# OXYGEN CONSUMPTION OF MASS-REARED QUEENSLAND FRUIT FLY *BACTROCERA TRYONI* (FROGGATT) PUPAE IN SEALED PLASTIC BAGS

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## Summary

The sterile insect technique (SIT) has been used for more than 50 years to manage a range of insects around the world. SIT is becoming a major component of many area-wide fruit fly management programs. Irradiation of immature life stages induces sterility in adults, and after a pre-release holding period, the emerged adults are distributed over large areas to mate with wild flies, resulting in no viable offspring. However, irradiation in normal air (about 21% oxygen ($O_2$)) results in declining adult quality. To optimise the quality of sterile adult flies, pupae are sealed in plastic bags for a pre-determined period before irradiation. It was assumed that this improved tolerance to irradiation due to depletion of $O_2$ inside the bags, thereby reducing metabolic activity of the pupae. In our trials, we measured the respiration rates for up to 84 minutes of Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) pupae inside sealed containers at 17ºC and 27ºC and before and after irradiation (four treatments). Respiration was minimised (<1ml.kg$^{-1}$.h$^{-1}$) at approximately 3% and 5.6% $O_2$ at 27ºC and 17ºC respectively. The model indicated that a standard barrier film bag containing 800g bag of Qfly pupae and 100ml headspace would fall to 6% $O_2$ within approximately 3h at 17ºC but only 30 minutes at 27ºC. Irradiation both suppressed and increased variability of $O_2$ consumption, with the greatest effects at 60-65 and 70-75 Gy in atmospheres close to air. This is consistent with previous observations that irradiation disrupts metabolic activity, reducing development rates and increasing variability of eclosion. Suppression of metabolic activity at relatively high $O_2$ partial pressures likely reflects a key role for $CO_2$ in suppressing pupal respiration. Our results are discussed in terms of optimising pupal fitness by reducing metabolic activity during irradiation, while avoiding negative impacts due to extended periods of $O_2$ deprivation.

**Key words***:* insect mass production, insect quality parameters, sterile insect technique, survival, hypoxia, Diptera, Tephritidae

## INTRODUCTION

The Sterile Insect Technique (SIT) is a biological control method for insects and originally postulated by Knipling (1955). SIT has been used in broad-scale management of some of the world's most economically and medically damaging pests (e.g., screwworm, fruit flies, tsetse fly, codling moth, and pink bollworm) (Krafsur 1998). SIT is ecologically attractive because it is highly targeted at a single species and is pesticide free (Hendrichs *et al*. 1983; Fisher *et al*. 1985; Fisher 1996; Suckling *et al*. 2014). Therefore, SIT has become a major component of many area-wide fruit fly management programs and systems approaches focussed on trade (Krafsur 1998; Dominiak 2019; Reynolds *et al*. 2012, 2022). In SIT programs, large numbers of sterile insects are released, overflooding the wild population. The wild population declines because the mating of wild and sterile flies produces no viable offspring (Meats 1996; Shelley and McInnis 2016; Mastrangelo *et al*. 2018). The continued introduction of sterile flies causes the eventual demise of the wild population.

All SIT programs rely on mass rearing facilities to produce large quantities of sterile insects. Standard procedures ensure that the insects produced are consistently high quality (Boller *et al*. 1981; Caceres *et al.* 2007; FAO/IAEA/USDA 1999, 2003). For fruit flies, pupae are the most commonly produced final stage and it is this lifestage that is irradiated and transported to field release centres. Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is the most economically important pest of fruit crops in eastern Australia (Dominiak and Mapson 2017). In 1996, a mass rearing facility was built at the Elizabeth Macarthur Agricultural Institute (EMAI), Camden in southern Sydney, to support SIT (Fanson *et al*. 2014). Procedures included using the bag method to create low $O_2$ atmospheres around pupae during irradiation.

Irradiation of immature life stages induces sterility in adults. However, the process needs to be managed carefully to limit insect stress. Irradiation in reduced $O_2$, or 100% nitrogen atmospheres can reduce formation of toxic free radicals and peroxides, improving insect quality (Ashraf *et al*. 1975; Ohinata

*et al.* 1977). For example, Nestel *et al*. (2007a) evaluated the effect of irradiation on *Ceratitis capitata* (Wiedemann) (Medfly) under different levels of $O_2$. They found that mating competitiveness greatly decreased when pupae were irradiated in open packaging with full access to normal air. In sealed bags, $O_2$ concentrations of 2% and 10% at the beginning of irradiation did not affect the mating competitiveness of males compared to males irradiated under maximum hypoxia. Irradiation in low $O_2$ atmospheres is an accepted method in most mass-rearing and sterilisation facilities (FAO/IAEA/USDA 2003). However, the mechanism still is not fully understood, despite considerable research (Nestel *et al*. 2007a).

Methods of reducing oxygen levels in pupal tissue have been studied in several species. For Medfly, nitrogen flushing and similar techniques were used only where rearing and irradiation occur at the same location (Ohinata *et al*. 1977; Nestel *et al*. 2007a). This equipment is not permitted inside the Australian Nuclear Science and Technology Organisation (ANSTO), Lucas Heights, in Sydney, which is about two hour's drive from the mass rearing at EMAI. Therefore at EMAI, sealing pupae in plastic bags offered a simpler method to decrease the level of $O_2$ in pupal tissue (bag method) (Nestel *et al*. 2007a). Oxygen consumption of Medfly pupae in plastic bags approached zero after 80 min, with the time to reach maximum hypoxia influenced by pupal age and temperature (Nestel *et al*. 2007a).

Dominiak *et al*. (2011) reported on eclosion changes for Qfly pupae held in plastic bags for up to 192h. However, the oxygen levels inside the bags were not tested. For normal operations at EMAI, pupae were simply sealed in plastic bags for 24h and it was assumed that $O_2$ levels were reduced to hypoxic levels before irradiation. Here, we report on tests conducted to evaluate $O_2$ consumption of Qfly at two different temperatures and at four irradiation treatments.

## MATERIALS AND METHODS

### *Trial establishment and management*

Tests were conducted on pupae from production batches between 16 November to 21 December 2011 (for production details, see Dominiak *et al*. 2008; Fanson *et al*. 2014). In each experiment, 800 g of 8-day-old pupae (at the 2-day pre-eclosion stage) were placed into 2L low density polyethylene plastic bags (approximately 150□m thick) and stored at 17°C. The tops of the bags were folded over several times and taped shut, making an airtight seal. About 18h after sealing, the samples were loaded into insulated white styrene foam boxes and transported (about 2 h one-way) in an air-conditioned passenger vehicle to ANSTO, Lucas Heights in Sydney. Previous observations indicated that the temperature inside the insulated boxes did not change significantly during this journey. The bagged pupae were irradiated by a $Co_{60}$ GATRI in-ground gamma irradiator at a nominal dosage of 50-55, 60-65 or 70-75 Gy and then returned to EMAI. Additional sealed 'control' bags were not irradiated or transported to ANSTO, but otherwise stored under similar conditions within the laboratory (approximately 20ºC) during assessments.

**Table 1. Schedule of experiments, the treatments, the number of replications, treatment temperature and time of evaluation.**

| Date | Treatment | Number of replications | Treatment temperature | Time of evaluation (minutes) |
|---|---|---|---|---|
| 16 Nov 2011 | Unirradiated | 4 | 27 | 32 |
| | 60-65 | 6 | 27 | 38 |
| | 70-75 | 6 | 27 | 38 |
| | Unirradiated | 6 | 17 | 84 |
| 23-24 Nov 2011 | Unirradiated | 6 | 27 | 38 |
| | 60-65 | 6 | 27 | 38 |
| | 70-75 | 6 | 27 | 38 |
| 21 Dec 2011 | Unirradiated | 6 | 27 | 38 |
| | 50-55 | 6 | 27 | 38 |
| | 70-75 | 6 | 27 | 38 |

***Oxygen consumption***

Oxygen assessments (unless stated otherwise) were conducted in the EMAI quality control laboratory which was maintained at 27 ± 1 °C and 65 ± 5% RH with artificial lighting (L12:D10, with 1 h ramping up and down to simulate dawn and dusk (Fanson *et al*. 2014)). Initial calibration of equipment occurred on 10 November 2011. Two experiments were conducted. Experiment 1 (16 Nov 2011) assessed the oxygen consumption and respiration rates of unirradiated pupae at 17°C (6 reps) and 27°C (4 reps). Experiment 2 was conducted at 27°C over 3 days (Table 1), and assessed the oxygen consumption and respiration rates of unirradiated pupae (16 reps) compared to irradiated pupae: 50-55 Gy (6 reps), 60-65 Gy (12 reps) or 70-75 Gy (18 reps) (Table 1).

For each assessment, all bags (irradiated and unirradiated) were opened at the same time and placed in open trays for at least ten minutes to allow pupae recover from the altered atmospheres inside the bags and reach room temperature. Pupae have a high surface area relative to their volume, so it seemed probable that internal gas concentrations would approximate those in air within a minute or two of opening the bags. Nestel *et al*. (2007a) considered $O_2$ and $CO_2$ levels to have stabilised after 10 minutes when testing anoxia in Medfly pupae. It was assumed that 10 minutes was also sufficient for pupal respiration to recover once the atmosphere returned to normal, but this was not assessed.

Two subsamples of 250 g (from each 800 g irradiated sample) of pupae were placed inside 600 mL tubs. The head space of the closed container was estimated to be 432.5 ml (pupal volume = weight x 1.83). The KE-25 $O_2$ sensors (GS Japan Battery Co. Ltd, Osaka) were sealed into the tub lids using silicone sealant. These sensors have a mV output proportional to the partial pressure of $O_2$, reading approximately 12-14mV in air (21% $O_2$). Sensors reading 10mV or less in air were not used as this indicated the electrochemical sensor had aged. KE-25 sensors have a fast response time, making them ideal for measuring respiration. However, they must be calibrated daily to adjust for changes in atmospheric pressure. Sensor readings were taken using a digital voltmeter. Readings were taken every two minutes for up to 84 minutes. At the end of each run, the tubs were opened and pupae discarded.

***Data analysis***

All analyses were conducted using Rv3.6 (R Core Team (2017)). For each model, assumptions were assessed using graphical assessment of the model residuals, checking for homoscedasticity, normality, outliers and remaining relationship between predictors and residuals. Initial readings were discarded to allow time for the internal atmosphere to settle. We calculated respiration rates (RR) ($\mathrm{ml}^{-1}.\mathrm{kg}^{-1}.\mathrm{h}^{-1}$) as follows:

$$\text{respiration rate} = \frac{\text{change in oxygen (\%)} \times \text{container head space (ml)}}{100 \times \text{weight (kg)} \times \text{reading interval (h)}}$$

Data was approximately linearised through taking the natural log of $O_2$ consumption rate.

First, the relationship between respiration rates and oxygen (%) in the containers at two different temperatures (17°C and 27°C) was modelled. We ran a linear mixed model (LMM). Respiration rate was the response variable. Oxygen (log-transformed), temperature and the interaction were included in the model. We included a random slope and intercept for each container to account for the correlation within each container. This model was compared to the null model (no temperature included) using AIC (Akaike Information Criteria) to assess if temperature affected RR.

Next, we asked if irradiation affected pupal metabolism indicating a decline in pupal quality. We performed a similar LMM to the temperature model, except temperature was replaced with irradiation dose. Also, date and date-by-oxygen interaction was included as experiments were replicated over three separate temporal periods. To better assess whether irradiation dose affected, we compared the following models by comparing AIC: (1) model with four doses (unirradiated, 50-55Gy, 60-65Gy, 70-75Gy); (2) three doses (unirradiated, 50-55Gy, 60-65Gy/70-75Gy); (3) two doses (unirradiated vs. irradiated); and (4) no dose (null model).

## RESULTS

***Temperature effects***

Not surprisingly, temperature affected respiration rates ($\Delta AIC = 51.4$; Table 2). Oxygen levels decreased more slowly at 17°C, reaching 9.0% (SD: 0.93) after 84min. In contrast, at 27°C oxygen reached the 5% threshold at the 34min mark in all containers. Respiration rates ($O_2$) were more than four times higher at 27ºC than at 17ºC, regardless of partial pressure of $O_2$. At 27ºC, respiration was rapidly inhibited as $O_2$ levels declined.

The relationship of respiration rate to $O_2$ partial pressure was accurately described by the equation

$RR(O_2) = [28.38 \times \ln(\%O_2)] - 30.081$; $R^2 = 0.9709$. The relationship between partial pressure and respiration rate (RR) could be approximated by the equation $RR = [9.6708 \times \ln(\%O_2)] - 15.702$, but $R^2$ was only 0.5409. Nevertheless, this modelling indicated that $O_2$ consumption approached zero ($<1 ml.kg^{-1}.h^{-1}$) at 3% and 5.6% $O_2$ at 27°C and 17°C respectively.

**Table 2: ANOVA results for the statistical models. Temperature model had two temperatures: 17°C and 27°C. Irradiation dose was the fourdose model: Unirradiated, 50-55, 60-65, and 70-75Gy.**

| Type | Variable | F value | df | p-value |
|---|---|---|---|---|
| Temperature | oxygen | 64.7 | 1, 39.4 | <0.001 |
| | temperature | 528.1 | 1, 15 | <0.001 |
| | oxygen:temperature | 128.4 | 1, 25.4 | <0.001 |
| Irradiation | oxygen | 488.7 | 1, 42.8 | <0.001 |
| | irradiation | 55.5 | 3, 24.2 | <0.001 |
| | group | 6.8 | 1, 24.1 | 0.016 |
| | oxygen:irradiation | 74.9 | 3, 34.2 | <0.001 |
| | oxygen:group | 11.6 | 1, 44 | 0.0014 |

***Irradiation effects***

Similarly, irradiation dose affected the rate of change in $O_2$ over time ($\Delta AIC = 60.0$ compared to no dose model, see Table 2). Respiration rates of irradiated pupae were significantly lower than those of the unirradiated pupae (Figure 1). The magnitude of the irradiation dose (50-55 vs 60-65 vs 70-75Gy) did not affect respiration rates, as indicted by two dose model (unirradiated vs. irradiated) having the lowest AIC ($\Delta AIC = 5.5$ compared to four dose model). The difference between $O_2$ consumption rates of irradiated and unirradiated pupae was greatest as $O_2$ partial pressures approached those of normal air. As $O_2$ decreased close to 5%, rates of $O_2$ consumption by irradiated pupae approached those of the unirradiated pupae.

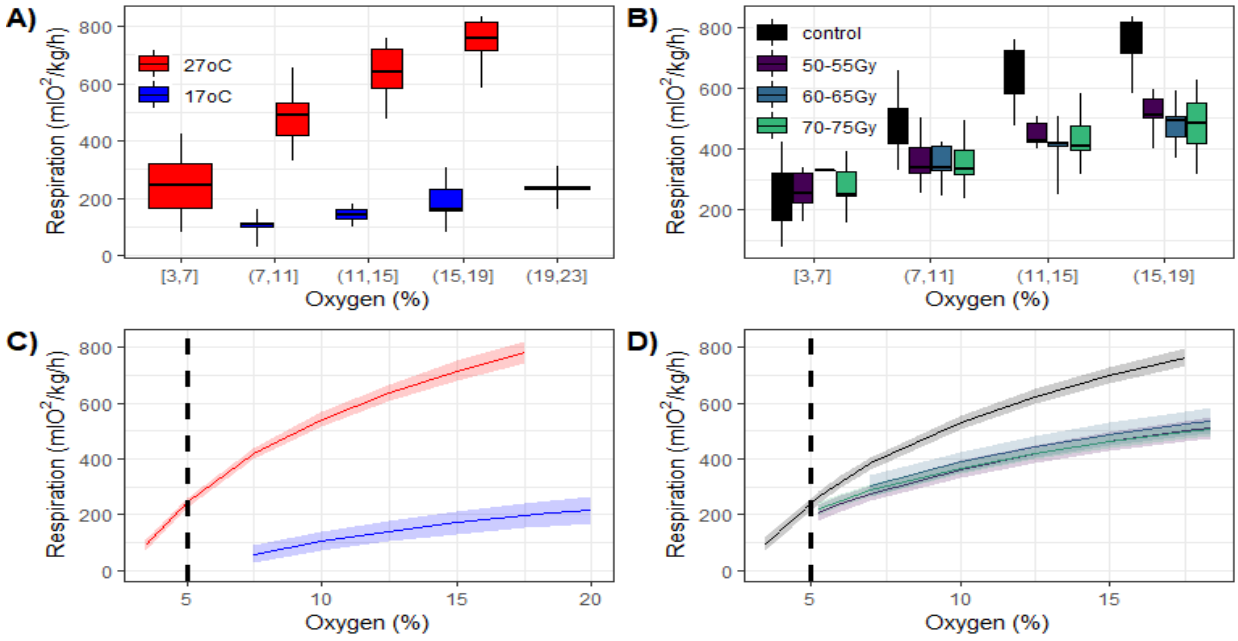


**Figure 1: Relationship between respiration rates and oxygen (%) levels. Panels A and B show boxplot of raw respiration rates (binned by oxygen) for temperature and irradiation experiments, respectively. Panels C and D show the modelled relationships. Error bars are 95% confidence intervals. Vertical dashed line shows 5% threshold.**

## DISCUSSION

Mammals and birds can survive only a few minutes without oxygen, but many insects are adapted to withstand damage from severe $O_2$ deprivation (Visser *et al.* 2018). In the case of fruit flies, late instar larvae feed by tunnelling into environments with little or no available $O_2$ (Callier *et al.* 2015). Also, fruit flies pupate in the soil, where wet conditions can lead to extended periods of hypoxia. Therefore, fruit flies have a relatively high tolerance to $O_2$ stress. For example, larvae of Oriental fruit flies (*Bactrocera dorsalis* Hendel) can survive up to 24h of anoxia without significant reductions in survival. However only 4% survived after 60h and there was 100% mortality at 84h (Deng *et al.* 2018). Similar results were reported for *Anastrepha ludens* (Loew), with 72h anoxia achieving 100% mortality (Lara-Perez *et al.* 2019). In contrast, nearly 83% of Qfly pupae survived 72h of low $O_2$ conditions (Dominiak *et al*. 2011).

Dominiak *et al*. (2011) speculated that Qfly were relatively tolerant of anoxic conditions due to their origin in the wet tropics, where soils are frequently saturated. However, this does not explain why Qfly appears to be better adapted to anoxic conditions than Oriental fruit fly, even though the latter is a lowland pest found in high rainfall areas (Ekesi *et al.* 2006). Comparing the two species directly, using the same experimental conditions, could answer whether this observation is an experimental anomaly or a true difference between species.

Brief exposure to hyoxia can provide cross tolerance to other stresses, including the extreme oxidative stress incurred during irradiation. Fruit fly pupae irradiated in low $O_2$ or no $O_2$ environments perform better than flies irradiated in normal air (Zumreoglu *et al.* 1979; Lopez-Martinez *et al.* 2016). For example, only 1h of exposure to full anoxia increased emergence and flight ability of irradiated Caribbean fruit flies (*Anastrepha suspensa* Loew), and this result was attributed to stimulation of antioxidant enzyme activity (Lopez-Martinez and Hahn 2012). There was significantly improved tolerance by *B. dorsalis* to irradiation in 0% or 2% $O_2$, but above 4% $O_2$ there was no difference from ambient air (Zhan *et al.* 2020).

Since 1996 at EMAI, Qfly pupae were held at 17°C to compress seven days of production into one day's irradiation treatment. Pupae were placed in plastic bags for about 18h and stored overnight at 17°C because the time taken to reach hypoxia at 17°C was not known. Our results indicate that 800g pupae, sealed inside a bag made of barrier film and with 100ml headspace, would deplete the atmosphere to close to 5% $O_2$ within approximately 3h at 17°C but only 30 minutes at 27°C. However, at 17°C, respiration was almost completely inhibited once $O_2$ concentration fell to 6-7%, and that level that would be reached in approximately 2h in the model above. Frequently, headspaces inside the bags used were less than 100ml, which would further reduce the time required to minimise respiration and, therefore metabolic activity.

Because of the extreme inhibition of respiration that occurs at a relatively high $O_2$ partial pressure, we suspect that $CO_2$ may be more important than $O_2$ in suppressing metabolic activity by Qfly pupae. In our trial, $CO_2$ accumulation was not measured. However, assuming a respiratory quotient close to 1, a decrease in $O_2$ to 7% should be associated with an increase in $CO_2$ to approximately 14%. Although fruit flies were highly tolerant of a 100% nitrogen atmosphere, an atmosphere containing 1% $O_2$ and 15% $CO_2$, when combined with high temperature, was an effective disinfestation treatment against western cherry fruit fly (*Rhagoletis indifferans* Curran) (Neven and Rehfield-Ray 2006). Similarly, a short exposure to 95% $CO_2$ reduced the time needed to achieve 100% mortality of Qfly (Golding *et al.* 2012), whereas a similar exposure to 1% $O_2$ had no effect.

Similar to $O_2$ consumption, $CO_2$ emission is a function of respiration rate, so $CO_2$ emission increases at high temperatures and is reduced at atmospheres that deviate significantly from air. In addition, Nestel *et al.* (2007b) found that $CO_2$ emission followed a quadratic function with high metabolic activity in the first hours of metamorphosis, a lower level in the mid-pupal period and increasing up to adult emergence. The role of $CO_2$ in irradiation tolerance requires further research.

Many facilities use procedures to decrease $O_2$ or to increase $CO_2$ to minimise the adverse effects of irradiation. Based on our results, sealing pupae inside bags 18h prior to irradiation is likely to result in hypoxic conditions for 48h or even longer, this being the interval between initial bagging and receival at release centres. Sealing pupae inside bags on the day of irradiation, rather than the day before will still result in depletion of $O_2$ to 5-6% or less prior to irradiation. This could reduce the total time pupae remain hypoxic by about 16 hours. Benelli *et al.* (2021) recommended avoiding prolonged periods of hypoxia for Qfly pupae due to effects on adult fly vigour.

Further research should examine whether punching an air-hole in bags post-irradiation, further reducing the time pupae remain hypoxic, can improve pupal development. Already, there is evidence that increasing the time inside sealed bags reduces fitness; Dominiak *et al.* (2011) reported eclosion rates of 86.3% after 32h and 83.8% after 48h in pupae stored at 17ºC in sealed plastic bags. Also, additional research is required to verify the $CO_2$ and $O_2$ levels that accumulate inside the bags prior to and post irradiation as it is unclear whether the bags used are full barrier films or somewhat permeable to gas exchange.

Additionally, our results demonstrate that irradiation both suppresses and increases variability of pupal respiration. We suspect that irradiation damages a range of metabolic functions, even when pupae are irradiated at the recommended maturity (2 days pre eclosion). This is unsurprising considering the purpose of irradiation is to irreversibly damage reproductive function. Respiration is a measure of metabolic activity, and we suggest that irradiation reduces the rate of pupal maturation, and likely contributes to variable eclosion rates. Lower doses of irradiation, that still result in 100% sterility, may optimise fitness. Some research has been conducted (Collins *et al.* 2009; Dominiak *et al.* 2014; Bloomfield *et al.* 2017) but did not result in lower irradiation rates used operationally at the EMAI facility before its closure in 2018. We predict that the combination of shorter anoxic periods and lower irradiation doses could improve field competitiveness. Our results should be adopted where feasible and further explored in ongoing research to improve SIT programs in Australia.

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