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Packaging and Shipping of Irradiated Mexican Fruit Fly (Diptera: Tephritidae)-Infested Citrus Fruits for Detector Dog Training

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ABSTRACT

The United States Department of Agriculture's National Detector Dog Training Center (NDDTC) mission is to inspect passenger baggage, cargo, and parcels to detect prohibited agricultural commodities. Detector dogs can locate and help prevent the spread of invasive insects, including the Mexican Fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), which infest fruit and are detrimental to the citrus industry. Training detector dogs with a target insect species not endemic to the region where the canine training occurs poses a problem. The Science and Technology Insect Management and Molecular Diagnostics Laboratory (IMMDL) located in Edinburg, Texas prepares and packages shipments of *A. ludens* larvae-infested fruit which are then irradiated to provide training aids for the NDDTC in Newnan, Georgia. *Anastrepha ludens* larvae were collected and placed with forceps into grapefruit using 20 2nd instar larvae per fruit. Two to three days post inoculation, the infested fruit were irradiated at ~140Gy dose from a ^{60}Co source to safeguard and to ensure sterile larvae. The fruit was packaged in specially designed sealed PVC canisters to prevent larvae from escaping during transport. Two types of shipping containers, a styrofoam box and Igloo™ hard-sided ice chest, were compared for the survivorship of living larvae during transportation. A total of thirty-three shipments containing training aids were sent. The styrofoam box had a survivorship rate of 81.5 ± 10.9% across 22 shipments, while the Igloo™ hard-sided ice chest had a survivorship of 92.3 ± 3.3% across 12 shipments. The ice chest increased survivability and overall fitness of the larvae during transit.

Additional index words: Mexican Fruit Fly, Detector Dogs, Gamma Radiation

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The Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), is a serious recurring invasive pest to agricultural in the southern United States (Dupuis et al. 2019, Thomas and Mangan 2005). *Anastrepha ludens* has the potential to be transported and possibly become established in the United States through imported fruits, such as citrus, mango, and avocado from various native regions in Mexico and Central America (Sequeria et al. 2001). Infestations of *A. ludens* can trigger quarantines that can cause millions of dollars in additional cost to growers (Conway et al. 2019) through increased production costs, loss of market opportunities, and fruit damage (USDA-APHIS 2008). The developing *A. ludens* larvae inside the fruit are a severe threat since the larvae are naturally protected from surface-applied insecticides (Heve et al. 2016) and difficult to detect at ports of entry and inspection stations (Thomas and Hallman 2011).

Targeted screening using detector dogs can potentially reduce the number of wild flies' introductions and help prevent the spread of invasive pests that are destructive to native ecosystems and local agricultural commerce (Moser et al. 2020). Detector dogs have demonstrated the ability to accurately detect a variety of materials, such as invasive brown tree snakes (Engeman et al. 2002), bed bugs (Pfiester et al. 2008), red imported fire ants (Lin et al. 2011), and explosives through the dog's olfactory system (Brooks et al. 2003). Most insect-detection dogs are trained with live targets for all, or part, of the training process (Lehnert and Weeks 2016). However, training dogs to detect an exotic insect species not endemic to a region poses a problem (Moser et al. 2020).

The primary goal of the United States Department of Agriculture's National Detector Dog Training Center (NDDTC) in Newnan, Georgia is to train detector dog teams to find prohibited agricultural items. In May 2018, NDDTC began exploring the use of detector dogs to detect *A. ludens* larvae inside citrus fruit. Two pilot studies conducted at S&T Insect Management and Molecular Diagnostics Laboratory (IMMDL) demonstrated that a trained canine could detect the

odor of Mexican fruit fly larvae inside the fruit. The success of the pilot studies extended into a longer arrangement between the parties. Due to the very low odor profile of the larval stage and the need for the canines to alert on only live larvae, the determination was made that live larvae were the most efficient and effective target for the canine training program. Therefore, NDDTC and S&T IMMDL developed a method to ship live irradiated *A. ludens* larvae within the host fruit to Newnan, Georgia. S&T IMMDL routinely provide NDDTC canine handlers with training aids in the form of infested citrus fruit with sterile *A. ludens* larvae.

This work presents the procedures used in handling and shipping training aids to NNDTC in Newnan, Georgia. Two different shipping methods are compared based on the percentage of irradiated *A. ludens* larvae that survive shipment for training detector dogs.

MATERIALS AND METHODS

Study Insects. The 2nd instar larvae used in this study were obtained from the S&T Mexican Fruit Fly Containment Facility located at Moore Air Base in Edinburg, Texas. The colony originated from infested fruit collected April 2008 during an outbreak of wild *A. ludens* in Willacy County, TX (Thomas et al 2014). Insects were reared on an artificial meridic diet adjusted from Spishakoff and Hernandez-Davila (1968) for use in methods development for the Sterile Insect Technique (SIT) as part of the Mexican fruit fly Preventative Release Program in the citrus-producing regions of south Texas.

Fruit Infestation. From July 2020 to January 2021, twelve grapefruit (*Citrus* × *paradisi* Macfad*)* were harvested weekly from a mature citrus grove located at Moore Air Base (MAB) in Edinburg, Texas. In February 2021, an uncharacteristic freeze for this region occurred resulting in all the fruit freezing and dropping from the trees. The resulting fruit on the ground were not usable as training aids. Beginning in February 2021, fruit used in this work were purchased from local stores. Fruit was selected for uniformity in size and outward ripeness and transported to Arthropod Quarantine Facility at MAB to be triple washed with Dawn Dish Soap™ and rinsed with water to eliminate residues of insecticides, herbicides, and possible pathogens on the rind.

Samples of 100 to 240 2nd instar larvae were obtained from the S&T Mexican Fruit Fly Containment Facility, Edinburg, Texas. The larval samples were collected into a 200ml polyurethane cup (Highland Plastics, Shepherd, MI) mixed with 50g meridic diet and sealed. The sealed polyurethane cup was then placed into a Ziploc® quart sized bag to ensure no larvae escaped. Safeguarding was implemented using a chain of custody document with Permit # P526P-20-02321 when transferring the *A. ludens* larvae from the Fruit Fly Containment Facility to IMMDL Arthropod Quarantine Facility.

Once the larvae and fruit were in the Arthropod Quarantine facility, a 12mm diameter hole was bored approximately 50mm deep with a cork-borer (Cole-Parmer, Vernon Hills, IL) into the center of the fruit to allow artificial infestation of 2nd instar larvae. Twenty larvae per fruit were inserted into the bored holes (Figure 1A) with blunt featherweight forceps (Bioquip Products, Rancho Dominguez, CA). The fruit rind plug (Figure 1B) from inside the borer was placed into the hole and the hole was covered with labeling tape. The labeling tape contains information for each fruit including, the number of larvae infested per fruit and the date of infestation.

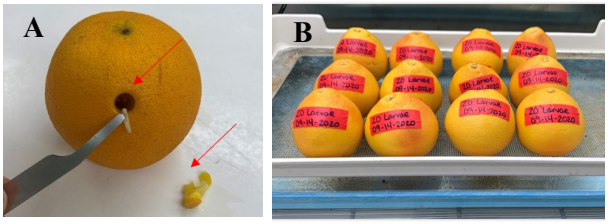

Fig. 1. (A) Second instar *A. ludens* larvae artificially introduced into the grapefruit. The fruit rind plug (arrow) was inserted back into the bored hole to prevent larval escape. (B) Infested grapefruit labeled and stored in an Environmental Growth Chamber for 72 hours.

The unused larvae were autoclaved and disposed. Infested grapefruit were placed onto a screened fiberglass tray (77.2 cm x 40.3 cm x 6.7 cm) (Fiberglass Tray Co., Linesville, PA) stacked on a solid fiberglass tray and stored in an environmental growth chamber (EGC, Chagrin Falls, OH) for 72 hours at 25° ± 1°C and 65 ± 5% relative humidity (Figure 1B).

Irradiation Process. Four to five larvae-infested fruit were placed into a designated radiation bag containing a Sterin™ indicator patch (Ashland LLC, Covington, KY). When exposed to gamma radiation, the Sterin™ indicator patch changes color from red to black (Figure 2 A&B). Each fruit and radiation bag were given a sequential number to ensure all tested material were accounted for and properly irradiated prior to shipping (Figure 2C).

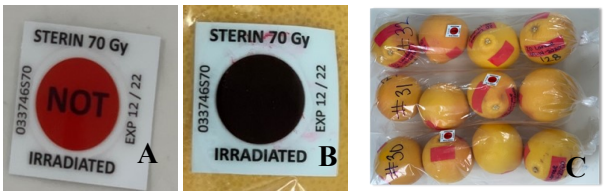

Fig. 2. SterinTM indicator strip on radiation bags. (A) Represents a non-irradiated indicator patch. (B) Represents irradiation at minimum ~70 Gy dose rate from a ^{60}Co source. (C) Infested fruit and radiation bag were labeled with a sequential tracking number.

The irradiation bags containing the infested fruit were

placed into a secondary bag to provide a double seal to ensure no larvae escaped.

Mass rearing personnel placed infested fruit, still within the radiation bags, into metal canisters used to convey material though the irradiator. The fruit filled bags then underwent the irradiation process. Infested fruits were irradiated twice to ensure larval sterility by providing a ~140Gy dose total from a ^{60}Co source. Each radiation bag was confirmed by the color change of the Sterin™ indicator patch going from red to black. The initials of both the Mexican fruit fly irradiation personnel and S&T personnel were documented into a logbook. Irradiated fruit was returned to the Arthropod Quarantine Facility with the same safeguarding procedure as described above. The fruit were packaged in specially designed PVC canisters that were sealed to prevent larvae from escaping the fruit during transportation.

Gamma radiation treatment has been found to be effective in controlling adult *A. ludens* populations, provided the radiation occurs during the pupal stage. For this study, it was crucial to obtain viable larvae that underwent the sterilization process. When the larvae were sterilized within the grapefruit, the larvae did not die immediately. However, when these sterilized larvae underwent the pupation stage, no adult emergence was observed due to the radiation treatment that was administered during the larval stage. (Thomas and Hallman 2011).

Preparing Shipments. The initial shipping container was a styrofoam cooler placed within a cardboard box (0.762m x 0.558m x 0.432m) [30in x 22in x 17in] capable of transporting three PVC canisters (Figure 3A).

Fig. 3. (A) The cardboard shipping container measures (0.762m x 0.558m x 0.432m) [30in x 22in x 17in] with an inner styrofoam box and multiple icepacks. (B)The secondary shipping container (0.635m x 0.457m x 0.457m) [25in x 18in x 18in] for one PVC canister stored in an Igloo™ hard-sided ice chest.

The second shipping container was an Igloo™ hard-sided ice chest (Nostalgia Products LLC, Green Bay, WI) (0.635m x 0.457m x 0.457m) [25in x 18in x 18in] suitable for transporting one PVC canister (Figure 3B).

Larvae-infested fruit were sealed inside the radiation bags and placed into the specially designed PVC containment canisters with lock-tight ends (Figure 4). The PVC canisters contained 0.40mm mesh stainless steel screen allowing air movement but small enough to keep any loose larvae inside the canister. Bubble wrap rolled into bundles cushioned the canisters and prevent them from shifting within the Styrofoam and IglooT^{M} hard-sided ice chest. Packaging reduced movement and minimizes the risk of larvae escaping from the fruit during transportation. Ice packs were placed at the bottom of each cooler to maintain a lower temperature and reduce larvae movement during shipment. Once the packaging was completed, the shipment was weighed to determine shipping costs and transported via United Parcel Service (UPS) – Next Day Air Early™ AM for earliest transport time to the NDDTC.

Fig. 4. The fruit were packaged in specially designed PVC canisters to contain any escaped larvae from the fruit during transportation.

Post-Shipment Process. Once the shipment arrived at the NDDTC facility the following morning (~8-10h transit time), canine handlers inspect the shipping container for any physical damage that might have occurred during UPS transport. The container was opened, and the PVC canisters were removed and placed on a sterile table. The canisters were opened and the sealed radiation bags containing the fruit were removed. The outer portion of the fruit and radiation bag was carefully inspected for any dead or alive *A. ludens* larvae that may have escaped from within the fruit but were still within the radiation bag. The radiation bag was opened, and the irradiated fruit dissected to inspect and collect the larvae.

RESULTS

Survivorship. The styrofoam shipping container had a larval survivorship rate of 81.5 ± 10.9% across 22 shipments. The total amount of larvae provided for training purposes was 4,120 from July 2020 to June 2021. The Igloo™ hard-sided ice chest resulted in survivorship of 92.3 ± 3.3% (t(33)=-3.291:P= 0.0024) across 12 shipments. (Figure 5). JMP 13 (SAS) was used to analyze the T-test values for this study.

The total amount of larvae shipped in the Styrofoam cooler was 4,120 larvae across twenty-two shipments from July 2020 to June 2021. The total amount of larvae shipped in the Igloo™ hard-sided ice chest was 1,200 across twelve shipments from July to December 2021 with higher survivorship when compared to the Styrofoam cooler. The number of dead

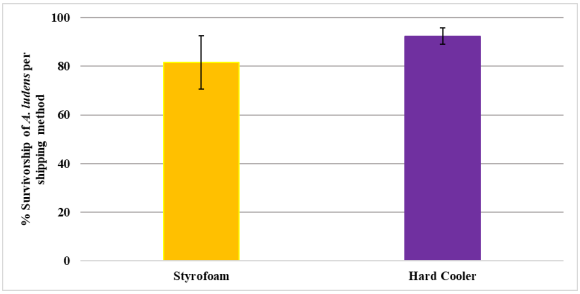

Fig. 5. Percent survivorship of *Anastrepha ludens* for each shipment method. The Igloo™ hard-sided cooler had a survivorship of 92.3 ± 3.3% (t(33)=-3.291:P= 0.0024), while the styrofoam cooler had an 81.5 ± 10.9% survivorship.

larvae also contribute to the number of larvae found outside of the fruit when received by the NDDTC Canine Handlers. The movement of the grapefruit during transit would cause the mortality (Table 1).

Table 1. Percent survivorship of *Anastrepha ludens* per shipment.

Date	Shipping container	# of larvae shipped	# of larvae alive	# of larvae dead	% Survivorship
22-Jul-20	Styrofoam	120	117	3	97.5
29-Jul-20	Styrofoam	120	99	21	82.5
5-Aug-20	Styrofoam	120	90	30	75
12-Aug-20	Styrofoam	120	96	24	80
19-Aug-20	Styrofoam	120	104	16	86.2
26-Aug-20	Styrofoam	240	214	26	89.2
2-Sep-20	Styrofoam	240	199	41	82.9
9-Sep-20	Styrofoam	240	170	70	70.8
16-Sep-20	Styrofoam	240	230	10	95.8
23-Sep-20	Styrofoam	240	140	100	58.3
30-Sep-20	Styrofoam	240	221	19	92
7-Oct-20	Styrofoam	240	206	34	85.8
21-Oct-20	Styrofoam	240	194	46	80.8
28-Oct-20	Styrofoam	240	221	19	92.1
2-Dec-20	Styrofoam	240	229	11	95.4
10-Mar-21	Styrofoam	160	105	55	65.6
24-Mar-21	Styrofoam	160	125	35	78.1
21-Apr-21	Styrofoam	160	147	13	91.9
12-May-21	Styrofoam	160	113	47	70.6
26-May-21	Styrofoam	160	100	60	62.5
9-Jun-21	Styrofoam	160	128	32	80
23-Jun-21	Styrofoam	160	113	47	70.6
18-Jul-21	Igloo hard-sided	100	85	15	85
28-Jul-21	Igloo hard-sided	100	88	12	88
11-Aug-21	Igloo hard-sided	100	85	10	85
25-Aug-21	Igloo hard-sided	100	97	3	97
8-Sep-21	Igloo hard-sided	100	93	4	93
26-Sep-21	Igloo hard-sided	100	100	0	100
20-Oct-21	Igloo hard-sided	100	94	6	94

DISCUSSION

This is the first known study to determine the survivorship of *A. ludens* larvae after shipping. Methodology and protocols were specifically developed to prepare, package, and send *A. ludens* infested and irradiated fruit with the goal of minimal larval mortality during transportation. Science and Technology – Insect Management and Molecular Diagnostics Laboratory provided a total of 321 training aids in the form of larval infested and irradiated grapefruit to the NDDTC. The training aids allowed year-around training thus eliminating the need for costly personnel and canine travel from Georgia to Texas.

In this study, a protocol was developed (Figure 6) to ship citrus that have been artificially infested with Mexican fruit fly larvae to a NDDTC to allow the canines to be trained on living specimens. Sweet oranges, *Citrus sinensis,* were requested by NDDTC for uniformity of size and consistency of host fruit to train the canines. However, we chose grapefruit and oranges because they are commonly grown citrus in south Texas and were readily available (Conway and Forrester 2007).

Artificially infesting fruit resulted in uniform larval sizes and a known number of larvae in each shipment. In contrast, fruit naturally inoculated by multiple female fruit flies results in variably aged larvae and an unknown starting number within the fruit. Artificially infesting the fruit with a known number of larvae allowed the receivers to accurately determine mortality during shipping.

Radiation treatment for *A. ludens* is effective but does not kill larvae inside fruit outright. The larvae die later or during the pupation process, effectively causing the adult *A. ludens* to not develop at all (Thomas and Hallman 2011). Mortality in *A. ludens* from a radiation treatment typically occurs during a major developmental transition, usually one involving ecdysis. Early instars fail to develop into later instars or to pupariate (Thomas and Hallman 2011). The irradiated larvae continue moving for a period after irradiation making them ideal for use as training aids for detector dogs. Thomas and Hallman (2011) found that over a range of sublethal doses of gamma radiation applied to third instar Mexican fruit flies infesting grapefruits, the great majority of treated larvae arrest development at pupal ecdysis, the transformation from a cryptocephalic to a phanerocephalic pupa.

From July 2020 to June 2021, the styrofoam container was used to transport irradiated larvae infested citrus from Texas to Georgia. Frequently, the styrofoam insulation would result in damage during transit. Specifically, the styrofoam insulation would be cracked or crushed (Figure 7). Damage was caused during transit by delivery personnel delivering and/or storing package on the wrong side of box or through rough handling of packages. Damaged styrofoam insulation caused the cool temperatures to rise within the shipping box, allowing the larvae to become mobile and subsequently, resulting in higher mortality due to the mobile larvae escaping and being crushed by the fruit in transit. The high mortality caused by styrofoam damage also resulted in more frequent shipments due to the unreliability of survivorship and frequent replacements of the styrofoam container. A final deci-

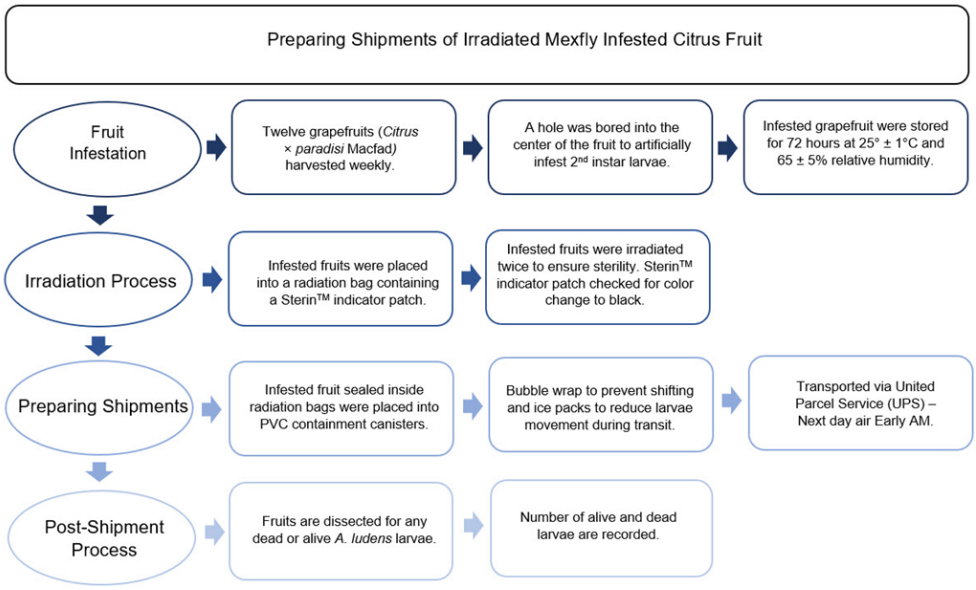

Fig 6. Schematic workflow of steps to prepare a shipment of irradiated Mexfly infested citrus fruits.

sion was made to switch to the Igloo™ hard-sided ice chest instead of the styrofoam container.

Fig. 7. Examples of observed damage to styrofoam shipping container. (A) Several areas of the box damaged when container was delivered standing on short ends. (B) Damage of styrofoam container border in transit.

Shifting to the Igloo™ hard-sided ice chest eliminated the damages experienced previously with the styrofoam container. The size allowed the transport of five fruits per shipment. The Igloo™ hard-sided ice chest had similar shipping costs to the styrofoam shipments due to the weight of the packages. Compared to the Styrofoam cooler, the larvae mortality rate for the Igloo™ hard-sided ice chest was relatively low. The Igloo™ hard-sided ice chest also improved the survivability and reported quality of the larvae received by the NDDTC team.

The research conducted will allow other researchers in areas where *A. ludens* is not endemic to obtain live *A. ludens* larvae to complete research that may contribute to the control and eradiation of wild populations across the southern United States without the risk of furthering the establishment of *A. ludens* populations.

With the success of the Igloo™ hard-sided ice chest, a larger hard-sided ice chest could allow successful transports of more PVC cannisters, thus allowing more fruit and larvae to be shipped at a single time. Future research would investigate the survivorship of *A. ludens* larvae in larger shipments.

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