Section 7.5.3.1.3 Effects on reproduction of birds
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			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2005) Final report: Avian Reproduction Toxicity Test of Difenacoum Technical in the Japanese Quails (Coturnix coturnix Japonica) XXXXX. Study Code: 03/779-206FU	x
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD guidelines Section 2: Effects on Biotic Systems. No 206; Avian Reproduction test (1984) and OECD guidelines for testing of chemicals, Avian reproduction toxicity test Draft Guideline October 1998.	x
2.2	**GLP**	Yes	
2.3	**Deviations**	None	
		3 METHOD	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
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	Must be kept in a cool dry place away from light.	
3.1.6	Method of analysis	N/A	
3.2	**Administration of the test substance**	Ethanol abs and deionised water.	
3.3	**Testing procedure**		
3.3.1	Test organisms	Japanese quail (see table A7_5_3_1_3-3)	
3.3.2	Test system	(see table A7_5_3_1_3-3)	x
3.3.3	Diet	Not stated	x
3.3.4	Test conditions	(see table A7_5_3_1_2-4)	x
3.3.5	Duration of the test	Six weeks	x
3.3.6	Test parameter	Effects on reproduction (test parameters are listed in 3.3.6)	

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3.3.7	Examination / Observation	Clinical Observations All adult birds and hatchlings were observed daily from test initiation to test termination. A record was maintained of all mortality, signs of toxicity, or abnormal behaviour. Body Weight Measurement Adults: Individual body weights were measured at the start pre-treatment, at the start of treatment and at the end of the treatment period. Individual weights of the birds that died or were sacrificed during the test were measured. Hatchling: Individual body weights were measured by pen at hatching and at the end of the rearing period. Necropsy Adults: All birds were examined for gross pathological changes after mortality or at termination of the study. The appearance of the tissues and organs were observed macroscopically. Hatchlings: Not examined. Organ Weight Liver, spleen and testes of every adult bird was measured. The number of follicles were recorded. Data on Reproduction Eggs laid per day, Cracked/brokenm eggs Eggshell thickness Mean weight of eggs set Egg abnormalities Fertile/infertile eggs Early embryonic deaths Late embryonic deaths Viable live embryos Eggs that hatch Surviving birds (14 days old)
3.3.8	Statistics	The groups were compared with corresponding values in the control group and with data collected from each bird during the test using adequate statistical methods. The hetrogencity of variance between groups was checked by Barlett's homogeneity of variance test. Where no significant hetrogeneity was detected, a one-way analysis of variance was carried out. If the obtained result was positive, Duncan's Multiple Range test was used to assess the significance of intergroup differences. Where significant hetrogeneity was found, we examined the normal

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distribution of data by Kolmogorov-Smirnov test. In case of not normal distribution Kruskai-Wallis One-way analysis of variance was applied. If a positive result, the intergroup comparisons were performed using Mann-Whittney U- test.

4 RESULTS

4.1 Limit Test / Range finding test

Performed

4.1.1 Concentration

5 separate preliminary tests were performed. The preliminary that gave the best results for the level of toxicity that should be used in the main study was one which mixed the test item with drinking water.

4.1.2 Number/ percentage of animals showing adverse effects

Dose µg/kg-bw	Concentration µg/mL	No. of animals	Time of death
35	0.2	1 M + 2 F	-
70	0.4	1 M + 2 F	-
140	0.8	1 M + 2 F	-

4.1.3 Nature of adverse effects

In the 4th preliminary test the test item was mixed with SSNIFF SM quail diet in concentrations of 16, 32, 64, 128 ppm and death occurred in all dosing groups apart from the 16 ppm group.

4.2 Results test substance

4.2.1 Applied concentrations

Doses applied were 70, 140, 280 and 0 µg/kg

4.2.2 Effect data (Mortality and reproductivity)

Mortality

No mortality occurred in the 70 µg/kg/bw during the test. Treatment-related mortality occurred in the nominal dose of 140 and 280 g/kg-bw. In these two groups all dead animals were females.

Reproductive data

Eggs laid:

The exposure to the test item had no effect on egg number/surviving female birds.

Eggs mass:

No significant egg weight changes appeared during the study.

Cracked/broken egg:

No concentration related effect.

Eggshell thickness:

No effect.

Percentage fertile eggs of eggs set (fertility):

No effect

Percentage viable embryos of eggs set (viability):

No effect

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		Hatchlings **Number of hatchlings** The number of hatchlings/surviving female birds was not affected. **Percentage hatchlings of eggs set (hatchability):** No test item effect was observed. **14-Day old survivors:** No differences were observed at the lowest dose group compared to the control. 14-day survivors decreased in the treatment period at 140 and 280 µg/kg-bw. The dose-related relation is well demonstrated (the deviation was greater than 30%). *(*see table A7_5_3_1_3-5) The reproductive NOEC is 0.4 mg/L water concentration (equivalent to 70 µg/kg-bw, 58 µg/kg-bw measured and the value ofthe reproductive LOEC is 0.8 mg/L water concentration (equivalent to 140µg/kg-bw nominal, 115 µg/kg-bw measured
4.2.3	Body weight	When compared to the control there was no significant increases in body weight in any of the testing groups.
4.2.4	Food consumption	During the 6 weeks treatment the average food consumption of the exposed animals showed statistically significant difference in a few cases, but these differences were not considered test item related effects.
4.2.5	Results of residue analysis	N/A
4.2.6	Other effects	N/A
4.3	**Results of controls**	
4.3.1	Number/ percentage of animals showing adverse effects	No animals died in the control group.
4.3.2	Nature of adverse effects	Only fluffed feathers were observed in a few cases in the control group.
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	Three different nominal dose groups, 70, 140, 280 (measured: 58 115, 241) µg/kg-bw (body weight) and one control group were tested un the study. Each dose group consisted of 12 male and 12 female birds, which were 9 weeks old at the beginning of the test. Test birds were housed indoors by dosage groups in pens. Each pen consisted of one male and one female. Birds were exposed to drinking water containing the test item for a period of six weeks. Effects on adult health, body weight gain food consumption, pathological changes and reproductive parameters were monitored and evaluated (eggs laid, fertility, viability, hatchability eggs cracked/broken, egg mass, eggshell thickness, embryonic death) furthermore the 14-day old survivors, their body weights, food

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		consumption and genera state of health were observed. Birds received the drinking water prepared with test item ad libitum. The applied nominal concentration levels in the drinking water was: 0.4, 0.8 and 1.67 mg/L. These water concentrations are equal to nominal 70, 140 and 280 µg/kg-bw dose groups.
5.2	**Results and discussion**	
5.2.1	NOEC	The reproductive NOEC is 0.4 mg/L water concentration (equivalent to 70 µg/kg-bw, 58 µg/kg-bw measured and the value ofthe reproductive LOEC is 0.8 mg/L water concentration (equivalent to 140µg/kg-bw nominal, 115 µg/kg-bw measured
5.3	**Conclusion**	Validation The concentration of the test item was constant in the water for 4 days. There was no mortality during the acclimatisation and there was no mortality in the control during the test.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

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Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	4.12.2006
Materials and Methods	2.1: The test methodology is a mixture of on the OECD 206 (Avian reproduction test) and Draft Guideline. The number of animals is taken from the OECD 206 whereas the treatment periods are taken from the draft guideline. The treatment period in the draft guideline is 6 weeks whereas it is 20 weeks in the OECD 206. The number of pairs required in the OECD 206 and draft guideline are 12 and at minimum 16, respectively. Further, the exposure time in the draft guidance may be too short for bioaccumulating substances and a steady state may not be reached sufficiently early in the test. It has been agreed that avian reproduction test for the second generation anticoagulants should be performed with the current OECD 206 guideline (TMII04). 3.3.2, Table A7_5_3_1_2-3: Number of animals per dose is 24 (12 males and 12 females). 3.3.2, Table A7_5_3_1_2-3: Test period after egg-laying was 6 weeks (+ 2-weeks observation of hatchlings). 3.3.2, Table A7_5_3_1_2-3: Eggs were collected daily throughout the test (2 weeks pre-treatment + 6 weeks treatment). 3.3.3: Diet has not been described in the test report. Source, composition, manufacturer's nutrient analysis and any supplements and carriers used should be described in the test report. This information is needed for both adult and hatchling diet. Birds were fed *ad libitum* during the test. (SSNIFF SM quail diet mentioned in Table A7_5_3_1_2-3 is mentioned in connection to range finding tests without any further explanation.) 3.3.4, Table A7_5_3_1_2-4: Temperature in the animal room was reported to be 16-27 °C and relative humidity 46-80%. 3.3.5: Test duration was 2 weeks pre-treatment + 6 weeks treatment period. Before pre-treatment period birds were acclimated for 17 days. 3.3.8: Statistics have not been performed on adult and hatchling mortality, number eggs/female/day, number of abnormal eggs including cracked or broken eggs, fertility, viability and hatchability data. Power of the statistical tests has not been reported.

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Results and discussion	Difenacoum increased mortality of adult female birds. 4 and 5 females out of 12 died at the test concentrations of 0.63 and 1.34 mg/L (corresponding to doses 115 and 241µg/kg-bw). The dead birds had also clinical symptoms of anticoagulant poisoning (haematomas and haemorrhages). The female birds at the two highest treatment groups had increased weights of liver and spleen and the effects were dose related. Anticoagulant related effects were not observed in surviving birds in the two highest treatment groups. At lowest test concentration and control all birds survived and no anticoagulant related macroscopic changes were observed in the post mortem examination. Difenacoum did not affect body weights, food or water consumption of adults in any treatment group. Neither did difenacoum affect food consumption or body weights of hatchlings. Difenacoum increased mortality of 14-day hatchlings in dose related way during two last treatment weeks in the two highest treatment groups. Percentage of hatchlings that survived to 14 days is shown in Table A7_5_3_1_3-5. There seems to be a declining trend in number of eggs laid/hen/day at the two highest treatments. Viability of eggs shows a slightly declining trend during the test period at the highest difenacoum treatment, whereas in control and lower difenacoum treatments the viability increase during the treatment period. Statistical significance of these findings is unknown as no statistics have been performed. Difenacoum do not have any significant effects on the eggshell thickness, body weight or food consumption of chicks. Difenacoum does not appear to have any effect on hatchability (no statistics performed). The test substance in concentrations of 0.2-0.8 mg/L was reported to be stable for 7 days. The measured difenacoum concentrations in water ranged from 73 to 90%. The limit of determination was 0.2 mg/L. The validity criteria of the OECD 206 were fulfilled except maintenance of measured concentrations of at least 80% of nominal concentrations. This is not regarded as a problem as the results are based on the measured concentrations. The validity criteria of the draft guideline of *at least 16 breeding pairs of control birds that have produced eggs must be available at the end of the 6-week treatment period* was not filled as there was only 12 pairs of birds in the beginning of the test. Of the 12 pairs in the controls two did not produce any egg during the 6-week treatment period. NOEC = 0.31 mg/L based on the measured concentrations in water NOEL = 58 µg/kg-bw based on the measured concentration in water
Conclusion	Difenacoum is very toxic to Japanese quail in the long-term exposure. Due to short exposure duration it is possible the NOEC/NOEL would have been lower if the exposure period has been continued with additional 14 weeks.
Reliability	2/3 Test should have been performed according to the OECD 206, in particular the exposure duration should have been longer.
Acceptability	Acceptable The RMS view is that this test is on the borderline whether it can be accepted. We have preliminary decided to accept the test because we are reluctant to require to repeat the test with vertebrate animals.

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Remarks	1.1. The author name is Gaty, S. Adult body weight, food and water consumption, organ weights, necropsy findings or mortality have not been reported in the study summary. Egg fertility, egg weight, body weight and food consumption of hatchlings have not been reported in the study summary. The calculation method of the percentages presented in the summary tables (Appendices) should be explained. Some of the percentages reported in Appendix 5 (summary of organ weight data) do not correspond to the values from which they are derived from.
	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	Yes, deionised
Organic carrier	Ethanol abs
Concentration of the carrier [% v/v]	N/A
Other vehicle	N/A
Function of the carrier / vehicle	N/A

Table A7_5_3_1_3-2: Test animals (if more than one species is used, for each species one table)

Criteria	Details
Species/strain	Japanese Quail (Coturnix coturnix japonica)
Source	Dezso Rokolya quail breeder, Csavoly, Szent Istvan u. 83, H-6448, Hungary
Age (in weeks), sex and initial body weight (bw)	9 weeks old birds at the initiation of the test, 48 males and 48 females, with reserve birds (8males and 8 females). Body weight range; males 167-211g, females 201-261g
Age range within the test	See above
Breeding population	Not stated in report
Amount of food	N/A; test substance in drinking water.
Age at time of first dosing	9 weeks
Health condition / medication	All the birds appeared in good health during the acclimation period. No medication of the adults was carried out during the entire period of the test.
Pre-treatment	Acclimation period of 16 days followed by a pre-treatment period of 14 days. No illness or mortality appeared during the acclimation period. No observations stated for the pre-treatment period.

Table A7_5_3_1_2-3: Test system

Criteria	**Details**
Test location	Indoor in holding pens
Holding pens	Parent birds: Each pen had a floor space of 50cm x 50cm. Ceiling height was 40cm. External walls, ceiling and floors were constructed of galvanised wire. Hatchlings: Brooder pen with a floor space measuring 1. x 1.0 m. and ceiling height of 40cm. External walls, ceiling and floors were constructed of galvanised wire.
Number of animals (male/female)	Male: 48 Female:48
Number of animals per pen [cm²/bird]	2 (1 male and 1 female)
Number of animals per dose	2/sex/dose
Pre-treatment / acclimation	Untreated layer diet was administered *ad libitum*
Diet during test	SSNIFF SM quail diet
Dosage levels (of test substance)	70, 140, 280µg/kg/bw (nominal) 58, 115, 241 µg/kg/bw (measured)
Replicate/dosage level	24 birds per dose level
Dosing method	Drinking water prepared with test substance *ad libitum*.
Dosing volume per application	Volume of drinking water was 200ml per cage.
Frequency, duration and method of animal monitoring after dosing	Following test initiation until termination adult birds and hatchlings were observed daily for clinical observations
Time and intervals of body weight determination	Adults: Individual body weights were measured at the start of pre-treatment, at the start of treatment and at the end of treatment. Hatchlings: Individual body weights were measured by pen at the end of the rearing period.
Incubation, storing and hatching	Eggs were incubated in PL MASCHINE incubator and hatched in PL MASCHINE hatcher.
Test period after egg-laying	
Turning of eggs	Yes during incubation with automatic device
Collection period for eggs	Eggs were collected daily throughout the test period

Table A7_5_3_1_2-4: Test conditions (housing)

Criteria	Details
Test temperature	Ranges as below
Shielding of the animals	Not specified
Ventilation	With HELIOS –HS type ventilator, maximal aeration 18times/hour
Relative humidity	Ranges as below
Photoperiod and lighting	Photoperiod was 7-8 hours per day at beginning of acclimation period then during two weeks it was continuously increased to 16-18 hours per day. Lighting was artificial, minimum 10 lux at the fooder of birds.
Storing, incubation and hatching conditions for eggs	Eggs were stored not more than 7 days at the temperature of 13.3 – 16.0°C and 52 - 64% relative humidity. Temperature in the incubator was 37.5-37.7°C, and relative humidity was 51-65%. Temperature in the hatcher was 37.0-37.4°C, relative humidity was 71-72%
Environmental conditions for young birds	Temperature: 30-38°C Relative humidity of 50-73%. Photoperiod: 16hours of light per day Ventilation: as above

Table A7_5_3_1_3-5: Values of reproduction ability for Japanese Quails *Coturnix coturnix Japonica*

Parameter	**Test substance dosage level (µg/kg bw)**	**Pre-treatment (weeks -2 and -1)**		**Treatment (weeks 1-6)**					
		-2	**-1**	**1**	**2**	**3**	**4**	**5**	**6**
Egg production (eggs laid per hen/day)	Control (0)	0.85	0.85	0.94	0.75	0.82	0.81	0.83	0.86
	70	0.88	0.93	0.82	0.90	0.80	0.89	0.93	0.90
	140	0.95	0.90	0.98	0.94	0.97	0.86	0.83	0.81
	280	0.92	0.83	0.86	0.77	0.86	0.77	0.79	0.69
Percentage of cracked eggs	Control (0)	15.4		13					
	70	6.5		9.2					
	140	4.5		8.3					
	280	9.5		10.4					
Viability (per cent viable embryos of eggs set): Results are expressed as the % of dead embryos	Control (0)	13.0	15.8	4.9	8.9	14.8	29.8	25.5	30.9%
	70	39.3	18.8	8.1	9.0	16.4	25.4	20.0	29.5
	140	11.6	17.6	7.2	12.9	12.9	21.6	19.1	38.5
	280	25.8	14.0	16.1	12.7	19.2	11.9	20.0	17.9
Hatchability (per cent hatching of eggs set)*	Control (0)	87	84.2	95.1	91.1	85.2	70.2	74.5	69.1
	70	60.7	81.2	92	91	83.6	74.6	80	70.5
	140	88.4	82.4	92.8	87.1	87.1	78.4	80.9	61.5
	280	72.2	86	83.9	87.3	80.8	88.1	80	82.1
Percentage of hatchings that survive to 14 days **	Control (0)	96.4	96.5	97.1	95.1	93.8	91.7	92	88.9
	70	95.5	95.5	94.1	94.5	90.9	91.1	96.8	92.3
	140	91.8	94.0	89.5	91.8	89	87.7	82.8	69.2
	280	89.7	100	87.9	87.9	81.7	77.8	71.7	57.4
Number of 14-day old survivors per hen ***	Control (0)	3.8	3.8	4.7	4.1	3.2	3.1	3.3	2.8
	70	2.9	4.5	4.5	4.8	3.5	3.6	4.2	3.3
	140	4.7	4.4	4.8	4.7	**4.6**	3.5	**3.6**	**2.1**
	280	3.7	4.1	3.6	3.4	3.4	**3.2**	**2.9**	1.9
Eggshell thickness mean (mm)	Control (0)	0.25	0.25	0.26	0.27	0.22	0.25	0.22	0.24
	70	0.25	0.25	0.24	0.25	0.23	0.24	0.23	0.25
	140	0.24	0.24	0.24	0.24	0.20	0.23	0.21	0.24

	280	0.24	0.24	0.24	0.24	0.22	0.23	0.21	0.23

* The hatchability has been calculated as follows:

$$\frac{\text{number of hatchings}}{\text{number of incubated eggs}} \times 100$$

** The percentage of hatchlings that survived to 14 days was calculated as follows:

$$\frac{\text{number of 14-day old chicks/female/day}}{\text{number of 0-day old chicks/female/day}} \times 100$$

*** The number of 14 day old survivors per hen has been calculated as follows:

$$\frac{\text{number of chicks}}{\text{number of females}}$$

Table A7_5_3_1_3-6: Validity criteria for bird reproduction test according to OECD 206

	Fulfilled	**Not fulfilled**
Mortality of control animals <10%	**X**	
Average number of 14-day-old survivors per hen in controls ≥ 14, 12 and 24 for mallard duck, bobwhite quail and Japanese quail	**X**	
Average eggshell thickness for the control group ≥ 0.34, 0.19 and 0.19 mm for mallard duck, bobwhite quail and Japanese quail	**X**	
Concentration of the test substance in the diet ≥ 80 % of the nominal concentration throughout the test period		**X**