RESEARCH THESIS MRes

Project Title:

Improved larval diets for mass rearing of Queensland fruit fly (*Bactrocera tryoni*)

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Declaration

I wish to acknowledge the following assistance in the research detailed in this report:

A/Prof Phil Taylor for his supervision and support during all time of this study

Dr. Fleur Ponton for her collaboration for larval diet experiment and data analysis

Dr. Vivian Mendez for her collaboration in the aeration experiment

All other research described in this report is my own original work.

Tahereh Moadeli

Submission date: 9 October 2015

General abstract

The Queensland fruit fly or 'Q-fly', *Bactrocera tryoni* (Diptera: Tephritidae), is Australia's most economically important insect pest of fruit crops. The Sterile Insect Technique (SIT), which involves the mass rearing and release of millions of sterile flies to curtail reproduction of wild populations, is used as an environmentally benign approach to control Q-fly. High quality larval diets are essential for economical mass rearing and effective SIT. As a first step to development of improved diets, liquid larval diet formulations that have received substantial interest over the past decade ('Chang 2006' and 'Chang 2009') were compared with a conventional carrot-based solid diet. Results suggested that liquid diets are a promising alternative to carrot diet, but that their performance can be constrained by heterogeneity and high viscosity that can result in high larval mortality. To deal with these issues, the liquid diet formulations were mixed with agar (0, 0.25, 0.5, 1.0 and 1.5%) to produce gel diets. Significant diet by agar group interactions were found for most quality parameters, indicating that development of agar-based diets is a far more complex task that adding a specified proportion of agar to a suitable liquid diet formulation; the ideal proportion of agar to use depends on the diet formulation. Overall, around 1% agar provided the best balance of development rate, productivity and quality parameters. Diet experiments used eggs transported in water, and supplementary experiments confirm that the typical transit time of ca. 2 hours does not reduce hatching rate. These projects set the stage for more detailed investigation of gel diets as the most promising option for mass-rearing of Q-fly for SIT.

Keywords: Queensland fruit fly, *Bacterocera tryoni*, larval diet, sterile insect technique.

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Detrimental effect of immersion in water on egg hatching of *Bactrocera tryoni*, is ameliorated by aeration

Chapter one

Comparison between conventional larval diet for Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) and two liquid diets

Abstract

The Queensland fruit fly or 'Q-fly', *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), is Australia's most significant insect pest of fruits and vegetables. Sterile insect technique (SIT), a chemical-free approach for management of Q-fly, entails mass-rearing and releasing millions of sterile flies to mate with the wild population and induce sterility. Improved larval diets are urgently needed for increased efficiency in mass rearing of Q-fly for SIT. Carrot-based solid media is used in most small-scale rearing for Q-fly but, like all solid diets, generates substantial expense in storage, handling and waste. The present study compares the productivity and performance of two liquid larval diets ('Chang 2006' and 'Chang 2009') with carrot diet. The Chang 2006 diet was similar to carrot diet in terms of pupal number (with lower larval density), and the Chang 2009 diet showed promising flight ability performance, and this may be a reflection of the higher levels of wheat germ oil in this diet. Although promising, both liquid diet formulations were highly heterogeneous as heavier components settled out, and were also prone to high viscosity that was associated with high larval mortality. These are key issues to resolve in further development of these diets for mainstream use. Chemical analysis of yeasts and diets provide base information about nutritional composition of larval diets that will guide further development of these diets through varying composition and proportions of components.

Introduction

The Queensland fruit fly, *Bacterocera tryoni* (Diptera: Tephritidae), is one of Australia's worst horticultural pests, especially in eastern regions (Clarke et al., 2011; Dominiak et al., 2003; PBCRC, 2015; Sutherst et al., 2000). Sterile insect technique (SIT) is a chemical-free, cost-effective and promising method for management of Q-fly (Dominiak et al., 2003). In SIT, millions of flies are mass reared, sterilized, and released to mate with the wild population. Sterile males that mate with wild females induce reproductive failure, leading to reduced pest population levels in the next generation. A cost-effective mass-rearing method for production of high-quality flies is important for successful SIT (Chang et al., 2004; FAO/IAEA/USDA, 2003; Khan, 2013; Khan et al., 2011). Main mass-rearing procedures are feeding and holding adult stocks, collection, incubation and handling of eggs,

production of larvae and manipulation of pupae. Larvae are produced in an air-conditioned room using standard larval medium (Steiner and Mitchell 1966).

Solid larval diets have performed well worldwide for mass rearing of fruit flies. Fresh and fortified carrot diets were pioneered by Finney (1956). After that, the dehydrated carrot diets and squash diets were formulated for larval production of some tephritids (Steiner and Mitchell 1966). Wheat diets were also described by Tanaka et al. (1969) as a low-cost larval rearing medium for oriental and Mediterranean fruit flies. Nowadays, carrot diet is one of the best solid diets in terms of availability, price and quality parameters. Carrot-based media is used in most small-scale rearing for Q-fly (Ekesi & Mohamed, 2011; Khan et al., 2011). Liquid diets that replace biological bulking agents of solid diets with reusable substrates such as sponge cloth have received substantial attention over the past decade as potential replacements for solid diets (Chang, 2009b; Chang et al., 2007; Chang et al., 2004; Chang et al., 2006; Fay & Wornoayporn, 2002; Ekesi et al., 2014; Khan, 2013; Khan et al., 2011; Resilva et al., 2014; Vera et al., 2014). However, to date no large scale mass rearing programs have switched to using liquid diets. Liquid diets can increase the efficiency and convenience in mass rearing of flies for SIT owing to reduced need for storage of diet ingredients, reduced labor costs, and reduced waste (Chang et al., 2004). Liquid larval diets seem to be an effective potential alternative for solid diets that might be adapted for factory-scale production (Chang et al., 2006; Ekesi et al., 2014).

Development of new larval diets requires information about larval nutritional requirements. The common ingredients of larval diets include a nitrogen source (protein), carbohydrates, lipids, vitamins, minerals, preservatives, pH modifiers and water (Cohen, 2004; Nash & Chapman, 2014; Nestel et al., 2004; Rodrigues et al., 2015). Yeast products are the main nutritional component of fruit fly larval diets, providing all essential growth factors such as high levels of amino acids, nitrogen, carbohydrates and micro nutrients including vitamins, minerals and cholesterol (Chang et al., 2007; Fanson & Taylor, 2012). Insects need 8 to 10 essential amino acids (EAAs), which must be present in food. Removal of even one of the 10 EAAs from the diet can lead to no survivorship and no pupation (Chang, 2004; Chang et al., 2001; Nestel et al., 2004). In contrast, a deficiency of non-essential amino acids (NEAAs) in flies can often be compensated by the activity of symbiotic bacteria, with the exception of glycine (Nestel et al., 2004). Carbohydrate and lipids are needed for normal growth and development as well as energy (Chang et al., 2001). Wheat germ oil, which typically contains 20% saturated and 80% unsaturated fatty acids, and vitamin E, can promote growth and egg hatch (Chang & Vargas, 2007; Chang et al., 2006). Vitamins are required for normal development and survivorship (Chang et al., 2000).

In the present study, we compare 'Chang 2006' (Chang et al., 2006) and 'Chang 2009' (Chang, 2009b) liquid diet formulations with carrot-based solid larval diet to investigate the suitability of these liquid diets. Khan (2013) carried out some studies using the Chang 2006 diet, but this is the first study to assess the Chang 2009 diet with Q-fly.

Materials and methods

Eggs Production and Collection

Q-fly eggs were obtained from the Department of Primary Industry, Ourimbah, Australia. The eggs were collected in a plastic container that contained ca.40ml of water and was covered with fine punctures that the flies oviposited through. The eggs were rinsed into a beaker and then tipped into a vial for transport. The eggs were from F3 generation adults that had been reared on carrot-based larval diet. The eggs were collected at 8:30am (AEST) and transported by air-conditioned car for approximately one hour to Macquarie University. The experiments were carried in an environment-controlled laboratory (25 ± 0.2^0C, 65±3% RH and 11: 1: 11: 1 (light: dusk: dark: dawn photoperiod) at Macquarie University.

Diet Formulation

The effect of carrot diet and two liquid diets, 'Chang 2006' (Chang et al. 2006) and 'Chang 2009' (Chang 2009b), on the quality performance parameters of the Q-fly was evaluated. The streptomycin in 'Chang 2009' diet was removed because of negative effects of symbionts. Liquid diets were otherwise used in their standard form. These diets were composed of LBI2240 and FNILS65 yeasts (Lallemand, Bio ingredients, Montreal, QC, Canada), brewer's yeast (SF Health foods, Australia), torula yeast (HJ Langdon, Australia), sugar (Homebrand, Australia), nipagen (Southern Biological, Australia), sodium benzoate (Sigma, Australia), citric acid (Sigma, Australia), distilled water, wheat germ oil (Melrose laboratories PTY LTD, Australia), and diced carrot (HJ Langdon, Australia) (Table 1). Initial pH for carrot and Chang 2006 diets was 4, while that for Chang 2009 was 3.5.

Diet preparation

The diet mixtures (Table 1) were prepared by weighing all ingredients and blending in a 1.5-litre electronic blender (for liquid diets) or mixing by hand (for carrot diet) until the diet ingredients were

fully dissolved and homogenous (ca. 5 min). Two experiments were run, with differences only in size of tray, amount of diet, and number of eggs added to the diet (stocking density).

Experiment 1

50 ml of liquid diets and 50 g of carrot diet were added to plastic trays (10.5 cm long, 7.5 cm wide, 2.5 cm deep). For liquid diets, the trays contained yellow sponge (7.5 × 5 cm, Kalle USA Inc., Flemington, NJ), which provided the primary support matrix for feeding larvae, and polyethylene mesh (8.5 × 5.5 cm). The mesh was placed between the yellow sponge and the bottom of the tray. The yellow sponge was positioned 1 – 1.5 cm away from each of the four sides of tray, with the stripe-shaped pattern facing up. A strip of blue sponge cloth (5 × 1cm) was placed at the centre of the diet-soaked yellow sponge. After removing all excess water using plastic pipette so that only just enough water remained to cover the eggs, eggs (125 µl; approximately 14,000 eggs per ml) were transferred onto the wet blue sponge cloth using a 1000 µl pipette. The eggs were then sprayed gently with distilled water to spread them evenly across the sponge surface. The plastic rearing trays were then covered with plastic lids until the larvae began to exit the diet to pupate. The diets were made one day before the egg seeding and poured into the rearing trays ready for seeding with eggs the following day.

When the larvae were ready to pupate the lids of the plastic rearing trays were removed, and the rearing trays were placed onto larger plastic containers containing 1 cm deep layer of fine vermiculate. Pupae were collected daily until no larvae remained. Each day of pupal collection was weighed two days after collection.

During the last days of the larval period in the first experiment in which the recommended ratio of egg density to diet volume for fruit flies was used (Chang et al., 2006), high larval mortality was observed possibly because of food limitation and because of stickiness that impeded larval movement. Moreover, high density ingredients in liquid diets settled down to the bottom of the rearing tray and low density ingredient floated, resulting in inconsistent availability of diet components.

Experiment 2

Methods were identical to experiment 1 except for larger size of trays (17.5 cm long, 12 cm wide, 4 cm deep), main support sponges (13.5 × 7.5 cm), polyethylene mesh (15 × 10 cm), strip of sponge for seeding of eggs (9 × 1cm), amount of diet (150ml), and number of eggs (250µl, or ca. 3,500). Larval density was lower in Experiment 2 at 23.3 eggs/g compared with 35 eggs/g in Experiment 1. This change in method was adopted in response to apparent diet constraints in Experiment 1. Moreover, the

trays containing liquid diets were periodically shaken to improve consistency. Water was added as needed to reduce stickiness of liquid diets.

Evaluation of quality parameters

The quality parameters of flies reared on three different larval diets (one carrot-based and two liquid diets) were compared following methods of FAO/IAEA/USDA (2003) and Collins et al. (2008).

Pupal weight: Pupal weight was estimated by the mean weight of four sets of 100 pupae from each larval diet.

Pupal number: Pupal number was estimated by dividing total weight of all collected pupae by 100 and then multiplying by the mean weight of four sets of 100 pupae.

Developmental time: Egg-larval period was latency from the day of egg seeding until the day on which pupation was first observed.

Flight ability: Four sets of 100 pupae of the peak day of collection for each larval diet were counted and placed in separate 55 mm plastic Petri dish lids. The dishes of pupae were then centered on 90mm Petri dishes that were lined with black paper. A 100 mm tall black tube (89 mm inner diameter) with a fine coat of talcum powder on the interior (to prevent flies walking out) was placed onto the 90mm Petri dish lid. Each tube with pupae was placed in a mesh cage (30 × 30 × 30 cm) beneath a fluorescent tube positioned ca. 5 cm above the cage. To quantify fly-back - the number of flies that escaped from the tube and later returned and died inside - a second, empty, black tube was placed 6 cm away from the tube containing pupae. Flies that escaped from the tube were removed from the mesh cage every second day. When all emergences ceased (6 days after the first flies emerged) the remaining contents of the tubes were counted. The data were collected as six categories: (1) not emerged (inside unopened pupal case); (2) part emerged (a portion of adult body stuck in puparium); (3) deformed (fully shed the pupal case but with deformed or damaged wings); (4) non-fliers (morphologically normal flies that were collected from inside the first tube); (5) fly-back (the number of flies inside the second tube plus the same number of normal flies inside the first tube); (6) fliers (the number of flies that were collected from outside the rubes plus fly-back.

Percentage of fliers: Calculated as (N pupae - (N not emerged + N partially emerged + N deformed + N non-fliers)/N pupae)) × 100).

Percentage of adult emergence: Calculated as (N pupae - (N not emerged + N partially emerged)/N pupae) × 100.

Rate of fliers: Calculated as percentage fliers/percentage emergence × 100.

Sex ratio: *Sex ratio*: Calculated as N males emerged / N total flies emerged.

Chemical analysis

Amino acid and Monosaccharide profiles of diets prepared for Experiment 2 were analyzed by Australian Proteome Analysis Facility at Macquarie University.

Data analysis

Quality parameters of each larval diet were compared using two-way ANOVA and post-hoc Tukey tests in SPSS 22 software (SPSS, 2012). Comparisons for the parameters were also performed using SPSS 22 and descriptive statistics obtained as mean values ± one standard deviation.

Results

Evaluation of quality parameters

Pupal weight: Pupal weight was not significantly affected by the diet type in both experiments (Tables 2 and 3).

Pupal number: Pupal number was significantly affected by the diet type in both experiments. (Table 2).The number of pupae in carrot diet was substantially greater than liquid diets in Experiment 1, whereas in Experiment 2, in which larval stocking density was reduced, the number of pupae in Chang 2006 liquid diet was as high as carrot diet (Table 3). The number of pupae in Chang 2009 diet was however lower than for Chang 2006 and carrot diets in the second experiment (Table 3).

Developmental time: Pupal number was significantly affected by an interaction between egg-larval period and diet type for both experiments (Table 2). Number of pupae collected varied with egg-larval

developmental time. The peak day of pupal collection was also different among diets. The greatest number of pupae in the Chang 2006 diet was obtained one day earlier than in carrot and Chang 2009 diets (Figure 1).

Percentage of emergence: The percentage of emergence was significantly influenced by diet type in both experiments (Table 2). In Experiment 1, emergence was lower for Chang 2009 diet however, emergence was not different between Chang 2006 and carrot diet. In Experiment 2, carrot diet showed greater percentage of emergence than the liquid diets (Table 3).

Percentage of fliers: The percentage of fliers was not significantly affected by diet type in the Experiment 1, while there was a significant effect in Experiment 2 (Table 2). The percentage of fliers observed in Chang 2006 diet was lower than in the carrot diet but not different to Chang 2009 diet (Table 3).

Rate of fliers: The rate of fliers was not significantly influenced by diet type in either experiment, although there was a non-significant trend toward lower rate of fliers for the Chang 2006 diet in Experiment 2 that mirrored the results for percentage of fliers (Table 2 and 3).

Sex ratio: Sex ratio was not affected by the diet type in either experiment (Table 2 and 3).

Chemical analysis

The amino acid and monosaccharide profiles of three diets and their yeasts in the second experiment are shown in Table 4:

Yeasts

FNILS65 yeast showed the highest amount of amino acids, while brewer's yeast showed the lowest. The highest amount of glucose was observed in brewer's yeast which was approximately four times as high as torula yeast (Table 4).

Diets

While liquid diets were similar in terms of amount of amino acids and glucose, carrot diet showed lower amounts of amino acids and higher amounts of glucose (Table 4).

Discussion

Physical parameters including eggs/diet ratio and pH have already been shown as important parameters in larval diet performance (Chang et al., 2006). Similar obtained results from carrot diet and Chang 2006 diet in the second experiment might be because of similar and higher pH in these diets compared with the Chang 2009 diet (Vera et al. 2014; Ekesi et al., 2014). The lowest number of pupae was observed in Chang 2009 diet in which the pH was adjusted to 3.5 (Chang 2009a) with higher amount of citric acid in this diet (Table 1). The low number of pupae in the Chang 2009 diet might also be due to removal of streptomycin in our experiments.

Type and amount of yeasts can affect larval diet performance, and the amount used in the present study was based on what was found to work well for other fruit fly species reared using these diets (Chang et al., 2004; Chang et al., 2006; Ekesi et al., 2014). The amount of yeast in liquid diets appears sufficient for Q-fly since the pupal number, especially for the Chang 2006 diet in the second experiment, was similar to that for carrot diet despite inconsistency and stickiness of liquid diet. Moreover, the amount of amino acid in the Chang 2006 diet was more than in carrot diet. Brewer's yeast showed the lowest amount of amino acids and the highest amount of glucose among yeasts. Stickiness of the Chang 2006 diet might result from the high amount of carbohydrate in the brewer's yeast or from proliferation of the live yeast cells.

Brewer's yeast showed the lowest amount of glycine; lack of this amino acid can result in reduced adult emergence (Chang, 2004). Moreover, more non-fliers in the Chang 2006 diet may be explained by the lower amount of total fatty acid in brewer's yeast (Chang 2009a; Cho et al., 2013). Total amount of amino acid and proportion of wheat germ oil in the Chang 2006 diet was lower than in the Chang 2009 diet. Adding wheat germ oil or fatty acids could improve flight ability (Chang & Vergas 2007; Cho et al., 2013), and this should be a key area to consider in future studies. FNILS65 yeast in the Chang 2009 diet might be responsible for improved flight ability and reduced pupal recovery compared with the other studied yeasts by Chang (2009a). The chemical analysis of FNILS65 yeast showed the highest amount of amino acids in this yeast.

In medfly, amino acids in the larval diet tend to be a more limiting factor for optimal development and performance than carbohydrates (Nestel et al., 2004). Quantity and quality of protein is key during the larval phase, although carbohydrate plays an important role during the metamorphic phase of medfly (Nash & Chapman, 2014). Carrot diet was a comparatively protein poor diet (Table 4), with larval developmental time in carrot diet longer than liquid diets, especially Chang 2006 diet. Moreover,

developmental time in medfly, oriental fly and melon fly from diets with torula yeast is shorter than diets with FNILS65 (Chang, 2009a). Similar results have been observed with *Drosophila simulans* (Matavelli et al., 2015).

Ratio of sugar, wheat germ oil and brewer's yeast can affect performance of larval diets (Vera et al., 2014; Pascacio-Villafán et al., 2015). Sugar concentration can influence fly survival especially males (Chang et al. 2001). Although carrot diet was without added sugar, it showed the highest amount of monosaccharide from the diced carrot. The high fly emergence in this diet might be due to this sugar-rich ingredient.

The ratio between NEAAs and EAAs might also be an important factor in larval diet. Carrot diet showed the highest ratio, although the total amount of amino acids was lowest in this diet due to low percentage of torula yeast. Total amount of amino acid in liquid diets was approximately double that of carrot diet.

In conclusion, the Chang 2006 and Chang 2009 liquid diets show potential for rearing of Q-fly, although modifications will be needed to improve some quality parameters. In particular, pupal number and flight ability was promising in liquid diets; however, these diets were very heterogeneous and tended to become very viscous and sticky. The addition of gelling agents such as agar might resolve these issues. After that, manipulation of larval diet composition such as yeasts and wheat germ oil, adding fatty acid and Vitamin E and glycine can provide a broad understanding of larval nutritional demand for Q-fly and guide the development of optimal diets.

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Figure 1 Quantity (number) of pupae of Q-fly reared on ‘Chang 2006’ and ‘Chang 2009’ liquid larval diets and carrot solid diet according to egg-larval period in Experiment 2.

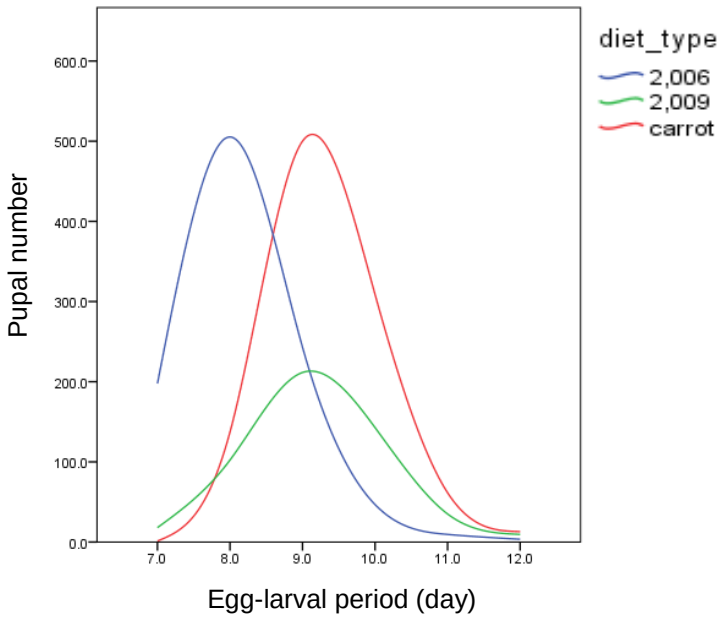

Figure 1

Tables

Table1 Composition of carrot, ‘Chang 2006’ and ‘Chang 2009’ larval diets

Ingredients	*Carrot-based solid diet*		*‘Chang 2006’ liquid diet*		*‘Chang 2009’ liquid diet*	
	%	g	%	g	%	g
Yeast LBI2240	-	-	-	-	10.96	15.03
Yeast FNILS65	-	-	-	-	3.65	5.1
Brewer’s yeast	-	-	15.06	20.40	-	-
Torula yeast	6.27	8.95	-	-	-	-
Sugar	-	-	8.99	12.18	8.72	12.18
Nipagen	-	-	0.15	0.20	0.14	0.20
Sodium banzoate	0.26	0.37	0.15	0.20	0.14	0.20
Citric acid	0.94	1.34	1.70	2.31	4.66	6.50
Water (ml)	70.04	100	73.81	100	71.62	100
Wheat germ oil (ml)	-	-	0.15	0.2	1% of water volume	1
Diced carrot	22.48	32.1	-	-	-	-

Table 2 Statistics of quality parameters of Q-fly reared on 'Chang 2006' and 'Chang 2009' liquid diets and carrot solid diet.

	First experiment	Second experiment
	Statistics	Statistics
Pupal weight	$F_{3, 12} = 2.96$; *P = 0.098*	$F_{3, 12} = 0.662$; *P = 0.059*
Pupal number	$F_{2, 12} = 7.98$; ***P = 0.010***	$F_{2, 12} = 7.67$; ***P = 0.011***
Developmental time	$F_{8, 60} = 2.64$; *P* = ***0.018***	$F_{10, 72} = 15.24$; *P* = ***0.000***
Emergence (%)	$F_{2, 12} = 10.86$; ***P = 0.004***	$F_{2, 12} = 18.68$; ***P = 0.001***
Fliers (%)	$F_{2, 12} = 0.974$; *P = 0.414*	$F_{2, 12} = 5.61$; ***P = 0.026***
Rate of fliers	$F_{2, 12} = 2.320$; *P = 0.154*	$F_{2, 12} = 3.48$; *P = 0.076*
Sex ratio (% male)	$F_{2, 12} = 0.224$; *P = 0.804*	$F_{2, 12} = 0.292$; *P = 0.754*

Table 3 The quality parameters of Q-fly reared on 'Chang 2006' and 'Chang 2009' larval diets and carrot diet. The descriptive statistics presented as mean ± SD. Different letters across rows for each parameter indicate significant Tukey's post hoc comparisons (P<0.05). Post hoc tests were run separately for each diet formulation (i.e., Chang 2006 and Chang 2009).

	First experiment			Second experiment		
	2006	2009	carrot	2006	2009	carrot
Pupal weight	9.40 ± 0.13	7.38 ± 0.67	7.74 ± 1.12	9.83 ± 0.44	9.56 ± 0.29	11.06 ± 0.42
Pupal number	353 ± 69b	388 ± 22b	747 ± 197a	1005 ± 179a	519 ± 158b	1021 ± 265a
Emergence (%)	89.50 ± 3.0a	77.75± 6.39b	90.75±2.62a	85.75 ± 4.34b	90.75 ± 2.21b	98.25 ± 1.25a
Fliers (%)	68.25 ± 6.65	69.25 ± 10.4	76.25 ± 9.03	54 ± 14.49a	74.25±11.32a	84.50 ± 13.27b
Rate of fliers	76.28 ± 7.36	88.72 ± 7.22	84.05 ± 9.89	62.73 ± 15.80	81.66 ± 10.82	85.90 ± 12.53
Sex ratio	51.82 ± 7.22	52.2 ± 10.59	55.64 ± 8.49	48.50 ± 11.16	51.78 ± 7.77	47.56 ± 4.12

Table 4 Chemical analysis of LBI2240, FNILS65, brewer's yeast and torula yeast, and 'Chang 2009', 'Chang 2006' and carrot larval diets

Amino Acids (mg/g)		Yeast				Diet		
		LBI2240	FNILS65	Brewer's	Torula	2009	2006	carrot
NEAA	Alanine	21.5	27.3	19.2	24.3	3.38	3.14	2.03
	Aspartic acid	37	51.7	31.2	41.1	6.04	5.17	4.01
	Cystine	-	-	-	-	-	-	-
	Glutamic acid	59.5	69.6	53.5	53	9.21	7.25	5.84
	Glycine	15.4	19.7	13.1	17	2.4	2.19	1.28
	Proline	16	19.7	15.9	15.2	2.48	2.44	1.14
	Serine	18.4	23.4	17	19.7	2.93	2.9	1.48
	Tyrosine	12.3	17.3	10.2	13.9	1.9	1.67	0.97
EAA	Arginine	19.5	23.2	15.7	22.2	2.94	2.75	1.73
	Histidine	8.3	11.4	7.3	8.8	1.32	1.21	0.66
	Isoleucine	17.6	25	15.1	22	2.84	2.54	1.63
	Leucine	26.4	35.5	23.2	33.1	4.23	3.82	2.42
	Lysine	27.8	38.5	23.4	32.3	4.53	3.89	2.18
	Methionine	5.6	7.9	4.9	5.5	0.78	0.76	0.38
	Phenylalanine	16.8	22.2	14.9	20.4	2.68	2.44	1.52
	Threonine	18.3	22.6	16.3	21.5	2.86	2.75	1.55
	Valine	21.3	30	18.4	24.3	3.43	3.11	1.92
	Tryptophan	-	-	-	-	-	-	-
Total amino acids		341.7	445	299.3	374.3	53.95	48.03	30.74
Carbohydrates (Glucose, µg/ml)		24.8	15.2	44.4	10.4	6.5	6.8	9

Chapter two

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High productivity gel diets for mass rearing of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)

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Short title: Larval liquid diet formulations combined with different percentage of agar for Q-fly

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Keywords

Liquid diet, semi-liquid diet, agar-based diet, sterile insect technique (SIT), flight ability, fliers, mortality under stress

Abstract

The Queensland fruit fly or 'Q-fly', *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), is Australia's most economically damaging insect pest of horticulture. The sterile insect technique (SIT) has been adopted as an environmentally benign approach for management of Q-fly through the mass rearing and release of millions of sterile flies to curtail reproduction of wild populations. New larval diets are needed for economical mass rearing of high quality flies for use in SIT. Liquid larval diets have recently been developed as an alternative to traditional solid diets that incorporate biological bulking agents, but liquid diets also suffer drawbacks that have limited their adoption. The present study considers whether some of the deficiencies of two promising liquid larval diets ('Chang 2006' and 'Chang 2009') might be overcome by incorporating agar (0, 0.25, 0.5, 1.0 and 1.5%) to create a gel diet. Overall, gel diets containing greater than 0.5% agar outperformed liquid diets (0% agar) and semi-liquid diets (0.25% agar) of identical nutritional composition, especially in terms of development rate and overall productivity. Semi-liquid diets performed especially poorly in terms of emergence and flight ability. The Chang 2009 larval diet outperformed the Chang 2006 diet in terms of flight performance, and this likely reflects the higher concentration of wheat germ oil in this diet, but might also reflect differences in the type of yeast used. For many performance metrics, significant interactions were detected between diet formulation and percentage of agar. Accordingly, in the search for an optimal diet there is a need to consider the ideal agar composition for each combination of nutritional components.

Introduction

Fruit flies (Diptera: Tephritidae) are important pests of fruit crops around the world. The Queensland fruit fly, *Bactrocera tryoni* ('Q-fly'), is Australia's most economically damaging insect pest of fruits and is highly polyphagous, attacking, for example, apples, citrus, pears, stone fruit, berries, and grapes (Sutherst et al., 2000; Dominiak et al., 2003; PBCRC, 2015). Traditionally, insecticides have provided a high level of protection from Q-fly (Clarke et al., 2011), but with regulatory restrictions on the use of organophosphate insecticides such as dimethoate and fenthion, there is a growing need for more sustainable approaches (PBCRC, 2015).

Sterile insect technique (SIT) is a promising method for management of fruit flies, and has been used to a limited extent with Q-fly over the past 20 years (Dominiak et al., 2003). In SIT, millions of male flies are reared, sterilized and then released in the field to mate with wild female flies. Females mated by sterile males suffer reproductive failure and, consequently, pest population levels are reduced in the next generation. Successful implementation of SIT relies on an efficient and economical mass-rearing method that produces high-quality flies (Chang, 2004; FAO/IAEA/USDA, 2003; Khan et al., 2011). Q-fly is currently mass reared using a traditional solid diet that includes a biological bulking agent. Most small-scale rearing uses a carrot-based media, and the sole factory-scale operation uses a lucerne-chaff-based diet (Khan, 2013). Such diets have several significant disadvantages that constrain their economy and productivity. The biological bulking agents pose problems for storage, handling, and disposal, and variation in quality of bulking agent can be an important contributor to variability in quality of produced flies. New larval diets are needed to improve the quality, economy and consistency of mass-reared flies.

Significant effort has been invested over the past years in development of larval diets for mass rearing of tephritid fruit flies (e.g., Finney, 1956; Steiner and Mitchell, 1966; Tanaka et al., 1969; Chang, 2004; 2009a,b; Chang et al., 2007; Chang et al., 2004; Chang et al., 2006; Ekesi et al., 2014; Khan et al., 2011; Resilva et al., 2014). Fresh and fortified carrot diets were pioneered by Finney (1956). After that, the dehydrated carrot diets, squash diets and low-cost larval rearing medium (wheat diets) were formulated for larval production of some tephritids (Steiner and Mitchell, 1966; Tanaka et al. 1969). Most of recent studies have especially focused on liquid diet formulations that maintain larvae in a liquid medium on a re-useable synthetic substrate, and such approaches have shown promise for use with Q-fly (Khan, 2013, 2014). While the overall concept is encouraging, some issues remain with the deployment of liquid diets in medium or large scale mass rearing operations. For example, liquid larval diets tend to settle, such that diet components become separated. Some liquid diets tend to

dry out or to become highly viscous, leading to high larval mortality. This then can require additional management such as periodically spraying the diet (Chang et al., 2004) or adding water (Chang, 2009b). Liquid diet formulations require a physical substrate - usually sponge and plastic mesh - to support the developing larvae. Cleaning and replacement of physical substrates adds expense and inconvenience. Liquid diets are a promising approach for small scale rearing of fruit flies, but remain impractical at this time for deployment in large-scale operations such as are required for SIT.

Gelling agents offer a potential solution to the current limitations of liquid diets. In addition to removing the need for physical substrates to support the larvae, gelling agents can improve insect diets by (1) modifying the high water content of the diet into a gel state so that insects do not die if their food collapses on them when tunneling, and enabling easy exit for pupation, (2) improving the consistency of diet by preventing more dense materials from settling down and less dense materials from floating, a common issue for liquid diets, and (3) inhibiting reactions between diet ingredients (Cohen, 2004). Various thickeners have been used in larval diets including gum Arabic, guar gum, locust bean gum, cellulose, agar, starch, pectin and gelatin (Cohen, 2004; Hanife, 2008; Pašková, 2007; Vera et al., 2014; Pascacio-Villafán et al., 2015).

With a view to developing larval diets suitable for both laboratory experiments and mass-rearing of Q-fly, we here consider whether performance of liquid diet formulations, such as those used by Khan (2013, 2014) might be improved by the addition of agar as a gelling agent.

Materials and methods

Q-fly eggs were obtained from the Department of Primary Industries, Ourimbah, New South Wales, Australia. The eggs were collected in a plastic jar that had numerous puncture holes for the females to oviposit through and contained ca. 40ml of water. Eggs were then rinsed into a beaker before tipping into a vial for transport. The eggs were from F3 generation from a culture reared on carrot-based diet. The eggs were collected at 8:30am (AEST) and transported by air-conditioned car for approximately one hour to Macquarie University. All experiments were carried in a controlled environment laboratory (25 ± 0.2^0C, 65±3% RH and 11: 1: 11: 1 light: dusk: dark: dawn photoperiod) at Macquarie University.

Diet Formulation

The performance of two liquid diets, ‘Chang 2006’ (Chang et al. 2006) and ‘Chang 2009’ (Chang 2009b), was evaluated both in their standard liquid form and after mixing with 0.25, 0.5, 1.0 and 1.5 % agar. Six replicates were completed for each of the 10 combinations of larval diet formulation and

percentage of agar used. Diet components included LBI2240 and FNILS65 yeasts (Lallemand, Bio ingredients, Montreal, QC, Canada), brewer's yeast (SF Health foods, Australia), sucrose (Homebrand, Australia), nipagen (Southern Biological, Australia), sodium benzoate (Sigma, Australia), citric acid (Sigma, Australia), distilled water, wheat germ oil (Melrose laboratories PTY LTD, Australia) and agar (MP Biomedical LLC, France) (Table 1). Initial pH for these diets was between 3.5 and 4. The diets were prepared one day before experiments commenced and were maintained overnight in the controlled environment room.

Diet preparation

Liquid diets (i.e., 0% agar) of both formulations were prepared by mixing all ingredients in blender. The mixed diet was then poured into clear plastic rearing trays (17.5 cm long, 12 cm wide, 4 cm deep). The trays contained yellow sponge (13.5 × 7.5 cm, Kalle USA Inc., Flemington, NJ), which provided the primary support matrix for feeding larvae, and polyethylene mesh (15 × 10 cm). The mesh was placed between the yellow sponge and the bottom of the tray. The yellow sponge was positioned 1 – 1.5 cm away from each of the four sides of tray, with the ribbed surface facing up. A ribbon of blue sponge cloth (9 × 1cm diameter) was placed at the centre of the diet-soaked yellow sponge. After tipping off all excess water so that only just enough water remained to cover the eggs, 250 µl of eggs (approximately 3500 eggs) was transferred onto the wet blue sponge cloth using a 1000 µl pipette. The eggs were then sprayed gently with distilled water to spread them evenly across the sponge surface.

To prepare gel diets (i.e., 0.25, 0.5, 1.0 and 1.5 % agar), dry ingredients were mixed in a blender with half of the water until the diet ingredients were fully homogenous (ca. 5 min). The agar was then mixed with the rest of the water and heated. After heating, the agar was added to the ingredients inside blender and mixed again until homogenous. Gel diets were then poured into the rearing trays to set. Diets with 0.5, 1.0 and 1.5% agar set as a firm gel, but diets with 0.25% agar remained as 'semi-liquid'. Eggs were seeded directly onto the surface of gel diets.

As soon as eggs had been added to the diets, the plastic rearing trays were covered with plastic lids until the larvae began to exit the diet to pupate. The lids of the plastic rearing trays were then removed, and the rearing trays were placed onto larger plastic containers (25 × 25.5 × 25.5 cm) that were closed by a lid that had a 10-cm diameter mesh-covered window for ventilation. The large plastic containers contained 30 × 30 cm sections of white fabric that the larvae pupated on. Pupae were collected daily until no larvae remained. Each day of pupal collection was weighed two days after collection.

Evaluation of quality parameters

The 10 different larval diets (two formulations, five agar concentrations) were compared following procedures of FAO/IAEA/USDA (2003) and Collins et al. (2008).

Parental egg hatch (%): Eggs were collected using a 100-µl pipette and counted under a stereomicroscope. Three sets of ca.100 eggs were spread on a 1 × 3.5 cm strip of wet blue sponge cloth for liquid diets and directly on agar-based diets, and incubated in covered 55 mm Petri dishes containing larval diet. The number of eggs that did not hatch after 4 days was recorded. To calculate mean percentage of egg hatch, the number of eggs hatched was divided by sum of unhatched and hatched eggs and then multiplied by 100.

Egg-larval duration: Latency from the day of egg seeding until the day on which pupation was first observed.

Egg-pupal duration: Latency from egg seeding until the day on which adult emergence was first observed.

Pupal period: Latency from the day on which pupation was first observed until the day on which emergence was first observed.

Pupal production period: The number of days on which pupae were produced.

Peak day of pupation: Latency from day of egg seeding to the day with highest production of pupae.

Pupal weight: Pupal weight was estimated by the mean weight of six sets of 100 pupae from each larval diet.

Pupal number: Pupal number was estimated by dividing total weight of all collected pupae by 100 and then multiplying by the mean weight of six sets of 100 pupae.

Pupal recovery: Percentage of pupal recovery was calculated as the total number of pupae produced from the predicted number of hatched eggs of each diet.

Flight ability: Six sets of 100 pupae from the peak day of collection for each diet were counted and placed in separate 55 mm plastic Petri dish lids. The dishes of pupae were centered on 90mm Petri dishes that were lined with black filter paper. A 100 mm tall black tube (89 mm inner diameter) with a fine coat of talcum powder on the interior (to prevent flies walking out) was placed onto the 90mm petri dish lid. Each tube with pupae was placed in a mesh cage (32.5 × 32.5 × 32.5 cm, Megaview BugDorm-43030F) beneath a 20-watt fluorescent tube positioned ca. 5 cm above the cage. To quantify fly-back (the number of flies that escaped from the tube and later returned and died inside) a second, empty, black tube was placed 6 cm away from the tube containing pupae. Flies that escaped from the tube were removed from the mesh cage every second day. When all emergence ceased (6 days after the first flies emerged) the remaining contents of the tubes were counted. The data were collected as six categories: (1) not emerged (inside unopened pupal case); (2) part emerged (a portion of adult body stuck in puparium); (3) deformed (fully shed the pupal case but with deformed or damaged wings); (4) non-fliers (morphologically normal flies that were collected from inside the first tube); (5) fly-back (the number of flies inside the second tube plus the same number of normal flies inside the first tube); (6) fliers (the number of flies that were collected from outside the tubes plus fly-back).

Percentage of fliers: Calculated as (N pupae - (N not emerged + N partially emerged + N deformed + N non-fliers)/N pupae)) × 100).

Percentage of adult emergence: Calculated as (N pupae - (N not emerged + N partially emerged)/N pupae) × 100.

Rate of fliers: Calculated as percentage fliers/percentage emergence × 100.

Sex ratio: Calculated as N males emerged / N total flies emerged.

Mortality under stress: After emerging in a cage, three sets of 100 flies (50 males and 50 females) from each larval diet were collected using manual aspirator and transferred into plastic Petri dishes (150 mm diameter). The lids of these Petri dishes had an opening of ca. 10 mm diameter, fitted with a stopper. The Petri dish containing flies was placed in a drawer without food, water and light for 48h. After this time, dead flies were separated by inverting the Petri dish, removing the stopper, and shaking out the

dead flies. The live flies were placed in a freezer for 2 hours to facilitate counting. Percentage mortality under stress was calculated as N dead at 48 hours/total number × 100.

Data analysis

Quality parameters for each diet were compared using two-way ANOVA and post-hoc Tukey tests in SPSS 22 software (SPSS, 2012), with diet formulation and percentage agar as fixed effects. Descriptive statistics are given as mean values ± one standard deviation.

Results

Parental Egg hatch: Parental egg hatch was not significantly influenced by diet formulation or percentage of agar (Table 2). Overall, the percentage of egg hatching was above 80% (Table 4).

Egg-larval duration: Egg-larval duration was significantly influenced by an interaction between agar concentration and diet formulation (Tables 2). Egg-larval duration was longest in semi-liquid diets, with this effect being especially pronounced in the Chang 2009 diet (Figure 1A, Table 4).

Egg-pupal duration: Patterns for egg-pupal duration closely resembled those for egg-larval duration, being significantly increased for the semi-liquid Chang 2009 diet (Tables 2 and 4). Egg-pupal duration was significantly increased on liquid and semi-liquid diets in the Chang 2006 diet (Tables 2 and 4), while it was significantly longer on semi-liquid diet only in Chang 2009 (Tables 2 and 4).

Pupal period: Pupal period was significantly influenced by an interaction between agar concentration and diet formulation (Tables 2). Pupal period was slightly longer for liquid diet compared to 1% agar diet in Chang 2006 diet (Figure 1B and Table 4). In Chang 2009 diet, it was significantly increased for semi-liquid diets (Figure 1B and Table 4).

Pupal production period: Pupal production period was affected by an interaction between agar concentration and diet formulation (Tables 2). The production period was shorter for diets with at least 1% of agar in Chang 2006 diet. In Chang 2009 diet, the production of pupae was also shorter for the liquid diet (Figure 1C, Tables 4).

Peak day of pupation: Peak day of pupation was affected by the interaction between agar concentration and diet formulation (Table 2). Peak of pupation was significantly delayed in semi-liquid diets, with this effect being especially pronounced in the Chang 2009 diet (Figure 1D, Table 4).

Pupal number, recovery and weight were all significantly affected by an interaction between diet formulation and percentage of agar (Table 2).

Pupal number: The number of pupae was substantially increased when agar concentration was equal to or greater than 0.5% in the Chang 2006 diet (Figure 2A, Table 2). At 0.5% agar, this effect was more pronounced for the Chang 2006 diet, whereas at 1.0 and 1.5% agar the effect was similar for the Chang 2006 and Chang 2009 diets (Table 4).

Pupal recovery: Results for pupal recovery closely mirrored those for pupal number, with substantial increases when 0.5% agar or more was added to the diet, and with this increase being more evident at 0.5% agar in the Chang 2006 diet (Tables 2 and 4). Pupal recovery was maximized at 1% agar for both diet formulations (Tables 2 and 4).

Pupal weight: Pupae from larvae reared in liquid and semi-liquid (0.25% agar) diets were lighter than those reared on diets containing higher percentages of agar in 2006 formulation, while in 2009 formulation, pupae from semi-liquid diet were lighter (Figure 2B, Tables 2 and 4).

Sex ratio: Sex ratio was significantly affected by the agar concentration (Table 3). It was slightly biased toward males in semi liquid diets compared to the other diets; however we did not detect any post-hoc significant differences (Table 4).

Percentage of emergence: The percentage of adult emergence was significantly influenced by an interaction between the percentage of agar and diet formulation (Table 3). For both diets, there was a significant increase in the percentage of non-emergence for the semi-liquid diet, but this effect was particularly pronounced for the Chang 2009 diet formulation (Figure 3A, Table 4).

Percentage of fliers: The percentage of fliers was significantly affected by agar concentration and also varied between the two diet formulations (Tables 3). Overall, percentage fliers was higher for the

Chang 2009 diet than for the Chang 2006 diet formulation, and for both diets was significantly reduced in semi-liquid diets (Figure 3B, Table 4).

Rate of fliers: The rate of fliers was significantly influenced by an interaction between the percentage of agar and diet formulation (Tables 3). The rate of fliers was generally lower in the Chang 2006 diet; however, this difference diminished with increasing concentrations of agar (Figure 3C, Table 4).

Not-emerged, partially emerged, *not-fliers and deformed flies*: The percentage of not-emerged flies was significantly influenced by an interaction between the percentage of agar and diet formulation (Tables 3). The greatest percentage of not-emerged flies was observed in semi liquid diets of both formulations, but this effect was particularly pronounced in the Chang 2009 diet (Figure 3D). The percentage of partially emerged flies was significantly affected by both agar concentration and diet formulation (Tables 3), with the percentage of partially emerged flies tending to be greater in liquid diet in Chang 2009 diet (Table 4). The percentage of non-fliers was only influenced significantly by diet formulation (Tables 3 and 4, and Figure 3E), generally being greater in the Chang 2006 diet. The percentage of deformed flies was not significantly affected by diet formulation and percentage of agar, but it was very close to being significant by percentage of agar. ANOVA tests showed a peak at 1% of agar for both formulations; however post-hoc test did not detect any significant differences (Tables 3 and 4).

Mortality under stress: Mortality under stress of male and female flies was significantly influenced by the interaction between the percentage of agar and diet formulation (Table 3). For the Chang 2009 diet, there was no evidence that agar concentration affected mortality under stress for females and mortality was slightly greater on 0.5% agar diet for males. In contrast, for the Chang 2006 diet there was a general pattern of increased mortality under stress as agar concentration increased for both sexes (Figure 4A, 4B; Table 4).

Discussion

The quantity and quality of mass-reared flies are important determinants of the overall performance of sterile insect technique (SIT) programs used to control tephritid fruit flies and other major insect pests. In turn, larval diets are important determinants of both quantity and quality of mass-reared insects, and have attracted substantial attention as part of an overall effort to improve the efficacy of SIT programs. Liquid larval diets have shown promise as a general approach for the rearing of

tephritid fruit flies, including Queensland fruit flies (Q-fly), eliminating the need for biological bulking agents that introduce expense and variability. However, liquid larval diets also suffer substantial drawbacks that have limited their implementation on a large scale. For example, in liquid larval diets the heavier diet components tend to settle to the bottom of the larval diet tray, leaving a dilute formulation above. Also, liquid larval diets require a synthetic substrate to support the developing larvae. We here investigated whether some of the limitations of liquid larval diets might be overcome through the use of agar as a gelling agent. Q-fly were reared on two different diet formulations, Chang 2006 and Chang 2009, with five concentrations of agar to create liquid diets (0% agar), semi-liquid diets (0.25% agar), and firm gel diets (0.5, 1.0 and 1.5 % agar).

Among the assessed agar concentrations in this study, the 0.5 and 1% concentration of agar in the Chang 2006 formulation and 1% in the Chang 2009 formulation showed the best performance almost in all parameters evaluated. High pupal number recovered from agar-based diets when agar concentration was equal to or greater than 0.5% for both formulations is most likely due to positive effects of agar on larval survival. The pupal numbers in agar-based diets were approximately 3 times higher than liquid diets. The lower pupal number and pupal recovery in liquid diets may be because of poor consistency. Similarly, larval survivorship was low in semi-liquid diets, apparently because the diet's stickiness makes it difficult for larvae to move and exit the diet to pupate, as has been reported in other studies (Panizzi & Parra, 2012; Pašková, 2007). Semi-liquid diets also had poor adult emergence, high proportion of partially emerged flies (especially in the Chang 2009 diet), and long developmental time, and this may be because of excessive energy demands of moving through and exiting these diets.

There were some notable differences between the diets, and interestingly significant differences between the diets in the effects of agar concentration. The Chang 2006 and Chang 2009 diets are broadly similar but differ in details that appear to influence the effects of agar concentration. The diets are similar in amounts of sugar, the preservatives sodium benzoate and nipagen, and water, excluding these as explanations for differences in larval performance. While the diets are similar in the amount of yeast used, the type of yeast is different and this is a likely source of differences in response of developing larvae to agar concentration. Furthermore, the Chang 2009 diet formulation contains approximately five times more wheat germ oil than the Chang 2006 formulation.

Flight ability is an important quality control parameter for mass-reared flies in SIT (Collins & Taylor, 2010), and has been discussed as a particular deficiency for Q-fly reared on liquid larval diets (Khan 2013, 2014). The greater amount of wheat germ oil in the Chang 2009 diet formulation might be responsible for the greater percentage of fliers in this diet compared to the Chang 2006 diet.

Absence of wheat germ oil in liquid larval diets can reduce flight ability in tephritids because of over expression of one flightless protein (Cho et al., 2013). Wheat germ oil contains protein, carbohydrates, fatty acids, vitamin E and sterols that might influence fly quality (Cohen, 2004). Polyunsaturated, saturated, monounsaturated and trans saturated fatty acids in wheat germ oil (Chang & Vargas, 2007) may contribute to development of flight muscles (Cho et al., 2013; Pascacio-Villafán et al., 2015), as may vitamin E (Chang and Vargas 2007). Cho et al. (2013) found that *Ceratitis capitata* (Mediterranean fruit fly) larvae reared on a diet deficient in fatty acids tend to look normal but have poor flight performance (Cho et al., 2013), and so differences in fatty acid content is a very likely source of the differences in flight performance of Q-fly reared on the Chang 2006 and Chang 2009 diet formulations. While wheat germ oil might be key to the greater flight performance of flies on the Chang 2009 diet formulation, it might also be responsible for the higher mortality under stress for flies reared on this diet formulation at some agar concentrations. Although the two diets tested in the present study differed in the type of yeast used, Ekesi et al. (2014) found no effect of yeast type on flight ability of *Bactrocera invadens* (*dorsalis*) (oriental fruit fly) and *Ceratitis fasciventris*. Accordingly, the contents of wheat germ oil offer a more compelling starting point from which to further investigate diet differences in flight ability.

For both sexes, mortality under stress was similar for all agar concentrations of the Chang 2009 diet formulation, but increased with agar concentration for the Chang 2006 diet formulation. To understand the causes of such differences in how the two larval diet formulations responded to the addition of agar will require experiments that systematically vary the likely sources of such variation, such as amount of wheat germ oil (and its constituents) and the type of yeast.

Despite the differences between the two diet formulations, the data obtained from some of the gel diets in this study were satisfactory and close to recommended quality control values for Q-fly (FAO/IAEA/USDA, 2003). Interestingly, the obtained pupal number is likely to outperform the conventional larval diets such as carrot or lucerne chaff (see Khan 2013). The question of how much agar is ideal depends somewhat on which larval diet formulation is considered and how the various quality control metrics are prioritized. Low levels of agar that result in semi-liquid diets (0.25%) performed poorly overall, with slow development, low productivity, and poor emergence. High levels of agar that result in firm gels (1 and 1.5%) were very similar in their performance in all metrics for both diets. Moderate levels of agar (0.5%) produced inconsistent results, with substantial differences between the larval diet formulations for some metrics (e.g., pupal number, mortality under stress) but importantly showing reduced mortality under stress at least for the Chang 2006 diet formulation.

The present study highlights key areas of research for future studies aimed at optimizing larval diets for mass rearing of Q-fly, including further investigating the role of wheat germ oil, and its constituents on performance parameters, understanding why different liquid diet formulations respond differently to addition of agar for conversion to a gel diet, and determining the comparative performance of economical alternatives to diet components investigated in the present study. Further, the addition of probiotic bacteria has yielded improved performance of larval diets for some tephritids (Augustinos et al., 2015), and the inclusion of probiotics in gel diets for Q-fly warrants investigation as an additional component. Finally, in addition to showing very compelling potential as an approach to mass rearing of Q-fly, gel diets offer new opportunities for experimental studies of diet and diet preferences, providing a far more consistent and defined composition than the alternatives of traditional solid diets or liquid diets.

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Figure legends

Figure 1. **Developmental time:** Egg-larval duration (A), Pupal period (B), Pupal production period (C) and Peak of pupation (D) of Q-fly reared on ‘Chang 2006’ and ‘Chang 2009’ larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

Figure 2. **Pupal production:** Pupal number (A) and Pupal weight (B) of Q-fly reared on ‘Chang 2006’ and ‘Chang 2009’ larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

Figure 3. **Flight ability:** Percentage of emergence (A), Percentage of fliers (B), Rate of fliers (C), Not-emerged (D), Not-fliers (E) of Q-fly reared on ‘Chang 2006’ and ‘Chang 2009’ larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

Figure 4. **Mortality under stress**: Male mortality under stress (A) and Female mortality under stress (B) of Q-fly reared on ‘Chang 2006’ and ‘Chang 2009’ larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

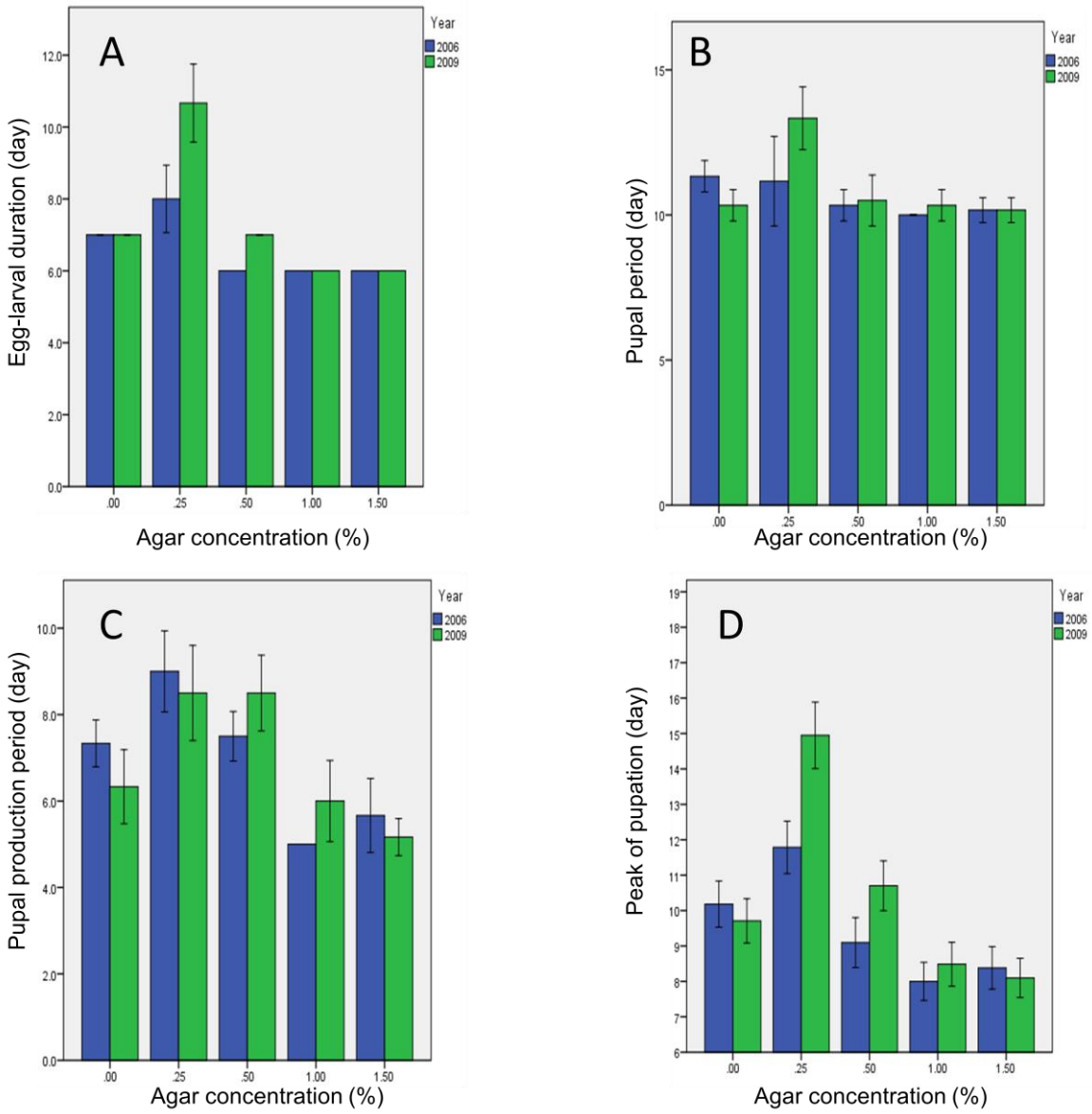
A
Year
2006
2009
Egg-larval duration (day)
Agar concentration (%)
.00
.25
.50
1.00
1.50
B
Pupal period (day)
C
Pupal production period (day)
D
Peak of pupation (day)

Figure 1

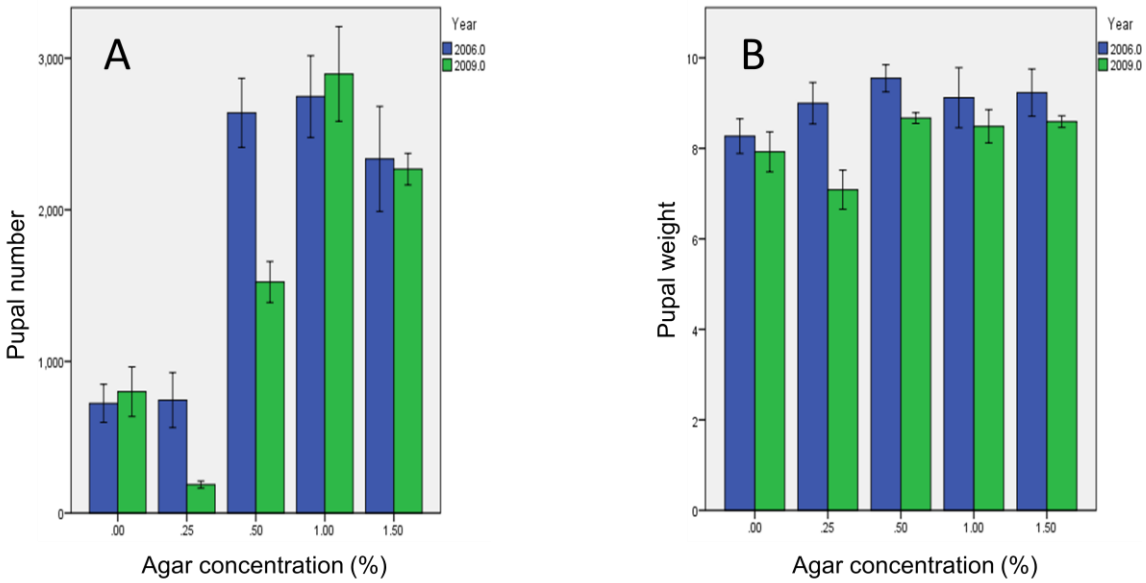
A
Year
2006.0
2009.0
Pupal number
3,000
2,000
1,000
0
.00
.25
.50
1.00
1.50
Agar concentration (%)
B
Year
2006.0
2009.0
Pupal weight
10
8
6
4
2
0
.00
.25
.50
1.00
1.50
Agar concentration (%)

Figure 2

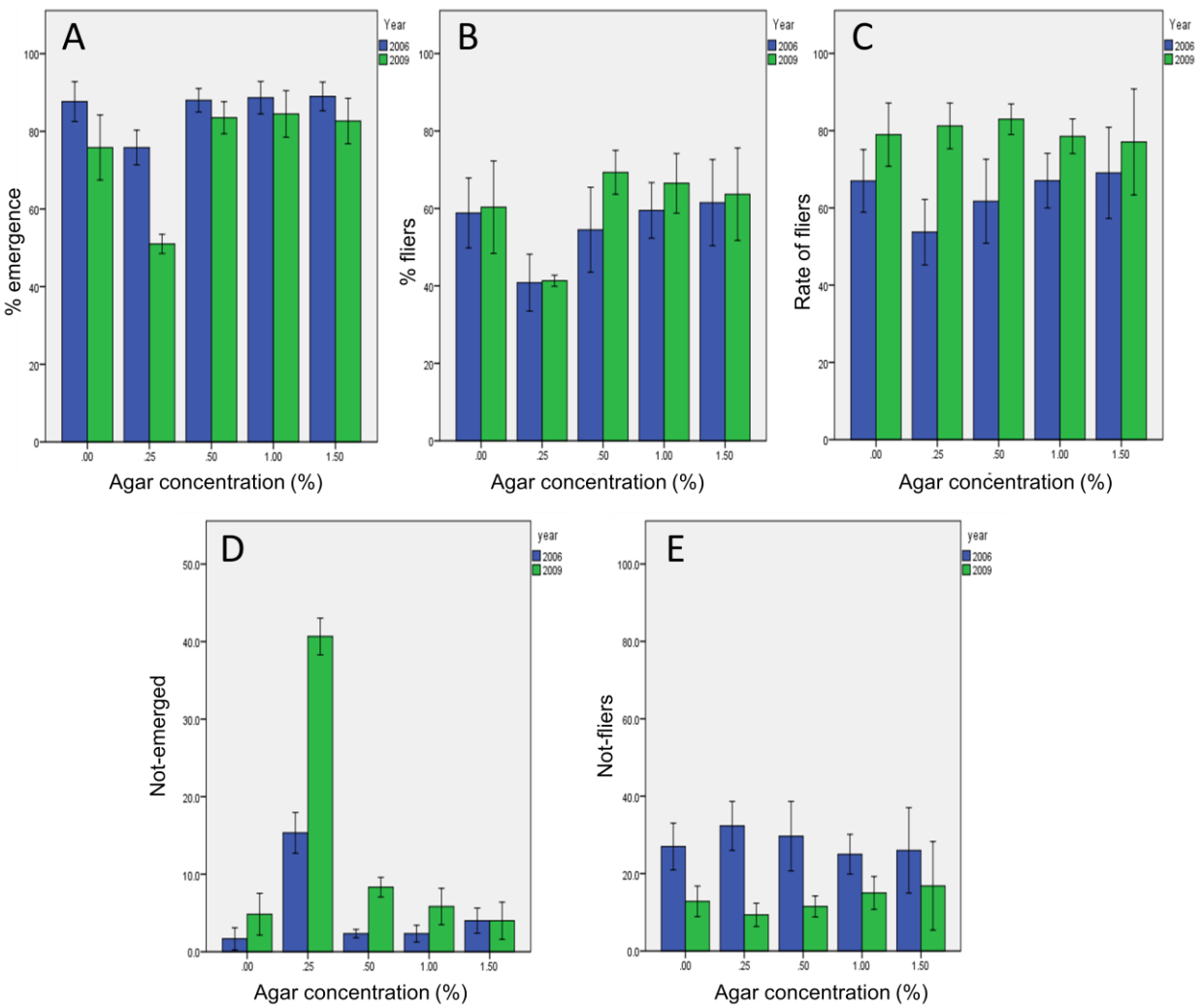
A
% emergence
Agar concentration (%)
Year
2006
2009
B
% fliers
Agar concentration (%)
C
Rate of fliers
Agar concentration (%)
D
Not-emerged
Agar concentration (%)
year
E
Not-fliers
Agar concentration (%)

Figure 3

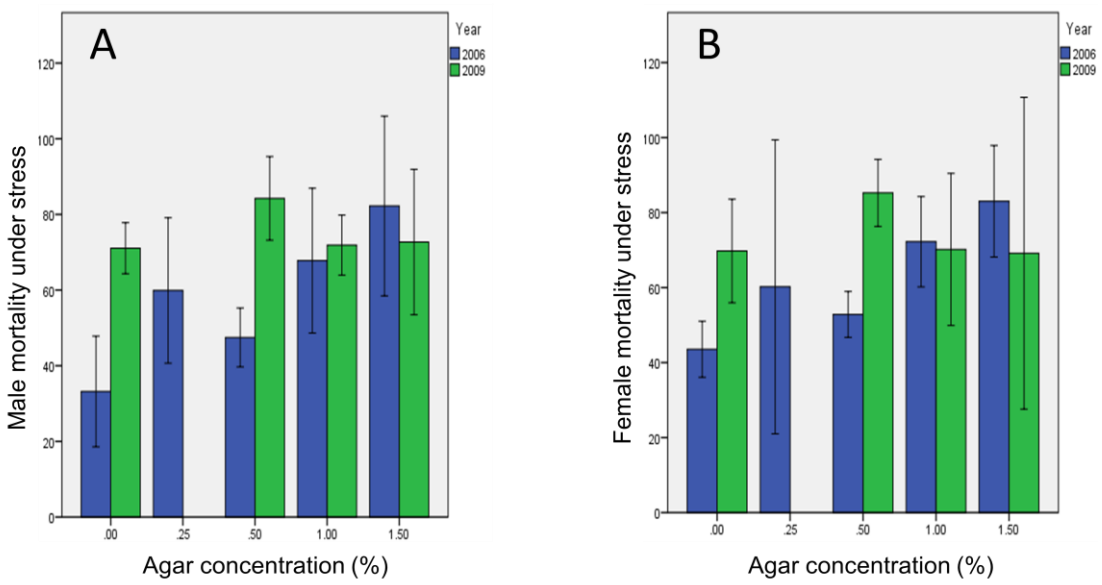
A
Year
2006
2009
Male mortality under stress
120
100
80
60
40
20
0
.00
.25
.50
1.00
1.50
Agar concentration (%)
B
Year
2006
2009
Female mortality under stress
120
100
80
60
40
20
0
.00
.25
.50
1.00
1.50
Agar concentration (%)

Figure 4

Tables

Table1 Composition of 'Chang 2006' and 'Chang 2009' larval diets with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

Ingredients	'Chang 2006' *liquid diet*		'Chang 2009'*liquid diet*	
	%	g	%	g
Yeast LBI2240	-	-	10.96	15.3
Yeast FNILS65	-	-	3.65	5.1
Brewer's yeast	15.06	20.40	-	-
Sugar	8.99	12.18	8.72	12.18
Nipagen	0.15	0.20	0.14	0.20
Sodium banzoate	0.15	0.20	0.14	0.20
Citric acid	1.70	2.31	4.66	6.50
Water (ml)	73.81	100	71.62	100
Wheat germ oil (ml)	0.15	0.2	1% of water volume	1
Agar	0	-	0	-
	0.25		1.25	
	0.5		2.5	
	1		5	
	1.5		7.5	

Table 2 Statistics of quality parameters of egg-pupal stages of Q-fly reared on 'Chang 2006' and 'Chang 2009' larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%).

	Parental egg hatch	Egg-larval duration	Egg-pupal duration	Pupal period	Pupal production period	Peak of pupation	Pupal number	Pupal recovery	Pupal weight
Agar × Diet	$F_{4, 30} = 2.47$ *P = 0.078*	$F_{4, 60} = 21.78$ ***P < 0.001***	$F_{4, 60} = 49.26$ ***P < 0.001***	$F_{4, 60} = 7.42$ ***P < 0.001***	$F_{4, 60} = 4.80$ ***P = 0.002***	$F_{4, 393} = 9.74$ ***P < 0.001***	$F_{4, 60} = 20.85$ ***P < 0.001***	$F_{4, 60} = 11.83$ ***P < 0.001***	$F_{4, 60} = 7.12$ ***P < 0.001***
Agar	$F_{4, 30} = 1.95$ *P = 0.141*	$F_{4, 60} = 123.57$ ***P < 0.001***	$F_{4, 60} = 199.64$ ***P < 0.001***	$F_{4, 60} = 17.14$ ***P < 0.001***	$F_{4, 60} = 48.27$ ***P < 0.001***	$F_{4, 393} = 72.75$ ***P < 0.001***	$F_{4, 60} = 307.53$ ***P < 0.001***	$F_{4, 60} = 326.00$ ***P < 0.001***	$F_{4, 60} = 18.67$ ***P < 0.001***
Diet	$F_{1, 30} = 0.20$ *P = 0.657*	$F_{1, 60} = 43.21$ ***P < 0.001***	$F_{1, 60} = 55.65$ ***P < 0.001***	$F_{1, 60} = 3.12$ *P < 0.083*	$F_{1, 60} = 0.00$ *P = 1.000*	$F_{1, 393} = 16.07$ ***P < 0.001***	$F_{1, 60} = 33.85$ ***P < 0.001***	$F_{1, 60} = 27.96$ ***P < 0.001***	$F_{1, 60} = 75.36$ ***P < 0.001***

Table 3 Statistics of quality parameters of adult Q-fly reared on 'Chang 2006' and 'Chang 2009' larval diet formulations with different percentage of agar (0, 0.25, 0.5, 1.0 and 1.5%)

	Sex ratio	Percentage of emergence	Percentage of fliers	Rate of fliers	Not-emerged	Partially emerged	Not-fliers	Deformed	Male mortality	Female mortality
Agar × Diet	$F_{4, 60} = 0.161$ *P = 0.957*	$F_{4, 60} = 9.916$ ***P < 0.001***	$F_{4, 60} = 1.440$ *P = 0.235*	$F_{4, 60} = 2.78$ ***P = 0.036***	$F_{4, 60} = 87.79$ ***P < 0.001***	$F_{4, 60} = 2.300$ *P = 0.072*	$F_{4, 60} = 2.27$ *P = 0.075*	$F_{4, 60} = 1.410$ *P = 0.244*	$F_{3, 27} = 21.63$ ***P < 0.001***	$F_{4, 27} = 9.304$ ***P = 0.001***
Agar	$F_{4, 60} = 3.17$ ***P = 0.021***	$F_{4, 60} = 50.97$ ***P < 0.001***	$F_{4, 60} = 14.26$ ***P < 0.001***	$F_{4, 60} = 0.979$ *P = 0.427*	$F_{4, 60} = 389.93$ ***P < 0.001***	$F_{4, 60} = 3.171$ ***P = 0.021***	$F_{4, 60} = 0.104$ *P = 0.980*	$F_{4, 60} = 2.50$ *P = 0.054*	$F_{4, 27} = 13.02$ ***P < 0.001***	$F_{4, 27} = 3.93$ ***P = 0.018***
Diet	$F_{1, 60} = 3.49$ *P = 0.067*	$F_{1, 60} = 70.45$ ***P < 0.001***	$F_{1, 60} = 5.52$ ***P = 0.023***	$F_{4, 60} = 55.104$ ***P < 0.001***	$F_{1, 60} = 246.60$ ***P < 0.001***	$F_{1, 60} = 4.25$ ***P = 0.044***	$F_{1, 60} = 75.43$ ***P < 0.001***	$F_{1, 60} = 1.549$ *P = 0.219*	$F_{1, 27} = 45.82$ ***P < 0.001***	$F = 8.63$ ***P = 0.009***

Table 4 The quality parameters of Q-fly reared on 'Chang 2006' and 'Chang 2009' larval diets with different percentages of agar (0, 0.25, 0.5, 1.0 and 1.5%). The descriptive statistics presented as mean ± SD. Different letters across rows for each parameter indicate significant Tukey's post hoc comparisons (P<0.05). Post hoc tests were run separately for each diet formulation (i.e., Chang 2006 and Chang 2009).

	Chang 2006 diet formulation					Chang 2009 diet formulation				
	Agar (%)					Agar (%)				
	0	0.25	0.5	1	1.5	0	0.25	0.5	1	1.5
Parental egg hatch	81.85 ± 10.67	91.33 ± 1.15	90.66 ± 2.30	80.66 ± 4.61	84.66 ± 3.05	88 ± 2.00	88 ± 4.00	80.66 ± 4.61	83.33 ± 3.05	85.21 ± 5.88
Egg-larval duration	7.00 ± 0.00a	8.00 ± 0.89b	6.00 ± 0.00c	6.00 ± 0.00c	6.00 ± 0.00c	7.00 ± 0.00a	10.66 ± 1.03b	7.00 ± 0.00a	6.00 ± 0.00c	6.00 ± 0.00c
Egg-pupal duration	18.33 ± 0.51a	19.17 ± 0.98a	16.33 ± 0.51b	16.00 ± 0.00b	16.17 ± 0.40b	17.33 ± 0.51a	24.00 ± 0.00b	17.50 ± 0.83a	16.33 ± 0.51c	16.17 ± 0.40c
Pupal period	11.33 ± 0.51a	11.17 ± 1.47a,b	10.33 ± 0.51a,b	10.00 ± 0.00b	10.17 ± 0.40a,b	10.33 ± 0.51a	13.33 ± 1.03b	10.50 ± 0.83a	10.33 ± 0.51a	10.17 ± 0.40a
Pupal production period	7.33 ± 0.51a	9.00 ± 0.89b	7.50 ± 0.54a	5.00 ± 0.00c	5.66 ± 0.81c	6.33 ± 0.81a	8.50 ± 1.04b	8.50 ± 0.83b	6.00 ± 0.89a	5.16 ± 0.40a
Peak day of pupation	10.18 ± 2.14	11.78 ± 2.64	9.09 ± 2.23	8.00 ± 1.43	8.38 ± 1.72	9.71 ± 1.90	14.94 ± 2.89	10.70 ± 2.47	8.48 ± 1.80	8.09 ± 1.51
Pupal weight	8.26 ± 0.36a	8.99 ± 0.43a,b	9.54 ± 0.28b	9.11 ± 0.63b	9.23 ± 0.49b	7.92 ± 0.42a	7.08 ± 0.41b	8.66 ± 0.11c	8.48 ± 0.35c	8.59 ± 0.12c
Pupal recovery	25.25 ± 4.18a	23.30 ± 5.41a	83.17 ± 6.83b	97.28 ± 9.11c	78.80 ± 11.14b	25.98 ± 5.05a	6.10 ± 0.72b	53.95 ± 4.56c	99.28 ± 10.20d	76.04 ± 3.31e
Pupal number	723 ± 119a	744 ± 173a	2639 ± 216b	2746 ± 257b	2335 ± 330b	800 ± 155a	188 ± 22b	1523 ± 128c	2895 ± 297d	2268 ± 98e
Sex ratio (% male)	48.42 ± 4.28	51.05 ± 6.44	46.54 ± 2.94	45.36 ± 3.48	46.72 ± 3.81	49.68 ± 5.69	54.55 ± 4.52	49.87 ± 5.27	47.61 ± 3.55	47.89 ± 6.18
Adult emergence	87.67 ± 4.88a	75.83 ± 4.26b	88 ± 2.89a	88.67 ± 3.9a	89± 3.52a	75.83 ± 7.98a	51± 2.36b	83.50 ± 3.93a	84.50 ± 5.71a	82.67± 5.57a
Fliers	58.83 ± 8.61b	40.83 ± 6.99a	54.50 ± 10.42a,b	59.50 ± 6.83b	61.50 ± 10.59b	60.33 ± 11.37a	41.33 ± 1.36b	69.33 ± 5.39a	66.50 ±7.34a	63.66 ± 11.37a
Rate of fliers	66.99 ± 7.7a,b	53.72 ± 8.07b	61.73 ± 10.35a,b	67.05 ± 6.7a,b	69.06 ± 11.24a	78.97 ± 7.82	81.24 ± 5.65	82.96 ± 3.78	78.54 ± 4.28	77.06 ± 13.06
Not-emerged	1.66 ± 1.36a	15.33 ± 2.50b	2.33 ± 0.51a	2.33 ± 1.03a	4 ± 1.54a	4.83 ± 2.56a	40.66 ± 2.25b	8.33 ± 1.21a	5.83 ± 2.22a	4 ± 2.28a
partially emerged	10.66 ± 5.64	8.83 ± 3.97	9.66 ± 2.94	9 ± 3.74	7 ± 3.09	19.33 ± 9.47a	8.33 ± 0.51b	8.16 ± 3.31b	9.66 ± 6.40a,b	13.33 ± 6.43a,b
Non-fliers	27 ± 5.76	32.33 ± 6.02	29.66 ± 8.57	25 ± 4.89	26 ± 10.50	12.83 ± 3.76	9.33 ± 2.87	11.50 ± 2.58	15 ± 4.04	16.83 ± 10.88
Deformed	1.83 ± 1.94	2.66 ± 2.42	3.83 ± 2.48	4.16 ± 3.48	1.50 ± 0.83	2.66 ± 1.75	0.33 ± 0.51	2.66 ± 1.50	3± 1.67	2.16 ± 1.32
Mortality (% female)	43.55 ± 3.00a	60.2 ± 15.7a,c	52.83 ± 2.45a	72.22 ± 4.84b,c	83.00 ± 5.99b	69.76 ± 5.56	-	85.25 ± 3.60	70.17 ± 8.16	69.17 ± 16.73
Mortality (% male)	33.19 ± 5.88a	59.89 ± 7.7b,c	47.47 ± 3.13a,b	67.79 ± 7.70c,d	82.24 ± 9.56d	71.07 ± 2.71a,c	-	84.23 ± 4.44b,d	71.87 ± 3.18c,d	72.68 ± 7.73c,d

Chapter 3

This chapter is written in the form of a journal article from:

Journal of Applied Entomology

Detrimental effect of immersion in water on egg hatching of *Bactrocera tryoni*, is ameliorated by aeration

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Short title:

Detrimental effect of water on egg quality of Q-fly

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Abstract

Bactrocera tryoni (Diptera: Tephritidae), the Queensland fruit fly or 'Q-fly', is Australia's most economically important pest of fruit crops. The Sterile Insect Technique (SIT) is an increasingly important approach to managing Q-fly outbreaks. SIT involves the mass rearing and release of millions of sterile flies to reduce pest populations in the next generation. Substantial attention has been given to larval diets as an influence on production, but the preceding operational steps of collecting and storing of eggs has attracted little attention. Eggs are commonly collected and stored in water, and we here considered whether egg hatching is affected by this practice and whether such effects can be ameliorated by aeration. Eggs were held in water for 6, 12 and 24 hours with and without aeration and were then seeded onto damp filter paper to assess proportion hatching. Without aeration, hatching success of eggs declined significantly, but with aeration no decline was detected even after 24 hours. Generally, minimal periods of holding in water are recommended to minimize reductions in hatching and potential sub-lethal effects on quality, with aeration strongly recommended especially for periods over 6 hours.

Keywords

Aeration, egg storage, mass rearing, tephritid, sterile insect technique

Introduction

The Queensland fruit fly, *Bacterocera tryoni* (Diptera: Tephritidae) ('Q-fly'), is the most economically important insect pest of Australian horticulture (Clarke et al. 2011; Sutherst et al. 2000; PBCRC 2015). Chemical control options have become increasingly constrained such that more environmentally benign approaches, including the Sterile Insect Technique (SIT), are increasingly important. In SIT millions of flies are reared, sterilized and released to mate with wild females and, by reducing fertility of eggs and survival of embryos, decrease population levels in the next generation.

Vast numbers of flies are required for effective SIT and so the mass-rearing process is critical. Substantial effort has been invested in the development of effective larval diets (Chang 2004; Chang et al. 2004, 2006, 2007; Chang 2009 a,b; Chang and Vargas 2007; Khan 2013; Resilva et al. 2014; Ekesi et al. 2014;Vera et al. 2014). However there are some quite significant gaps in our understanding of how preceding production steps - especially collection and storage of eggs - can impinge on overall productivity of mass rearing systems.

Mass rearing programs sometimes maintain fruit fly eggs immersed in water for periods ranging for a few minutes to many hours. Eggs may also be stored in water for transport and sexing (Robinson, 2002). Since egg hatching rate is an important determinant of productivity, we here consider the effects of immersion in water on hatching of Q-fly eggs. Several studies mention aeration of eggs in water either in mass-rearing programs (Schwarz et al. 1985) or in experimentation (Mamán and Cáceres 2007; Vera et al. 2014). While aeration is suggested to enhance hatching rates (Andrew Jessup, pers comm), there is very little evidence in the literature to support this practice.

Materials and methods

Q-fly eggs were obtained from New South Wales Department of Primary Industries, Ourimbah, New South Wales, Australia. The eggs were collected in a plastic jar that had numerous puncture holes for the females to oviposit through and contained ca.40ml of water. Eggs were rinsed into a beaker and then tipped into a vial for transport. The eggs were from adults of F3 generation that had been reared on carrot-based diet. The eggs were collected at 8:30am (AEST) and transported by air-conditioned car for approximately one hour to Macquarie University. All experiments were carried in a controlled environment laboratory (25 ± 0.2^0C, 65±3% RH and 11: 1: 11: 1 light: dusk: dark: dawn photoperiod) at Macquarie University.

Eighteen 150 mL glass Schott bottles were each filled with 100 ml of tap water (pH: 5.5). After tipping off excess water from the vial of eggs until only enough remained to cover the eggs, 500µl of

eggs (approximately 7,000 eggs) were transferred to each jar using a 1000 µl pipette, the tip of which had been cut to enlarge the opening and thereby prevent eggs from sticking. Three bottles of water containing eggs were aerated for each of 6, 12 and 24 hours at 25 ºC using an aquarium pump to force 2000 ml/min of air through an aquarium stone placed in the water. Aeration created turbulence that prevented eggs from settling on the bottom of the jar. Three additional bottles were kept without aeration for each of 6, 12 and 24 hours.

After each of the immersion periods, eggs were collected from each jar using a 100 µl pipette and dispensed into a Petri dish under a stereomicroscope. 100 eggs were counted and, using a fine camelhair brush, were transferred onto a moistened sector of black filter paper that was positioned in a 55 mm Petri dish atop two discs of moistened 55 mm white filter. As a baseline, additional dishes of eggs were set up on filter paper as soon as eggs were received. Because of variation in delivery time, eggs were set up after two baseline periods in water during transport. For two batches of eggs the experiments were set up ca. 2 hours after egg collection and for another two batches of eggs the experiments were set up ca. 5 hours after egg collection. Egg hatching was assessed 96 hours after eggs of the final treatment (24 h) were transferred to filter paper.

A mixed model was used to compare percentage egg hatch amongst baseline, the three time points of aerated eggs and the three time points of un-aerated eggs as fixed effects. The model included the two starting times (2 and 5 hours after egg collection) as a fixed effect and the experimental batch nested within starting time as a random effect. Data were analyzed using JMP (SAS Institute).

Results

Significant differences were found amongst experimental treatments (0, 6, 12, 24 hour aerated or unaerated; $F_{6,74} = 23.465$, $p < 0.001$) (Figure 1), but no significant difference was found between the two experiment starting times ($F_{1,74} = 0.200$, $p = 0.699$). Tukey HSD tests did not detect a significant change in proportion of eggs hatching after 6 or 12 hours in water for un-aerated eggs, but after 24 hours there was a significant decrease in the proportion of eggs that hatched. In contrast, for aerated eggs we found no evidence of changes in hatching over 24 hours. After 6 hours of immersion there was no significant difference in proportion of eggs hatched in aerated and un-aerated water, but there were significant differences at both 12 and 24 hours.

Discussion

The present study found significant reductions in percentage of Q-fly eggs hatching as a consequence of sustained immersion in water, and also demonstrated that such negative effects of immersion in water can be ameliorated by aeration.

Over the first six hours of experimental immersion in water there was no difference in the proportion of eggs hatching from aerated and un-aerated treatments. After this time, the proportion of eggs hatching reduced for un-aerated eggs but was maintained at baseline levels for aerated eggs. In situations where aeration is not feasible, our results suggest that eggs can be held in water for up to six hours without significant reductions in hatching, but that aeration is required to prevent losses if eggs are held in water for longer.

Developing embryos contain nutrition needed to sustain their development but must acquire required oxygen through the eggshell (Helvik and Walther 1993; Murillo and Jirón 1994; Woods et al. 2005). An air-filled tube, the aeropyle, is thought to be responsible for gas exchange between the embryo and its environment (Mouzaki and Margaritis 1986; Woods et al. 2005). Aeration of water containing the eggs might increase gas exchange for the developing embryos. The present study used a single airflow rate that was sufficient to prevent eggs from settling on the bottom of the jar of water in which they were held. This being the case, we cannot ascribe the effects of aeration to oxygen exchange or physical movement of eggs, as these manipulations were always imposed in concert. In halibut, strong and weak aeration has been associated with lower and higher percentage hatching of larvae, respectively (Helvik and Walther 1993), and it would be interesting to consider quantitative effects of aeration rates in fruit fly eggs as well as disambiguation of the effects of oxygenation and physical movement.

It is important to note that the present study focused only on proportion of eggs hatching. However, decrements in health are likely to precede death of embryos, so it is likely that quality of developing embryos is reduced over much shorter time frames in water than is evident in our existing data. Further studies are needed to investigate sub-lethal effects of immersing eggs in water for periods within which decrements in proportion hatching are not evident.

Acknowledgements

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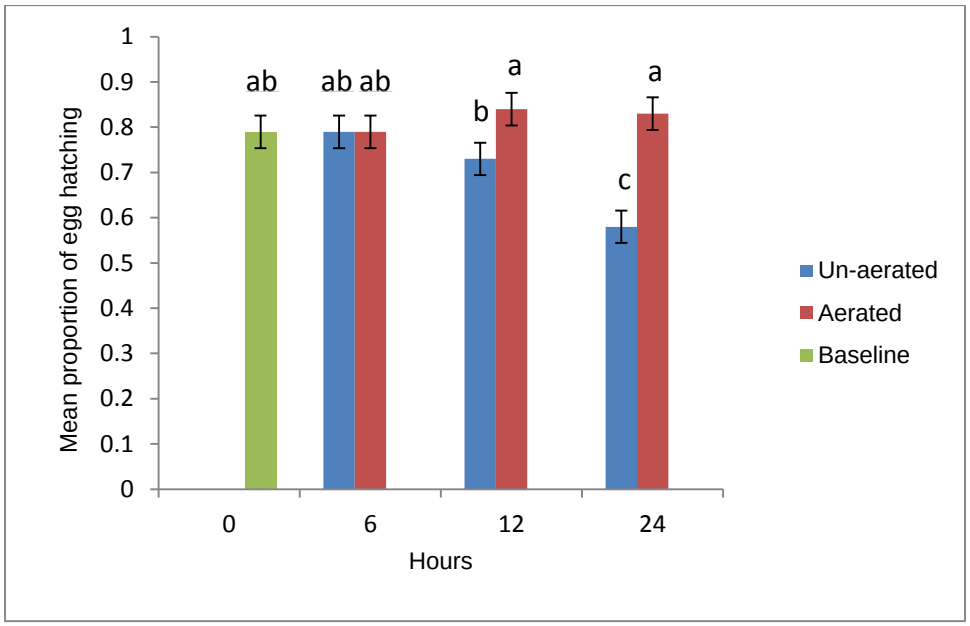

Figure 1 Mean proportion of egg hatching of Q-fly in water with and without aeration for 6, 12 and 24 hours. Treatments that share the same letter are not significantly different to each other (Tukey's HSD)

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