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Report

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Test for potential protective effects of Catosal? on mortality of fish due to temporary hypoxia

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Summary

Studies with several fish species have shown that fishes during transport are exposed to different stressful stimuli that may cause adverse physiological reactions, which may lead to mortality. Given the fact that ornamental fish represent a high economical value, reduction of stress in ornamental fish during transport to minimise mortality is essential.
It has been evidenced that the application of Catosal? reduces stress in pigs and cattle. It was therefore decided by Bayer HealthCare AG to give the Netherlands Institute for Fisheries Research an assignment to test whether their commercial product, Catosal? , would also be suitable to reduce stress in fish during transport and thereby minimise mortality.
The overall objective of the study was to establish whether Catosal? could, in principle, minimize mortality due to hypoxic stress in fish. In order to find the "proof of principle" the experimental approach was as follows.

- ?? Establish conditions for asphyxiation to achieve at least 40% mortality
- ?? Assess the effects of fish exposure to Catosal? prior to asphyxiation in order to reduce mortality.

Farmed sole (*Solea solea*) with an average live weight of 20.6 g (12 months of age) was used as model species. Groups of ten soles were tested together under each test condition assessed.
It appeared that at least 90 min asphyxiation of sole in water was required to induce 40% mortality. In a first experiment with a three hours pre-exposure to Catosal? , this rate of mortality could be confirmed, and there was a trend (p=0.0833) for a protection of the soles against hypoxia-induced mortality under these conditions. The lowest concentration of Catosal? used, i.e. 1 mg/l (referring to the active ingredient, butaphosphane), did not reduce mortality, while there appeared to be dose-dependent protection in the range of 10 to 1000 mg/l (n= 10 soles per concentration). This result could, however, not be reproduced in two identical replication experiments. In these experiments, control mortality was only 20-30%.
The results show that the model is not reliable enough to draw firm conclusions whether or not the application of Catosal™ reduces stress in the soles.

1. Introduction

Bayer is interested to investigate the applicability of an existing product (Catosal?) for fish. This product has been shown to decrease the stress-response in pigs and cattle and could be of interest in fish. The first application that comes to mind is with the transport and handling of fish, which are known to induce stress and mortality. Especially, in the trade of ornamental fish, which is a large business (millions of animals are transported yearly), such a product could be useful to reduce mortality. It is obvious that for reduction of mortality during transport of fish, mortality should be used as the parameter to establish the effect of Catosal? in the experimental work.
It is known that a fish species such as sole (*Solea solea*) is susceptible to handling as stressor and therefore this species was chosen for the study. Given the fact that there are many similarities in physiological and behavioural responses to stressors, it is foreseen that when Catosal? can be applied successfully to reduce mortality during transport of sole, it is of interest to study the efficacy further with other fish species.
The overall objective of the study was to find “proof of principle” for the product in fish. The information to be generated should be available on a zootechnical level e.g. by measuring effects on mortality. Underlying mechanisms were not of interest at this stage.
The following experimental approach was proposed:

- Establish conditions to achieve at least 40% mortality. In order to optimise the control in our study, a range of emersion times to asphyxiate the fish will be tested to establish the conditions to achieve at least 40% mortality
- Establish optimal Catosal? concentration. The fish will be exposed for three hours to Catosal? at 5 different concentrations, including a control condition with no Catosal? . After exposure the fish will be stressed by applying the previously established conditions
- Establish optimal exposure time to Catosal? . An exposure time of 3 hours is rather long. Therefore, a range of shorter exposure times at the established concentration of Catosal? will be tested.

2. Materials and methods

Sole (*Solea solea*) reared a the site of investigation with an average live weight of 20.6 ± 9.4 g were kept in recirculation (figure 1) tanks filled with seawater (160 l volume, pH =7, T= 19°C, O_2= 8 ppm) and not starved prior to the start of the experiment. The animals were caught with a dip net (15 cm).
The sex of the fishes cannot be determined on basis of outer appearance. The fishes were 12 months old at the start of the experimental work.

2.1 Standardisation of asphyxiation test

A procedure to achieve 40% mortality due to asphyxiation was established in the following experiment. We used the water of the tank in which the soles were kept. This water was used to fill small white buckets (volume 4 l, see figure 2), which were closed by a lid. Each lid is equipped with a tube, which brings nitrogen gas into the water (see figure 3). We flushed the water with nitrogen to remove oxygen (until oxygen level 0-0.5 ppm is reached). Subsequently, the fish are placed in this water, which is flushed continuously with nitrogen gas. Batches of 10 fish were asphyxiated for 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150 min. Asphyxiation by net confinement outside of the water (designated as emersion from the water in the introduction) appeared to give problems, as the animals escaped from the net, which could not be closed sufficiently by folding it, and therefore this was abandoned.

In addition, we observed that immediate observation of behaviour by administering stimuli was not sufficient to determine mortality, as the animals were exhausted due to the asphyxiation but alive. When a fish did not respond to administered stimuli this could be due to either exhaustion or death. Therefore, we left the fish overnight in the holding tanks (batches were kept separated) and after 18-19 h the animals were observed again and classified as dead (no escape behaviour, swimming and breathing) or alive (able to escape from the net or showing attempts to do so).

2.2 Assessment of asphyxiation

Sensitivity

In order to establish whether the fish lost consciousness or death occurred, responses to administered stimuli were recorded. Immediately after asphyxiation these responses or absence thereof were registered. When the responses were absent the fish was classed as not sensitive. We have to emphasise that absence of sensitivity did not imply that a fish was unconscious or dead, as the fish might be not able to respond due toe exhaustion.
We decided to use a simplified version of the protocol, which was published by Kestin, Van de Vis and Robb (2001), for the experiments, as the fish appeared to be too tiny to establish whether a vestibular ocular response or response to 6 V electrical stimulus applied to the mouth region, using a technique available at RIVO, were present. Observation of the vestibulo ocular response is performed to assess whether the position of the eyes is compensated for changes in the body posture. The simplified protocol is presented in table 1 and 2, and figure 4 and 5.

Mortality

The next day the fish, which were kept in recirculation tanks, were observed for swimming behaviour and breathing. If both were absent, we classed the fish as dead. When the fish were observed the next day a period of 18-19 h had elapsed after asphyxiation. Observation the next day is essential, as immediately after asphyxiation the animals could be exhausted only and because of that they might be wrongly classified as dead. Furthermore, deleterious effects of hypoxia may become manifest as delayed mortality.

2.3 Treatment with Catosal?

Dose-response

The exposure to Catosal? was performed in three trials. We exposed the fish for three hours to varying concentrations (0, 1, 10, 100 and 1000 mg/l) in 4 l buckets, containing aerated water. Group size was 10 soles per bucket. Then the batches were transferred to the buckets with oxygen free water with the same Catosal? concentrations and the fishes were asphyxiated for 90 min by flushing continuously with nitrogen gas at 1 bar. The buckets were closed with a lid to prevent escape. Subsequently, the fish were observed as quickly as possible, after transferring them to aerated seawater, by registering responses to the administered stimuli and monitoring the absence or presence of breathing. Thereafter the animals were kept overnight in the tanks, which were fed with seawater (without Catosal?) from the recirculation system.
We observed that keeping the animals overnight in the buckets with aerated water was more stressful than storage in the usual recirculation tanks. Mortality was increased when the animals were kept in buckets, compared to the holding tanks, and therefore we decided to keep the animals overnight in the holding tanks. We could not administer Catosal? to the recirculation system as it is one large system and therefore the water is used for other soles (not used in our experiments) as well.

Time-dose response

The exposure-time of 3 hours is rather long in order to guarantee an adequate uptake of the product into the plasma. Therefore, a range of shorter exposure times (0, 0.5, 1, 2, 3 hour) at a concentration determined previously should be tested. The five experimental groups of 10 sole should be tested in duplicate.

2.4 Statistical analysis

The obtained data were analysed using logistic regression. In SAS software GENMOD was used for processing of the data. It was decided to apply a Type 3 approach, as this is preferred for revealing significant differences. A 95% confidence limit was used.

2.5 Ethics

A governmental ethical committee approved the experiments beforehand.
The start of the experimental work was delayed for several months, as the governmental ethical committee rejected the project plan. A new version of the project plan was therefore made. In this version the rationale behind the project was presented in more detail. The experimental set up was not changed.
Due to this delay the size of the soles increased. The average live weight was 20.6 ± 9.4 g instead of an average live weight of 5 g, as chosen in the project plan.

3. Results and discussion

3.1 Standardisation of asphyxiation

It appeared that the soles were much more resistant to asphyxiation than expected when the project plan was written. At first it was assumed that asphyxiation for not more than 8 min was sufficient to provoke sufficient stress to induce mortality. In this study we observed that asphyxiation for a substantially longer period was needed to achieve at least 40% mortality. The reason for this might be that conditions for farming of sole were optimised during the past year, resulting in decreased susceptibility to lack of oxygen.
In two trials it was observed that after 60 min asphyxiation 2 out of 11 soles were dead. Decreased periods (15, 30 and 45 min) for asphyxiation did not result in any mortality at all (results not shown). Therefore, it was decided to test asphyxiation for 75, 90, 105, 120, 135 and 150 min. We found that asphyxiation for 90 min was required to achieve at least 40% mortality (see figure 6). A period of 90 min was therefore selected as fixed parameter for the asphyxiation.
Figure 6 also shows that observation after 18-19 h is necessary, as immediate observation of the soles that had been asphyxiated for 120 min were not able to respond to the administered stimuli (sensitivity 0%). Observation after a stay of 18-19 h in a recirculation tank revealed that mortality was 70% for this batch.

3.2 Treatment with Catosal?

Trial 1 (see figure 7) revealed a trend suggesting a reduced mortality at higher concentrations of Catosal? . However, statistical analysis revealed that application of Catosal? did not significantly reduce mortality, under conditions used (see table 4). Therefore, the trial was repeated twice (designated in figure 7 as trial 2 and 3). Analysis of data obtained in trial 2 and 3 revealed no significant effect for Catosal? , under conditions used. It appeared that weight as variable had a significant effect on mortality (see table 5). It was decided not to sort the soles by weight as size of ornamental fish may also vary substantially. Moreover, sorting prior to the start of the experiment is an additional stressor, which will probably influence the outcome of the experimental work.
Immediately after asphyxiation observation of behaviour was performed to establish whether the soles were sensible. No significant effect of the application of Catosal? could be established, under conditions used (see table 5). The weight of the individual soles was not determined immediately prior to or after asphyxiaton to avoid the addition of an extra stressor in the experimental set up.
As no significant effect of the application of Catosal? could be observed, experimental work to establish a time-dose response was not performed.

4. Conclusions

Our study did not reveal a reduction in mortality of asphyxiated soles by applying Catosal? , under conditions used. However, these test conditions did not yield a reliably robust control effect to exclude potential Catosal? effects. Since live weight of the soles had a reproducible significant effect on mortality, this variable could also have influenced the test results.

5. Acknowledgements

The authors thank Mr. W. Dekker M.Sc. for given advice on the statistical analysis and Mr. Jan van der Heul for managing the facilities in the laboratory.

6. References

Kestin, S.C., Van de Vis, J.W. and Robb, D.F.H. (2001): A simple protocol for assessing brain function in fish and the effectiveness of stunning and killing methods used on fish. *Veterinary Record*, 150, 320-307.

Table 1. Tests used to assess sensitivity of sole

Test	Ability to right itself	Breathing	Response to a needle scratch
Protocol	Place the fish with its blind side facing up	Observe the fish undisturbed	Scratch tail firmly
Location	In water	In water	In water
Observation	Ability or attempts to return to a natural position	Movements (existence and rhythm) of the gills opercula	Attempts to escape

Tests are listed according to the rank in which they were chronologically performed (on each observation time).

Table 2. Scores attributed according to the observations made for each test described in table 1.

	Ability to right itself	Breathing	Response to a needle scratch
0	No attempts	No rhythmic opercular movements	No response
1	Sluggish or delayed attempts	Opercular movements either irregular, reduced, deeper or faster than normal	Muscular contraction observed visually
2	Immediate response	Regular slow opercular movements	Attempts to escape

Table 3. Conditions during with treatment with Catosal? and asphyxiation: trial 1*

.		during treament with Catosal			conditions of asphyxiation		
sole (n)	Catosal concentration	pH	T	O_2 (ppm)	pH	T (°C)	O_2 (ppm)
10	0	7,44	19,6	7,7	7,86	17,9	0,2
10	1 mg/l	7,44	19,6	7,7	7,96	17,7	0,2
10	10 mg/l	7,44	19,6	7,7	8,1	17,5	0,2
10	100 mg/l	7,44	19,6	7,7	8,15	17,6	0,1
10	1000 mg/l	7,44	19,6	7,7	7,77	17,9	0,1

*The conditions for the two other trials were similar

Table 4. Effect of various Catosal? concentrations on mortality of sole: statistical analysis.

Trial 1

Parameter	**df**	**estimate**	**chisquare**	**P**	
Genmod					
Intercept	1	0,3608	26,46	<0,0001	model mortality = catosal
Catosal	1	-0,0003	3	0,0833	
Genmod					
Intercept	1	1,0189	49,66	<0,0001	model mortality = weight
Weight	1	-0,0385	28,08	<0,0001	

Trial 2

Parameter	**df**	**estimate**	**chisquare**	**P**	
Genmod					
Intercept	1	0,2762	15,04	0,0001	model mortality = catosal
Catosal	1	-0,0001	0,21	0,6453	
Genmod					
Intercept	1	0,7796	29,07	<0,0001	model mortality = weight
Weight	1	-0,0259	15,04	0,0001	

Trial 3

Parameter	**df**	**estimate**	**chisquare**	**P**	
Genmod					
Intercept	1	0,3521	23,23	<0,0001	model mortality = catosal
Catosal	1	-0,0002	2,08	0,1490	
Genmod					
Intercept	1	0,6110	20,37	<0,0001	model mortality = weight
Weight	1	-0,0133	6,62	0,0101	

Table 5. Effect of various Catosal? concentrations on sensibility in sole: statistical analysis.

Trial 1

Parameter	df	estimate	chisquare	P	
Genmod					
Intercept	1	0,3255	19,73	<0,001	model sensibility = catosal
Catosal	1	-0,0000	0,02	0,8926	

Trial 2

Parameter	df	estimate	chisquare	P	
Genmod					
Intercept	1	0,2442	11,40	0,0007	model sensibility = catosal
Catosal	1	0,0002	1,00	0,3161	

Trial 3

Parameter	df	estimate	chisquare	P	
Genmod					
Intercept	1	0,2347	13,31	0,0003	model sensibility = catosal
Catosal	1	-0,0002	1,19	0,2755	

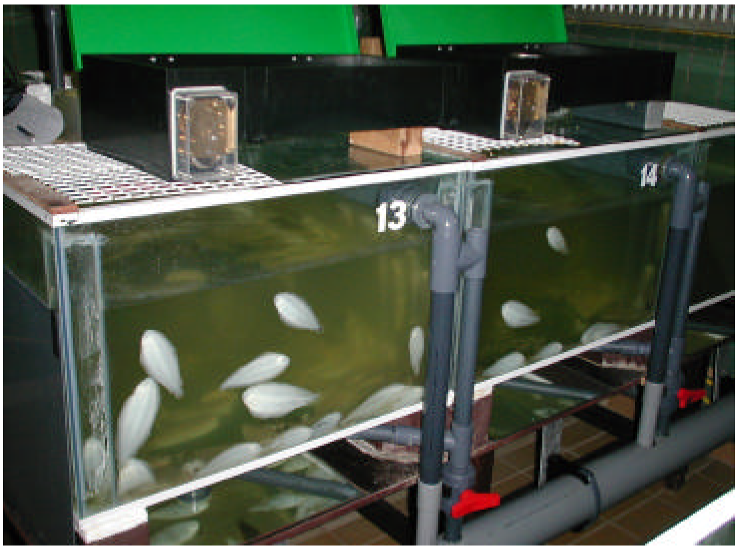


Figure 1: Fish kept in recirculation tanks

Figure 2: Bucket with soles prior to asphyxiation

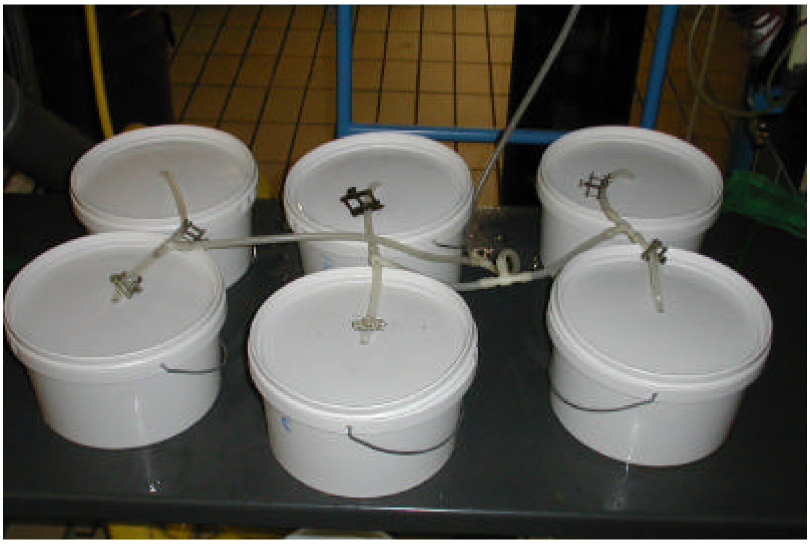


Figure 3: Asphyxiation of sole

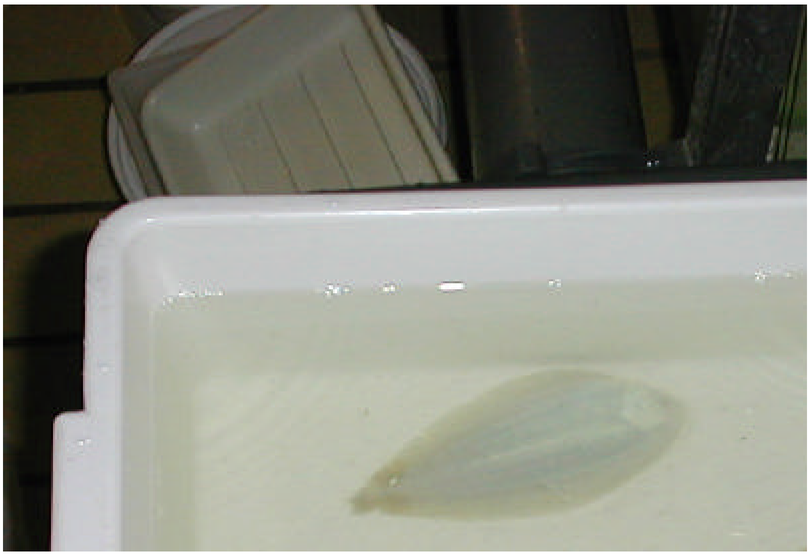


Figure 4: Observation of behaviour: ability of sole to right itself

Figure 5: Observation of behaviour: ability of sole to respond to a pain stimulus

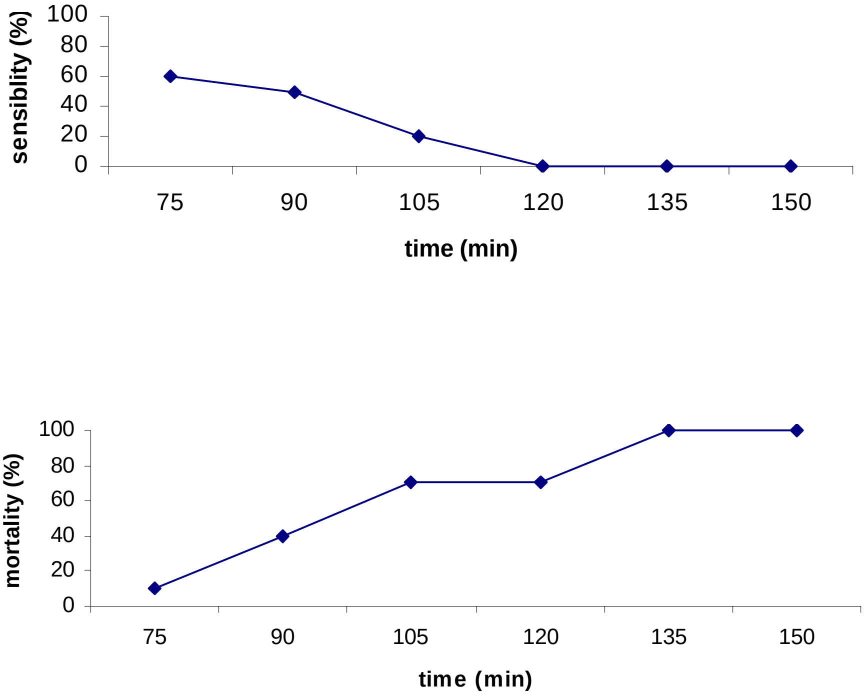
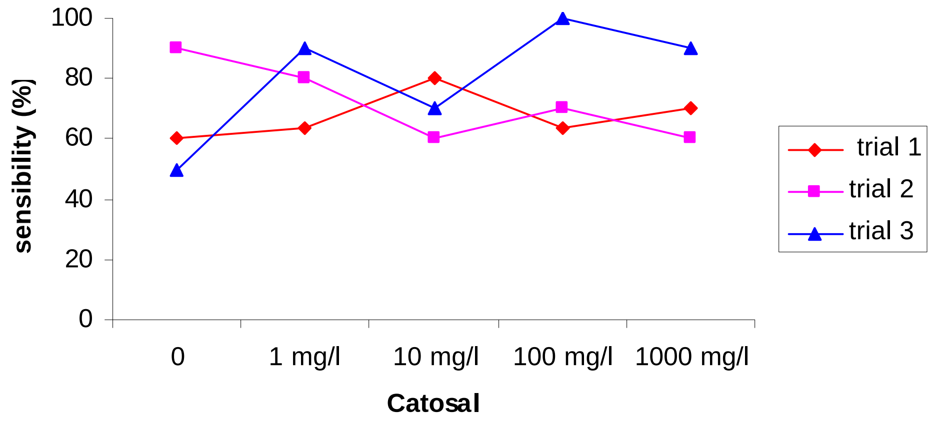


Figure 6: Relationship between duration of asphyxiation and sensibility in and mortality of sole



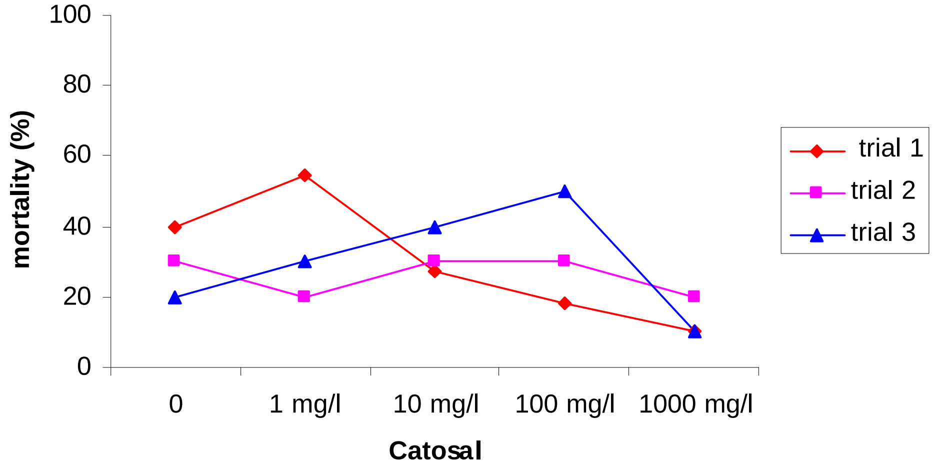


Figure 7: Effect of various Catosal? concentrations on sensibility and mortality in sole