AUSTRALIA 643595 P/00/001
Patents Act 1990 Section 29

PATENT REQUEST: CONVENTION PATENT

We, being the person identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification.

Full application details follow:-

Applicant:	ROUSSEL-UCLAF
Address:	35 Boulevard des Invalides, 75007, Paris, France
Nominated Person:	ROUSSEL-UCLAF
Address:	35 Boulevard des Invalides, 75007, Paris, France
Invention Title:	A METHOD OF COMBATTING STRESS OR INCREASING THE RESISTANCE TO STRESS IN AQUACULTURE BY USE OF 4,9,11-TRIENIC STEROIDS
Name(s) of actual Inventor(s):	Saburo KUBOTA
Address for service in Australia:	CALLINAN LAWRIE, 278 High Street, Kew 3101, Victoria, Australia
Attorney Code:	CL

Convention Details

Basic Applicant	Application Number	Country	Country Code	Date of Application
Nippon Uclaf K.K.	3-40648	Japan	JP	13.02.91

Drawing number recommended to accompany the abstract - Fig. 1.

D A T E D this 8th day of September 1993.

ROUSSEL-UCLAF

By their Patent Attorneys:

CALLINAN LAWRIE

Michael J. Hourihan.

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AUSTRALIA

PATENT

NOTICE OF ENTITLEMENT

We, ROUSSEL-UCLAF, of 35 Boulevard des Invalides, 75005, Paris, France, being the applicant and the person nominated for grant of patent in respect of the Application for an invention entitled THE USE OF T3A AS ANTI-STRESS DRUG IN AQUACULTURE state the following:-

STANDARD CONVENTION FILING

The person nominated for the grant of the patent has entitlement by virtue of an assignment of the invention from Nippon Uclaf K.K., the applicant of the basic application, who is, in turn, a person who would, if a patent were to be granted upon an application made by the said actual inventor, be entitled to have the patent assigned to it.

The basic application listed on the request form is the first application made in a Convention country in respect of the invention.

Jeffrey A. Ryder
Registered Patent Attorney

11 February 1992
Date

To: The Commissioner of Patents

AU9210870

(12) PATENT ABRIDGMENT (11) Document No. AU-B-10870/92
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 643595

(54) Title
A METHOD OF COMBATTING STRESS OR INCREASING THE RESISTANCE TOSTRESS IN AQUACULTURE BY USE OF 4,9,11-TRIENIC STEROIDS

International Patent Classification(s)
(51)[5] **A61K 031/57**

(21) Application No. : **10870/92** (22) Application Date : **11.02.92**

(30) Priority Data

(31) Number	(32) Date	(33) Country
3-040648	**13.02.91**	**JP JAPAN**

(43) Publication Date : **27.08.92**

(44) Publication Date of Accepted Application : **18.11.93**

(71) Applicant(s)
ROUSSEL-UCLAF

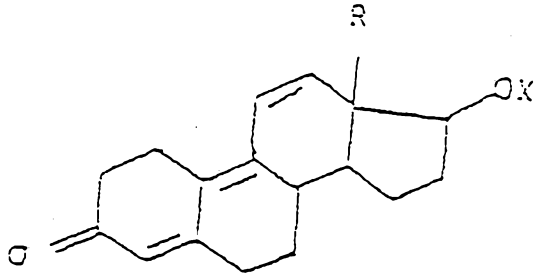
(72) Inventor(s)
SABURO KUBOTA

(74) Attorney or Agent
CALLINAN LAWRIE , Private Bag 7, KEW VIC 3101

(56) Prior Art Documents
GB 1035683

(57) Claim

1. A method for combatting stress of fish in fish farming which method comprises administering to said fish an effective amount of compounds of formula (I):

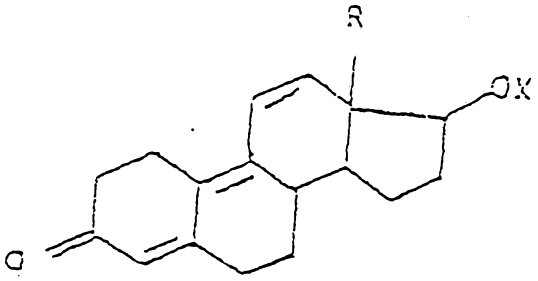


(I)

in which R represents an alkyl radical containing 1 to 4 carbon atoms and X represents an acyl radical derived from a carboxylic acid containing 1 to 18 carbon atoms.

2. A method for increasing the resistance to fungi, viruses, bacteria and other pathogenic agents in fish which method comprising administering to said fish an effective amount of compounds of formula (I):

.../2



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AUSTRALIA

PATENTS ACT 1990

COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

TO BE COMPLETED BY APPLICANT

Name of Applicant: ROUSSEL-UCLAF

Actual Inventor(s): Saburo KUBOTA

Address for Service: CALLINAN LAWRIE, 278 High Street, Kew, 3101, Victoria, Australia

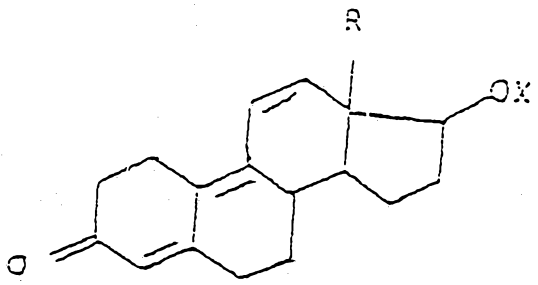
Invention Title: A METHOD OF COMBATTING STRESS OR INCREASING THE RESISTANCE TO STRESS IN AQUACULTURE BY USE OF 4,9,11-TRIENIC STEROIDS

The following statement is a full description of this invention, including the best method of performing it known to me:-

---

A METHOD OF COMBATTING STRESS OR INCREASING THE RESISTANCE TO STRESS IN AQUACULTURE BY USE OF 4,9,11-TRIENIC STEROIDS

The present invention relates to a method for combatting stress of fish in fish farming which method comprises administering to said fish an effective amount of compounds of formula (I)



(I)

in which R represents an alkyl radical containing 1 to 4 carbon atoms and X represents an acyl radical derived from a carboxylic acid containing 1 to 18 carbon atoms, ~~for the manufacture of anti-stress drugs intended for administration to fish.~~

In a second embodiment of the invention there is provided a method for increasing the resistance to fungi, viruses, bacteria and other pathogenic agents in fish which method comprises administering to said fish an effective amount of the compounds of formula (I) as defined above.

R preferably represents a methyl radical.

O X preferably represents an acetoxy, amyloxy or hexabenzyloxycarbonyl radical.

In a preferred embodiment compounds of formula (I) is trenbolone acetate (TBA) where R is a methyl radical and OX represents an acetoxy radical.

The fish is an animal which is difficult to rear; its health is directly linked to the medium in which it evolves. Therefore, any variation of the medium can be a stress factor for the fish.

Among the stress factors, there can be mentioned:

- Physical factors, such as variations of water temperature, variations of gas dissolved in the water;

- Mechanical factors due to transport, overcrowding of fish in a given place;
- Chemical or biological factors due to bacteria, viruses and parasites.

The fish is a very stress sensitive animal. Every time one transfers it from tank to tank, tank to pond or during the grading, using nets, one creates important stress reaction.

The stress reaction induces important modifications:

1) Internal: Discharge of catecholamines and

corticosteroids which greatly modify blood parameters, metabolism and excretion.

2) External: The epidermis of fishes is much less thick than upper vertebrate and does not contain keratin, the epidermis covers the scales and is very easily damaged and scratched during manipulation.

So very often after handling, diseases outbreaks and mortality occurs with variable intensity depending on the species and the age of the fish and the know-how of the farmer.

Reducing this manipulation mortability could be a break-through in aquaculture.

A more particular subject of the invention is the use of trenbolone acetate (TBA) for the manufacture of anti-stress medicated feed intended for administration to fish.

It has just been discovered that it is possible to obtain excellent results in fresh-water fish, sea water fish and brackish fish.

Using the medicaments of the invention, excellent results have been obtained in Salmonidae such as trout or salmon, in Anguillidae such as eels, in Cyprinidae such as carp, in cyclid fish such as tilapia and in sea fish such as sea bass or sea bream.

From the experimental work exposed hereafter, the following histological results have been obtained.

Pancreas: Less fatty degeneration in the TBA treated group than in control.

--> Better irrigation of the organ:

-Improved insulin secretion from Langerhans islets.

-Improved digestive enzymes production which ameliorates the food utilization.

Liver: Less fatty deposition in the treated group improved irrigation and thus, amelioration of liver functions:

-Depuration
-Synthesis

Skin: Thickening of the epidermis.
Decrease in the fatty infiltration of the subcutaneous muscle.

In a preferred implementation, the use is carried out by oral route, the product being able to be for example incorporated to a fish feed.

As fish feed, commercially sold mixtures containing fish meals, proteins, yeasts, soya meal and vitamins, which are often presented in the form of powders or granules, can be used.

A more particular subject of the invention is the use characterized by the fact the administration is carried out for salmon, trout or eel.

Notably a subject of the invention is the use characterized by the fact the product of formula (I) is incorporated to a fish feed at the rate of 1 to 20 ppm of weight of active ingredient per weight of fish feed, for example at the rate of 5 to 15 ppm.

A more special subject of the invention is the use characterized by the fact the product of formula (I) is used to increase the resistance of fish to stress in fish farming.

Notably the object of the invention is to increase resistance to fungi, viruses, bacteria and other pathogenic agents.

A more particular subject of the invention is the invention characterized by the fact the pathogenic agent is Saprolegnia. This is quite remarkable because it is a pathogenic agent which is difficult to treat.

Very good results have been obtained using the medicament of the invention as a treatment 20 to 30 days before the factor which will induce stress, for example before tank transfer.

The daily dosage can be from 0.1 to 0.5 mg per kg of fish body weight per day.

Examples:

a) A powder was prepared, intended for administration to the fish, containing the TBA product at a rate of 10 mg of TBA product for 1000 g of mixture.

b) A fish feed was prepared containing:

-1000 g of flour, 700 g of water, 70 g of arachid oil, 17.7 mg of TBA.

The mixture is made homogeneous by mechanical agitation.

The prepared feed contains 10 mg of TBA active ingredient per kg.

BIOLOGICAL STORY

1) Test method:

The fishes, Oncorhynchus kisutch (Coho Salmon) raised in the aquaculture pond were released into the rearing tanks on day 0, in a way that each group would consist of 70 fishes. All the fishes were apparently normal and similar with each other in body size (with extremely large or small ones excluded), with the average body weight being 22.2-22.4 g in each tank. The fish was baited while being acclimated. The fishes were fed in a way that they would take feed which, in total, corresponds to 1.8% (in weight) of the total body weight of the fish in each tank. On day 6, feed intake by the fish was completely stabilized, and so, on that day, TBA administration was started. The administration was terminated on day 25. During those 25 days, feed was given on 21 days (excluding Sundays). Feed was given at the amount corresponding to 1.8% of the body weight of fish, both in the treated groups and the control groups. Feed was prepared in the following way:

To the feed for trout (powder), water which corresponds to half of the feed powder (in volume) and specified amount of salad oil were added. The mixture was put in a polyethylene bag, and then crumpled up so that it would become homogeneous. Next, the mixture was put through a garlic squeezer for the purpose of forming and then dried by air.

When preparing feed for the TBA-treated group, TBA was first dissolved in salad oil at specified rate so that the content of TBA would finally become "10 mg/1000 g of feed (10 ppm)". It corresponds between 0.2 to 0.5 mg of TBA per 1000 g body weight".

Rearing conditions:

Fish were released in tanks of "30 cm x 70 cm x 29 cm" (water depth: 25 cm) each, with 70 fishes per tank. Water temperature was kept at 8.8 - 10.2 C and water flow rate was kept at 3 - 5 liters/minute.

Saprolegniasis test method:

In accordance with Dr. Hoshiai's "AMIMOMI" Method (mecanical stress or net shaking stress test) stress was forced on the fish and epithelial damages were made on them. In order to enhance the reliability of the study, "AMIMOMI" was conducted in varied conditions: the number of fish per "AMIMOMI" was 5 or 10, and "AMIMOMI" time was 30 seconds, 1 minute, or 2 minutes. In order for the fishes to undergo the attack of Saprolegnia zoospores, the fishes were released in water containing "2 x 10 zoospores/liter of water". During the test period, water in the "60 cm x 30 cm x 45 cm (water depth: 40 cm)" tanks was wholly changed every 3 days. Zoospore attack and fish observation lasted for 14 days, from September 22nd to October 6th. During the period, water supply was stopped; aeration was continued; and water temperature was kept at 12 - 13 C. During the rearing period, water was supplied through 2 pipes to take into account the risk of the possible damage of a pipe by accidents, such as heavy rainfall, etc.

2) Results and Discussion Concerning the Infection Test.

Test results are shown in figures 1 to 6 as follows:

Each figure displays the number of infected fishes in the control group and the TBA treated group, and the number of dead fishes (only among the control fishes, no mortality having been recorded in the TBA treated group).

Day 0 is the day of the stress test.

Figure 1: stress test: 30s on 5 fishes.
Figure 2: stress test: 30s on 10 fishes.
Figure 3: stress test: 60s on 5 fishes.
Figure 4: stress test: 60s on 10 fishes.
Figure 5: stress test: 120s on 5 fishes.
Figure 6: stress test: 120s on 10 fishes.

a) Trend observed in the control groups:

Given the same number of fish per "AMIMOMI", the longer the "AMIMOMI" time, the higher the rate of infection was, the earlier the symptoms developed, the higher the mortality was, and the quicker they died. It is because the longer the "AMIMOMI" time was, the higher the stress and the more serious the traumas were. Given the same "AMIMOMI" time, the smaller the number of fish per "AMIMOMI" was, the quicker the symptoms developed and the higher the incidence of infection was. The same trend was observed regarding the following parameters,

environmental conditions, such as the size of tanks, the number of zoospores per tank, rearing water temperature, etc.; the smaller the number of fish per "AMIMOMI", the higher the number of attacking zoospores per fish.

b) Trend observed in the TBA-treated groups:

The trend is generally similar to that observed in the control groups. In the following points, however, the TBA-treated groups showed large differences from the control groups. Among the fish which underwent "AMIMOMI" as a group of 5, infection was confirmed first on the 3rd day and it disappeared on the 6th, regardless of the duration of "AMIMOMI". The longer the "AMIMOMI" was, however, the higher the rate of infection was. In the case of the fish which underwent "AMIMOMI" as a group of 10 and where "AMIMOMI" lasted for 30 seconds, infection was confirmed on the 4th day and disappeared on the 9th day. In the fishes which underwent "1-minute AMIMOMI" as a group of 10, infection was confirmed 1 day earlier, i.e. on the 3rd day, but recovered to normal on the 9th day. In the latter group, rate of infection was higher than that of the "30-second AMIMOMI" group. The "2-minute AMIMOMI" group showed a trend which was almost the same as that shown in the "1-minute AMIMOMI" group, with all the fishes recovering from Saprolegniasis except the one in which the disease was still confirmed on the final day. Moreover, rate of infection was lower than in the "1-minute AMIMOMI" group.

According to the analysis where conditions of infection were compared among the fish of the same AMIMOMI time group:

In the "2-minute AMIMOMI group", rate of infection was much higher than others in the "5-fish AMIMOMI" group. In the 30-second and 1-minute AMIMOMI groups, there was no difference except that the 100% recovery was achieved later than in the 2-minute group.

Based on the above, the author concluded that when attacked by zoospores, the TBA-treated fish are once infected but soon get rid of the hypha and then recover. Thus, by being treated with TBA, prophylaxis against Saprolegniasis is achieved.

c) Differences between groups, both in the 5-fish AMIMOMI group and the 10-fish AMIMOMI group, infection was observed 1 - 2 days later and the degree of infection was remarkably smaller compared with the control groups, regardless of the AMIMOMI duration. If the two groups are compared under the same conditions, in the TBA-

treated group, rate of infection was lower, recovery appeared earlier, and there was no death. In the TBA group (10 fishes), 2-minute AMIMOMI, only 1 fish did not recover at the completion of the study.

The brief explanation of the drawings:

Figure 1 displays the results of antifungal activity of TBA when forcing stress on the group of 5 fishes for 30 seconds.

Figure 2 displays the results of antifungal activity of TBA when forcing stress on the group of 10 fishes for 30 seconds.

Figure 3 displays the results of antifungal activity of TBA when forcing stress on the group of 5 fishes for 1 minute.

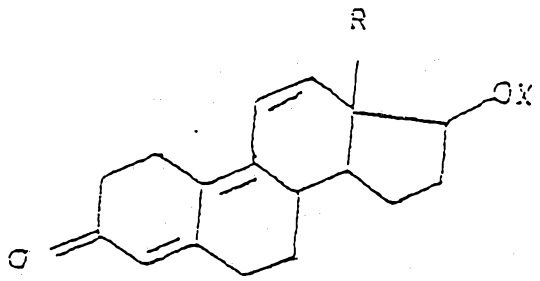
Figure 4 displays the results of antifungal activity of TBA when forcing stress on the group of 10 fishes for 1 minute.

Figure 5 displays the results of antifungal activity of TBA when forcing stress on the group of 5 fishes for 2 minutes.

Figure 6 displays the results of antifungal activity of TBA when forcing stress on the group of 10 fishes for 2 minutes.

The claims defining the invention are as follows:

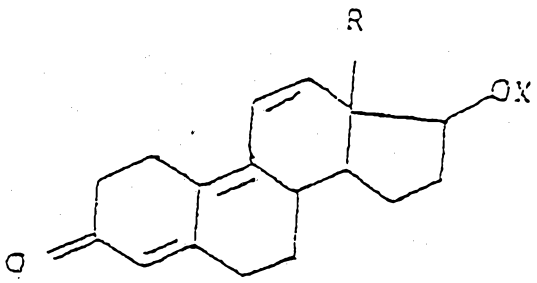
1. A method for combatting stress of fish in fish farming which method comprises administering to said fish an effective amount of compounds of formula (I):



(I)

in which R represents an alkyl radical containing 1 to 4 carbon atoms and X represents an acyl radical derived from a carboxylic acid containing 1 to 18 carbon atoms.

2. A method for increasing the resistance to fungi, viruses, bacteria and other pathogenic agents in fish which method ~~comprising~~ comprises administering to said fish an effective amount of compounds of formula (I):



(I)

in which R represents an alkyl radical containing 1 to 4 carbon atoms and X represents an acyl radical derived from a carboxylic acid containing 1 to 18 carbon atoms.

3. The method according to claim 1 or claim 2, wherein R is a methyl radical and OX represents an acetoxy radical.

4. The method according to any one of claims 1 to 3, wherein said compounds of formula (I) are administered by oral route.

5. The method according to any one of claims 1 to 3, wherein said compounds of formula (I) are incorporated with a fish food.

6. The method according to claims 5, wherein said compounds of formula (I) are incorporated with a fish food at a rate of 1 to 20 ppm of weight of active ingredient per weight of fish food.

7. The method according to any one of claims 1 to 6, wherein the administration is carried out for Salmonidae, Anguillidae, Cyprinidae, cycloid fish and sea fish.

8. The method according to claim 7, wherein Salmonidae is trout or salmon; Anguillidae is eel; Cyprinidae is carp; cycloid fish is tilapia; and sea fish is bass or sea bream.

9. Method for combatting stress of fish in fish farming which method is substantially as herein described with reference to any one of the Examples and accompanying drawings but excluding any Comparative Examples.

10. Method for increasing the resistance of fungi, viruses, bacteria and other pathogenic agents in fish which method is substantially as herein described with reference to any one of the Examples and accompanying drawings but excluding any Comparative Examples.

DATED this 16th day of August 1993.

ROUSSEL-UCLAF

By their Patent Attorneys:

CALLINAN LAWRIE

Michael J. Hourihan.

ABSTRACT

4,9,11-trienic steroids are provided as compounds to combat stress in fish by increasing their resistance to stress, particularly in fi arming. The invention also relates to fish food compositions including such 4,9,11-trienic steroids and to methods of increasing resistance to pathogenic agents in fish by the use of such steroids.

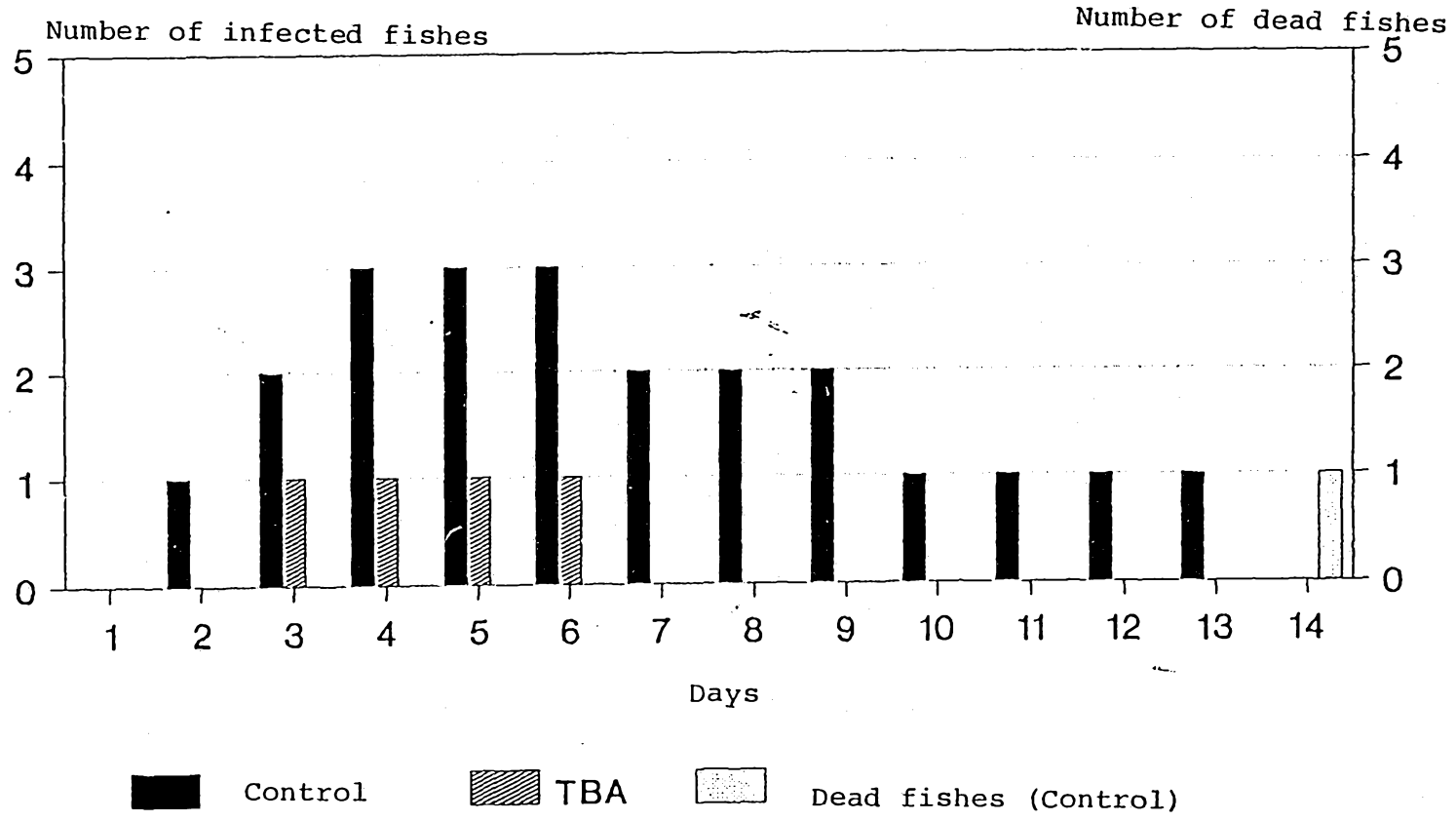
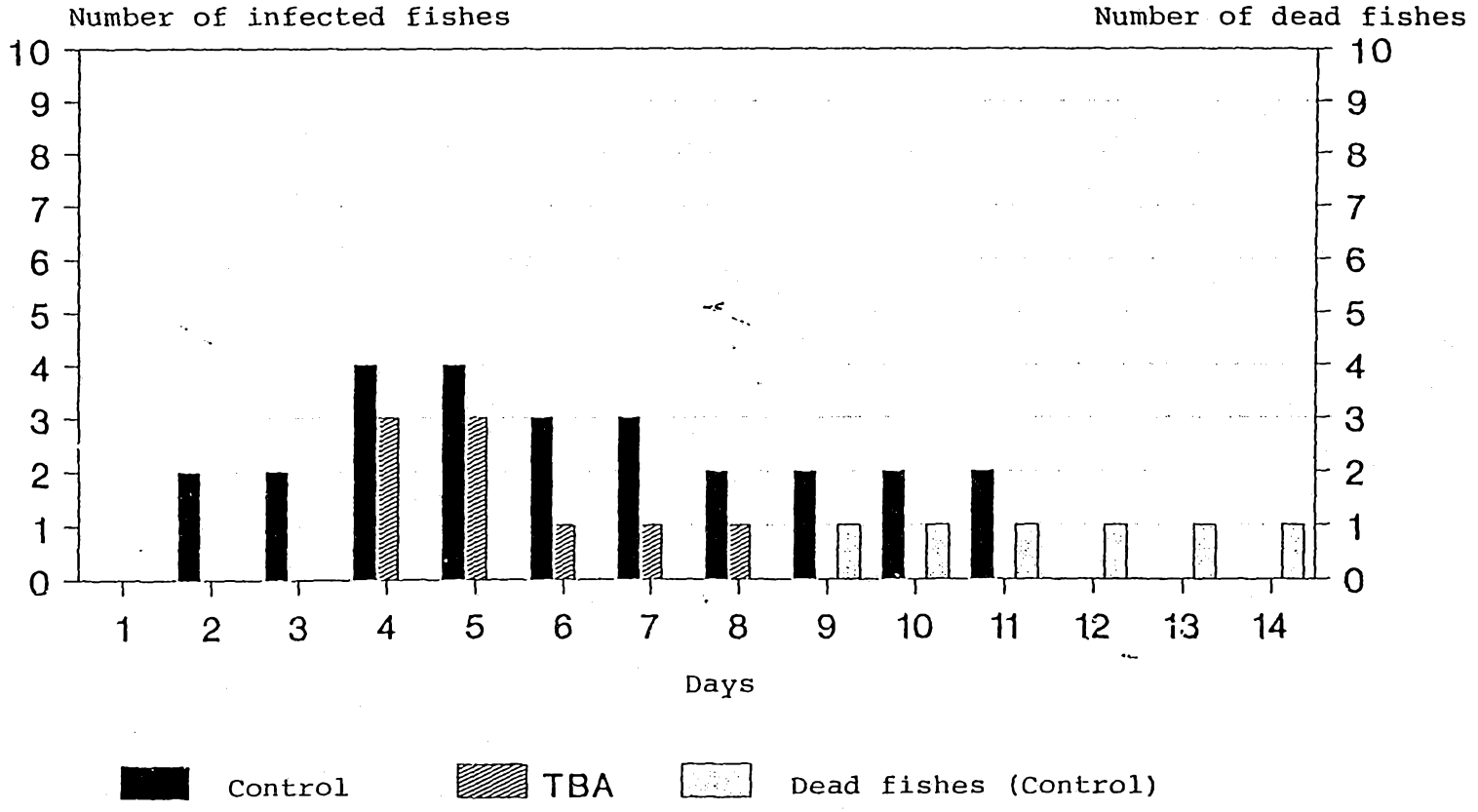

Number of infected fishes
Number of dead fishes
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days
Control
TBA
Dead fishes (Control)

Figure 1


Number of infected fishes
Number of dead fishes
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12
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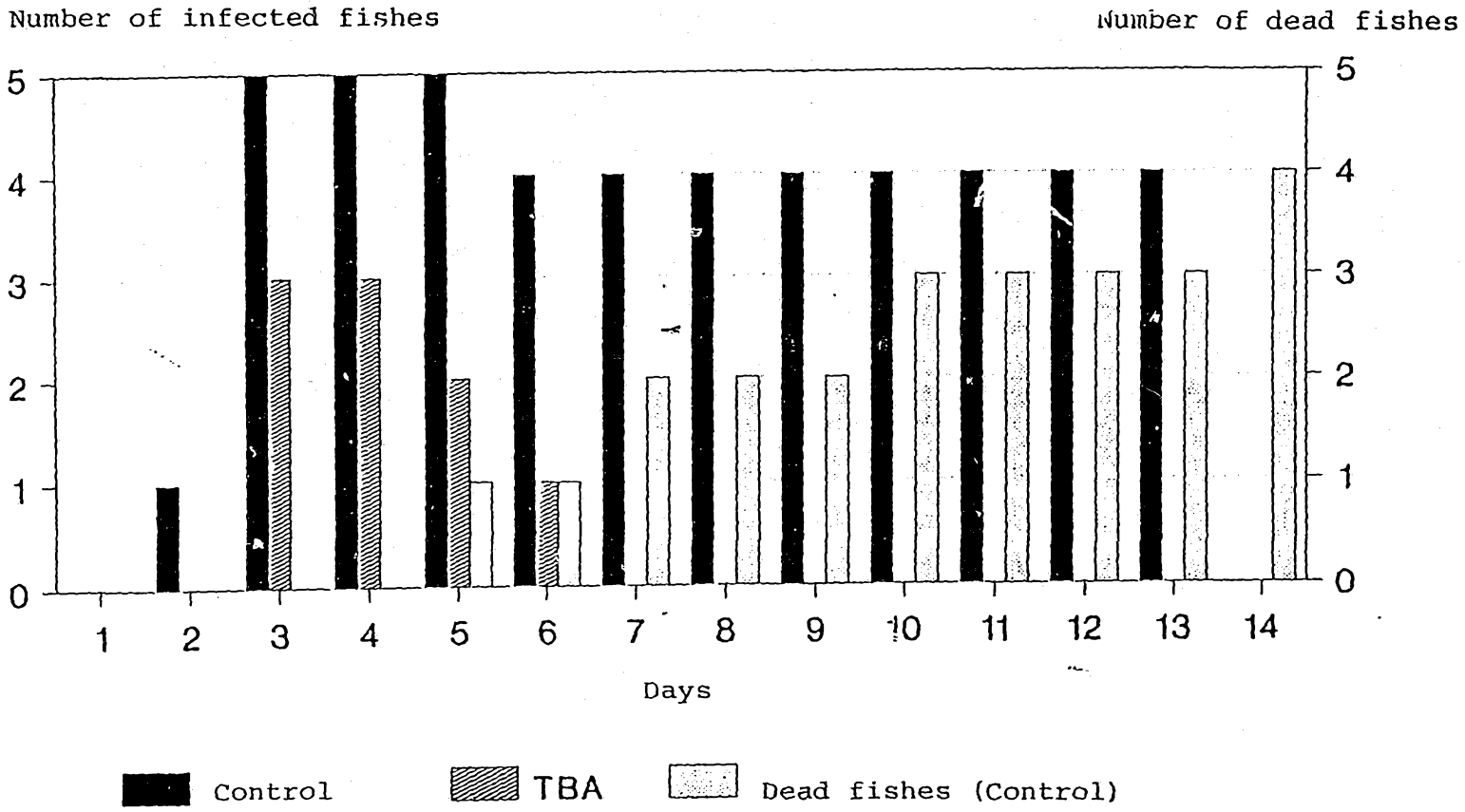
14
Days
Control
TBA
Dead fishes (Control)

Figure 2


Number of infected fishes
Number of dead fishes
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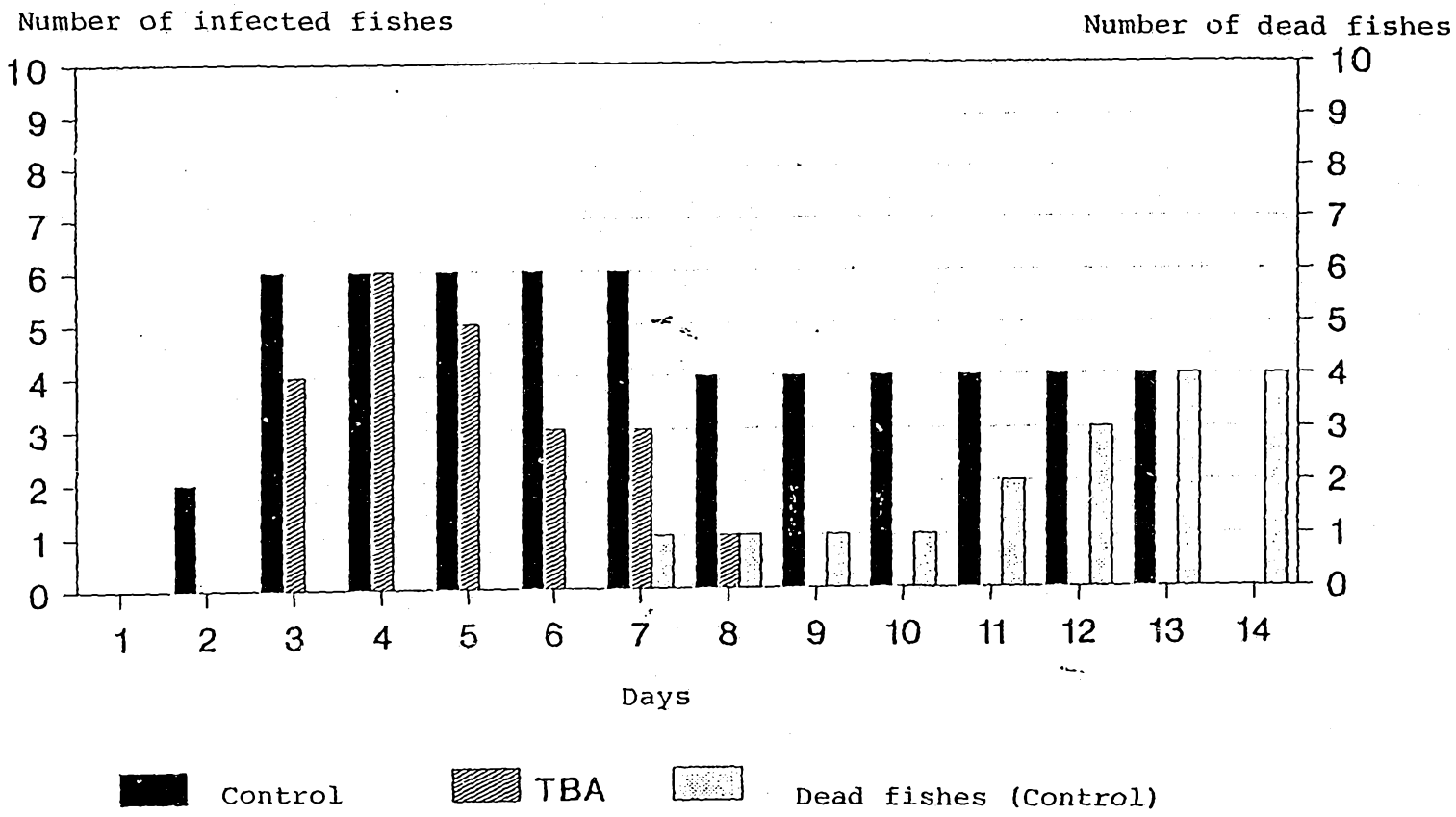
14
Days
Control
TBA
Dead fishes (Control)

Figure 3


Number of infected fishes
Number of dead fishes
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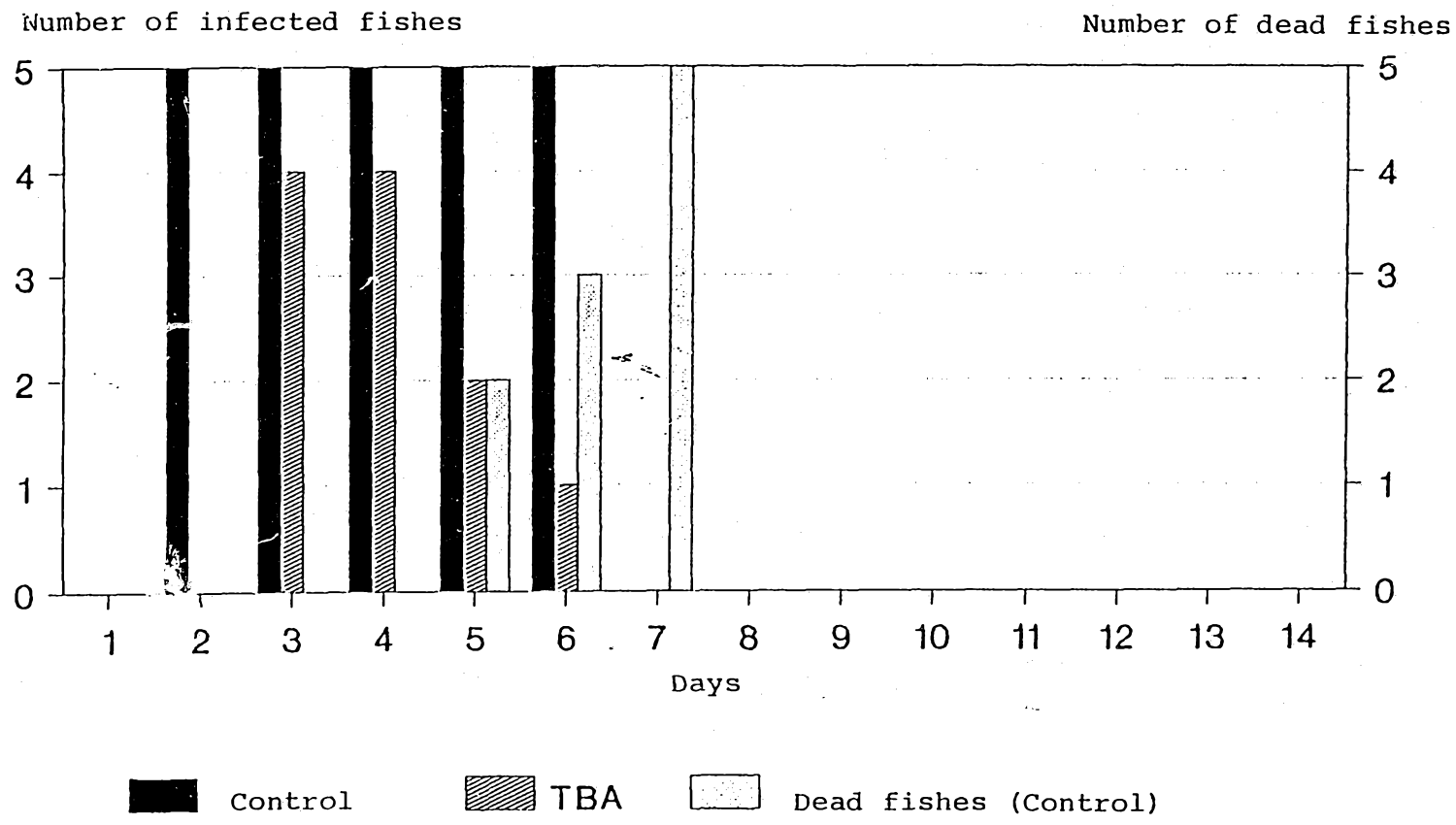
14
Days
Control
TBA
Dead fishes (Control)

Figure_4


Number of infected fishes
Number of dead fishes
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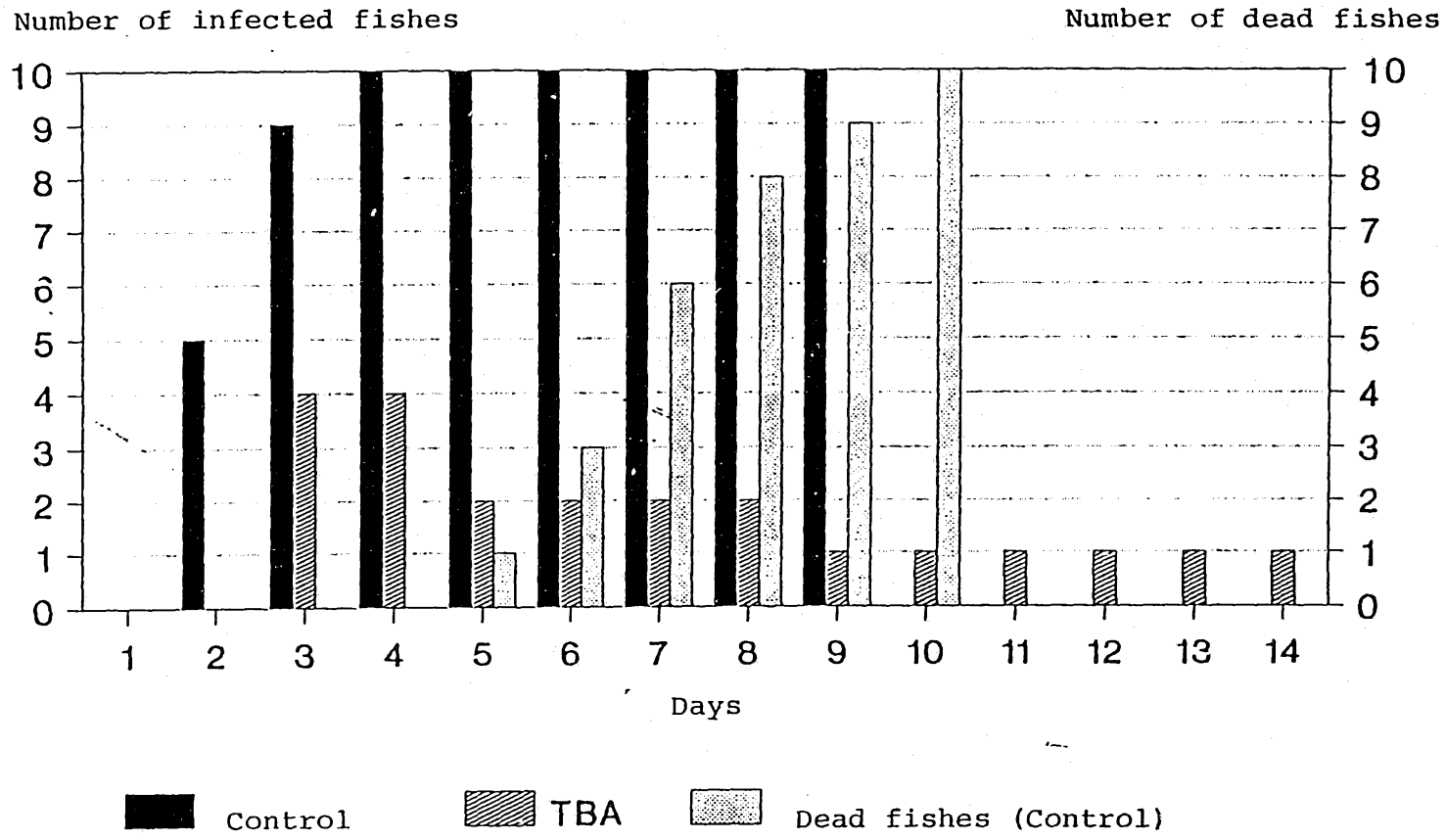
Days
Control
TBA
Dead fishes (Control)

Figure 5


Number of infected fishes
Number of dead fishes
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Days
Control
TBA
Dead fishes (Control)

Figure 6