperus dives*), which are natural host plants of *E. saccharina* (Conlong *et al*., 2016). The strategic use of plants producing deterrent volatile chemicals, such as Molasses grass, *Melinis minutiflora* P.Beauv (Poaceae; Poales), further drive *E. saccharina* out of sugarcane fields toward their natural habitat (Conlong and Kasl, 2000). By doing this, stimulo-deterrent diversion (SDD) or push-pull (Conlong and Kasl, 2000) is effectively applied. Using this push-pull technique further allows for inter-planting bands of Bt-maize within cane fields, to which ovipositing adults are attracted and their neonate offspring culled at the onset of feeding (Conlong and Kasl, 2000).

Successful reduction of *E. saccharina* population numbers using an AW-IPM approach increases the potential success for population suppression with the sterile insect technique (Dyck *et al*., 2006).

1.4. STERILE INSECT TECHNIQUE (SIT)

The SIT is a method of releasing sterile insects of a certain pest species within an area containing an existing wild population in an effort to reduce the target population's numbers through the introduction of sterility into the wild population (Dyck *et al*., 2006). For greater efficacy, a high ratio of sterile insects to their wild competing counterparts is sought, resulting in an increased probability of a wild female mating with a sterile male (Klassen, 2005). This high ratio is achieved by implementation of various auxiliary area-wide integrated pest management (AW-IPM) practices prior to release (habitat management, cultural and chemical control as discussed), aimed at lowering the pest's wild population (Dyck *et al*., 2006).

SIT was independently conceived in the 1930's and 1940's by A.S. Serebrovskii at Moscow State University, F. L. Vanderplank at a tsetse field research station in rural Tanzania, and E.F. Knipling of the United States Department of Agriculture (Van der Vloedt and Klassen, 1991). Serebrovskii investigated the use of chromosomal translocations to induce partial inherited sterility on *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Serebrovskii, 1940). Following the principles of Mendalian genetics, Serebrovskii (1940) and his colleagues experienced perpetual resistance from the ruling Union of Soviet Socialist Republics (USSR) who questioned the validity and use of modern genetic sciences (Serebrovskii, 1940; Klassen and Curtis, 2005).

Discovering that crossing two similar tsetse fly species, *Glossina morsitans* (Westwood) and *Glossina swynnertoni* (Austen) (Diptera: Glossinidae) rendered hybrid males and females, sterile and partially sterile, respectively. Vanderplank (1944) proposed this sterility may be used as a control method for the tsetse fly. After Jackson (1945) documented random mating between these two species in the field, Vanderplank (1947) organised

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the mass collection of *G. morsitans* pupae for release in an isolated 26 km^2 area occupied by *G swynnertoni*. *Glossina morsitans* virtually eliminated the less numerous *G. swynnertoni*. Hybrids were found for a period after release, and also declined in numbers. Due to the predicted unfavorable arid conditions for *G. morsitans*, its numbers also declined, finally allowing for total eradication using localized cultural practices (Curtis, 1968).

Runner (1916) first demonstrated that exposure to X-rays induced sterility in cigarette beetles, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). Muller (1928) at the University of Texas, used a dentist's X-ray machine to induce mutations in the chromosomes of the vinegar fly, *Drosophilia melanogaster* (Diptera: Drosophilidae). Muller (1928) further discovered the genetic mechanism of induced sterility, due to X-ray exposure that essentially contributed to the breakage of chromosomes. Broken chromosome ends ('sticky ends') tend to adhere to other complementary ends forming a dicentric chromosome that has two attachment sites (centromeres) for spindle fibres. When dicentric chromosomes are then still able to divide, meiotic cell division occurs and the resulting two daughter cells will end up with a deficiency and duplication of genes, respectively. Resulting gametes will have a normally matured phenotypical appearance. Resulting sperm cells will still have strong competiveness against normal sperm cells but with inadequate genetic information for function after fertilization. Resulting fertilization of a viable egg cell with an irradiated sperm cell will thus, due to inadequate genetic information and other chromosomal events, culminate in early death during embryonic development (Dyck *et al*., 2006).

Development of the first laboratory rearing technique for the New World screwworm (NWSW), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) on a simple ground meat feeding medium by Melvin and Bushland (1936), permitted the production of large numbers of this serious livestock pest to be available for research. Knipling (1937), knowledgeable in the aggressive sexual competitiveness of the male NWSW, and the fact that female NWSW tend to mate only once prior to oviposition, conceptualized that if males could be mass-reared and sterilized before release into the relatively low-density wild population, the NWSW population in the United States could be reduced to inconsequential numbers. Knipling (1955; 1959) devised a simple numerical model, one that foreshadowed most future modelling developments in this field, abstracting the proportion of a population that, under optimal conditions, will produce fertile eggs for a set ratio of fertile males to all males in the population (or females, assuming a 1:1 sex ratio). On this simple probability basis, Knipling (1937) demonstrated that released sterile male NWSW flies of manageable numbers and frequencies could successfully outcompete wild fertile males in breeding with females, to result in significant population suppression. After the war, Muller (1950) published an article on the dangers of atmospheric atomic testing, stating that it might induce harmful mutations in human populations, outlining the general detrimental effects of radiation on genetic material by graphically depicting the high progeny mortality ratio from matings of irradiated *D. melanogaster*. Knipling's attention was drawn to this by A.W. Lindquist who recognized that Muller devised a method of sexually sterilizing

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insects. This method proved to be effective on NWSW when exposing six-day old male pupae to an irradiation dose of 50 Gy (Klassen and Curtis, 2005). Trials further confirmed Knipling's hypothesis, with all eggs of females mated with irradiated males being sterile and with irradiated males and untreated males competing about equally (Klassen and Curtis, 2005). In 1954, Knipling proceeded with a release program on the island of Curaçao (435 km^2). A higher density and frequency of irradiated flies were needed for release due to few other management practices implemented, with a resulting large number of livestock bearing untreated wounds (Klassen and Curtis, 2005). Biweekly releases of 155 sterile males per km^2 were done and eradication was accomplished in 14 weeks (Klassen and Curtis, 2005). More eradication programs followed in the United States of America (USA), until finally, by 1966, the entire USA was declared free of NWSW, with population containment being the only reason for continual release to prevent the re-influx of NWSW from Mexico. By 2001, eradication was further achieved in the whole of Northern- and Central America with a permanent sterile fly buffer zone of 30,000 km^2 at the Darien Gap in Panama being maintained by the weekly release of 25–50 million sterile males [Dyck *et al*., 2006: 129,130]. The current annual cost benefits for producers in previously infested areas is estimated to exceed US$ 1 billion (Dyck *et al*., 2006). Maintenance of the buffer zone amounts to roughly an eighth of the total cost savings (US$ 124 800 000).

SIT has proven to not only have an extremely low environmental impact, but also has long term economic benefits when mass rearing and logistics for implementing SIT for a specific pest has been optimized (in relation to cost per individual insect and cost per area versus total area to be treated) (Dyck *et al*., 2006). Since the mid 1960's, successful SIT programs have been developed for the control of various insect species in the Orders Diptera, Coleoptera and Lepidoptera, that pose a threat as agricultural pests and human and animal disease vectors (Dyck *et al*., 2006).

The implementation of SIT within an AW-IPM system can prove beneficial even if eradication is not a viable option. The aim of such SIT projects is to aid in the exclusion or suppression of pest populations (Klassen, 2005). When successful, SIT programs aimed at population suppression lead to lower overall production costs whilst increasing yield due to lower infestation levels on an area-wide basis (Klassen, 2005). Lowering a seasons' rate of population growth can also provide an extended growing season for certain crops that are harvested before optimal physiological maturity due to pest populations that threaten to exceed economic thresholds (Klassen, 2005).

Inherited sterility (IS) is induced in insects by sub-sterilizing dosage levels of ionizing radiation (Potgieter *et al*., 2016). Partially sterile males that mate with wild females carry radiation-induced detrimental mutations over to the F1 generation. Inheritance of this mutation causes reduced egg hatch and resulting offspring that are both highly sterile and predominately male (Potgieter *et al*., 2016). This high ratio of males in the progeny is due to

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the greater ability males have for surviving large numbers of chromosomal breaks, compared to their female counterparts (Walton, 2011). Due to the high radiation dosages required to achieve full sterility in Lepidoptera, lower radiation dosages used to induce F1 sterility increases irradiated insect quality and competitiveness (Walton, 2011). Improved competitiveness is measured by improved dispersal after release, increased mating ability, and superior sperm competition (Walton, 2011). Due to the debilitating effect of fully sterilizing irradiation dosages on lepidopteran pests (Carpenter *et al.,* 2006), radiation biology studies are focused on providing partially sterile males that will provide sterile, male-only offspring when mated with wild females (Carpenter *et al.,* 2006). The induction of inherited sterility gives rise to the possibility to release the resulting sterile F1 progeny from mating reared, semi-sterile males with un-irradiated reared females. Not only will this exclude the effect of damage caused by sterile F1 larvae in the field but require a less intensive cold chain due to the possibility of mainly transporting eggs and pupa which are less easily damaged than adults.

1.5. CURRENT STATUS (GLOBALLY AND LOCALLY)

Many lepidopteran species are prominent agricultural pests that cause serious losses in yield and quality of food and forage crops, forests, and stored products (Suckling *et al*., 2017).
There are several new problems that arise when applying SIT against lepidopterans i.e. resistance against dominant lethal induction by ionizing radiation due to holokinetic chromosomes of Lepidoptera; production of eupyrene and apyrene sperm; reduction in sterilized male's mating ability; spermatophore formation and complex transfer of sperm (Dyck *et al.,* 2006).

It is also noted that the mass rearing of lepidopteran pests is generally more challenging than that of dipterans. In British Colombia, Canada, Proverbs and Newton (1962) and Proverbs (1982) developed a SIT program to suppress Codling Moth *Cydia pomonella* (Linnaeus,) (Lepidoptera: Tortricidae) using IS. Releases ensued in 1994 in the Okanagan Valley and lead to successful suppression. Releases continue, successfully suppressing the population on an area-wide basis, resulting in major cost benefits to pome fruit growers in the area (Klassen, 2005).

Codling moth is widely distributed throughout temperate fruit producing areas in North America, Europe, New Zealand, and Africa (Willet *et al*., 2009), and can enter diapause at low temperature, allowing for stockpiling of adults for activation and release in synchrony with the wild population. It can also be transported intercontinentally for trial and full-scale releases while in diapause (Suckling *et al*., 2017). This is not the case for most subtropical lepidopteran species i.e. the false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), further complicating the logistics of SIT programs. Nonetheless, a successful population suppression SIT program for FCM has been developed and implemented on a commercial scale by

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XSIT (Pty) Ltd (Citrusdal, South Africa). The success of this program can be attributed to the intensification and optimization of both rearing and handling technologies. The initial release program began in the geographically well isolated area of Citrusdal (32° 36' 0" S, 19° 1' 0" E), in the Western Cape province during the 2007-2008 growing season (Barnes *et al*., 2015). Successful suppression of wild populations in the release areas was notable in the following season and led to lower fruit infestation compared to surrounding untreated areas (Groenewald, 2009). Due to the fact that the rearing and radiation facility was erected in Citrusdal, the cold chain consisted of a short transportation period to the infected orchards. The optimization of these cold chain technologies had to be achieved, before expanding releases to citrus orchards in the Sunday's River Valley in the Eastern Cape (Nepgen, 2014). Adults had to be transported via road in refrigerated conditions over 729 kilometers (km) to Addo, Eastern Cape province lasting between 8 and 10 hours (Nepgen, 2014). This prolonged cold chain caused a reduction in adult moth quality at release. This effect could be monitored by using temperature logging throughout the cold chain and visually observing sampled adults for damage (Nepgen, 2014). When damaged adults of a certain severity were observed (specifically damaged wings, that hinder flight ability), investigation could be made into the cold chain temperature data to identify and rectify any occurrence of a break in the maintained temperature (Nepgen, 2014). The success of suppressing FCM after transportation over such a long distance and time without this subtropical pest's biology allowing for the induction of full diapause gives promise for controlling lepidopteran pests with similar biologies and distribution patterns e.g. *E. saccharina* (Walton, 2011). As with FCM, *E. saccharina* is widely distributed throughout Sub-Saharan Africa and is polyphagous, feeding on a wide variety of hosts (Walton, 2011). Similarly, *E. saccharina* and FCM had well established AW-IPM strategies but limited success (Carpenter *et al*., 2007). These established AW-IPM strategies e.g., habitat management, biological control (egg parasitoids, entomopathogenic fungi, entomopathogenic nematodes), cultural control (sanitation), pheromone mating disruption, monitoring and insecticide applications, aided in FCM wild population suppression prior to implementation of the SIT, increasing the over-flooding ratio of sterile versus wild males (Boersma *et al*., 2018). The current AW-IPM strategies implemented for lowering *E. saccharina* infestations (Rutherford, 2015) is thus comparable to that used for FCM, as it aims to suppress the overall wild populations, and although having limited effect, will be crucial for the success of the SIT as a component of the greater AW-IPM program.

All above mentioned lepidopteran pests on which SIT is currently implemented have comparable mating behaviors in the sense that males are attracted to females using pheromone signaling, which is not the case for *E. saccharina* (Atkinson, 1981). This allows for the reliable use of pheromone traps. Pheromone traps distributed in a network over an area can provide invaluable information regarding native pest population densities and importantly, dispersal data of sterile individuals (Atkinson, 1981). This release-recapture data provides dependable input regarding the field performance of released individuals. Field performance can then be related to the effect of the pre-release process, seasonal and other environmental effects (Mudavanhu *et al*., 2016).

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Seasonal environmental effects can also then further be minimized by modifying the pre-release process as to better acclimatize adults for environmental conditions.

Eldana saccharina's mating behavior can be compared to that of tephritids, as males attract females for mating within a lek formation, but unlike *E. saccharina*, tephritid males can be trapped for obtaining release-recapture data (Enkerlin, 2003). Furthermore, dispersal plays a crucial role in the life history of a tephritid adult (Baker and Chan, 1991). The role of dispersal ability for the field performance of male *E. saccharina* is less clear as there have been no studies that investigate the relationship between male dispersal ability and mating success. It is obvious that irradiated *E. saccharina* males must be fit at the point of release so as to seek refuge and initiate calling, but it is not clear if adult *E. saccharina* fitness can be directly related to dispersal (Mudavanhu *et al*., 2016). Until the development of a reliable trapping system for *E. saccharina* is developed, that would provide accurate information on field performance of released males, conventional destructive sugarcane stalk monitoring is suggested in treated sugarcane fields to provide this information (Rutherford, 2015). Monitoring data can then be compared to prediction models, as was developed by Potgieter *et al.* (2016), to estimate the impact of the SIT over time (Mudavanhu *et al*., 2016).

Walton (2011) investigated the general biology, parental and inherited sterility (IS) of *E. saccharina* and established that dosages of 200 and 250 Gy respectively led to sterile females and males with F1 sterility. Higher radiation doses negatively affected overall fitness and mating behavior. The large number of sterile male offspring resulting from IS males mating with wild females also increased the effectiveness of the SIT. Mudavanhu (2012) concluded that the mass-reared *E. saccharina* strain currently produced at the SASRI insect rearing unit was suitable, concerning thermal tolerance and mating competitiveness, for use in SIT-based projects. Mudavanhu (2012) further established that a radiation dosage of 200 Gy did not impact the mating behavior nor general fitness of adult *E. saccharina* males and induced partial sterility that produced high numbers of sterile male F1 progeny when mated with wild females. Success of a SIT program greatly relies on effective quality assurance tests for monitoring and providing feedback on insect performance during each step of production, treatment, handling and release (Vreysen *et al.*, 2016). Thus far, fitness assessments of irradiated *E. saccharina* have mainly been conducted in laboratory environments, with effects of handling and transport being minimized.

Focus must be set on maintaining the performance and competitiveness of transported and released sterile insects. Transport and release technologies and methods must therefore be developed concurrently with methodologies for the routine assessment of the performance and competitiveness of sterilised insects. Mudavanhu (2012) and Kleynhans (2014) established the CT_{min} of the laboratory reared *E. saccharina* strain to be 4.4 ± 0.4 °C. Thus, it is acceptable to say that the temperature of packaged adults could be lowered to 5 °C during storage and transport, minimizing the respiration rate and movement of the moths while avoiding the detrimental effects caused below the CT_{min}. It is important to strictly maintain the cold chain for transport of quality sterile *E. saccharina* adults

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from the rearing and radiation source to the point of release. Packaging must minimally impact moth quality whilst holding large volumes of moths for efficiency of transport resources. There is also the need for non-destructive monitoring such as trapping methods with which release-recapture trials can be conducted. Currently this impact of sterile releases could be deduced from conventional destructive monitoring methods (i.e. cutting cane and determining % infested internodes), through a reduction of these parameters in the fields where SIT is practiced.

1.6. AIM AND OBJECTIVES

Aim:

To provide quality control parameters for critical cold chain processes for a successful SIT program aimed at providing competitive semi-sterile *E. saccharina* male adults in the field, starting with collection after larval rearing, sexing, immobilization, high density packaging and storage for irradiation and transport.

Objectives:

1. Investigate possible sex separation by exploiting weight difference between sexes.
2. Investigate use of low radiation exposure of mating pairs to obtain a male only colony.
3. Measure if an automated collection box could effectively collect emerged adults.
4. Determine the impact of cold storage over time on male fitness.
5. Identify a good insulating buffer substrate and determine effect on male fitness during packaging and cold storage.
6. Compare conditions provided by refrigerated transport with transport using cooler box and ice packs.
7. Determine effect of transport of high-density packaged males with and without physical/insulating buffers.
8. Investigate possible quality control methods that can reliably indicate male competitiveness at the point of release.

Each of the chapters are written as individual scientific publications, and therefore some repetition may occur.

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CHAPTER 2: INVESTIGATION OF POSSIBLE SEX SEPARATION- AND COLLECTION METHODS

2.1. INTRODUCTION

Because of the worldwide threat that lepidopteran larvae pose to crop loss (Bloem *et al*., 2005), use of the Sterile Insect Technique (SIT) to control various lepidopteran pests have been investigated (Steinitz *et al*., 2016). In comparison to dipteran and coleopteran species, lepidopteran species require much higher levels of ionizing radiation to obtain full sterility (LaChance and Graham, 1984). Female lepidopterans are generally more sensitive to radiation compared to their male counterparts (Soopaya *et al*., 2011). For this reason, adult lepidopterans are irradiated at a dose that fully sterilises females (to avoid unwanted infestation) whilst leaving males partially sterile to preserve field competitiveness (North, 1975; LaChance, 1985). Partially sterile males exhibit inherited sterility (IS) and when mated with a wild female counterpart, the first-generation (F1) offspring are usually male biased with elevated levels of sterility due to genetic damage caused by sub-sterilising irradiation doses (Carpenter *et al*., 2005). Partial sterility may improve the efficacy of a SIT program due to this resulting sterile F1 offspring which further help in overflooding the wild population by further passing on the radiation-induced deleterious effects inherited by the F1 generation (Knipling, 1970). The lower dose of radiation used to induce F1 sterility also increases the quality and competitiveness of the released lepidopterans (Dyck *et al.,* 2006). In lepidopteran SIT programs, both males and females are typically irradiated and released over a target area (Grefenstette *et al*., 2009; Tabashnik *et al*., 2010; Vreysen *et al*., 2010). Sub-sterile male only releases will further theoretically improve the efficacy of a SIT program, compared to bi-sexual releases (Anisimov and Shvedov, 1996).

The key component of a conventional SIT program is sterile or semi-sterile males for release, thus sex separation to obtain males only, for irradiation and release, could improve overall efficiency (Papathanos *et al*., 2009). Development of a sex separation technique to obtain males only during or after production, will lead to lower production and post-collection handling costs, as less material and space will be taken up for a given number of sterile males (Papathanos *et al*., 2009). Females obtained during adult sex separation can also be added back into the brood-stock colony (Keil *et al*., 2001). Furthermore, there is the possibility of preferential mating between released steriles and the fact that sterile females do not directly diminish wild populations by inducing sterility (Vreysen *et al*., 2006). A possible benefit of released sterile females may be that they can distract wild males and act as a “sperm sink” (Hight *et al*., 2005).

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Papathanos *et al.* (2009) outlined a few basic rules that should be considered when an optimal sex separation method is sought. These rules are listed as the "7 S's" (viz. small, simple, switchable, stable, stringent, sexy and sellable):

Small: Early separation to obtain males only during early development frees up resources, enabling the production of more males while reducing expenses.

Simple: Mass production processes are more robust when kept simple. A once off blanket treatment that effectively separates sexes such as heat or chemical exposures would be more robust compared to methods involving individual sorting, inter-species/strain crossing or the lengthy use of unstable chemical treatments.

Switchable: Within the production process, non-separation is also needed to maintain an adequate size brood-stock colony for maintained production.

Stable: To ensure reliability and predictability over time, the laboratory reared colony must be stable and not result in the accumulation and high frequency occurrence of undesirable, exceptional individuals that are incompatible with the sex separation process, as well as the whole mass rearing process.

Stringent: The amount of female contamination after sex separation must be minimized. For disease vectors e.g. *Anopheles arabiensis* Patton (Diptera: Culicidae) this is critical as 0.05% female contamination during a 100:1 over flooding ratio release will result in a 100% increase of females in the field. For *Eldana saccharina* Walker (Lepidoptera: Pyralidae), female contamination will be less severe, but when released with males when treated at sub-sterilising doses for females (≤150Gy) (Mudavanhu *et al.*, 2011), mating with wild males and further unwanted infestation will result.

Sexy: Males bound for release must still be desirable to wild females. The production and sex separation process must not negatively affect male field performance. A simple and robust quality control method is needed to quantify/indicate the effect of sex separation and other handling processes on male field performance.

Sellable: This specifically applies to transgenic methods of sex separation, which has not yet been investigated for *E. saccharina*. Political and public support may vary depending on the seriousness of the threat posed by a specific pest/vector as well as public perception relating to transgenic technologies. When transgenic methods for obtaining males only is investigated, the mode of action and application of this technology must be well communicated with sugar industry members, and the general public to avoid unwanted stigma.

Sex separation techniques can be grouped into two main categories viz. biological and genetic methods (Papathanos *et al.*, 2009). Genetic methods include the development of genetic sexing strains, sex-specific repressible lethality and fluorescent markers and transgenic methods. Marec *et al.* (2005) proposed genetic sexing strain development for Lepidoptera by taking advantage of Lepidoptera's sex chromosomes, with most lepidopteran species female's having heterogametic sex in the form of a WZ sex chromosome pair and with males being ZZ. Thus, if a conditional lethal gene can be inserted into the female W chromosome, then all females should die after the application of the restrictive condition associated to the lethal gene. Biological methods

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include sexual dimorphism at various life stages such as weight/volume differences; visual differences such as pupal shape and 5th larval instar coloration (*Lobesia botrana*, Denis and Schiffermüller (Lepidoptera: Tortricidae)); pupal colour (*Ceratitis capitata* Wiedemann (Tephritidae; Diptera)); protandry (difference in developmental time); and behavioural differences at various life stages, such as male swarming.

For the SIT, separation must be done on a large scale and hand separation will not be possible (Krishnamurthy *et al.*, 1962). Steinitz *et al.* (2016) investigated methods to separate male and female *L. botrana*. These were: (i) the number of abdominal segments of the pupae, (ii) the colours of wandering larvae, (iii) protandry, and (iv) the lengths of the respective pupae. Although the numbers of abdominal segments were the most reliable separation method, it could not practically be automated for large scale use. Both colour variation and protandry could significantly separate males but overlapping caused unwanted loss of males as well as unwanted female contamination for collected males. Male *L. botrana* pupa were significantly shorter than females and 86% of males could be collected when limiting the maximum collection size to 5.4 mm. Furthermore, 100% females for production purposes could be obtained by limiting the minimum collection size to above 6.2 mm. Because *E. saccharina* spin themselves into cocoons during pupation (Graham and Conlong, 1988), separation up until eclosion will be more challenging compared to *L. botrana* and dipteran pests that display sexual dimorphism during their pupal stage, which is not within a cocoon (Philippos, 2009). There have been unsuccessful attempts at breeding an *E. saccharina* colony that produce only naked pupa for easy separation (Conlong, personal communication, 28 June 2016). Separation of *E. saccharina* at the adult stage would therefore currently be the most practical method for large scale sex separation. Because male *E. saccharina* start lekking and mating shortly after eclosion (Atkinson, 1981), male swarming (lekking) and other behavioural differences at the adult stage would not be plausible for sex separation, as virgin adults are sought for better field competitiveness for wild mates. A sex separation method based on volume or weight differences which is integrated into the rapid collection process is identified as the best area for currently investigating separation of newly emerged *E. saccharina* adults. Furthermore, obtaining a male only colony by using classical genetic or transgenic methods could be highly effective but requires intensive research and development to establish, i.e. the development of a genetic sexing strain (GSS) (Curtis, 1979).

Walton (2011) found that *E. saccharina* males and females treated at 100 Gy irradiation and paired for mating resulted in a 100% male progeny and a survival percentage like that of the untreated control. If adult *E. saccharina* pairs produce 100% male offspring whilst maintaining adequate fecundity and fertility for large scale production when treated with a specific irradiation dosage, adults could possibly be collected prior to the onset of mating and exposed to an established level of irradiation to result in a 100% male colony that is semi-sterile and suitable for SIT release. If this hypothetical strategy is found plausible, irradiation of these F1 males prior to release can be

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excluded, shortening the handling and transport process. Predominantly, the exclusion of females will improve overall production efficacy as discussed above.

Another possible option for obtaining *E. saccharina* males only for irradiation is by developing a collection method that would separate adult sexes based on a possible difference in the average weight/volume between males and females. Although females will be present during production, separation of pupa or adults by weight would still provide benefits post collection. Except for the increase in males/volume that would allow for larger numbers of males per shipment, males bound for irradiation and release will also not stand the chance of mating with accompanying females during post-collection and post-release stages. More females will also be available to supplement production (Papathanos *et al*., 2009; Steinitz *et al*., 2016)).

It is optimal to collect adult moths as soon as possible after emergence (Boersma and Carpenter, 2016). This reduces the risk of physical damage and prevents newly emerged adults from mating prior to irradiation. For this purpose, a plenum type collection system in which pupated larvae are placed and eclosed adults collected, is used at XSIT Pty (Ltd) at Citrusdal, South Africa and the El Paso/Trans Pecos Pink Bollworm Cooperative Eradication Program in the USA (Hofmeyr and Pretorius, 2010).

In this chapter the possibility of obtaining males from the adults produced in the SASRI mass rearing facility only for release was investigated. Firstly, the effect of parental (P1) (male and female) irradiation levels on the first-generation offspring (F1) sex ratio was investigated. Obtaining adequate numbers of 100% semi-sterile F1 males by exposing parents to low levels of irradiation will not only provide a method of obtaining males only, but also exclude the irradiation step in the handling and transport process due to inherited sterility (IS). Secondly, the difference in male and female weights was investigated as a possible trait to be exploited in a large-scale sex separation method, as female pupae of *E. saccharina* are close to twice the mass of male pupae (Ngomane *et al*., 2017), and it thus stands to reason that the female moths will be heavier than the male moths after eclosion.

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2.2. MATERIALS AND METHODS

F1 sex ratio of irradiated *Eldana saccharina* adult pairs

Adult *E. saccharina* males and females were treated at low irradiation levels (0 Gy; 50 Gy; 100 Gy) and paired for mating. The resulting offpring were reared through to adults, and those emerging were sexed, to determine F1 sex ratios. Surviving adult F1 sex ratio results, as well as total numbers of adult F1 offspring per treatment could then be compared between different treatments, as described below.

A total of 3000 pupae obtained from the routine *E. saccharina* mother colony (Walton and Conlong, 2016) were packed singly per cell (to ensure eclosed moths stay virgin) in plastic multicell trays (4 x 8 cells/tray) at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal. Pupa were still contained within cocoons which provided protection for them against shaking inside cells during transport. The 100 trays containing the pupae were wrapped using perforated clingwrap (Handywrap®) to seal the cells and prevent the pupae from falling out, before being packaged in boxes with shredded newspaper around them as a buffer, and couriered to Department of Conservation Ecology and Entomology Stellenbosch University, Western Cape for adult irradiation. On arrival, pupae were placed in the departmental insectary and kept at ~24 °C with a 12h:12h Dark:Light (D:L) photoperiod. When more than 30 less than one-day-old adult males and females were observed to have eclosed, they were collected from their individual cells. Freshly emerged males and females were kept separately in 2L containers to prevent mating. The 2L containers each contained a 30cm section of folded paper towel for extra surface for moths to perch on. Ten individuals were kept per container in order to conduct three irradiation treatments. Treatments were 0 Gy (control treatment), 50 Gy and 100 Gy, and were conducted at the ARC-Infruitec/Nietvoorbij (FruitFly Africa), Stellenbosch, Western Cape irradiator. After exposure to the respective ionizing irradiation levels, 10 pairs of males and females per treatment were individually packaged in 500 mL paper cups, each containing a pleated yellow cardboard oviposition substrate (50x10 mm when pleated five times), secured at one end with a paperclip to maintain the pleats, and a 10mm dental cotton roll (supplied by Shanghai Ristea Industries Co,. Ltd.) soaked with distilled water to provide moisture. Oviposition substrates were replaced daily for five consecutive days (Walton and Conlong, 2016). Upon removal, oviposition substrates were placed in marked, sealable plastic bags and sent back to SASRI, where upon arrival, eggs were stored in an incubator (24 ± 2 °C; 75 ± 5% RH; 0h:24h D:L photoperiod) for 5 days as being done during routine mass rearing (Way, 1995). On a sterilized laminar flow bench to prevent unwanted infection of neonate larvae as well as cross contamination between treatments, hatched neonate larvae were placed using a fine bristled paint brush in marked clear plastic 25mL vials containing ~8 mL diet and sealed with a mesh lid to provide aeration (2 larvae/vial). Between inoculation of each treatment the laminar flow bench and room were thoroughly disinfected using Dismed D-Germ (0.5% Chlorhexidine Gluconate in 70% Alcohol) and paper towel sheets to avoid cross

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contamination. Thus, extra caution was taken as a result of previous contamination challenges experienced by Walton (2011). A maximum of 30 vials per paper substrate were inoculated (i.e. 60 neonate larvae: 2 per vial). Both the 50 Gy and 100 Gy treatments resulted in less than 60 neonate larval offspring per replicate and thus less than 30 vials were inoculated. Inoculated vials were placed in separate rooms to avoid cross contamination. The three separate rooms shared the same temperature control (~27°C) and 12h:12h D:L photoperiod. At the onset of adult emergence, vials were inspected every two days to collect emerged adults and determine sex. After emergence stopped completely, the F1 sex ratio for 0 Gy, 50 Gy and 100 Gy treated pairs could be calculated. The total F1 progeny of each treatment also gave a rough indication of parental fecundity as, on average, less than 60 larvae were inoculated per replicate for the 50 Gy and 100Gy treatments versus more than 60 larvae observed for each of the control treatment replicates. The viability of using low irradiation levels to induce a male-only colony for SIT could then be investigated.

Statistical analyses: To determine if a relationship existed between the different treatments and adult sex ratio, a chi-squared test ($\chi 2$ test) was conducted in Statistical Package for Social Sciences (SPSS) version 20. The null hypothesis (H_0) states that there is no correlation between irradiation dosage and F1 sex ratio; the alternative hypothesis (H_1) states that there is a correlation between irradiation dosage and F1 sex ratio. Lastly, a likelihood ratio test was done to compare the goodness of fit between H_0 and H_1. The likelihood ratio (LR statistic) was used to determine a p-value to confirm the hypothesis accepted under the chi-square test. A two-way contingency table with corresponding chi-square statistics showing the relationship between the parental (P1) irradiation dosage and the resulting first generation (F1) sex ratios was used to display the results. The total numbers of male and female F1 adults from each P1 treatment could further be compared to determine the combined effect of P1 fertility and F1 survival to adulthood of the F1 generation.

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Difference in *Eldana saccharina* pupal male and female weights as possible morphological trait for sex separation

Naked male and female pupa (removed from cocoons by hand) reared on both conventional (Walton and Conlong, 2016) and a newly developed rabbit pellet diet (Ngomane *et al.*, 2017) were sexed, individually weighed using an analytical balance (Mettler Toledo New Classic MF Model Ml54) to an accuracy of 0.0001g and then placed in multicell trays at SASRI. Emerged adults were removed daily and frozen to immobilize them. They were then removed from the freezer and individually weighed using the same analytical balance to an accuracy of 0.0001g. Due to condensation on the thawing moths after removal from the freezer, adult weights were discarded and the mean difference between 30 male and 30 female pupal weights for the conventional diet treatment and 60 male and 60 female pupal weights for the rabbit pellet diet treatment (i.e. conventional and rabbit pellet diets) were compared. A larger sample size was measured for the rabbit pellet diet and the whole sample was included for statistical comparison with the smaller conventionally reared sample size.

These weight differences between male and female pupa give an indication of the difference in male and female adult *E. saccharina* weights, which if as distinct as the pupal weights are (Ngomane *et al.*, 2017), could be further investigated as a possible sex separation trait.

Statistical analyses: Factorial ANOVA was performed in SPSS version 20., with weight being the dependent variable and treatment and sex being the independent variables. An LSD *post hoc* test was used to separate means. Homogeneity of variances was tested using Levene's test, and where data were not normally distributed, a bootstrap multiple comparison was done (Cahoy, 2010).

Using Microsoft Excel 2016, the hypothetical percentage females collected for the rabbit pellet and conventional diet was determined when setting a maximum weight limit 0.001g above that of the heaviest male individual of the two samples of 0.0850g (This would thus ensure that 100% of males would be collected with a possible degree of female contamination).

If male and Female *E. saccharina* travel different distances after exiting the moth collection pipe of the plenum type moth collection device, due to weight and size differences, this occurrence could be exploited to obtain a significant degree of sex separation. To calculate if male and female *E. saccharina* travel different distances during collection with the prototype collection box, both male and female speed was assumed to be 10 m/s (the same speed as the airstream) at the time of exiting the collection pipe.

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The effect of both gravity and drag on the deceleration of the moth for every 0.1 m/s after leaving the collection pipe was calculated up until the moth has fallen for 1.5 m where it touches down on the bottom of the collection tray. The formula for force exerted on the moth by drag is given as:

$F_D = 1/2\rho v^2 C_D A$

Where:

- F_D : The total force exerted on the moth
- ρ : The density of the air (assumed to be 1.204 kg/m^3)
- v : The speed of the moth relative to the air
- C_D : The coefficient of drag (0.85)
- A : The area of the moth on which the air is exerting drag

The gravitational force is given as $F = mg$, with $g = 9.81$

Newtons law of acceleration was used to further calculate the deceleration of the moth with the following formula:

$a = F_D M$

- a = acceleration of the moth
- F_D = The total force exerted on the moth
- M = The mass of the moth (using the average male weight of 0.0700g the average female weight of 0.1332g)

Using Matplotlib v 3.3.3 (www.matplotlib.org, 2020), a simulation of the male and female flight paths was run for a male moth (using the average male weight of 0.0700g) and Female moth (using the average weight of 0.1332g). The script that was used is shown in Appendix 1.

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Testing efficiency of collection box

Overview

A collection box based on the design used at XSIT Pty (Ltd) (Hofmeyr and Pretorius, 2010), was constructed (Figure 2.1) and modified to cater for the biology of *E. saccharina*. The dimensions of the collection box are shown in Figures 2.2 and 2.3. and the mechanisms of the box illustrated in Figure 2.4. During full usage, 4 L plastic pupal trays containing 2 L diet and pupated *E. saccharina* in the diet, were placed on 1 mm rods that served as shelves in a sealed steel box (10 x 10 mm stainless steel square tube covered with 2 mm sheet metal) with two funnels (Figure 2.3) (made from 2 mm sheet metal and crevices where moths can hide were sealed with foam and silicon) in the bottom. It was designed so that eclosed moths could move out of their pupal trays and drop to the collection funnel at the bottom of the sealed box. These vertical collection funnels led into a horizontal moth collection pipe (6.5 cm PVC) in which an axial flow induction fan blew an airstream (10 m/s) to move moths through the pipe toward a cold room for immobilization. Moths were blown along this pipe through a calibrated plenum breaking chamber (3 mm steel sheet around a 10 x 10 mm stainless steel square tube frame with the face sealed with removable transparent Perspex in order to better observe), where air movement exiting from the moth collection pipe was drastically slowed by being drawn off by an exhaust fan. Due to their momentum, moths move through the plenum into the extension moth collection pipe and exit this pipe into a cold chamber set at 5 °C. The near halting of airflow in the calibrated plenum prevents any air from penetrating the cold room, lowering the occurrence of condensation and temperature fluctuations in the cold room (Hofmeyr and Pretorius, 2010). Moths also decelerated when they moved through the plenum and the risk of physical damage when exiting into the cold room was thus lowered. In the 5 °C cold room, moths were collected in an open container. The 5 °C temperature immobilises adult *E. saccharina* just above their CT_{min} of 4.2 °C (Mudavanhu *et al.*, 2011). After chilling, *E. saccharina*, as with *T. leucotreta*, must be packaged for irradiation and transport whilst kept at a constant 5 °C chilling temperature to avoid increased activity, that results in physical and physiological damage. This cold chain is the subject of the further investigations described in this thesis.

XSIT Pty (Ltd) determined that *T. leucotreta* adults exiting the collection pipe into the collection and cold chamber at speeds above 12 m/s experienced physical damage (Boersma and Carpenter, 2016). The custom designed collection box for *E. saccharina* had three different speeds at which the fan could be set. Air flow at the lowest setting (I) was 10 m/s. This airspeed was used in the trial as it was, based on the work done by Boersma and Carpenter (2016), assumed it would cause the least damage to *E. saccharina* adult moths.

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Figure 2.1: Photograph of the assembled collection box. The adult emergence box (iii) lid (ii) is open to display the racks (iii) on which trays containing pupa are placed as well as two vertical square stainless steel funnels (iv) leading into the transporting pipe below (v) which has an airstream (10 m/s) supplied by the axial flow induction fan (i). The airstream flows through a plenum box system (vi) which halts airflow where moths exit via the exit pipe (vii)

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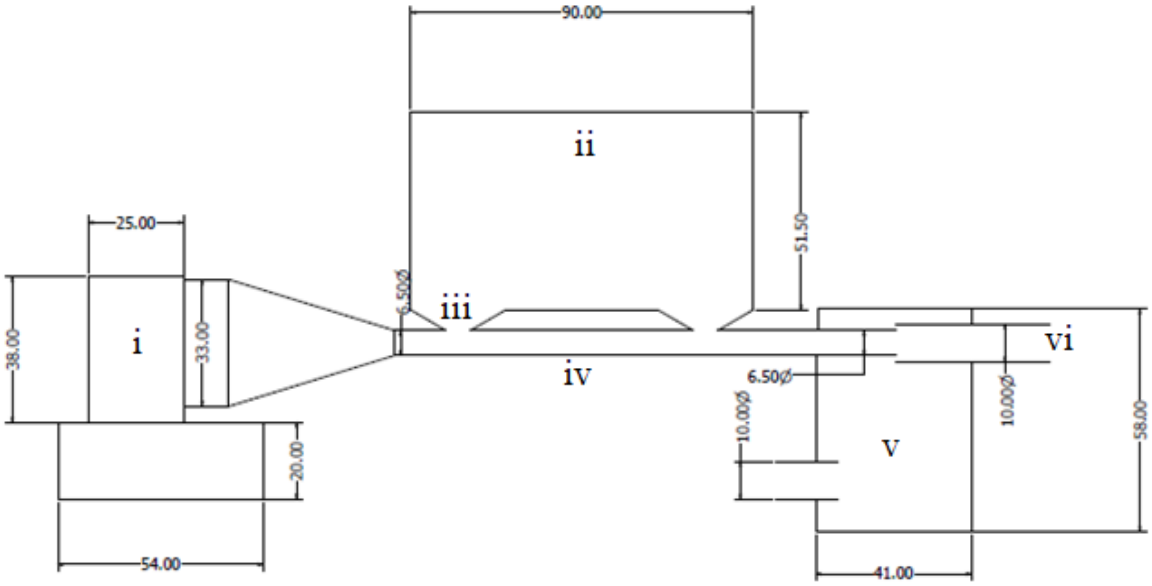

Figure 2.2: Measurements of the collection system prototype for newly eclosed *Eldana saccharina* adults. The axial flow induction fan (i) is connected to the collection pipe (iv) into which the two vertical square stainless-steel funnels (iii) lead from within the emergence box (ii). The airstream from the fan blows moths into the plenum system (v) where air is halted, but moths proceed through the exit pipe (vi) into the cold chamber. Note that the collection box (ii) is 46 cm wide and the plenum (v) 35.5 cm wide - This is not presented on the figure.

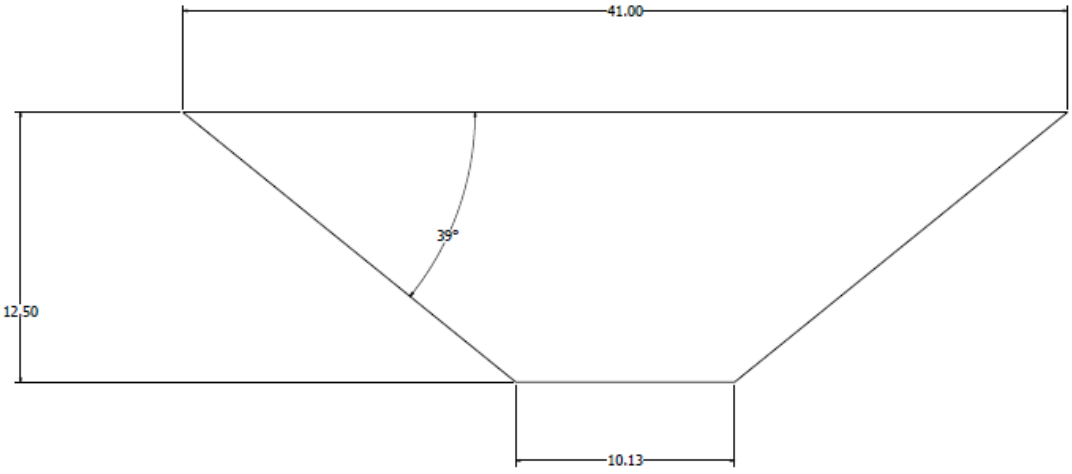

Figure 2.3: Dimensions of the vertical square stainless-steel funnels (two) at the bottom of the collection box that lead into the 6.5cm collection pipe with a 10 m/s airstream provided by an axial flow induction fan.

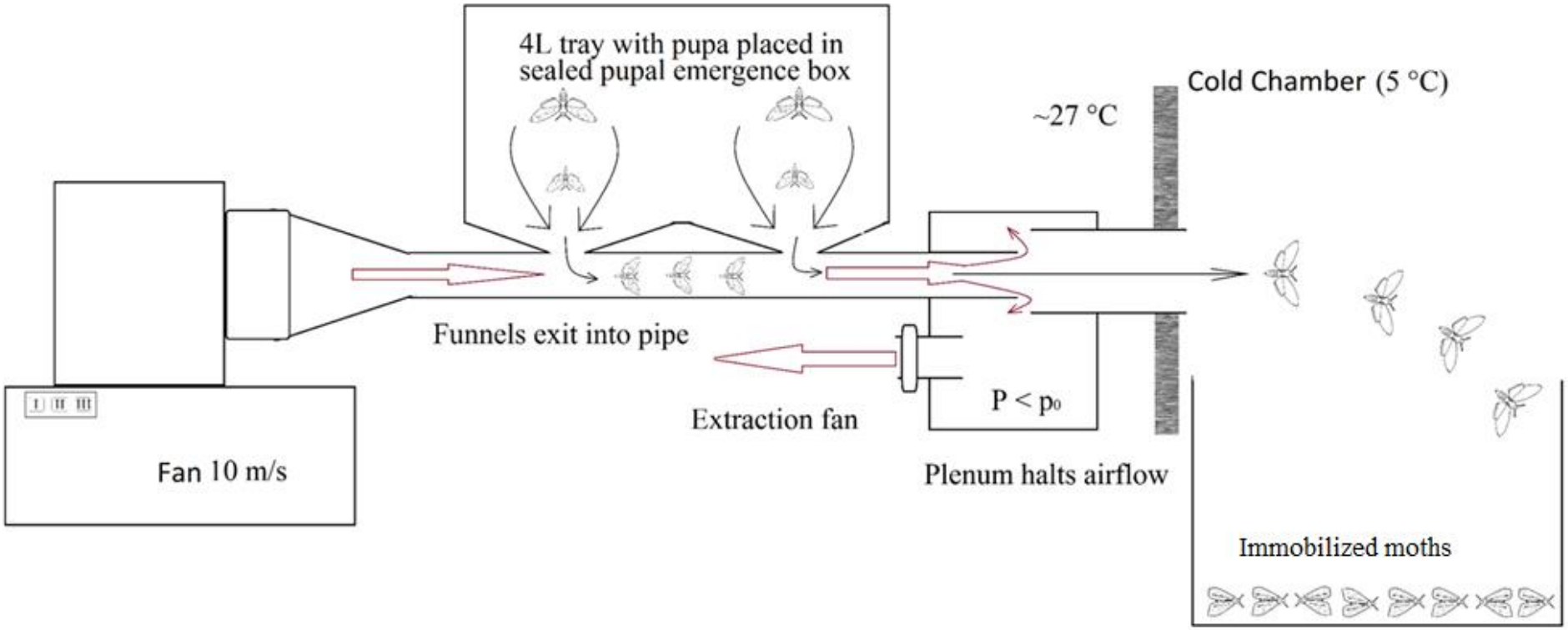

Figure 2.4: A diagrammatical representation of the custom-made collection box for *Eldana saccharina* consisting of a axial flow induction fan providing an airstream (10 m/s) into the collection pipe which is connected to two vertical square stainless steel funnels leading down from the pupal emergence box into the collection pipe. The collection pipe leads into a plenum that halts airflow and allows decelerating moths to move into a cold chamber set at 5 °C. Thick (red) arrows signify airflow through the system, whilst thin arrows signify the movement of eclosed moths through the system.

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Experimental design

For each of the three replicates conducted for this experiment, an empty 4L plastic tray with 200 (1:1 sex ratio) less than one-day-old *E. saccharina* adults were placed in the sealed pupal emergence box at 6pm. These adults were obtained from emergence boxes during conventional daily collection of adults for the mother colony (Ngomane *et al.,* 2017). Because this prototype was not installed in a cold room where moths could be immobilized for packaging, mating could take place. To get an indication of the need to collect adults as soon as possible after eclosion, females that were collected daily during conventional production from eclosion boxes, to be transferred to mating and oviposition boxes, were sampled to inspect for mating status. These females eclosed within 24 hours prior to being sampled. The temperature in the room was set at ~25 °C and the room was kept dark. The light was only kept on for the time of collection and replacement of the collection bag. Testing was conducted overnight due to the nocturnal nature of *E. saccharina* adults (Atkinson, 1981). The cotton bag at the end of the collection pipe collected the emerged moths. This bag was inspected every 2 hours for the number and sex of collected moths. Cumulative two hourly male and female collection percentage was calculated from this. The last count was conducted after 12 hours at 6am the next morning. This experiment was repeated three times on three separate consecutive days (every second day) in order to obtain an accurate average.

Statistical analyses: The number of males and females collected over cumulative time intervals were compared using Least Square means for each 120-minute time interval ranging from 0 to 720 minutes. The analysis was conducted using Statistica version 13 (StatSoft, Inc. 1984-2017).

Mating status of adult female *Eldana saccharina* at conventional daily collection at the SASRI insect rearing facilities

During current rearing of *E. saccharina* at the SASRI insect rearing facilities, pupae were collected and placed in sealed but ventilated plastic boxes called emergence boxes (29 cm x 29 cm x 135 cm). Emerged adults were collected daily by hand and placed in oviposition boxes (also 29 cm x 29 cm x 135 cm plastic container) where mating and egg oviposition onto paper towel sheets took place. The mating status of moths collected from these emergence boxes was unknown as they were by the time of daily collection less than one day old from eclosion and were given further time to mate and lay eggs in oviposition boxes to which they were transferred. Because conventionally collected moths were used to test the current automated collection box, the mating status of these moths must be known by dissection to determine mating status by inspecting for the presence or absence of one or more spermatophores in the bursa copulatrix as described by Walton (2011). If a high degree of these moths were observed to be virgins after collection, the occurrence of mating within the automated collection system could be investigated.

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To determine adult mating status shortly after emergence during conventional production procedures of *E. saccharina* at the SASRI insect rearing facilities (Graham, 1990), 50 newly eclosed females were sampled during daily adult collection from emergence boxes. These sampled females were placed in marked 2 L plastic containers sealed with a steel mesh lid and killed by freezing before being dissected with the aid of a dissection microscope to determine mating status by inspecting for the presence or absence of one or more spermatophores in the bursa copulatrix (Walton, 2011). The percentage females mated for each day was calculated by taking the number of females mated (as determined by the presence of a spermatophore) out of the 50 sampled daily and multiplying it by two.

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2.3. RESULTS

F1 sex ratio of irradiated *Eldana saccharina* adult pairs

The Chi square test showed that the F1 sex ratio was dependant on the parental (P1) irradiation dosage ($\chi2 = 8.691$, df = 2, $\chi2/df = 4.35$, $P(\chi2 > 8.691) = 0.0130$), therefore the null hypothesis (H_0 = F1 sex ratios are not dependant on parental irradiation dosages) was rejected (Table 2.1). The likelihood ratio test (LR statistic = 9.617; P = 0.008) further supported the rejection of H_0.

The 0 Gy and 50 Gy treated pairs produced 52.87 % (276 F1 males out of 522 total offspring) and 52.98 % (178 F1 males out of 336 total offspring) adult male offspring, respectively, and the 100 Gy treated pairs produced 83.33 % (20 F1 males out of 24 total offspring) male offspring. As irradiation doses increased on the parental adults, fewer F1 adults were produced. (ie from 522 individuals that reached the adult stage for the 0 Gy treated pairs, to 336 individuals for the 50 Gy treated pairs, to only 24 F1 adult individuals at the 100 Gy treated pairs.

Table 2.1.: Contingency table of the dependence between parental irradiation dosages' (0 Gy; 50 Gy and 100 Gy) effect on the sex ratio of the surviving adult F1 *Eldana saccharina* generation ($p < 0.01$). True values are displayed in **bold,** expected values are displayed in *italics*. Individual $\chi2$ values are displayed in parentheses.

	P1 Radiation F1 sex ratios		
	Number of Males	Number of Females	TOTAL
0 Gy	**276** *280.53* ($\chi2 = 0.07$)	**246** *241.47* ($\chi2 = 0.09$)	**522**
50 Gy	**178** *180.57* ($\chi2 = 0.04$)	**158** *155.43* ($\chi2 = 0.04$)	**336**
100 Gy	**20** *12.9* ($\chi2 = 3.91$)	**4** *11.1* ($\chi2 = 4.54$)	**24**
TOTAL	**474**	**408**	**882**

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Difference in *Eldana saccharina* pupal male and female weights as possible morphological trait for sex separation

An overview of the factors used in the analysis of this study are given in Table 2.2.

Table 2.2.: Between-Subject Factors

			N
Gender	1	Male	90
	2	Female	90
Diet	1	Rabbit Pellet	120
	2	Conventional	60

Overall, mean weights were significantly different between male and female *E. saccharina* pupae ($F_{(1, 118)}$=268.55, $p < 0.01$) (Figure 2.5). The average male pupal weight from the conventional diet was 0.0700 g and an average female pupal weight was 0.1332 g. There was a more pronounced difference in weight with the experimental rabbit pellet diet with males weighing on average 0.0589 g and females on average 0.1427 g (Figure 2.5).

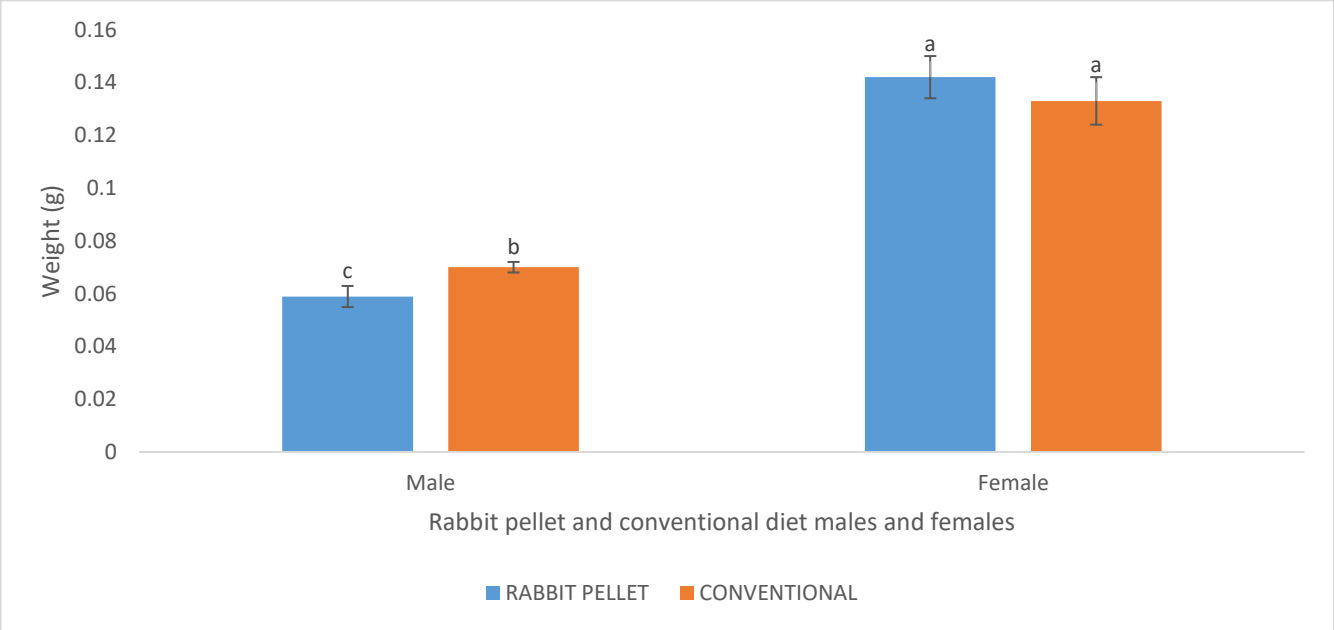

Figure 2.5.: Bootstrap mean weights of 30 pupal *Eldana saccharina* males and 30 females reared on conventional diet and 60 *E. saccharina* males and 60 females reared on rabbit pellet diet. Error bars denote 95% confidence intervals with significant difference indicated with letters. Same letters indicate no significant differences, different letters indicate significant differences. Note that a larger sample of rabbit pellet diet pupa was measured in this analysis for comparison of means

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The diet treatment used had no significant effect on pupal weights ($F_{(1, 176)} = 0.050$; $P = 0.823$). Female *E. saccharina* reared on rabbit pellet and conventional diets were significantly heavier than their male counterparts proving that sex had a statistically significant effect on pupal weights ($F_{(1, 176)} = 407.26$; $P < 0.001$). The interaction of diet and sex had a statistically significant effect on pupal weights with male *E. saccharina* reared on the conventional diet being significantly heavier than males reared on the rabbit pellet diet ($F_{(1,176)} = 7.97$; $p = 0.005$).

The total R Squared for this model is $R^2 = 0.743$, meaning that the combination of these variables accounted for 74.3% of the variance (or 73.9% if adjusted for the bias).

To demonstrate that pupal *E. saccharina* weight (and therefore probably adult weight) could be linked to pure one sex collections, all pupae weighing below 0.0850g were collected from the rabbit pellet and conventional diet treatments and sexed to determine if 100 % male collection for both diet treatments was attained. There were 3.33 % of the female pupa collected from the rabbit pellet diet and 0 % of the female pupa from the conventional diet, whilst 100 % of the males were collected for each treatment (Figure 2.6).

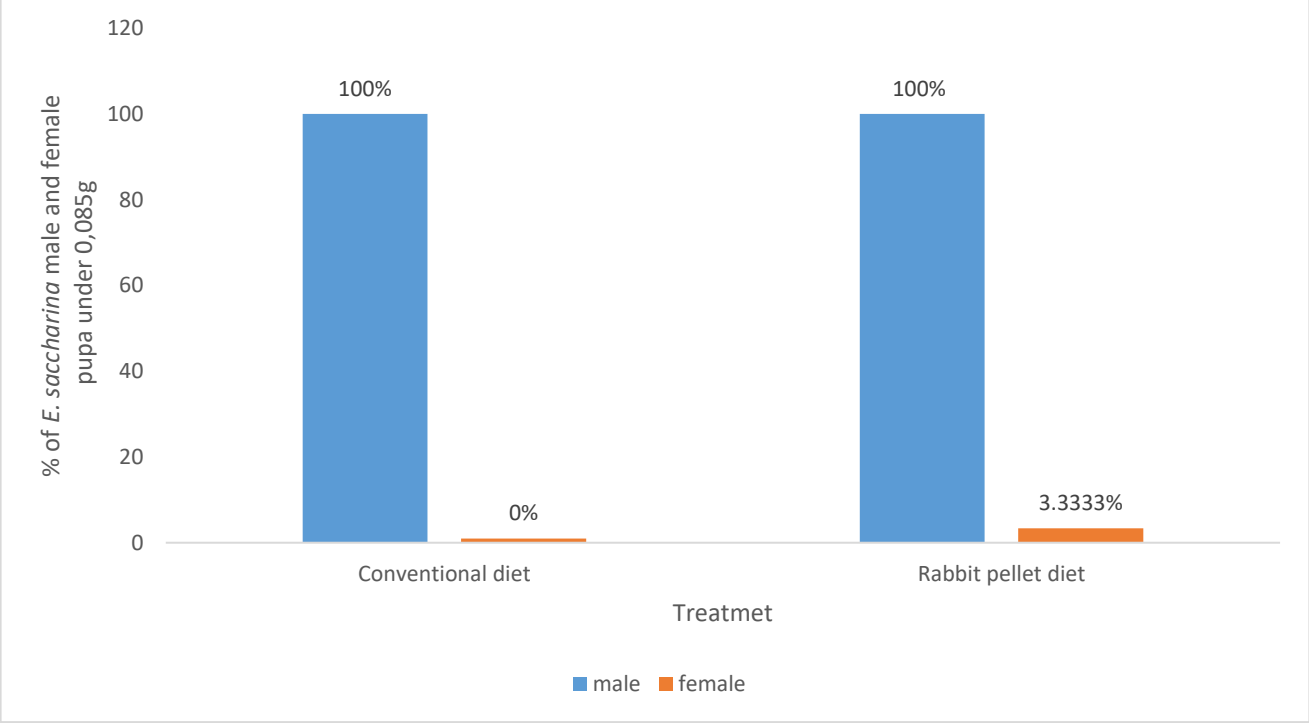

Figure 2.6: Respective percentage of total male and total female *Eldana saccharina* pupa that weighed below 0.0850g for conventional and rabbit pellet diets.

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When *E. saccharina* male and female pupa reared on both conventional and rabbit pellet diets were presented on a frequency distribution histogram by weight, the low level of overlapping is apparent (Figures 2.7, 2.8). For the Conventional diet, the discrimination of weight was highly significant (Wilks' lambda = 0,24876, $F_{(1, 58)} = 175.15$; $p<0.001$).

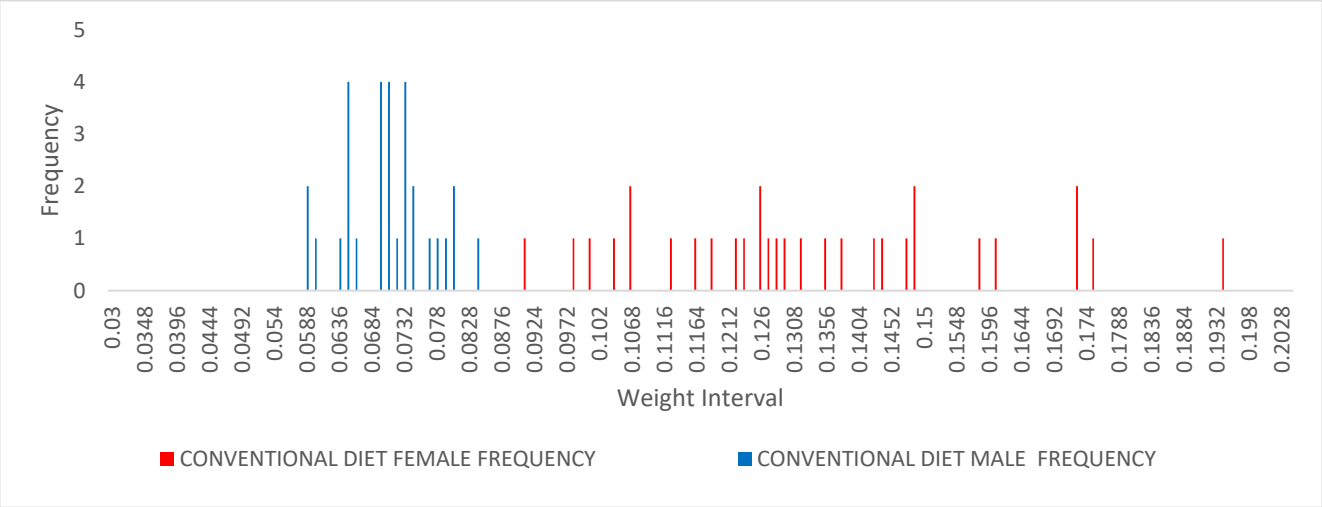

Figure 2.7: Frequency distribution histogram of the weights (g) of 30 male and 30 female pupal *Eldana saccharina* reared on the conventional diet.

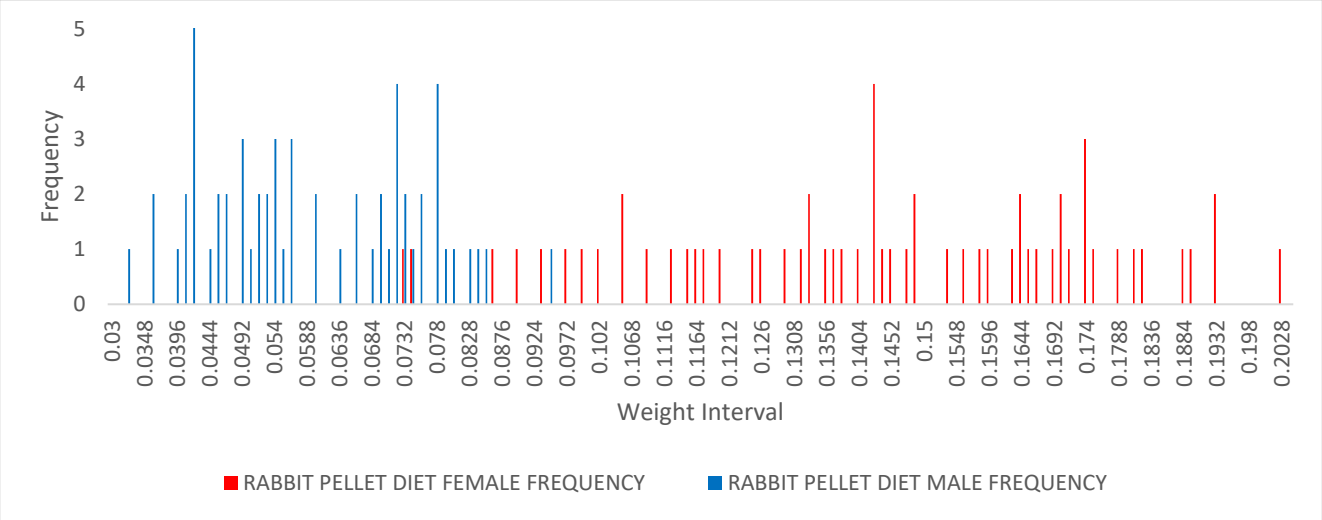

Figure 2.8: Frequency distribution histogram of the weights (g) of 60 male and 60 female pupal *Eldana saccharina* reared on rabbit pellet diet.

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In the simulation of airspeed vs adult weight, for the average weight *E. saccharina* male adults (0.0700g) and female adults (0.1332), the acceleration simulation showed for the same surface area (5 cm^2) for both males and females, that males travel 102cm away from the exit pipe when exiting the pipe at 10 m/s, versus females that travel 66cm further (168 cm) (Figure 2.9).

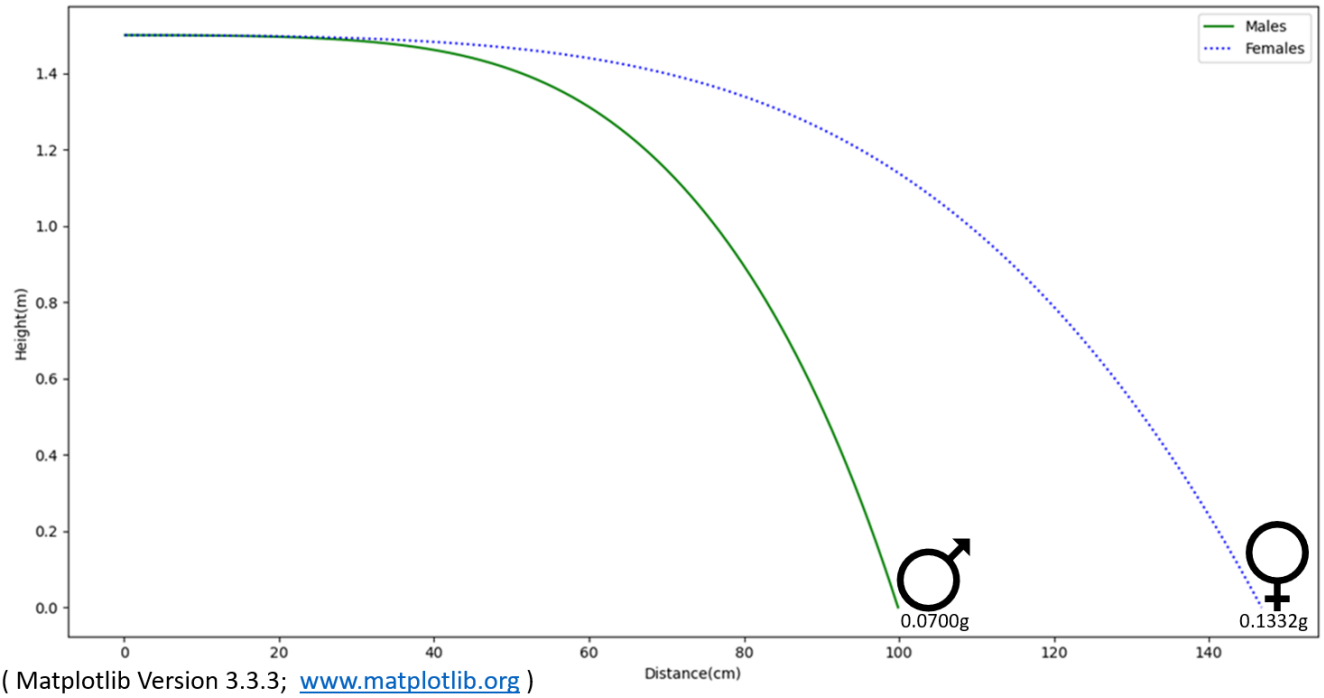

Figure 2.9. Calculated travel path for every 0.1 ms for average weighted male (0.0700 g) and female (0.1332 g) *E. saccharina* with the same surface area (5 cm^2). Moths exit the collection box pipe at 10 m/s at a height of 1.5m from the ground (Y(m)) – Horizontal travel distance are calculated by factoring in drag, gravity, male/female mass (Newtons law of acceleration: $a = F_D M$)

Efficiency of collection box

There was no significant difference at each 120-minute time interval between male and female collections over the 12 hour collection period (Figure 2.10: $F_{(6,28)} = 0.34189$, P=0.90862). A significant increase in total adult collection percentage was observed over time (Figure 2.10): ($F_{(6,28)} = 174.36$; P<0.01).

At the 720 minute collection period, average male collection percentage was significantly higher (average of 86 males collected out of 100) versus female collection percentage (average of 75 females collected out of 100) (Figure 2.10: $F_{(1,28)}$=16.860, P<0.01).

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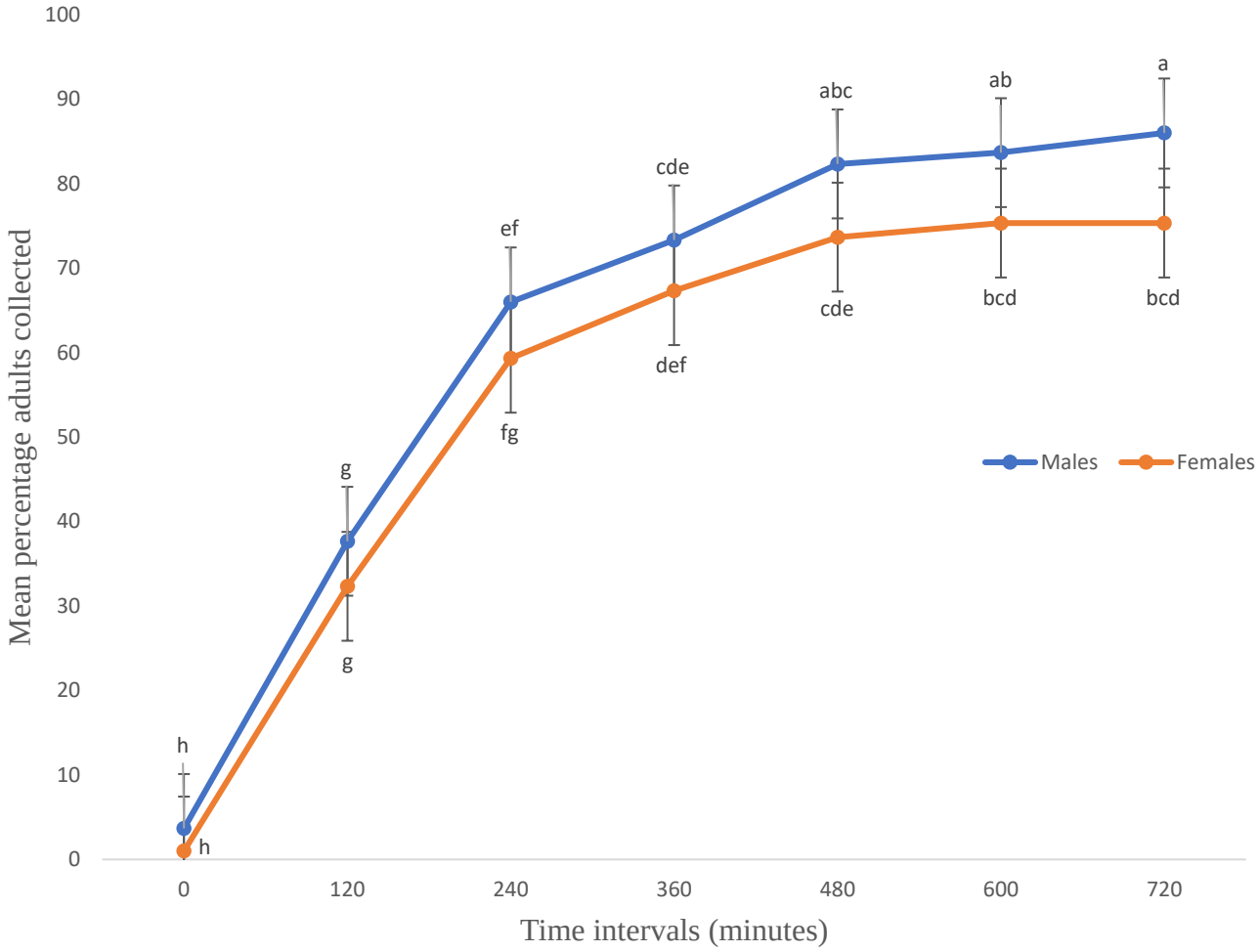

Figure 2.10: Cumulative mean counts of male and female adult *Eldana saccharina* collected over 720 minutes (12 hours) in 120-minute intervals starting at 0 minutes. Error bars denote 95% confidence intervals. Different letters indicate significant difference between data points.

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Mating status of adult female *Eldana saccharina* during conventional daily collection

During the conventional daily collection (SASRI rearing facility) of emerged adults from pupal trays for placement in oviposition trays, the average percentage of the 350 females collected over seven days that had already mated, was 98% (Figure 2.11).

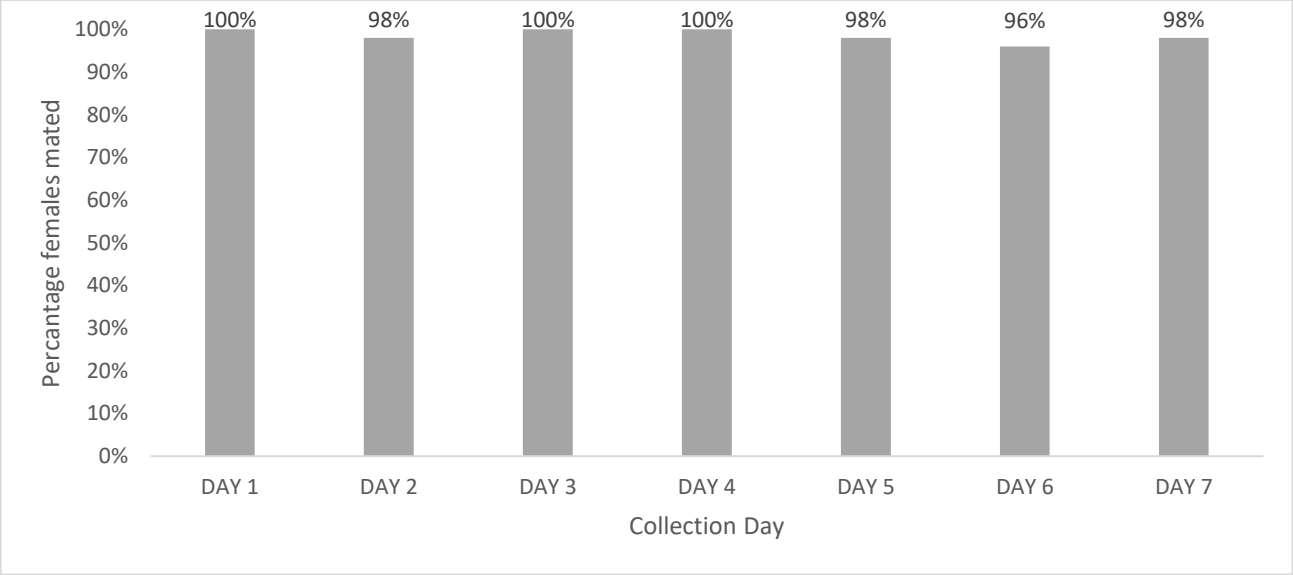

Figure 2.11: Percentage (n=50) of female *Eldana saccharina* adults already mated during daily collections from pupal emergence boxes over seven days.

2.4. DISCUSSION

F1 sex ratio of irradiated *E. saccharina* adult pairs

This study showed that although an increase in *E. saccharina* parental (P1) irradiation dose resulted in a male bias for the surviving first Generation (F1) adults, the highest dose used (100 Gy) did not result in a male-only F1 adult generation, as observed by Walton (2011). Out of the 24 surviving F1 offspring from the five 100 Gy treated *E. saccharina* pairs, 20 (83.3%) were male. P1 irradiation of both sexes at 100 Gy to obtain a 100% male, semi-sterile F1 colony for release was thus not achieved. An 80% plus male biased F1 progeny is nonetheless promising. Further investigation of F1 female sterility and F2 fecundity from F1 males are needed if the above-mentioned proposed method is to be considered. The apparent impact of the P1 irradiation level on fecundity could decrease the attractiveness of exploring irradiation as a method of obtaining a male bias, semi-sterile, F1 colony. The direct cause of decreased F1 adult numbers at higher irradiation dosages could not be determined in this study. Increased

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irradiation of both parental pairs significantly reduced fecundity, fertility and % F1 survival to adulthood in the more detailed irradiation study done by Walton (2011).

Walton (2011) found that *E. saccharina* P1 male and female pairs treated with 100 Gy irradiation each before mating resulted in 100% male F1 offspring. Despite the low fertility and fecundity of this cross, which resulted only in one individual male F1 adult (Walton, 2011), this low-level irradiation of adult *E. saccharina* pairs prior to mating to obtain a male only F1 generation was nevertheless further investigated. The reason for this was that, if successful, it would provide males-only for use in the SIT. The correct P1 irradiation dosage could provide 100% F1 males with inherited partial sterility. These F1 males would have a simplified handling and transport process versus when sterile or semi-sterile irradiated P1 males are bound for release due to exclusion of the irradiation step for the F1 males. Furthermore, 100% male pupa could be obtained for transport up to release sites, further reducing the need for an intensive handling and transport system, which is needed to immobilize and preserve adult moth fitness (Nepgen, 2014)~~F1~~

Although P1 pairs treated at low irradiation dosages did not result in a male only F1 generation, the possibility of mating irradiated, semi-sterile P1 males with either irradiated or non-irradiated female counterparts to obtain semi sterile F1 adults for field release can further be investigated as an option of simplifying the handling and transport process of F1 adults or pupa. Not only will F1 insects be exposed to less stress prior to field release, but P1 pairs could be irradiated at an off-site irradiation facility and the resulting eggs, or pupae, transported back to the rearing facility for rearing and release (Conlong pers. comm., 2017). There is currently no irradiation facility close to the SASRI rearing facility, which is a large limiting factor for the development of the SIT for *E. saccharina* (Walton, 2011; Mudavanhu, 2012). The establishment of an *E. saccharina* broodstock colony at an irradiation facility could allow for the irradiation of P1 pairs. Resulting F1 eggs from mating of irradiated P1 males and unirradiated females could then be transported back to the SASRI rearing facility for hatching and semi-sterile offspring rearing. In this way, rearing of enough semi-sterile F1 *E. saccharina* adult males for implementation of a successful SIT program could exclude the need for an expensive irradiation facility.

Difference in *E. saccharina* pupal and adult male and female weights

Sex separation for an operational SIT program is a large scale process, and must thus be simple and automatable to be effective (Papathanos *et al.*, 2009). Furthermore, separation must be accurate so as not to lose males in the process and prevent female contamination (Papathanos *et al.*, 2009). Steinitz *et al.* (2016) concluded that pupal length of *L. botrana* could effectively be used to separate males and females when limiting pupal length to 5.4 mm when collecting, achieving collection of 86 % of males and 22 % of females (male biased sex ratio of 0.77) and was identified as a suitable sex separation method. This method was modified by using pupal mass rather than

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pupal length, for potentially separating naked pupae of *E. saccharina* . It was shown that all pupae under 0.085 g in weight, were only males.

Eldana saccharina can be accurately sexed by removing pupa from their cocoons and observing anatomical differences on the ventral surface of the last segment of the abdomen, using a stereo microscope (Atkinson, 1980). However, this method is labour intensive and not suited for large scale, automated separation for SIT. Because reared *E. saccharina* spin cocoons of varying sizes and weights within and on top of the feeding media, further separation during pupal stages using pupal weight or length is complicated.

Therefore, pupal male and female *E. saccharina* weights were recorded as an indicator of adult weights to investigate the possibility of using mass as a sex separation tool during the adult phase. For the SIT, an automated collection method should be used to collect newly emerged adults into a cold room which immobilises the moths without impacting on their fitness, for packaging, irradiation, and transport (Hofmeyr *et al*., 2010).

The large difference recorded between male and female *E. saccharina* pupal weights is promising for sex separation development, as there are many methods of gravity separation that can be investigated (Falconer, 2003). Gravity separation is a method of practically separating two components by exploiting their differing specific gravities (Falconer, 2003). Gravity separation is the literal separation of the "wheat from the chaff" which in its simplest form, involves throwing a mixture of grain or seeds and chaff into the air so that the wind blows away the lighter chaff, while the heavier grains fall back down for recovery. The lighter males can be separated in a similar manner, as the airstream of the adult collection device would have different effects on negative and positive acceleration for differing moth weights and wind resistance (Hofmeyr *et al*., 2010), as shown in the Matplotlib acceleration simulation (Matplotlib Version 3.3.3, 2020). An alternative method, also using gravity separation is a cyclone system. Reared adult *C. pomonella* (and *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) for SIT purposes are collected using such a cyclone system. Here, newly eclosed, heavier moths quickly lose speed after collection and the lighter, looser scales are separated within the cyclone device for moth quality and human health and safety reasons (Stewart, 1984; Wolf and Stimmann, 1971). For collection of *T. leucotreta* a similar cyclone system was initially used, but due to *T. leucotreta's* differing morphology, the system was not effective, primarily due to build-up of scales and fatty deposits in the cyclone system (Hofmeyr and Pretorius, 2010). A new system was engineered (Hofmeyr *et al*., 2010) that collected eclosed moths out of a sealed emergence box using funnels leading into a pipe with constant airflow provided by an axial flow induction fan, as described in the Materials and Methods section of this Chapter. This system causes minimal damage to the moths, as from entering the collection pipe, there are no bends in the pipe.

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It is during the collection process with either a plenum or cyclone collection system that a prominent difference in male and female weights could be exploited. With 100 % males being collected and no female contamination for the conventionally reared *E. saccharina* under 0.0850 g (Male biased sex ratio of 1). An engineered system that could exploit this weight difference on a larger scale could provide sex separation that is more accurate than the optimal parameter of pupal length (≤5.4 mm) for separating *L. botrana* sexes during collection that result in collection of 86 % of males and 22 % of females (male biased sex ratio of 0.77). The simulation showed that the difference in distance travelled from the collection box between average weighted males and females is pronounced. This could be a possible phenomenon during collection through a plenum system that can be exploited to obtain male biased moths for collection after immobilization. This exploitation could be as simple as placing a physical barrier after which 100 % females are collected for replenishment of the broodstock colony in the rearing facility. Such a method of separation must rather focus on collecting all available males with some female contamination in order to optimize male collection for use in the SIT program (Papathanos *et al.*, 2009)

Efficiency of current collection box

For the three replicates, a mean of 80.67 % ± 4.56 % adults were collected when placing 200 adult moths (male : female = 1:1) into the prototype collection system overnight for 12 hours. Although moth collection numbers were significant over time, clear problem areas were found that can be improved. The main area where collection was impeded was within the emergence box, where there were too many crevices where moths could crawl into. The funnels at the bottom of the collection box also had crevices where remaining moths would be perched after the 12-hour collection time for the replicates. A steeper funnel system is proposed (Figure 2.12) that would also prevent moths from finding grip within the collection box. The 10 m/s air speed provided by the axial fan in the collection pipes was effective in transporting moths down the pipe, through the plenum system and into the collection bag fixed to the end (the collection pipe did not enter a cold room for this experiment).

Due to the cryptic nature of *E. saccharina* (Conlong, 1994), a simple emergence box that is smooth and has no crevices and which has steep funnels at the bottom that enter the collection pipe is thus identified as the main change that could improve the current collection box. This observation is similar to the identified issues in the collection system developed by XSIT (Pty) Ltd. where unnecessary bends in the originally tested cyclone collection system caused scale build up and damage to adults, reducing the efficacy of the collection system, and thus a simpler plenum system was developed (Hofmeyr and Pretorius, 2010). When these changes have been made and the collection box has been installed into a cold room, this experiment can be repeated to assess for improved collection over time. The difference in distance that male and female moths travel in relation to their weight before landing in the collection tray in the cold chamber can also be assessed, for the possibility of

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obtaining male biased moths for irradiation and release is discussed in this chapter and showed in the simulation (figure 2.9).

More males than females were collected after 12 hours (86.0 ± 6.44 out of 100 versus 75.33 ± 6.44 out of 100) but this was not significant. Future improvements to the collection system must keep the possible effect of protandry in mind as it may be that males are more active as they initiate lekking, whereas females are less active and only respond to already formed male leks (Atkinson, 1981). This effect of protandry could add to a systems approach to obtain a male biased population for irradiation and release. Another type of protandry is where male pupa are often first to eclose before those of females - In many lepidopterans and other insect families, protandry occurs where males mature sexually prior to females (Wiklund and Fagerström, 1977; Harari *et al.*, 2000). This has not been recorded for difference in male and female *E. saccharina.* Collection must also be investigated when pupae yet to eclose (instead of adults as done in this experiment) are placed in the collection system. Steinitz *et al.* (2016) found that *L. botrana* showed protandry, with significantly more adult males emerging during the first days of pupation (0.88 male biased sex ratio), but that sex separation using only this method would not be efficient due to a larger fraction of males that emerge later (74 %) during the onset of female emergence.

In the tested system, mating probably occurred after collection in the "collection bag" that was placed at the end of the collection system to capture exiting moths. The high percentage collection over 12 hours indicated that adults leave the tray quickly after being placed in the box, and got into the collection pipe and blown through to the collection bag. The mating status of females emerging from the collection box after collection and into cold storage must thus still be investigated. The percentage mating that occurred overnight in the conventional routine *E. saccharina* emergence box was high (99 %), further prompting the necessity for ensuring mating did not occur during the emergence and collection of adults for SIT application.

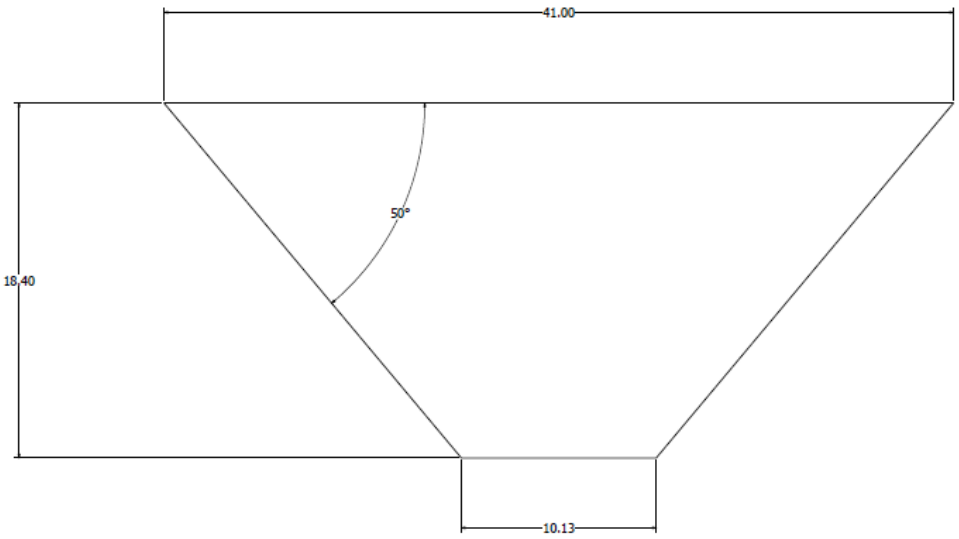

Figure 2.11: Proposed modified funnel with a steeper incline for more effective adult *E. saccharina* collection than the current one described in the materials and methods section. Measurement units are presented in cm.

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2.5. CONCLUSION

When irradiating adult *E. saccharina* pairs, there was a clear reduction in parental fertility as treatment irradiation levels increased from 0 Gy (control) to 100 Gy. This was not definitively measured due to a lack of vials containing feeding media in order to inoculate all neonate larvae. Eggs were also not counted, but these parameters should be measured in future. The high male to female ratio makes this method of obtaining a male biased F1 colony for use in the SIT promising. The sterile nature of these F1 males (Walton, 2011) was further attractive as rearing sterile F1 males and excluding the irradiation process for these males when collecting for transport to release sites simplified the handling process up to release, reducing risk of possible deleterious effects to competitiveness to wild males.

The significant difference in pupal male and female weights with little overlap gave a strong indication that an automated sex separation technique during the pupal or adult stages of development would be effective. A high degree of sex separation with minimal male losses and maximal female exclusion could be incorporated into the adult collection process. This would allow more males to be irradiated at one time and thus also more males to be transported per shipment to the field. Sex separation could also decrease the risk of mating occurrence before collection as well as preferential mating in the field.

A high degree of adult collection was obtained at all three replicates where 200 (M:F = 1:1) adult moths in pupal trays were placed in the collection box overnight (6 pm-6 am). The high collection percentage after 12 hours indicated that adults leave the tray quickly after being placed in the box. But it was observed that funnels were inadequate to rapidly lead moths into the collection pipe.

The mating status of adults after emergence from pupa and collection into cold storage must still be investigated. The percentage mating that occurred overnight during the routine *E. saccharina* adult collection operation was high (99 %), further prompting the necessity for ensuring mating did not occur during adult emergence and collection for SIT application. Further investigation on what impact mating prior to collection might have on *E. saccharina* males that show the ability to mate multiple times is necessary.

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CHAPTER 3: EFFECT OF COLD EXPOSURE, PACKAGING AND TRANSPORT ON *ELDANA SACCHARINA* MALE FITNESS

3.1 INTRODUCTION

For the successful implementation of the Sterile Insect Technique (SIT) on lepidopteran pests, laboratory reared adults must be collected after emergence, irradiated, packaged, and transported to release sites, whilst still maintaining competitive fitness (Calkins and Parker, 2005). Chilling of collected adults and further maintenance of an unbroken cold chain until release reduces adult activity and respiration rates, practically immobilising moths to enable transport over prolonged time in high density packaging, without negatively impacting on adult fitness (Boersma and Carpenter, 2016). An unbroken cold chain is defined as an uninterrupted succession of refrigerated production, storage and distribution undertakings, together with the associated technology and logistics, which maintain a set low-temperature range in order to ensure a preserved and extended shelf life of products (Gyesley, 1991). This is desired to maintain fitness of irradiated *Eldana saccharina* Walker (Lepidoptera: Pyralidae) male adults for field release in the implementation of the SIT. Immobilization of collected adults by chilling allows high density packaging for irradiation and transport without significantly impacting male adult field competitiveness, but exposure to rapid chilling and low temperatures up until release could still lead to partial loss of field performance. (Terblanche *et al.*, 2008). Codling moth, *Cydia pomonella* Linnaeus (Lepidoptera, Tortricidae) adults are chilled at 2 °C for 24-78 hours and packaged in Petri dishes for irradiation and transport to release sites (Bloem *et al.*, 2006). Similarly, packaged False Codling Moth (FCM), *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), are kept at an ambient temperature of 4-6 °C during the 729 kilometre (km) road transport from the XSIT Pty (Ltd) production and irradiation facilities in Citrusdal, Western Cape, to Addo in the Eastern Cape (Boersma and Carpenter, 2016); One cardboard box packaging (130 x 130 mm) holds around 14,000 [+ or -] 200 *T. leucotreta* adults at a 1:1 male to female ratio (Boersma and Carpenter, 2016). The same packaging holds only about 4000 *E. saccharina* adults (Serfontein. Unpublished data). Packaged adult *T. leucotreta* transport temperatures were monitored within packaging and remained around 2 °C higher than the cold chamber's ambient temperature (Boersma and Carpenter, 2016). In the XSIT operational program, should *T. leucotreta* transport temperatures within packaging exceed 8 °C during the storage period, they are not dispatched from the rearing facility, as increased activity during these higher temperatures caused adult damage above the minimal acceptable level of quality (Boersma and Carpenter, 2016). Maintenance of a rigid cold chain is thus critical to minimize physical damage due to increased adult activity at higher temperatures. However, if temperatures decrease to below the insects CT_{min}, chilling injury will occur, decreasing the field competitiveness of the sterile insects (Stotter and Terblanche, 2009).

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Physical damage caused by mechanical shock during handling and transport has not been studied for SIT implementation but is a factor that must be considered in the development of handling and transport protocols (Seck *et al*., 2015). To mitigate the effect of mechanical shock to insect life stages during handling and transport, cotton is used by the Centre International de Recherche-Développement sur l'Elevage en zone Subhumide (CIRDES) and sawdust and/or vermiculite by the Slovak Academy of Sciences (SAS) as packaging substrates when transporting packaged *Glossina palpalis gambiensis* Vanderplank (Diptera: Glossinidae) pupa (Dyck *et al*., 2006).

For any SIT program, the ability of released sterile males to adequately compete with wild males and successfully mate and inseminate target wild females is of highest concern (Dyck *et al*., 2006). Important components of male field performance are sterile male mating competitiveness and compatibility, adult dispersal ability to locate nourishment, shelter, mating arenas and wild females, successful sperm, and accessory gland fluid transfer as well as good survival percentage and longevity of the mass-reared sterilized males (Koyama *et al.*, 2004). Research and development of the SIT is thus continually aimed at enhancing these factors (Calkins and Parker, 2005). To monitor these behavioral and biological factors that affect male field performance, adequate quality control (QC) methods are required. The basic principles of QC are applicable to any product that must fulfil a specific function. Feigenbaum (1961) defined total QC as:

"an effective system for integrating the quality-development, quality-maintenance and quality-improvement efforts of the various groups in an organization so as to enable production and service at the most economical levels which allow for full consumer satisfaction."

The *control* in quality control is a management tool and therefore quality control is often called quality management. The Deming Cycle is a short summary of what quality management entails, i.e., Plan; Do, Study, Act (Deming, 2018). Quality standards must be set and based on factual grounds. These standards must further be applied and monitored from pre-production through rearing and up to release in the field. If the standards are not met, appropriate action must be taken. Standards must also strategically be improved continuously. It must be understood that the limiting factor to large scale SIT insect production is not the scale of a rearing operation, but the field performance of released sterile males (Dyck *et al*., 2006). If the rearing process is not kept to standard, there will be no use in applying optimal quality standards at later stages, as prior damage done can rarely be reversed. Successful implementation of such an approach would improve production whilst decreasing costs.

Quality control can be divided into three categories (Dyck *et al*., 2006): (1) Production quality control, where rearing inputs are considered, e.g. diet ingredients and equipment, (2) Process quality control, monitoring how rearing procedures are conducted, e.g. preparation of diet, environmental conditions (lighting, humidity, temperature), infestation rate, larval separation, pupal holding and irradiation dose, and lastly, (3) Product quality

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control, where the insects produced are evaluated for effectiveness in completing the purpose for which they are required. Categories 1 and 2 have been addressed in the Standard Operational Procedures of the SASRI Insect Rearing Unit, which are continually updated as improvements become available, as suggested by Dyck *et al.* (2006). This chapter will aim to establish options for category number 3, i.e., product quality control. In this case the fitness of male *E. saccharina* that have been reared, packaged, irradiated, and transported in low temperature conditions to release sites will be evaluated.

Both production and process quality control have received most attention because of the longer history of insect mass rearing compared to the SIT (Dyck *et al*., 2006). There are strict process and production quality control measures in place at the SASRI mass rearing facility in Mount Edgecombe, ensuring optimal sanitary and environmental conditions, as recommended by Dyck *et al.* (2006). Routine quality control measures for mass reared *E. saccharina* include larval survival and pupation % after certain time periods, daily environmental control parameter checks and pupal weight estimates of insect produced from daily diet mixes (Walton and Conlong, 2016). Mudavanhu *et al.* (2016) concluded that these mass-reared *E. saccharina* males adequately compete with wild males for wild female mates when observed in laboratory and field cage trials. The effect of post-production conditions for the SIT (collection, cold exposure, high density packaging, irradiation, transport, field release) cannot be routinely measured using these conventional product QC methods used during mass rearing of *E. saccharina*. Quality control measures must now be focussed on retaining quality laboratory reared male *E. saccharina* fitness during collection, irradiation and transport up to the actual release.
In this Chapter, the establishment of an efficient collection and cold chain was investigated, using male longevity and male mating frequency as indicators of quality at each step in this collection and cold chain process. Measuring male mating frequency and longevity provide repeatable results but are labour intensive and results are only obtained a few days after release. As the release phase of the *E. saccharina* SIT program progresses, QC procedures as practiced by XSIT for *T. leucotreta* could be phased in, as outlined by Nepgen (2014).

Although there is little available literature on transportation of Lepidoptera as adult moths over long distances (Blomefield *et al.*, 2011), as well as on subsequent tools and methods to assess field performance (Vreysen *et al.*, 2016), SIT programs for *P. gossypiella*, (Dowell *et al.*, 2005) and *T. leucotreta* (Nepgen, 2014) all collect adult moths after emergence, immobilise adults by cold temperature for easier handling during packaging, transport and up to release. Upon arrival at their destination after long distance transport, *T. leucotreta* adults are sub-sampled and subjected to visual inspection as a QC measure (Nepgen, 2014). The percentage dead, damaged and deformed moths in the sub-sample are counted. This gives a good indication of what the field performance of the transported irradiated males will be. Visual cues importantly also indicate if any breach of the cold chain that could spoil quality occurred, quality being spoilt due to moths regaining activity and damaging each other within the high-density packaging in which they were placed after cold immobilization (Nepgen, 2014). Compared to testing

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longevity and mating frequency, visual inspections are rapid and easy, and allow for the quick response to any issues occurring during post-production handling.

Physical damage would not only impact flight ability of adult moths, but also general behavioural qualities (e.g., courtship rituals for successful mating and habitat finding for survival) (Dyck *et al*., 2006). In the case of *E. saccharina* males, physical damage to various body parts, such as pheromone signalling hair pencils, wings, eyes, and antennae, will impede desirability as a mate within a larger lek of competing males (Atkinson, 1981). Visual inspection of *E. saccharina* is thus one possible method that could indicate male field performance. A visual inspection method at the SIT packing facility or site of release is easy and more importantly quick, allowing for rapid response to issues during post-production. Visual observations should be quantifiable in order to be recorded and analysed. Upper and lower control limits can further be derived from long term data (Dyck *et al*., 2006).

The main objectives of this study were i.) To investigate the effect of exposure of adult male *E. saccharina*, to immobilizing cold temperatures over periods of time (that would reflect the collection up to release times in transit) on their fitness; ii.) To investigate possible transportation containers/carriers that would provide an adequate cold chain to preserve male moth fitness from facility collection and packing, up to release; iii.) To identify insulating packing substrates for *E. saccharina* adult males that would provide the best buffer against temperature fluctuations during the cold chain; iv.) To investigate the effect of the best above identified packing substrate on male moth fitness when exposed to chilling temperature for various periods of time, reflecting possible storage and transport times; v.) To investigate the effect of the identified packaging substrate on male moth fitness when exposed to simulated transport conditions; and vi.) To investigate the use of visual observations of packaged and transported male moths as a possible QC method.

3.2. MATERIALS AND METHODS

Effect of exposure of adult male *E. saccharina*, to immobilizing cold temperatures over time on mating frequency and longevity.

Less than one-day-old *E. saccharina* males were obtained by packaging pupa removed from diet during conventional rearing at the SASRI Insect Unit, singly in cells of plastic multicell trays (4 x 8 cells/tray) and wrapped with clingwrap. A pinprick was made above each cell to provide aeration. Packaging of between 500 and 1500 pupae daily for seven consecutive weekdays provided adequate numbers of less than one day old, emerged *E. saccharina* adult males for two treatments (90 adult males per treatment exposed to treatment conditions and sampling of 30 males for mating frequency and longevity testing) to be conducted at a time. Multicell trays containing pupae were kept in 12:12 (L:D) conditions at 75 % RH and 25 °C ± 2 °C at the SASRI Insect Facility

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and checked daily for adult emergence. Cells containing daily-emerged moths were marked with the date of emergence and sexed until 180 less than one-day-old males could be collected from a single day's emergence. For each treatment, 90 less than one day old males were placed in one-litre plastic cylinders (this lower density packaging eliminated the effect of high density packaging in order to measure the effect of cold exposure only) containing a 30 cm section of paper towel (to serve as extra perching surface) and closed with lids aerated with steel mesh. One container was placed in an incubator at 5 °C for 24 hours and the other in a separate incubator at 25 °C for the same period as a control. Thereafter, 30 males per treatment were randomly removed to measure longevity and mating frequency. They were individually placed in 500 mL paper cups containing pleated cardboard (50x10 mm when pleated five times) for extra resting surface and with a 10mm dental cotton roll (supplied by Shanghai Ristea Industries Co, Ltd.) soaked with distilled water to provide moisture. Cups were sealed using plastic lids. Each male was paired with a less than one-day-old female collected from the multicell trays. Females were replaced every 24 hours until males died, and male longevity was recorded. Removed females were placed in vials marked for each specific male, frozen at -5 °C, and their bursa copulatrix dissected to determine mating status (Walton, 2011). Male mating frequency (MF) was calculated for each male by determining the number of females he mated with before his death. Male longevity (days) was determined from the time of its removal from the temperature treatments until its death. In addition to the males that were kept at the two temperatures for 24 hours, the above procedure was repeated for males kept at 5 °C for 48 and 72 hours, respectively.

Average male longevity and mating frequency could be calculated from the individual measures obtained and used as an indicator of male fitness between the four treatments to determine the effect of exposure to 5 °C over 24, 48 and 72 hours compared to the control treatment of exposure to 25 ºC over 24 hours.

Comparing temperature over time in transportation carriers

To measure temperature over time in three different transportation carriers Thermochron® iButton® (Dallas Semiconductors, Model DS1920; 0.5 °C accuracy) data loggers were set to measure ambient temperature (°C) in 1-minute intervals.

i.) Incubator, to simulate optimal temperature regulation: An incubator set at 5 °C, as used for testing cold effect on male fitness, was measured so as to display an optimal temperature curve with minimal temperature fluctuation over time. To measure the temperature over time, an iButton was suspended from the top of the incubator using a 5 cm piece of string and pieces of Sellotape, and measurements were taken as soon as the iButton was placed in the already 5 °C set incubator.

ii.) Cooler (Coleman 48 Quart Chest Cooler) with ice blocks: which is a cheap, conventional method of transporting insects : Twelve ice bricks (Seagull Ice bricks, model 31174, 15 cm x 8.5 cm x 3 cm) were taken

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from a freezer (-5 °C) and placed in a Coleman 48 Quart Chest Cooler to line the bottom and sides of the inside of the cooler. To measure the temperature over time, an iButton was suspended from the lid using a 5 cm piece of string and pieces of Sellotape and measurements were taken as soon as the cooler was packed with the ice blocks.

iii.) Empty transportable chest freezer: An empty transportable chest freezer was set at 5 °C prior to placing an iButton inside, also suspended using a 5 cm piece of string and pieces of Sellotape, in the empty freezer. Larger transportable freezers/fridges are an option when doing commercial scale releases far from irradiation sources.

iv.) Transportable chest freezer filled with bran: Another temperature curve (temperature over time in one-minute intervals) was measured using an iButton inside the freezer when packed with 12 x 350 g commercial Snowflake wheat bran packs to simulate a more thermally insulated environment, compared to air, during transport due to the lower thermal conductivity of bran. The iButton was placed in the middle of the Bran packs to be fully surrounded by the insulating bran.

Investigating insulating properties of packing substrates for best buffer effect against temperature fluctuations.

Insulating properties of four possible packaging substrates, namely: Snowflake Bran, Course vermiculite, fine vermiculite, and a blank (air) control were measured. Robinson and Hendrichs (2005) remarked that for future developments for SIT, packaging materials should aim to maintain correct temperature and atmospheric conditions. These possible substrates were selected to achieve such goals. In addition, they are cheap, and environmentally friendly .

Three 250 mL paper cups per treatment were filled with: i.) commercial Snowflake wheat bran, ii.) course vermiculite, iii.) fine vermiculite and iv.) air as a control, and sealed with plastic lids. A Thermochron® iButton® data logger was suspended with string and Sellotape 4 cm into the contained substrate from the middle of each cup's lid. Temperatures were logged at one-minute intervals. Cups were taken from room temperature (25 °C) and placed at 5 °C for four hours. The cups were then removed and placed back at 25 °C for four hours.

The average times for each treatment to reach ambient temperature (after placement from 25 °C to 5 °C *vice versa*) were calculated and compared between treatments. The treatment that took the longest time to reach ambient was identified as the most insulating substrate.

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Effect of packaging substrate on male moth fitness during high density packaging

The effect of using wheat bran as a packaging substrate on male mating frequency and longevity were measured against packaging of moths with no packaging substrate. Pupae were placed in multicell trays as described previously in this chapter. Five hundred less than two-day-old virgin males (less than one-day-old males would have been optimal, but due to the high numbers of males needed this was not practical) were collected and placed in five separate 1 L bottles sealed with a mesh lid, i.e., 100 moths per 1 L bottle (these bottles were large enough to immobilize moths at lower packaging densities) containing a 30 cm section of paper towel to serve as extra perching surface. Bottles were placed in an incubator set at 5 °C for one hour to simulate the pre-packaging cold immobilizing time in cold storage after automated collection as will be done after collection of eclosed moths as based on the XSIT Pty (Ltd) procedure for .*T leucotreta* (Nepgen, 2014). Two 250 mL cups were each containing 250 males to simulate high density packaging for irradiation and transport and one of these was further fully filled with bran. Cups were immediately placed back at 5 °C for 24 hours to simulate transport time. Thirty males were subsequently removed from each treatment to conduct longevity and mating frequency trials as described in this chapter. A 48-hour and 72-hour treatment was also subsequently conducted.

Treatments were:

i.) No-bran packaged males exposed to 5 °C for 24 hours

ii.) Bran packaged males exposed to 5 °C for 24 hours

iii.) No-bran packaged males exposed to 5 °C for 48 hours

iv.) Bran packaged males exposed to 5 °C for 48 hours

v.) No-bran packaged males exposed to 5 °C for 72 hours

vi.) Bran packaged males exposed to 5 °C for 72 hours

For treatments v.) and vi.), 30 additional males were sampled per treatment and frozen for future visual observation rating exercises.

Effect of packaging substrate on male moth fitness during transport

Male moth fitness was measured when simulating storage and transport in the transportable chest freezer. Five hundred less than two-day-old virgin males (optimally less than one day old should be used, but there was limited availability) were collected and placed in five separate 1 L bottles containing a 30 cm section of paper towel for additional perching surface. Bottles were placed in an incubator set at 5 °C for one hour to simulate cold immobilization time in cold storage after automated collection, as will be done after automated collection of eclosed moths as based on the XSIT Pty (Ltd) procedure for *T. leucotreta* (Nepgen, 2014). Two 250 mL cups were each filled with 250 males and one of these cups was further filled with bran. Cups were placed in the transportable chest freezer calibrated as close to 5 °C as possible for a further 72 hours to simulate maximum

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transport time. The chest freezer was placed on a trolley for the last 45 minutes of the 72 hours and pushed back and forth for 45 minutes over an electrical wire on tiles, crossing the wire twice per second, to simulate shaking during road transport. Thirty males were subsequently removed per treatment to conduct longevity and mating frequency trials, as described previously.

Investigating the use of a visual observation rating system as a quality control method for high density packaged and cold stored *E. saccharina*

To measure the quality *T. leucotreta* before and after transport, Nepgen (2014) visually sampled 100 male and female moths to determine the number of deformed, damaged, and dead moths. For a reliable visual inspection method for *E. saccharina,* a more intensive visual inspection than the *T. leucotreta* field inspection is investigated, as more factors than just damage to wings could play a significant role in field performance. It is important that such inspections are not only accurate, but easily repeatable by different persons and as simple as possible.

Visual rating systems enable one to quantify the severity of damage which is visually observable. Such systems are regularly used to quantify the severity of damage caused by a pest to a crop (Davis and Williams, 1992). In this section a visual rating system was conceived to measure lost or loose scales on various body parts of high density packaged *E. saccharina* males placed in cold chain conditions. Scale loss after packaging was apparent on the pronotum of *E. saccharina* males. These loose scales could further be observed covering the males eyes and ventral surfaces. Scale loss from the pronotum as well as scale coverage on eyes and ventral surfaces were thus identified as easily quantifiable for a visual ratings system.

Thirty bran and no bran packaged male moths sampled and frozen from the 72-hour bran and no bran treatments experiment were thawed before being photographed under a stereo microscope. A close-up photograph was taken of the eyes and pronotum, as well as a full dorsal and ventral view of each adult. The percentage scale cover on the eyes was estimated and rated from 0 to 5 (0= <1 %; 1= 1 %-20 %; 2= 20 %-40 %; 3= 40 %-60 %; 4= 60 %-80 %; 5= 80 %-100 %). The same rating method was applied for estimated percentage loose scale cover on the ventral surface and percentage scale loss from the pronotum. The visual rating results of the no bran packaged moths, which had a significantly lower mating frequency versus bran during the previous experiment, could then be compared to bran packaging. This indicated if there is a possible relationship between physical appearance of males and their longevity and mating ability, i.e., fitness.

Statistical analyses: Male longevity and mating frequency for the 24 h at 25 °C control and the 24 h, 48 h and 72 h at 5 °C treatments; the 24 h, 48 h and 72 h at 5 °C bran and no bran packaged male moth treatments; and the 72 h at 5 °C in the chest freezer bran and no bran packaged male moth treatments were each separately compared

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using one-way ANOVA with a LSD post hoc test used to separate means, following Levene's test for homogeneity of variances.

For the comparison of visual ratings as a quality control measure, the non-parametric Mann-Whitney U test was used to compare differences, following Levene's test for homogeneity of variances, which confirmed heterogeneity of the visual observations rating data (p = 0.4960). A box plot was drawn to visualise the ratings data. All data analyses were conducted in Statistica version 13 (StatSoft, Inc. 1984-2017).

3.3. RESULTS

Effect of cold exposure over time on male *Eldana saccharina* longevity and mating frequency

There were no significant differences in mean longevity for males held for 24 h at 25 °C (Control) and those held for 24, 48 and 72 h at 5 °C ($F_{(3, 116)} = 2.2307$, P=0.09) (Figure 3.1). The highest mean longevity was with the control treatment (8.567 days) and the lowest at 72 h at 5 °C (7.233 days).

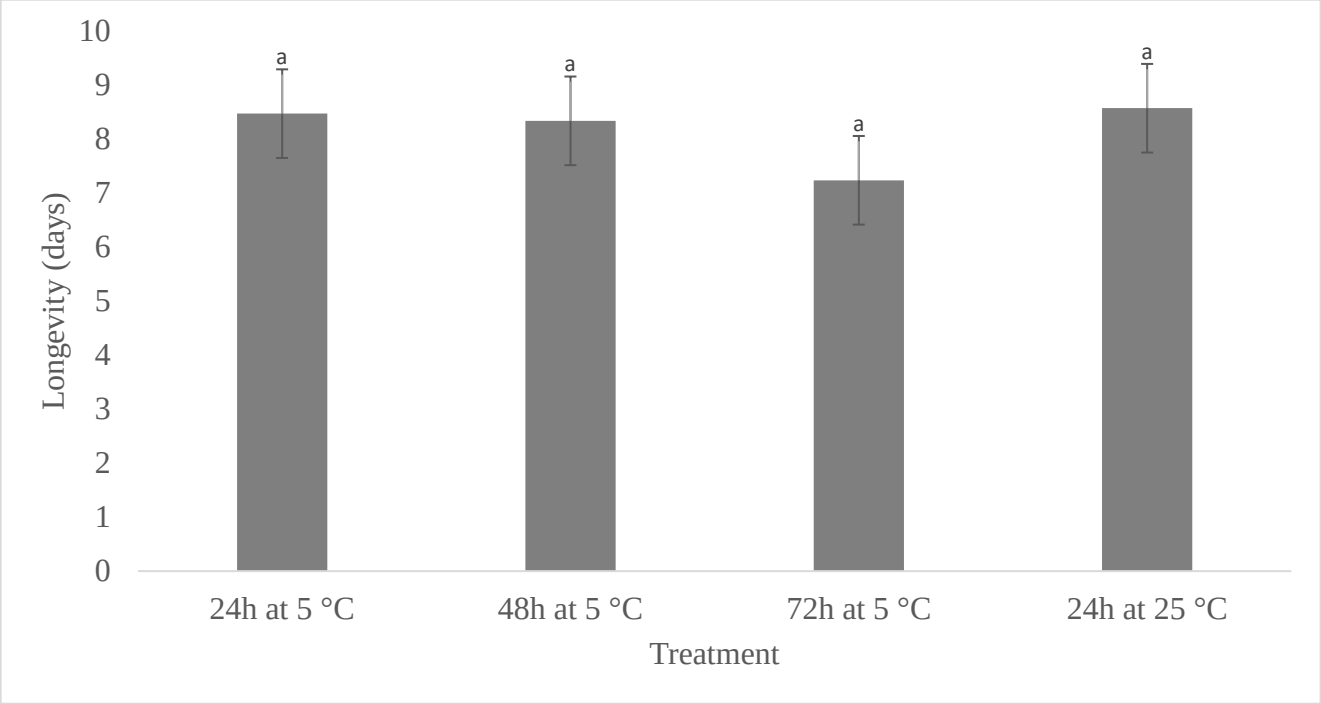

Figure 3.1 Male *Eldana saccharina* mean longevity after exposure to 5 °C for 24, 48 and 72 hours, compared to a control treatment kept at 25 °C for 24 hours. Error bars denote 95% confidence intervals. Bars with the same letter above each column are not significantly different.

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Significant differences were, however, found when comparing mating frequency between treatments ($F_{(3, 116)}$ = 6.0400, P=<0.01) (Figure 3.2). The adult males held for 72 h at 5 °C had a significantly lower mean mating frequency (mean 2.7 females mated) than the other treatments (24 h at 5 °C = 4.9; 48 h at 5 °C = 4.4; 24 h at 25 °C = 4.3) (Figure 3.2). Adult males held for 24 h at 5 °C had the highest mean mating frequency of 4.9 females per male compared to only 2.7 females per male held for 72 h at 5 °C.

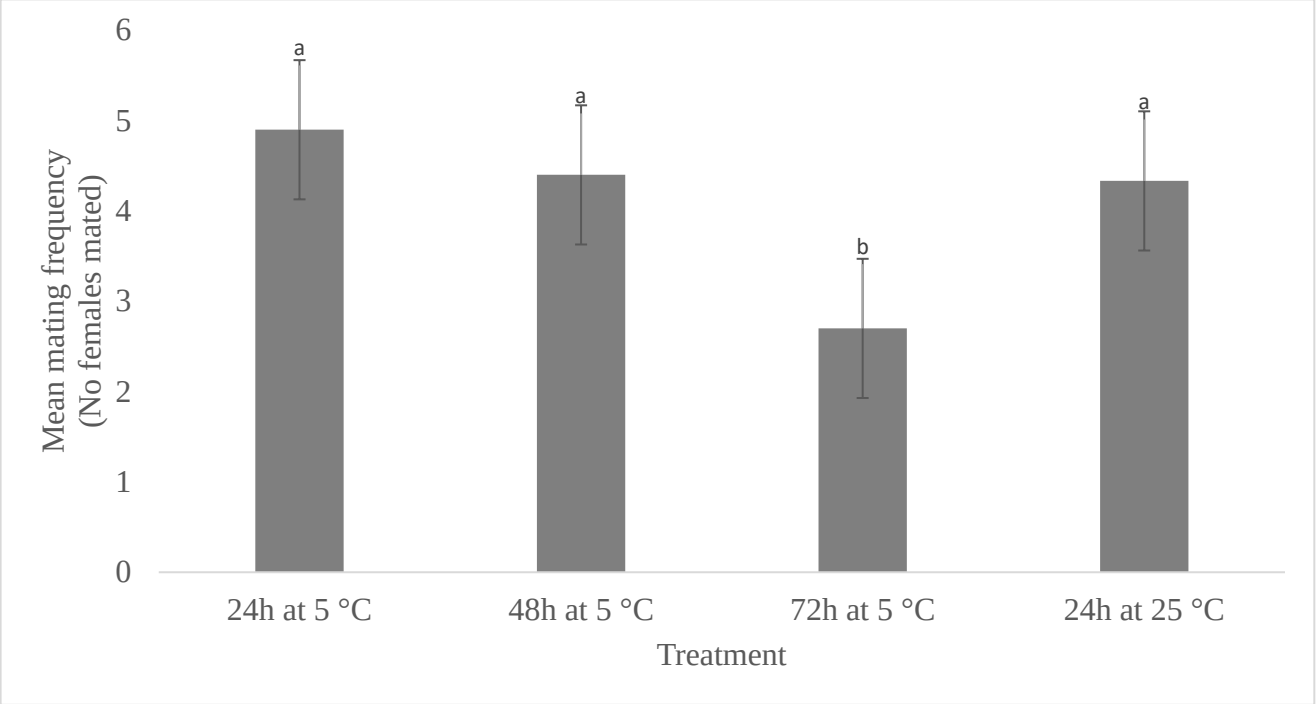

Figure 3.2: Adult male *Eldana saccharina* mean mating frequency after exposure to 5 °C for 24, 48 and 72 hours, compared to a control treatment kept at 25 °C for 24 hours. Error bars denote 95% confidence intervals. Bars with the same letter above each column are not significantly different.

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Comparing temperature over time in possible transportation carriers for transporting high density packaged *Eldana saccharina*

The temperature in the incubator set at 5 °C increased to 9 °C at the start of measurement due to the opening of the door for placing the iButton, but after 20 minutes, 5 °C was reached and maintained within 1 °C for the remainder of the 24-hour period.

The iButton placed in the Coleman 48 Quart Chest Cooler with twelve ice bricks took 6 hours to cool down to a minimum of 6 °C from 15 °C before gradually heating up, reaching 9 °C after 24 hours.

For the transportable chest freezer temperature curves, stocking of the freezer with 12 x 350 g commercial Snowflake wheat bran packs offered greater temperature buffering. The empty freezer set at 5 °C had an average ambient temperature of 2.80 °C with maximum and minimum temperatures of 6 °C and 0 °C. The freezer stocked with bran, also set at 5 °C had maximum and minimum temperatures of 5.5 °C and 1.5 °C, thus considerably decreasing peaks in temperature extremes. This fluctuation was apparent throughout the 24-hour period with peaks and troughs of the empty freezer being on average 50 minutes apart versus the freezer filled with bran having peaks and troughs which were on average 75 minutes apart, thus extending the time between temperature extremes (Figure 3.3).

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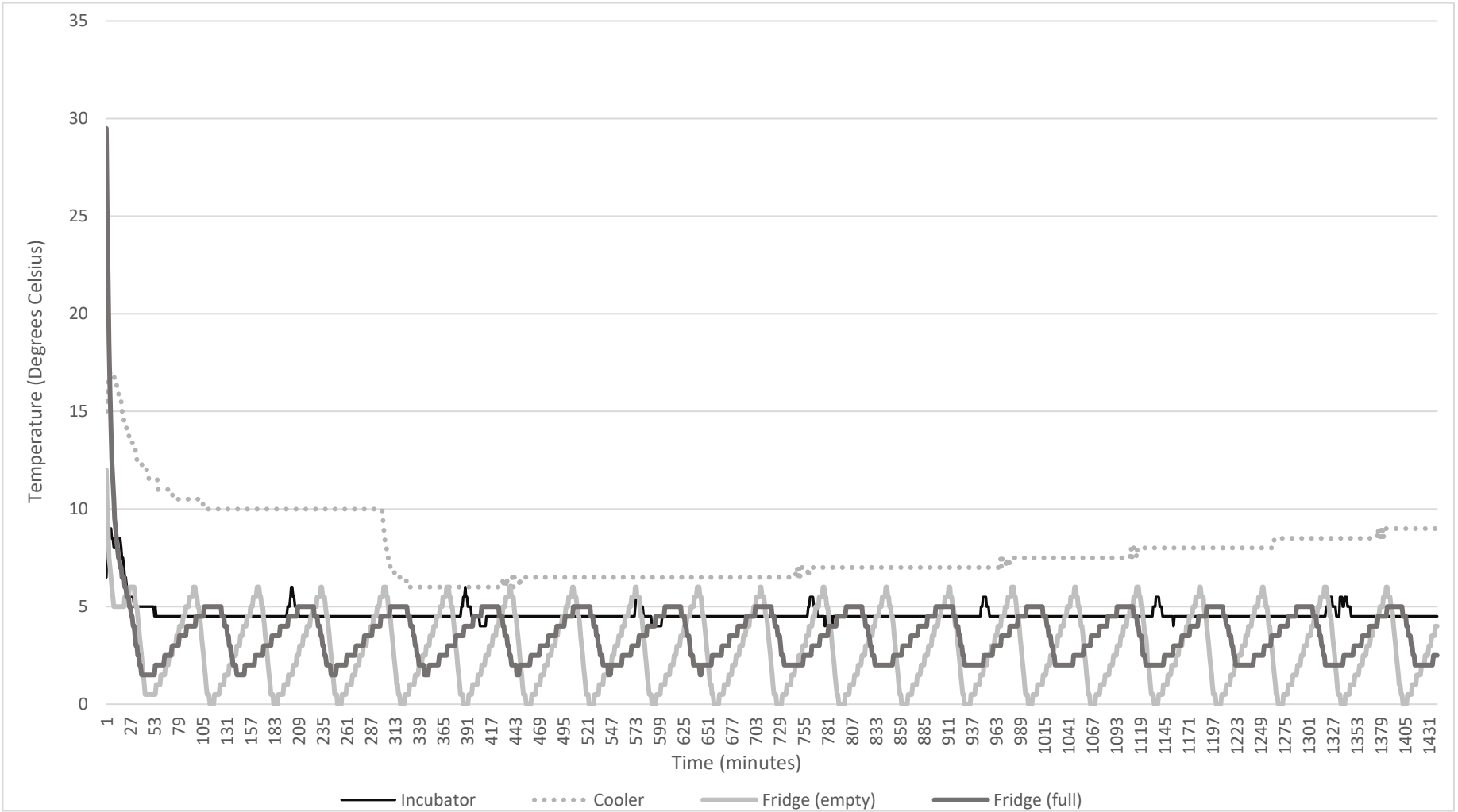

Figure 3.3. Ambient temperature curves of i.) Incubator set at 5 ℃; ii.) Coleman 48 Quart Chest Cooler with twelve ice bricks from a freezer set at -5 ℃ ; iii.) portable chest freezer (empty) set at 5 ℃ and iv) Portable chest freezer (containing 12 x 350g commercial Snowflake wheat bran packs) set at 5 ℃, temperature was measured with iButtons at 1-minute intervals over a 24 hour period and temperature curves were drawn up from obtained data. Note that the Critical minimum temperature (CT_{min}) for the lab reared *Eldana saccharina* is 4.4 °C ± 0.4 °C (Mudavanhu *et al.*,2012)

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Identifying best temperature insulating packaging substrate

The data was error free at a 95 % confidence interval and thus all differences between treatments were considered to be of significance (Figure 3.4). The bran treatment took an average of 156.7 minutes to cool down when cooled from 25 ˚C to 5 ˚C, compared to 87.3 minutes for the blank (air) control treatment. The bran treatment took 88.7 minutes to reheat to 25 ˚C compared to 32.3 minutes for the blank control. Both vermiculite treatments offered better insulation than the blank control by having slower cooling and reheating rates. Bran offered significantly more insulation during cooling (bran cooling period to 5 ℃ from 25 ℃ was 156.7 minutes versus 123.3 minutes and 112 minutes for fine- and course vermiculite respectively). Bran also provided better insulation during heat accumulation from 5 ℃ to 25 ℃ compared to both fine- and course vermiculite (bran heat accumulation period to 25 ℃ from 5 ℃ was 88.7 minutes versus 53.3 minutes and 55.3 minutes for fine- and course vermiculite, respectively). Bran was thus chosen as the insulation medium for further trials.

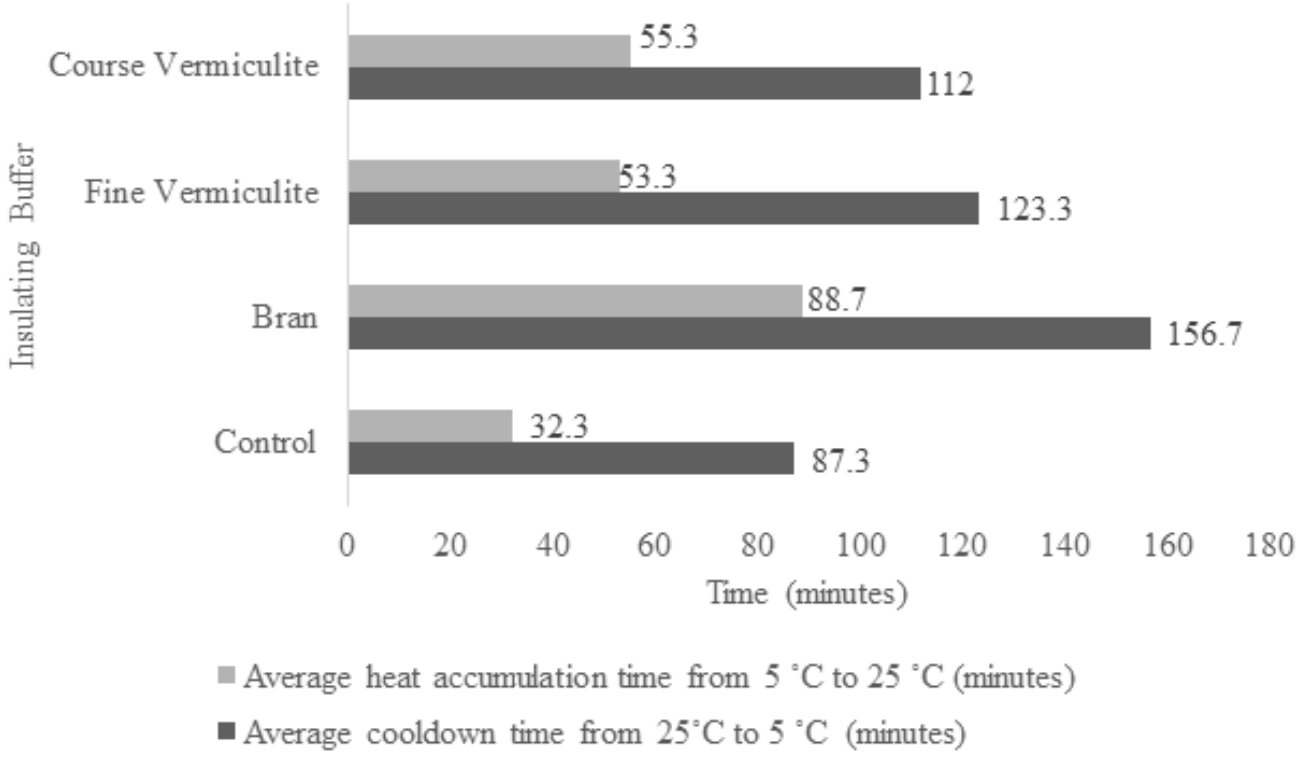

Figure 3.4. Average heat accumulation at room temperature (from 5 °C to 25 °C) and cooldown in incubator (from 25 °C to 5 °C) time for 500 mL paper cups containing course vermiculite, fine vermiculite, bran and a blank control.

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Effect of packaging substrate on male moth longevity and mating frequency

There was no significant difference in longevity of males held at 5 °C for 24, 48 or 72 hours with and without bran ($F_{(2, 174)}$ = 0.11689, P=0.88815) (Figure 3.5).

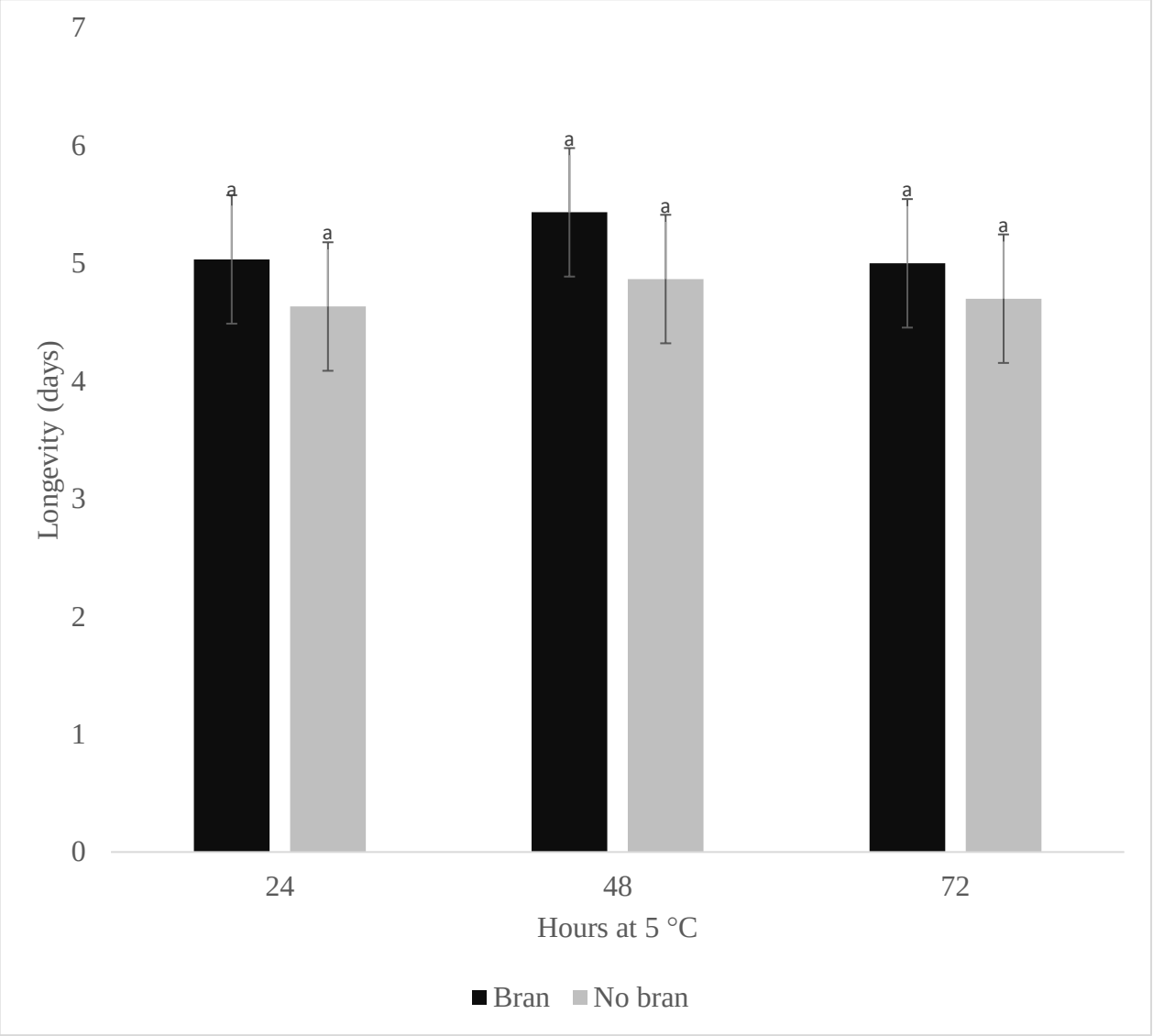

Figure 3.5. Mean male longevity for *Eldana saccharina* males packaged in Bran and No Bran held for 24, 48 and 72 h at 5 °C. Error bars denote 95% confidence intervals. Bars with the same letter above are not significantly different.

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Male mating frequency was constantly higher for individuals when packaged in bran compared to individuals in no bran packaging (Figure 3.6). These differences were significant in the 24 and 72 hours at 5 °C treatments ($F_{(2, 174)}$ = 1.0665 P=0.34047). Males packaged in bran and kept at 5 °C for 24 hours had a mean mating frequency of 3 females mated compared to 2.1 females mated for the no bran treatment kept at 5 °C for 24 hours. Males kept in bran for 72 hours at 5 °C had a mean mating frequency of 2.6 females mated compared to the significantly lower 1.5 females mated for the no bran packaged males kept at 5 °C.

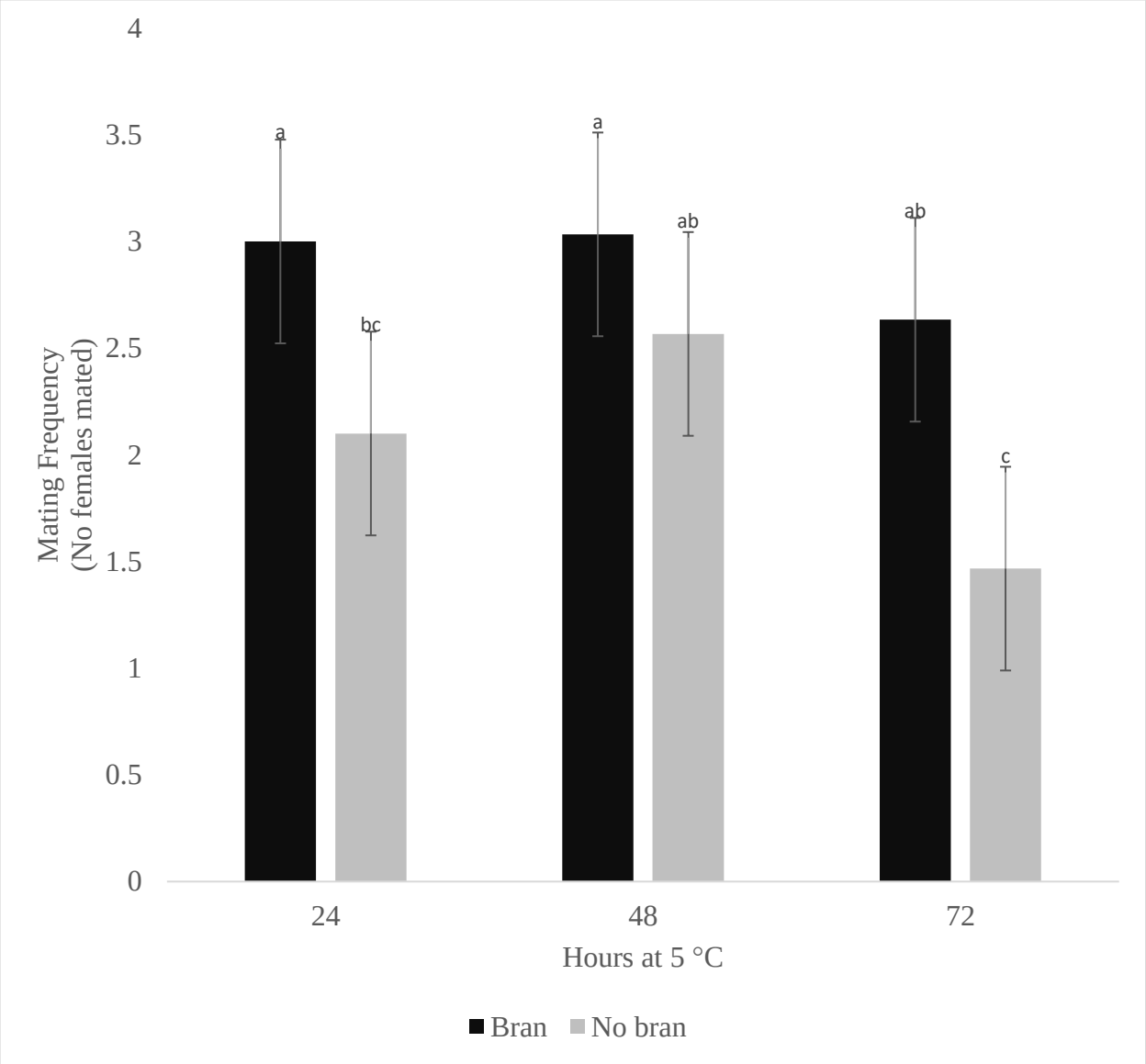

Figure 3.6. Mean male mating frequency for *Eldana saccharina* males packaged in Bran and No Bran held for 24, 48 and 72 h at 5 °C. Error bars denote 95% confidence intervals. Bars with the same letter above are not significantly different.

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Effect of packaging substrate during simulated transport on male moth longevity

There was no significant difference in less than two-day old male longevity between bran and no bran packaged moths when measured after 72 hours in the chest freezer set at 5 ℃ ($F_{(1, 58)}$ = 0.536, P = 0.46). The 250 males packaged in bran in a 250 mL container and kept for 72 hours in the 5 ℃ set chest freezer had a mean longevity of 4.267 days after removal from the treatment (Compared to 5 days for the 72 hour bran packaged incubator treatment). The no bran packaged males had a mean longevity of 3.833 days after removal from the treatment (Compared to 4.7 days for the 72 hour no bran packaged incubator treatment) (Figure 3.7).

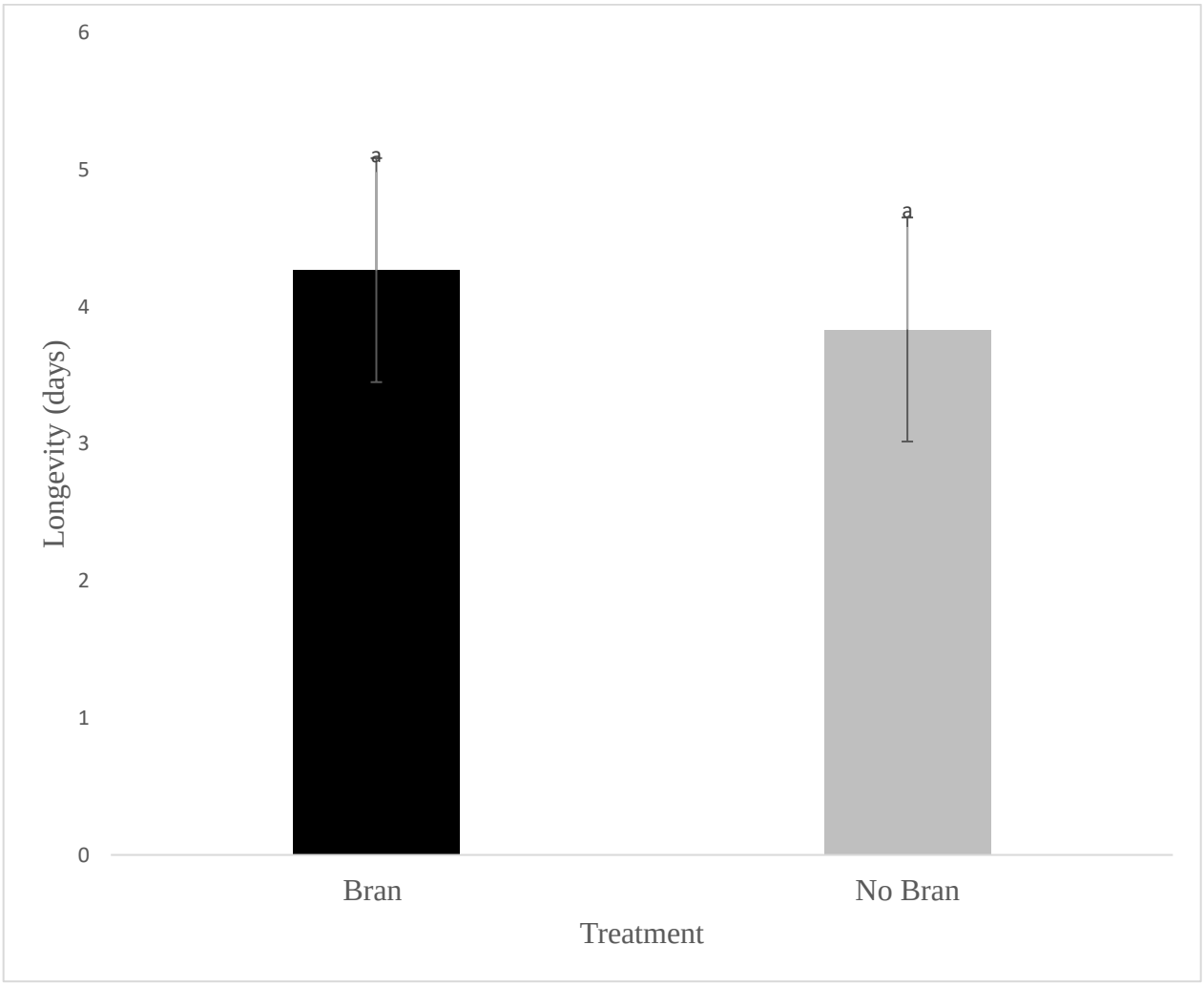

Figure 3.7. Mean longevity (days after removal from treatment) for less than two-day old virgin *E. saccharina* males packaged in bran and no bran, respectively, and held for 72 hours in the transportable chest freezer set at 5 °C. Error bars denote 95% confidence intervals. Bars with the same letter above are not significantly different

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There was also no significant difference in less than two-day old male mating frequency ($F_{(1, 58)} = 2.9383$, P = 0.09184). Males packaged in bran had a mean mating frequency of 1.833 females (Compared to 2.633 females mated for the 72 hour bran packaged incubator treatment). No bran packaged males had a mean mating frequency of 1.067 females (Compared to 1.467 females mated for the 72 hour no bran packaged incubator treatment) (Figure 3.8). Although no significant difference was measured, the trend of bran packaged moths having higher mating frequency versus no bran packaged moths were similar to that of the previous experiment's incubator bran versus no bran packaged treatments.

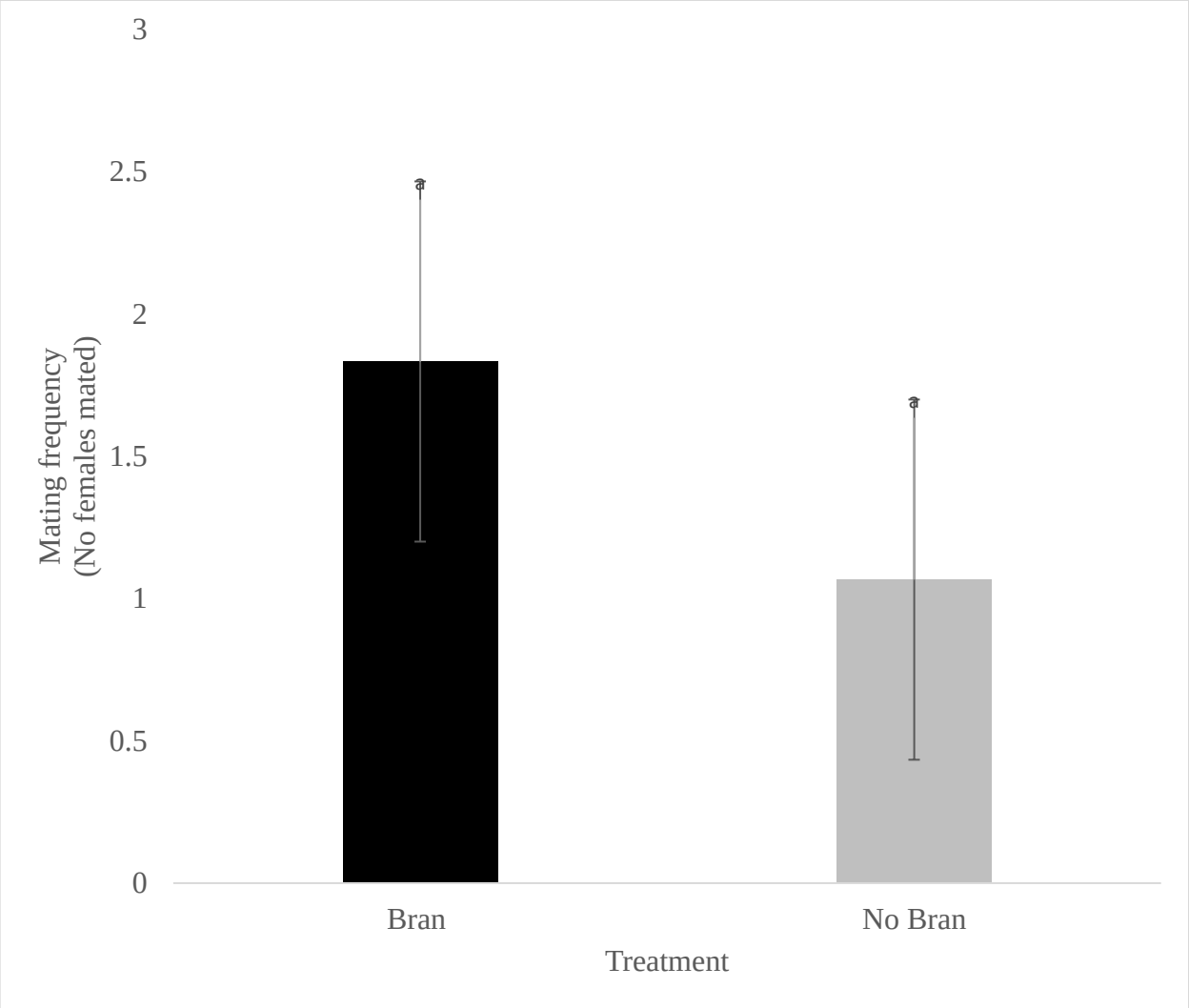

Figure 3.8. Mean mating frequency (days) for males packaged in bran and no bran respectively and held for 72 hours in the freezer set at 5 °C. Error bars denote 95% confidence intervals. Bars with the same letter above are not significantly different.

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Investigating the use of a visual observation rating system as a quality control method for high density packaged and cold stored *E. saccharina*

Differences in visual ratings (Figures 3.9, 3.10) between bran and no bran-packaged moths kept in an incubator set at 5 °C were significant when comparing:

- Loose scale cover on eyes ($Z = -5.337$; $P < 0.05$)).
- Loose scale cover on ventral surface ($Z = -6.0912$; $P < 0.05$)
- Loose scale cover on dorsal surface ($Z = -5.4702$; $P < 0.05$)
- Scale loss from pronotum ($Z = -4.9676$; $P < 0.05$)

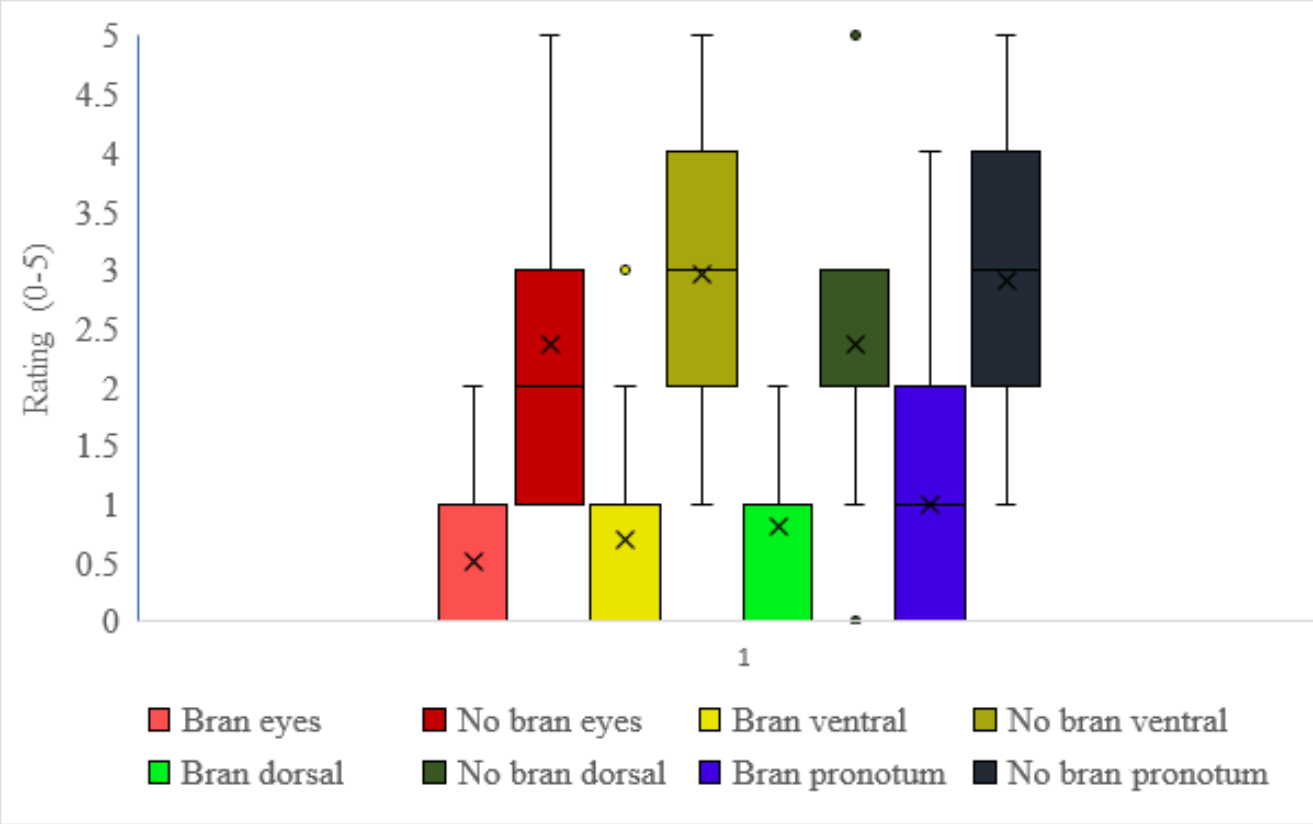

Figure 3.9: Boxplot indicating visual ratings (0 – 5) of loose scale cover on adult male moths' eyes, loose scale cover on the full ventral and dorsal surfaces of the adult male moths as well as scale loss from the adult male moth pronotum – for both bran and no bran packaged moths (250 moths packaged in a 250 mL paper cup with lid) stored for 72 hours at 5 ℃. "X" symbols in each box plot signifies the mean rating.

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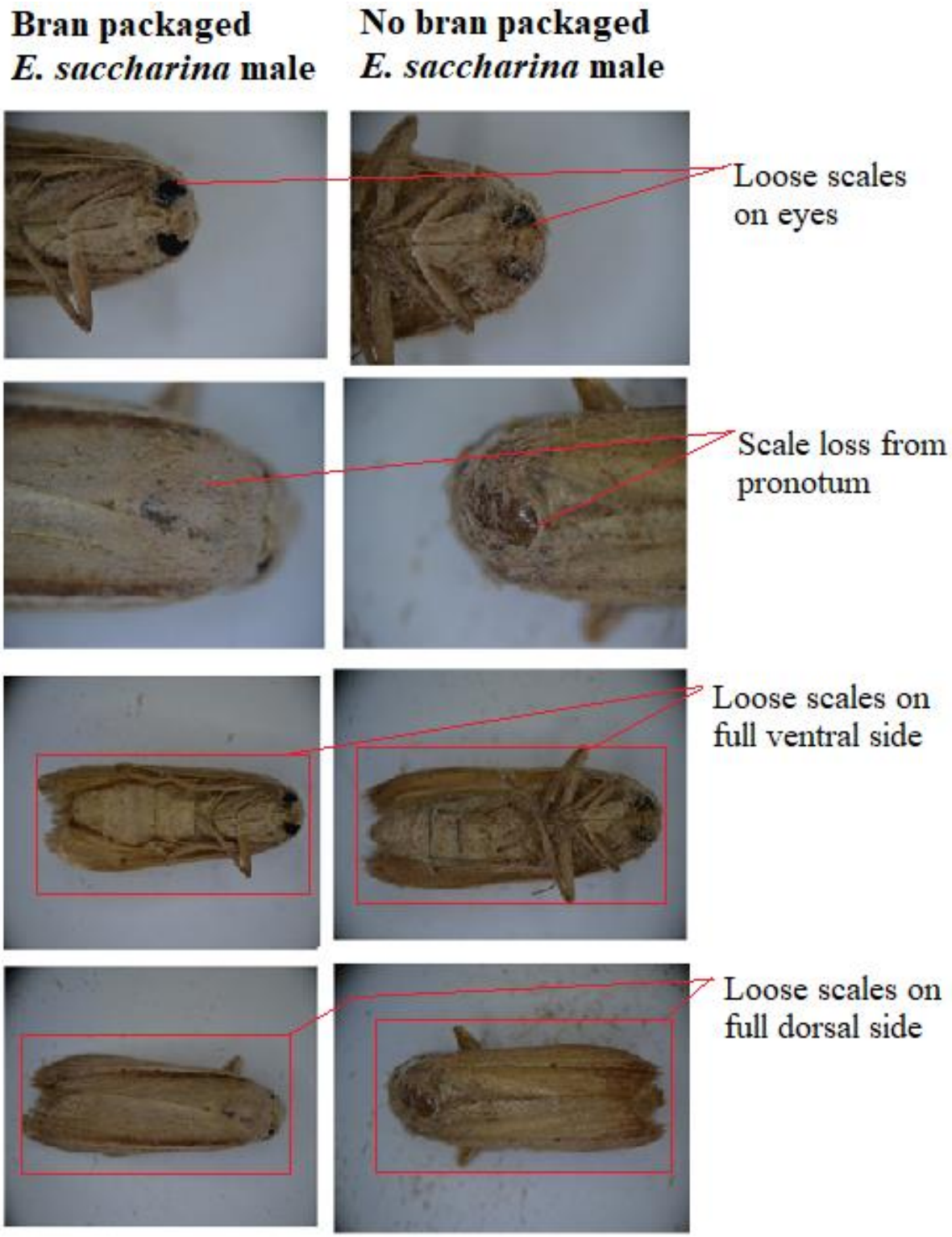

Figure 3.10: Typical comparison between bran packaged (Left) and no bran packaged (Right) adult male *Eldana saccharina* kept at 5 °C for 72 hours. Note difference in loose scales on the various body parts as well as scale loss from the pronotum.

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3.4. DISCUSSION

Effect of exposure of adult male *E. saccharina*, to immobilizing cold temperatures over periods of time on mating frequency and longevity

Mudavanhu (2012) and Kleynhans *et al.* (2014) found that for laboratory-reared *E. saccharina*, the CT_{min} of non-irradiated laboratory-reared individuals were 4.4 ± 0.4 °C but increased significantly when exposed to irradiation levels of 150 Gy and 200 Gy. Therefore, the chilling and storage temperature used after collection will have to be increased to accommodate for the effect of the specific irradiation dosage used. As non-irradiated laboratory-reared moths were used to conduct cold chain simulation tests during this investigation, the chilling and storage temperature was set at 5 °C, which is 0.6 ± 0.4 °C above the laboratory reared *E. saccharina* strain's CT_{min} of 4.4 ± 0.4 °C (Mudavanhu, 2012). Exposure to 5 °C significantly decreased male mating frequency after 72-hours but had no impact after 24 and 48 hours of exposure.

Bloem *et al.* (2006) investigated the impact of 0 h (control), 24 h, 48 h and 72 h storage times at 2 °C on motor activity of adult *C. pomonella*. After treatments, moths were placed in a chamber (25 °C) and motor activity was measured after 1 hour of acclimation using infrared actographs (Activity Monitor, Electronic Services Unit, University of New England, Armidale, New South Wales, Australia). The control treatment showed that the length of time kept at 2 °C did not influence mobility, but that all cold stored moths had significantly reduced mobility versus the control. This investigation can also be conducted for *E. saccharina* as it was observed that male *E. saccharina* stored at 5 °C for longer periods tended to have reduced first day mating success (unpublished data). In addition to mobility measurement after various periods in cold storage, first day mating success measurements could also indicate male fitness shortly after cold storage for prolonged periods.

Comparing temperature over time of possible transportation carriers for transporting high density packaged *Eldana saccharina*

Nepgen (2014) showed a reduced quality of *T. leucotreta* packaged in cardboard boxes containing around 3000 adults per box and transported for 729 km in insulated containers under an insulated vehicle canopy. His temperature records for the journey showed that the adult moths were subjected to up to a 20 °C variation in temperature in consignments transported in different seasons. Visual inspection of moths upon arrival showed increased damage for consignments that reached higher temperatures. These observations by Nepgen (2014) show the impact of temperature on moth quality if it is not carefully controlled.

The Incubator used as representation of an optimal transport carrier provided the most constant temperature of 5 °C over time with minimal peaks/dips in temperature. Bran packaged *E. saccharina* kept at 5 °C within the incubator for up to 72 hours showed no reduction in male mating frequency when compared to treatments over shorter periods, but when bran packaged *E. saccharina* were kept in the transportable chest freezer set at

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5 ℃ for 72 hours, there was a reduction. This was possibly due to more severe temperature fluctuations within this carrier.

A refrigerating transport carrier transporting packaged moths, with measured and controlled temperature over time, such as measured in the laboratory incubator, would be optimal in reducing risk that temperature fluctuations could pose detrimental dips below the *E. saccharina*''s CT_{min} of 4.4 ℃ ± 0.4 ℃ (Mudavanhu *et al*., 2012). Also, temperature peaks above temperatures where physical activity is regained (Nepgen, 2014) should not occur (*E. saccharina* was observed to constantly regain activity around 7 ℃ during trial conduction (Serfontein. Unpublished data) – This threshold must be further investigated), and consequent damaging occurs. The Coleman 48 Quart Chest Cooler with its bottom and sides lined with ice bricks showed a constant reduction in temperature to a minimum of 6 ℃ whereafter a gradual increase was observed for the remainder of the experiment This is similar to that observed by Nepgen (2014). Thus, although suitable for short periods, a prolonged cold chain of up to 48 hours would need a better temperature regulating carrier than the tested cooler and ice bricks. Seasonal temperature changes will also impact the effectiveness of such a transportation carrier as observed by Nepgen (2014). The transportable chest freezer showed a decrease in fluctuations from its calibrated 5 ℃ when fully filled with bran packs versus when empty. This indicated that bran could serve as a insulating buffer against temperature fluctuations, improving the reliability of the carrier. Nonetheless, both the bran packed, and empty freezer temperature curves showed pronounced temperature fluctuations which could risk either exposure to below CT_{min} or above temperatures where *E. saccharina* will regain activity and damage themselves if still in packaging.

Controlled exposure to extreme temperatures could also possibly increase moth fitness after field release in extreme temperatures as observed for *C. pomonella* by Chidawanyika and Terblanche (2011). This effect must be better investigated for *E. saccharina* before considering incorporation into the cold chain. Future transportation carriers (e.g., refrigerated canopies on utility vehicles) must also be investigated for temperature fluctuations by measuring temperature over time curves for the empty and fully stocked carrier.

Identifying best temperature insulating packaging substrate

Nepgen (2014) and Blomfield *et al.* (2011) both transported moths in cooler boxes containing ice packs during long distance transport of *T. leucotreta* and *C. pomonella* with focus on ensuring a maintained cold temperature. Nepgen (2014) showed that this method of packaging risks unwanted increases in temperatures during the hot South African summer seasons. It is therefore important to consider the resources necessary that would provide an optimal cold chain. The management of food cold chains is receiving increasing research attention to reduce wastage (Ndraha *et al.*, 2018). Vreysen *et al.* (2016) identified improved handling transport and release methods as a focus area for lepidopteran SIT programs. Aligning future lepidopteran SIT research with that of food cold chain principles could use established knowledge from this field that could help in preserving live moths as is being done for “live” fresh produce. A focus of the cold chain is to maintain a constant temperature in order to reduce stress and degradation in quality. One strategy of reducing temperature fluctuations is by using insulating materials such as the polyurethane cooler boxes (Nepgen, 2014). Filling

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containers with a substrate with insulating properties could further improve the cold chain by insulating packaged moths against temperature fluctuations. For the transport of rock lobsters a maintained cold chain is critical in preserving freshness, and for this purpose saw dust is used as an insulating buffer (Walker *et al.*, 1994).

Effect of packaging substrate on male moth fitness during high density packaging

This study confirmed Dick (1945), Betbeder-Matibet *et al.* (1977) and Walton's (2011) results that male *E. saccharina* can mate more than once in the laboratory. The effect of mating on male *E. saccharina* longevity is unknown, but mating could possibly decrease longevity as was observed in male *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae) (Clutton-Brock, 1997). In this study, male longevity measured in conjunction with male mating frequency did not serve as a sensitive indicator of male fitness across treatments as no significant decreases were observed, but this result could be different when tested separately from mating frequency tests (Clutton-Brock, 1997). The ability of male *E. saccharina* to mate more than once has important implications for calculating overflooding ratios for SIT (Walton, 2011). For SIT compatibility, it is not essential, but less ideal that females are monogamous (Barclay, 2005). Walton (2011) showed in her study that 56.7% of females only mate once within lab conditions where virgin males are provided. This trend together with the short lifespan of *E. saccharina* females of five to seven days during which mating, and oviposition must take place, makes it unlikely that female mating frequency will have any considerable effect on the success of a SIT program (Atkinson, 1981; Walton, 2011).

The effect of bran on increased male mating frequency after prolonged (72 hours) cold storage at 5 °C could be due to the temperature insulation effect as compared to no substrate conditions. Packaging substrate could also provide a cushioning that protects against physical damage during physical activity when the cold chain breaks to above a threshold temperature or shaking caused during handling and transport. Such debilitating physical damage observed by Nepgen (2014) for shipments .of *T. leucotreta* during increased temperatures during transport. It was also observed that when removing moths from cold storage during high ambient RH, moths not packaged in bran were wet due to condensation whereas bran-packaged moths were kept dry by bran absorbing moisture. This moisture absorbing quality of bran (and possibly also vermiculite) could be advantageous in the humid sugarcane growing regions of South Africa where moths will be removed from the cold chain after transport and released in humid field conditions.

Although male mating frequency was used as a measurement of male fitness with some measurable differences between treatments when exposed to various simulated handling, storage and transport conditions, this prolonged and labour-intensive measure is not practical for use during full scale implementation as it would take about a week to obtain the results of one release shipment. Presuming that a bi-weekly release program will be followed, this will mean that inferior quality males could be released multiple times before quality issues are detected. It will take more time to confirm that any identified and rectified issues in production or

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during the cold chain process were effective in restoring male quality at release. For this reason, a real time quality control (QC) measure must be developed.

Effect of packaging substrate during simulated transport on male moth longevity

When calibrating the transportable chest freezer at 5 ℃ and placing 250 males packaged in 250 mL paper cups with lids respectively with no bran and bran for 72 hours, no significant difference in mating frequency was observed between bran and no bran treatments. The mating frequency of 1.833 females mated for the bran treatment and 1.0667 females mated for the no bran treatment in the chest freezer is comparable to the no bran at 5 ℃ for 72 hours incubator treatment (1.467 females mated). The significantly higher mating frequency for the incubator bran treatment at 5 ℃ for 72 hours of 2.633 females mated indicated that temperature fluctuations in the current transportable freezer are too great and that bran is not adequate as a temperature buffer in these refrigerated conditions. This result highlights the importance of monitoring temperature curves over time of possible transportation carriers. Monitoring the cold chain temperature during commercial SIT programs will also provide information as to the expected fitness of sterile moths at their release point (Nepgen, 2014).

Investigating the use of a visual observation rating system as a quality control method for high density packaged and cold stored *E. saccharina*

Conventionally, a grid of pheromone traps in the field is used for the collection of dispersal data of sterile males such as *T. leucotreta* (Moore, 2011). This is also used as a further field quality control measure to indicate male fitness. Unlike other commercial lepidopteran SIT programs, there is no trapping method to record dispersal of released *E. saccharina* males. Only destructive monitoring of sugarcane stalks, and collecting and comparing immature life stage numbers found, and recording stalk damage (Rutherford, 2015). However, these measures are a delayed effect of the sterile insect releases (recapture data from sterile males versus data from infestations by following generations) and could be too late as a QC parameter to respond to and could mean that multiple releases were completed in vain due to inadequate male quality. Furthermore, percentage infestation is not a direct measure of released male fitness, but a combined result of environmental conditions and multiple cultural and other Integrated Pest Management (IPM) practices (Rutherford, 2015). The aim of an Area-Wide Integrated Pest Management program, that incorporates the SIT, is to decrease and continually suppress infestation (Dyck *et al*., 2006). The role of dispersal of *E. saccharina* males in obtaining a mate is also not well understood, as males aggregate to form leks shortly after emergence to call and compete for female mates (Atkinson, 1981). Carpenter *et al.* (2012) and Visser (2015) established the use of flight cylinder bioassays to measure *C. pomonella* flight ability in the laboratory before releases, as an alternative to recapture data. This cylinder system, whereby adult moths are placed in cylinders of various heights and widths (increasing difficulty for moths to escape higher, narrower cylinders) to record the ratio of escaped moths over time for each cylinder did not work when tested for *E. saccharina*. Adequately fit *E. saccharina*

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males placed in cylinders did not attempt to escape and disperse but rather started with their distinctive lekking behaviour inside the open cylinders (Serfontein. Unpublished data).

For this reason, a visual rating system was conceived whereby males were sampled from packaging after transport. In this case after exposure to 5 °C in an incubator for 72 hours whilst 250 individual males were packaged respectively in both bran and no bran inside 250 mL paper cups with lids. From the same treatment that males were visually rated, another 30 males per treatment were sampled to measure mating frequency and longevity. Although there was no significant difference between bran and no bran packaged moths when measuring longevity, bran packaged moths had significantly increased mating frequency. The significant differences for all visual parameters that rated bran packaged moths as less damaged (less loose scales and less scale loss from pronotum) versus no bran packaged males indicated that there may be a relationship between visual scale loss/cover and male fitness. The use of such a visual rating system, for sampled moths at the release point after handling and transport, together with the standard SASRI Entomology Department Insect Rearing Unit mass rearing quality control parameters (pupal weight, sex ratio, and rate of development) (Woods *et al.*, 2020) could be a valuable additional quality control parameter to observe for possible physical damage that could occur during the handling process. \

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3.5. CONCLUSION

Cold exposure over time (5 ℃ at 24, 48 and 72 hours) showed that male mating frequency decreases at 72 hours and thus the holding period at 5 ℃ must ideally be limited for up to 48 hours to still provide good male quality in terms of mating frequency. The transportable chest freezer tested proved to cause too much temperature fluctuation for even the insulating bran treatment to protect male fitness up to 72 hours, as was shown for the bran treatment at 72 hours at 5 ℃ for the incubator treatment. For the chest freezer, overall mating frequencies of bran and no bran treated moths were much lower than was observed for similar treatments conducted in a lab incubator.

Commercial Snowflake wheat bran was identified as an affordable substrate which could be used for packaging *E. saccharina* at high densities in terms of being a temperature insulating agent when compared with two texture grades of vermiculite and a blank control. Wheat bran could also serve as physical protection against shaking (e.g., on road transport) for active moths (during a break in the cold chain) during high density packaging. The current method of incorporating bran into the high density packaged moths were by filling the rest of the 250 mL paper cup with bran after placing 250 male moths into the paper cup. Future work can investigate the ratio of moths:bran in order to make use of the beneficial properties brand provides, whilst minimizing wastage and maximizing the number of moths per shipment.

Visual observations can further be investigated as an effective additional measure of male fitness at the point of release in terms of physical damage that could have occurred during the cold chain period.

This study can be refined by establishing the optimal size of a commercial container (also considering the number of moths contained) that could replace paper cups and lids, as well as the best ratio of bran:moths that will optimize usage of space whilst providing the measured benefits to male moth fitness from this study.

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CHAPTER 4: GENERAL DISCUSSION AND RECOMMENDATIONS

4.1. INTRODUCTION

It is important to put focus on the retention of adult quality (in the form of male compatibility to wild females; and competitiveness with wild males) throughout the mass rearing, irradiation and pre-release handling steps of *E. saccharina* SIT. Walton (2011) established that the mass-reared *E. saccharina* from SASRI are suitable candidates for the further development of a SIT programme using F1 male sterility, as female parents were rendered fully sterile at 200 Gy irradiation exposure and males were partially sterile. Mudavanhu (2012) further concluded that laboratory reared *E. saccharina* males currently produced at the SASRI insect rearing unit are suitable for use in SIT-based projects as they are compatible with wild females for mating, and competitive with wild males for wild female mates. There is little literature available on the effect of handling (collection and packaging) and transport of adult lepidopterans for the SIT (Blomfield *et al*., 2011). Boersma and Carpenter (2016) did confirm the finding by Nepgen *et al.* (2015) that the rapid chilling of *T. leucotreta* reduced male dispersal ability but was a necessary step to immobilize moths for high density packaging for irradiation and transport. Focus must now be set on developing a robust collection, handling, and transport parameters to preserve irradiated male competitiveness up until release in the field. Collection, handling, and transport conditions comparable to the commercial processes in various lepidopteran SIT programs were used for FCM (Nepgen *et al.*, 2015). Vreysen *et al.* (2016) identified both improved handling transport and release methods, and practical and effective methods for field quality assessment as two of the key issues to be further addressed by the Coordinated Research Project (CRP) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture entitled “Increasing the efficiency of Lepidoptera SIT by enhanced quality control”, which commenced in 2018.

Sex separation is another method of optimizing the handling and transport process by excluding the less functional females for SIT (Steinitz *et al.*, 2016). When packaging *E. saccharina* males and females at a 50:50 sex ratio, females would take up more than half the volume due to their larger mean weight. It is therefore necessary to pursue sex separation methods that can be automated for large scale use in a commercial SIT program.

To ensure quality and success of the SIT, it is crucial to assess potential quality degradation during each step of production, handling, processing, and release of the sterile insects (Boersma and Carpenter, 2016). Conventional field quality control (QC) methods for measuring male competitiveness from release onward relies on the collection of dispersal data from the field using pheromone or bait traps (e.g., *T. leucotreta* (Chidawanyikaand Terblanche, 2011) and *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Barry, 2004)). There are no established methods for capturing *E. saccharina* males nor females for monitoring purposes (Rutherford *et al.*, 2009). Also, *E. saccharina* is a poor dispersing insect (Rutherford, 2015) and the role of dispersal in mating is not well documented (Atkinson, 1982). Adjusted from the visual observations

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by Nepgen (2014) to determine the quality of transported *T. leucotreta* by investigating for the occurrence of damaged wings, a visual observation method was established in the current study to observe physical damage of *E. sacccharina* as an indicator of male quality (in terms of competitiveness) at the release point. This observation system entailed inspecting various bodyboard surfaces for the severity of either scale loss, or loose scale cover (i.e. scale loss from pronotum; loose scale cover on the eyes, the ventral surface and the dorsal surface).

4.2. SEX SEPARATION

Achieving a sex separation method is advantageous for an SIT program, as packaging only males for irradiation, transport and release frees up space and allows for more males to be "processed" per volume of storage/packaging/irradiation (Papathanos *et al*., 2009). Separation methods were thus investigated for the separation of females from males, so that they could again be added back into the broodstock colony for increased production efficiency, but also to maximise the collection of males for irradiation and release purposes to conserve resources (Steinitz *et al.*, 2016). Transgenic sex separation methods that enable the acquirement of males only from the neonate larval stage will also free up considerable space and resources for production of males for the SIT, but are not readily available for Lepidoptera, as transgenic methods require intensive research and development (Knipple, 2013). They should, however, be considered in the future *E. saccharina* SIT program, as knowledge on this approach for Lepidoptera develop.

In this study, however, the use of low irradiation dosages on parent pairs in order to obtain males only was investigated, based on preliminary work by Walton (2011). Her trials showed that exposing male and female *E. saccharina* to 100Gy irradiation levels resulted in sterile F1 offspring. However, survival of offspring was extremely low in her trial, and thus the results open to question. However, if the results could be replicated, with higher survival of the F1 offspring, this approach could provide similar advantages as those obtained from transgenic methods (Knipple, 2013).

Weight differences between adult male and female *E. saccharina* were investigated as a possible parameter for developing an adequate large-scale sex separation technique for use in a commercial SIT program. Male and female *E. saccharina* have distinctly different weights with heavier males rarely reaching the weight of smaller, lighter females. Females exiting the collection box at the same speed as males (10 m/s) will have greater momentum and simulations have shown that this momentum will cause females to touch down at the bottom of the collection tray further than the lighter males. This can be confirmed in practice and possibly exploited in order to achieve male biased moths for irradiation and release, which would not only improve efficiencies for the SIT by freeing up space that would have been occupied by females during packaging, irradiation and transport, but also providing females that could be used to replenish the broodstock colony.

There is evidence that the release of sterile females with partially sterile males can make a SIT programme more effective than releasing males only (North and Holt, 1971; Nguyen Thi and Nguyen Thanh, 2001; Hight *et al*., 2005). Sterile females act as a sperm sink by being mated with wild males, which then take them out of the competition with F1 partially sterile males for wild females (Hight *et al*., 2005). The fact that female

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E. saccharina searched for males that are lekking close to their larval food source (Atkinson, 1982), may enhance this possible positive effect. This aspect, by releasing sterile females during a SIT program, must be further studied in order to determine if the value added by sterile female releases to the success of the *E. saccharina* SIT program is worth including them within the handling, transport and release system.

F1 sex ratio of irradiated *Eldana saccharina* adult pairs

Walton (2011) observed that when 100 Gy irradiated male and female *E. saccharina* were crossed (5 pairs), a 100% male first generation progeny was obtained that survived to adulthood. Unfortunately only one F1 male from three original neonate larvae inoculated onto feeding media survived. Replication of this experiment with 10 parental pairs in this study (Chapter 2) produced a total of 24 surviving F1 adults (compared to 522 for the control) and 83 % were male. If surviving F1 females are fully sterile (this must be investigated), this could be a viable method of obtaining multiple semi- (or fully) sterile F1 males that can be transported as pupa to release sites due to sterility being inherited and thus irradiation of the adult moth would not be necessary. Fitness and sterility of the surviving F2 offspring is unknown.

Difference in Eldana saccharina male and female pupal weights

When limiting the collection weight of *E. saccharina* pupae to 0.085 g, 100% conventional lab and rabbit pellet reared males were collected (Chapter 2), and no female pupal contamination took place for the conventionally reared *E. saccharina*, because the females were always heavier than this set weight. This high level of separation using morphological differences between sexes is more accurate than the level of separation obtained when using pupal length for *L. botrana* sex separation. Steinitz *et al.* (2016) limited pupal length to 5.4 mm to separate sexes for this species, and collected 86 % of all males and 22 % of all females. Even though the separation was not complete for one sex, Steinitz *et al.* (2016) concluded that this level of separation would still provide great benefit in supplementing the broodstock with females and releasing mostly sterile males.

Separation of *E. saccharina* by weight is thus a viable option to obtain adults of just one sex. Operational SIT programs use different air collection systems (Chapter 2) to harvest their adults emerging from pupae, which, with further development to take cognisance of the *E. saccharina* adults weights, could provide a very adequate separation of sexes for an operational SIT program.

Reared adult *C. pomonella* (Linnaeus) (Lepidoptera Tortricidae) and *P. gossypiella* Saunders (Lepidoptera; Gelechiidae) are collected using a cyclone system, where newly eclosed, heavier adults quickly lose speed after collection in the suction air stream and the lighter, looser scales are separated from the adults (Stewart, 1984; Wolf and Stimmann, 1971). Such a gravitational separation method could be calibrated to separate adult male and female *E. saccharina*, as males are on average about half the weight of females (0.070 g versus 0.133 g; Chapter 3). If this method of separation cannot fully separate males and females, separation must be

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calibrated to be biased toward obtaining 100 % males, allowing a level of female contamination, in order to irradiate as many males as possible, without the added expense, space and time taken up in additional female irradiation, to achieve the highest possible and cost effective sterile to wild male overflooding ratio. Gravity separation is commonly applied when separating components in a mixture that have differing specific gravities and is commonly used in areas such as mining and grain harvesting and processing (Falconer, 2003). These various methods can be examined with the relevant expertise, when further investigating separation of male and female *E. saccharina* by exploitation of weight differences.

The other used collection system, which can be used to additionally separate *E. saccharina* adult sexes as described above, is a plenum-based system (Hofmeyr and Pretorius, 2010), which was tested in this study (Chapter 2), and is discussed in more detail below. The simulation showed that females travel further after exiting the collection box at 10 m/s when considering weight differences (ie. heavier females provide more momentum than the lighter males). This simulation can be refined by including accurate surface areas that experience drag. This simulation must also be confirmed in practice.

4.3. EFFICIENCY OF CURRENT COLLECTION BOX

The prototype plenum system collection box tested in this study is based on that described by Hofmeyr and Pretorius (2010) that was developed for the collection of *T. leucotreta*. The cyclone type collection system used for the collection of *C. pomonella* and *P. gossypiella* was not suitable for collecting *T. leucotreta* as the bends in the cyclone system damaged adults and caused a build-up of fatty deposits that rendered the system ineffective (Hofmeyr and Pretorius, 2010). The plenum type system was effective in collecting adult *E. saccharina* placed inside the eclosion box over a 12-hour period.

The less-than-one day old *E. saccharina* adult females that were used for testing the box were proven to have mostly mated prior to being placed in the emergence box it is thus important to ensure mating does not take place during the short period after eclosion up to collection into collection funnels should this system become part of an operational SIT system for *E. saccharina*. Although *E. saccharina* males can mate multiple times, mating before collection could impact longevity as observed with male *Glossina morsitans* Westwood (Diptera: Glossinidae) (Clutton-Brock, 1997). The effect of such mating on male mating ability during lekking whilst competing against virgin males are also not known. Therefore, one should take caution against possible mating within the emergence box before collection until its effects are better understood.

The eclosion box can be further improved by reducing coarse areas and corners where adults can grip and stay perched for prolonged periods of time. Such improvements could be by using less separate metal sheet parts, but rather folding a single sheet of metal to provide an almost totally smooth inside of the collection box. Another option is to conduct proper welding and sanding of any jointed metal sheets instead of relying on the use of ribbiting.

The plenum collection box must then further be tested by fully filling the box with plastic emergence trays containing pupated *E. saccharina* within feeding media, as will be done commercially, to collect newly

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eclosed adults with the plenum collection system must be assessed for mating status in order to take into account possible effects of such mating taking place.

4.4. EFFECT OF EXPOSURE OF ADULT MALE *ELDANA SACCHARINA* TO IMMOBILIZING COLD TEMPERATURES OVER PERIODS OF TIME ON MATING FREQUENCY AND LONGEVITY.

When exposing male *E. saccharina* to 5 ℃ for up to 48 hours, no significant difference in mating frequency and longevity was observed (Chapter 3). Mating frequency did decrease significantly after 72 hours though, indicating the limit for cold exposure to be between 48 and 72 hours to maintain the stored males mating competitiveness. This timeframe is important when planning sterile male release frequencies in pilot and eventually operational SIT programmes, as being under resourced and forced to hold adults for periods longer than 48 hours at 5 ℃ will decrease moth quality as was shown in this study in terms of mating frequency.

Based on the negative impact that exposure to 5 ℃ for 72 hours had on male mating frequency, it is recommended that planning of the *E. saccharina* SIT program must accordingly limit the adult holding period at 5 ℃ to 48 hours. The holding temperature must also be adjusted for irradiated adults due to the increased CT_{min} observed when exposed to increased irradiation temperatures (Mudavanhu, 2012)

There have been studies on larval thermal acclimation with the aim of enhancing performance in variable thermal environments (Hochachka and Somero, 2002; Angilletta, 2009) that show significantly increased survival and persistence of mass-reared sterile insects when released into field conditions with impacting high/low temperatures. This pre-exposure to sub-lethal temperature environments is referred to as rapid cold-hardening or (rapid heat-hardening) and could enable mass-reared strains of adult *E. saccharina* to survive otherwise lethal ambient temperatures (Loeschcke and Hoffmann, 2007; Slabber and Chown, 2005). Such temperatures in the field is less of a concern within *E. saccharina's* natural range (Walton, 2011), but this is not only applicable to field conditions but also to the tolerance of adults to being kept in the cold chain at sub-lethal low temperatures for prolonged periods of time. This capability of insects to adjust their thermal tolerance by acclimatization is termed phenotypic plasticity (West-Eberhard, 2003). It would be worthwhile to study this for *E. saccharina* as also recommended by Mudavanhu (2014).

4.5. COMPARING TEMPERATURE OVER TIME OF POSSIBLE TRANSPORTATION CARRIERS

When measuring temperature over time in different transportation carriers (Chapter 3), it was apparent that using a cooler with ice bricks will be logistically challenging as the optimal temperature of 5 ℃ is only maintained for a short period before increasing to a point where adult activity will resume, and damage will occur within high density packaging. Adult activity was observed to resume around 7 ℃ in this study, but this must be confirmed with future studies. The transportable freezer showed more temperature fluctuations

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than the incubator that had almost no fluctuations. These fluctuations were shown to be tolerable to male *E. saccharina* when comparing mating frequencies between incubator and freezer-stored adults for 72 hours. The transportable freezer was thus identified as an adequate transportation carrier. For any new transportation carrier that are considered, a temperature over time curve must be measured as in this study in order to measure the achievable consistency in the cold chain.

4.6. IDENTIFYING BEST TEMPERATURE INSULATING PACKAGING SUBSTRATE

Bran, course and fine vermiculite showed an increase in measured temperature buffering compared to air (Chapter 3). Bran was the best insulator measured and is cheap and biodegradable (Naveena *et al.,* 2005). The effect of bran when packaging adults at high densities were thus investigated further. Vermiculite also proved to provide insulating qualities and different texture grades can be compared to bran as a packaging substrate in future with cost considered together with efficacy. It is important to keep this packaging substrate in the cold room where adults are packaged, because warm substrate will reheat adults and take long to reach the cold ambient temperature as measured in this study. Using a packaging substrate will also protect adults against physical damage when shaking during handling and transport, when physical activity occurs during a break in the cold chain (Nepgen, 2014). It was also observed that bran acts as an effective moisture buffer, absorbing any condensation when adults are removed from the cold chain, preventing adults from becoming wet and subsequently being impeded from normal physical activity.

4.7. EFFECT OF PACKAGING SUBSTRATE ON MALE MOTH FITNESS DURING HIGH DENSITY PACKAGING

The identified optimal temperature buffer, bran, was further tested as a packaging substrate during adult high density packaging. When comparing mating frequency of high density packaged male *E. saccharina* (250 males in a 250 mL paper cup with a lid) in bran versus no bran, mating frequency was shown to significantly decrease after 72 hours at 5 °C for the no bran packaged male adults but not for the bran packaged adults (Chapter 3). This showed that bran packaged male adults can last longer in the cold chain versus no bran packaged male adults. The low plasticity in longevity that has been observed throughout this study has also been shown for other lepidopterans, e.g. *C. pomonella* (Bloem *et al.*, 2006) and *T. leucotreta* (Nepgen *et al*., 2015). When repeating this experiment, but without the high-density factor, it can better be determined if improved quality over time is attributable to temperature buffering, or protection against physical damage caused by high density packaging.

Throughout this study, longevity did not change between treatments within experiments and can thus be excluded as a possible measure of male fitness when measured in conjunction with mating frequency.

The mating frequency data could be reassessed for the percentage males mated in the first day as this could indicate the ability of males to regain a competitive level of activity before being predated upon or killed by

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other environmental factors after release. Preliminary reporting on this study showed that males exposed for 24 and 48 hours mated with a much larger proportion of females once back at 25 ℃ (87 %) compared to those exposed for 72 h (50 %). On the first night, 93 % of males mated in the untreated control treatment (Serfontein *et al.*, 2016). Another important benefit of measuring percentage first night mating success as a quality control measure would be that results can be obtained within one day, which enables rapid response to any reduction in sterile male competitive quality. This must be further investigated when improving on this study.

Mudavanhu (2012) observed that males which started with calling behaviour earlier generally also subsequently mated earlier. Males from the laboratory strain mated earlier than wild counterparts as also observed for *C. capitata* (Boake *et al.*, 1996) and this phenomenon was attributed to selection pressures within overcrowded mass rearing conditions (Briceno and Eberhard, 2002). This early mating success was observed to decline between 150 Gy treatments and 200 Gy treatments. Under field conditions, early mating behaviour by *E. saccharina* would be desirable for the SIT program as wild females would be more likely to mate with early calling sterile males with wild males more likely to miss their opportunity to mate (Mudavanhu, 2012). Thus, there is real promise in developing a quality control method that relies on measuring first day mating success of sterilized males that are sampled at their point of release for the SIT.

4.8. EFFECT OF PACKAGING SUBSTRATE DURING SIMULATED TRANSPORT ON MALE MOTH FITNESS

When calibrating the transportable chest freezer at 5 ℃ and placing 250 males packaged in 250 mL paper cups with lids respectively with no bran and bran for 72 hours, no significant difference in mating frequency was observed between bran and no bran treatments. The mating frequency of 1.833 females mated for the bran treatment and 1.0667 females mated for the no bran treatment in the chest freezer is comparable to the no bran at 5 ℃ for 72 hours incubator treatment (1.46 females mated). The significantly higher mating frequency for the incubator bran treatment at 5 ℃ for 72 hours of 2.633 females mated indicates that temperature fluctuations (as observed in chapter 3) in the current transportable freezer is too great and that bran is not adequate as a temperature buffer in these refrigerated conditions. This result highlights the importance of monitoring temperature curves over time of possible transportation carriers. Monitoring the cold chain temperature during commercial SIT programs will also provide information as to the expected fitness of sterile moths at their release point (Nepgen, 2014).

The incubator provided the optimal constant temperature when set at 5 ℃. The temperature fluctuations in the transportable freezer is considered too great and proves to cause reduced mating ability when comparing the same experiment conducted in the incubator. Thus a better transportation carrier should be sought which provides a more constant temperature over time curve.

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4.9. INVESTIGATING VISUAL OBSERVATIONS AS QUALITY CONTROL METHOD

Bran packaged moths had significantly less damage in the form of loose scale cover and scale loss (Chapter 3). This result correlated with the significantly higher mating frequency observed from the males held in bran (Chapter 3). This rating system can now be conducted in future treatments for comparing with other moth fitness measurements (e.g. first day mating percentage as recommended in this chapter). A simpler visual observation approach was also used by Nepgen (2014) where he only observed the percentage moths with visible damage to wings. This visual ratings would only provide information of physical damage caused which could impact moth fitness and it is therefore important to include other quality control parameters to measure for any other physiological stress that could occur during handling and transport (e.g., cold temperature stress will not cause scale loss but will impact male fitness).

When fully implementing the SIT, continual assessment of visual ratings will also set accurate standards and any deviation from this standard may indicate that there is a problem in the cold chain.

4.10. CONCLUSION

The results in this study provide new information for using the newly established parameters and given recommendations for the collection, sex separation, packaging, and cold storage/transport of *E. saccharina* from a mass rearing and irradiation source up to release points along the sugarcane growing belt of South Africa. The further development of these steps that will be built on the results obtained in this study will be vital for the success of a commercial SIT program for *E. saccharina*. Not only must future work be focussed on optimizing male competitiveness at release, but overall optimization of these processes to achieve a cost effective and successful SIT program is vital.

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5. Appendix 1

The Matplotlib v 3.3.3. script used to simulate as mentioned above (Chapter 2) is:

```
import matplotlib.pyplot as plt
import numpy as np

dt=0.001

class mot:
    def __init__(self,m,c,A):
        self.m=m
        self.c=c
        self.A=A
        self.x,self.y=0,1.5
        self.vx,self.vy=10,0
        self.ax,self.ay=0,0
        self.fx,self.fy=0,0
    def simulate(self,dt):
        xar=np.array([])
        yar=np.array([])
        while self.y>0:
            fy=0
            fx=0
            fy-=9.81*self.m
            fy+=0.5*1.204*(self.vy**2)*self.c*self.A
            fx-=0.5*1.204*(self.vx**2)*self.c*self.A
            self.ax=fx/self.m
            self.ay=fy/self.m
            self.vx+=self.ax*dt
            self.vy+=self.ay*dt
            self.x+=dt*self.vx
            self.y+=dt*self.vy
            xar=np.append(xar,[self.x*100])
            yar=np.append(yar,[self.y])
        return (xar,yar)

mannetjie=mot(0.07*(10**-3),0.85,5*10**-4)
wyfie=mot(0.1332*(10**-3),0.85,5*10**-4)
plt.ylabel("Height(m)")
plt.xlabel("Distance(cm)")
(xar,yar)=mannetjie.simulate(0.0001)
plt.plot(xar,yar,'-g',label='Males')
(xar,yar)=wyfie.simulate(0.0001)
plt.plot(xar,yar,':b',label='Females')
plt.legend()
plt.show()
```