Section 7.5.3.1.2 Short-term toxicity on birds

Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2006) Difenacoum Technical – Dietary Toxicity Test with the Japanese Quail (*Coturnix coturnix japonica*) XXXXX Study No. 13768.4100	
1.2	**Data protection**	Yes	
1.2.1	Data owner	PelGar International Ltd. And Activa/Tezza S.R.L	
1.2.2	Companies with letters of access	PelGar International Ltd. Activa S.R.L	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. / b.p. for the purpose of its entry into Annex I authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD Guideline Number 205 EU council Directive 91/414/EEC, Annex II, 8.1.2	
2.2	**GLP**	Yes	
2.3	**Deviations**	Yes 1. Humidity in the test room is recommended to be maintained at 50-75%. During the experimental period humidity ranged from 47 to 61%. 2. A maximum brooder temperature of 32°C for quails aged 8 to 14 is recommended. During this and the 8- to 14- day age range the maximum brooder temperature recorded was 34 °C *These deviations did not affect the results or interpretation of the test.*	
		3 METHOD	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	Batch No. 03652. CAS No. 56073-07-5	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	99.70% w/w	
3.1.4	Composition of Product	n/a	
3.1.5	Further relevant properties	n/a	
3.1.6	Method of analysis	All homogeneity, stability and verification samples were analyzed for difenacoum using HPLC/UV based on validated methodology. The HPLC analysis was conducted utilizing the following instrumental conditions:	

Section 7.5.3.1.2 Short-term toxicity on birds
Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

		Column: Supelco Discover c18, 150mm x 4.6mm Mobile Phase A: 0.1% phosphoric acid in purified reagent water Mobile phase B: 100% acetonitrile Flow rate: 1.00mL/minute Injection volume: 100µL Wavelength: 310nm Retention time: approximately 19 to 20 minutes Samples were diluted with methanol.	
3.2	**Administration of the test substance**	See table A7_5_3_1_2-1	
3.3	**Reference substance**	No	
3.3.1	Method of analysis for reference substance	n/a	
3.4	**Testing procedure**		
3.4.1	Test organisms	See table A7_5_3_1_2-2	X
3.4.2	Test system	See table A7_5_3_1_2-3	X
3.4.3	Diet	The birds were given commercially available feed Purina® Game Bird Startena® containing 0.35 to 0.4 ppm vitamin K which was provided *ad libitum.* Polypropylene glycol was used as the carrier. The test substance was mixed with the feed using a Hobart mixer for approximately 25 minutes to ensure thorough mixing.	
3.4.4	Test conditions	See table A7_5_3_1_2-4	
3.4.5	Duration of the test	8 days (5 days of exposure to the test substance followed by 3 days of observation)	
3.4.6	Test parameter	Mortality	
3.4.7	Examination / Observation	Body weights were measured for each test animal the day of, and prior to test initiation and on days 5 and 8 Feed consumption was determined for the initial 5 days (exposure period), and days 6 to 8 (post-exposure period). Each animal was observed 3 times on the first day of exposure, then twice daily, and once on day 8 for mortality, morbidity, and symptoms of intoxication. Post mortem gross pathological examinations on birds sacrificed *in extremis* or found dead during the test.	
3.4.8	Statistics	LC50, slope and intercept were determined via probit analysis using	

Section 7.5.3.1.2 Short-term toxicity on birds

Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

		TOXCALC (Oris, 1984 and Stephen 1984). Body weight data prior to start of treated feed were assessed for variance using Levene's Test (Weber et al 1989) and were tested for mean differences among cages using one-way analysis of variance (TOXSTAT, 1996) . Body weight changes were tested for normality using Chi-square Test (Weber et al 1989), homogeneity of variance using Levene's test. Change in body weight was analyzed using analysis of variance (ANOVA) followed by Bonferroni's t-test (Weber et al 1989) for pair wise comparisons when the data were normal and homogenous . Kruskal-Wallis and Dunns multiple comparisons test was used when data were non-normal or heterogenous.(TOXSTAT, 1996)
		4 RESULTS
4.1	**Limit Test / Range finding test**	Performed
4.1.1	Concentration / dose	0 (control) 0.050, 0.10, 0.50, 1.0, 3.0, 6.0 mg ai/kg feed.
4.1.2	Number/ percentage of animals showing adverse effects	Complete mortality was observed at the 6.0 ppm dietary concentrations by day 4. Mortality of 75% and 25% was observed at the 3.0 and 1.0 ppm dietary concentration by days 3 and 6.
4.1.3	Nature of adverse effects	Not stated
4.2	**Results test substance**	
4.2.1	Applied concentrations	0 (control) 0.40, 0.66, 1.1, 1.8, 3.0 and 5.0 mg a.i/kg feed *Calculated as:* (0, 0.083, 0.116, 0.213, 0.334, 0.5446 and 0.833 mg/kg bw/day)
4.2.2	Effect data (Mortality)	(see table A7_5_3_1_2-5); LC50 = 1.4mg a.i/kg feed with corresponding 95% c.l of 1.74 to 3.95 mg a.i/kg feed. NOEC (mortality) = 0.40mg a.i/kg feed

Section 7.5.3.1.2 Short-term toxicity on birds

Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

4.2.3 Body weight

Table 5. Body weight summary by treatment group during the Japanese quail (*Coturnix coturnix japonica*) dietary toxicity study with difenacoum technical.

Nominal Dietary Concentration (mg a.i./kg feed)		Mean Weight (g) Time 0 (Start of Treatment)	Mean Weight (g) Day 5 (End of Treatment)	Proportional Mean Weight Change Interval 0 - 5	Mean Weight (g) Day 8 (Test Termination)	Proportional Mean Weight Change Interval 0 – 8
Control [a]	Cage 1	57.5	85.7	0.5	110.4	0.9
	Cage 5	60.7	87.1	0.4	111.3	0.8
0.40	Cage 7	61.1	87.3	0.4	108.4	0.8
0.66	Cage 8	62.9	77.0	0.2[b]	98.0	0.6[c]
1.1	Cage 6	62.2	85.3	0.4	107.7	0.7[c]
1.8	Cage 3	63.8	86.6	0.4	120.7	1.0
3.0	Cage 2	59.7	68.7[c]	0.2	87.1	0.4[c]
5.0	Cage 4	61.3	74.9	0.2	95.7	0.5[c]

[a] Two cages were established for the control (12 birds each).
[b] Significantly different compared to the control (cage 1), based on Dunn's Multiple Comparison test.
[c] Significantly different compared to the control (cage 1), based on Bonferroni's test.

4.2.4 Food consumption

Table 6. Feed consumption (g) per bird per day during the Japanese quail (*Coturnix coturnix japonica*) dietary toxicity study with difenacoum technical.

Nominal Dietary Concentration (mg a.i./kg feed)	Day 1	Day 2	Day 3	Day 4	Day 5
Control	12.2	16.6	16.6	16.3	15.8
0.40	13.4	12.8	15.9	17.2	17.8
0.66	14.1	9.1	16.5	10.5	11.0
1.1	12.9	15.2	14.7	15.7	13.1
1.8	12.8	17.2	15.1	10.6	14.1
3.0	13.4	15.3	10.4	9.7	9.7
5.0	13.9	13.5	10.3	9.1	10.0

Nominal Dietary Concentration (mg a.i./kg feed)	Day 6	Day 7	Day 8
Control	21.6	24.4	19.4
0.40	20.9	25.8	18.2
0.66	18.4	24.2	14.3
1.1	15.1	19.2	21.8
1.8	15.6	24.1	25.6
3.0	7.9	15.9	18.4
5.0	7.6	21.7	24.6

NOTE: Total values were determined based on the original raw data and not the rounded daily values presented here.

Section 7.5.3.1.2 Short-term toxicity on birds

Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

4.2.5	Concentration / response curve	+ 2.84	x
4.2.6	Other effects	Abnormal behaviour including lethargy, piloerection, huddling, wing droop and unresponsiveness was exhibited in all treatment groups except the control group and the 0.40 mg a.i/kg feed. External signs of haemorrhaging (edema, purpura, haemorrhaging in the leg and thigh were observed in the 3.00 and 5.00 mg a.i/kg feed groups. Post mortem examinations showed haemorrhaging or signs thereof in all treatment groups. Frequency, severity and number of observations increased as the treatment level increased. Free blood was found and blood found externally on some treatment birds (possibly due to scratching) One bird at study termination (3.0 mg a.i/kg feed) showed signs of prior haemorrhaging from the kidneys, which had assumed a granular texture. One bird in the 3.00 mg a.i/kg feed group examined at study termination was markedly emaciated. One bird in the 5.00 mg a.i/kg feed group displayed a gall bladder that was exuding bile onto surrounding tissues.	
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	None	
4.3.2	Nature of adverse effects	N/A	
4.4	**Test with reference substance**	Not performed	
4.4.1	Concentrations	n/a	
4.4.2	Results	n/a	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The objective of the study was to determine the dietary median lethal concentration (LC50), Lowest Lethal Concentration (LLC) and No-Observed-Effect-Concentration (NOEC), if possible of difenacoum technical administered via the diet to Japanese quail. Sublethal effects, if any such as changes in body weight, reduced feed consumption, and changes in behaviour were monitored. The study was conducted in accordance with the following guidelines: OECD Guideline number 205 EU Council Directive 91/414/EEC, Annex II, 8.1.2	
5.2	**Results and discussion**	Measured concentrations (0.40, 0.66, 1.1, 1.8, 3.0 and 5.0 a.i/kg feed) ranged from 90.6 to 111% of the nominal concentration. Since stability data indicated that the test substance was stable at ambient temperature for 14 days, and that all of the test substance was accounted for, the nominal concentrations were considered representative of the actual exposure levels. All conclusions are based on the nominal concentration.	

Section 7.5.3.1.2 Short-term toxicity on birds

Annex Point IIIA XIII 1.2 Dietary Toxicity Test with the Japanese Quail *(Coturnix coturnix japonica)*

		No birds in either the control cage or in the 0.40mg a.i/kg feed group exhibited abnormal behaviour during the study.	
		Mortality of 0, 17, 33, 83, 83, and 83% were observed among quail exposed to nominal dietary concentrations of 0.40, 0.66, 1.1, 1.8, 3.0, and 5.0mg a.i/kg feed. No mortality was observed among the control birds.	
		Between day 0 (start of treated feed) and day 8 (end of test), mean body weight gain in the 0.66, 1.1, 3.0 and 5.0mg a.i/kg feed treatment groups was significantly less than that of the control groups.	
		The dietary LC50 for Japanese quail exposed to difenacoum technical was 1.4mg a.i/kg feed. The Lowest lethal concentration (LLC) was 0.66mg a.i/kg feed, and the No-Observed-Effect Concentration was 0.40 mg a.i/kg feed.	
5.2.1	LC_0	Not stated	
5.2.2	LC_{50}	1.4mg a.i/kg feed	x
5.2.3	LC_{100}	Not stated	
5.3	**Conclusion**	The test was considered to have met the validity criteria as the mortality in the control group was < 10% at the end of the test.	
5.3.1	Reliability	1	
5.3.2	Deficiencies	Yes LC100 value was not determined	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	26.6.2006
Materials and Methods	3.1.10: Aceton was used as solvent in addition to polyethylene glycol. 3.1.8, Table A7_5_3_1_2-2: The mean weight at start ranged from 57.5 to 63.8 g. 3.1.9, Table A7_5_3_1_2-3: The floor area per bird was 277 cm^2 assuming that the pen height was 25 cm. (Cage dimensions have been given 81 x 41 x 25 cm without specifying what is height.)
Results and discussion	4.1.8: The slope of the dose response curve was +2.84. LC50 (95 % confidence limits) 1.4 (1.74-3.95) mg a.i./kg feed.
Conclusion	Difenacoum is very toxic to Japanese quail in 5-day dietary exposure.
Reliability	1
Acceptability	Acceptable
Remarks	The diet contained 0.35-0.4 ppm vitamin K.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_5_3_1_2-1: Method of administration of the test substance

Carrier / Vehicle	Details
Water	No
Organic carrier	Yes polypropylene glycol
Concentration of the carrier [% v/v]	2% w/w
Other vehicle	No
Function of the carrier / vehicle	Solvent for test substance

Table A7_5_3_1_2-2: Test animals

Criteria	Details
Species/strain	Japanese quail
Source	Northwest Gamebirds, LLC,Kennewick, Washington
Age (in weeks), sex and initial body weight (bw)	14 days old. Sex not stated Initial body weights at start of treatment ranged from 49.2g – 70.5g
Age range within the test	All birds were 14 days old at experimental start
Breeding population	Obtained from a single hatch
Age at time of first dosing	See above
Health condition / medication	All birds were in apparent good health

Table A7_5_3_1_2-3: Test system

Criteria	**Details**
Test location	Indoor in holding pens
Holding pens	Cage dimensions were 81 x 41 x 25cm. The sides, floor and top of the cages were made of polycarbonate-coated welded wire mesh.
Number of animals	72 (tested) 24 (control)
Number of animals per pen [cm²/bird]	12 (300 cm^2) per individual.
Number of animals per dose	12
Pre-treatment / acclimation	Birds were acclimatised to test conditions for 13 days prior to experimental start and were fed a basal diet, *ad libitum*. They were housed in holding pens as described above with a photoperiod of 14 hours light 10 hours dark and a brooder temp of 30 – 35°C.
Diet during test	Birds were fed diet of Purina® Game Bird Stratena® and water.
Dosage levels (of test substance)	0 (control), 0.40, 0.66, 1.1, 1.8, 3.0 and 5.0 mg a.i/kg feed. *Calculated as :* (0, 0.083, 0.116, 0.213, 0.334, 0.5446 and 0.833 mg/kg bw/day)
Replicate/dosage level	n/a
Dosing method	*ad libitum*
Dosing volume per application	n/a
Frequency, duration and method of animal monitoring after dosing	**Food consumption** Feed consumption days 0 to 5 (exposure period), 5 to 8 (post-exposure period) and day 8 to the end of test. **Observations for mortality, morbidity and symptoms of intoxication** Each animal was observed 3 times on first day of exposure and twice daily, and once on day 8 for mortality, morbidity, and symptoms of intoxication.
Time and intervals of body weight determination	Body weight was measured for each test animal on the day of, and prior to test initiation. Body weight was also tested on days 5 and 8 and at death if prior to test termination.

Table A7_5_3_1_2-4: Test conditions (housing)

Criteria	Details
Test temperature	Ranged from 21 to 27°C
Shielding of the animals	Not stated
Ventilation	15.2 room exchanges per hour
Relative humidity	Ranged from 47 to 61%
Photoperiod and lighting	Sylvania Sunstick fluorescent bulbs at average light intensity of 27 foot candles at pen level. Photoperiod of 14 hours light and 10 hours darkness

Table A7_5_3_1_2-5: Mortality data after test termination

Test substance dosage level (mg/Kg/bw/day)	Test substance dosage level (mg a.i/kg feed)	Percent survival	
		Day 5	Day 8
Control	Control	100	100
0..083	0.40	100	100
0.116	0.66	92	83
0.213	1.1	75	67
0.334	1.8	42	17
0.5446	3.0	33	17
0.833	5.0	33	17
Temperature (range)		21-27°C	21-27°C
Relative humidity (range)		47-61%	47-61%

Table A7_5_3_1_2-6: Validity criteria for short-term toxicity test according to OECD 205

	Fulfilled	Not fulfilled
Mortality of control animals <10%	**X**	
Test substance concentration > 80 % of nominal concentration throughout the dosing period	**X**	
Lowest treatment level causing no compound-related mortality or other observable toxic effects	**X**	