Original Research Article

Randomized response surface pathway design with skewed starting point and stochastic dose window

Trond Holand[1]*, Øystein Evensen[1], Sagita Dewi[1,2], Stig Larsen[1]

[1]Department of Production Animal Clinical Sciences, Norwegian University of Life Science, Faculty of Veterinary Medicine, Oslo, Norway
[2]Bali Indera Hospital, Government of Bali Province, Indonesia

*Correspondence:
Dr. Trond Holand,
E-mail: trholand@online.no

ABSTRACT

Background: The aim was to introduce response surface pathway (RSP)-design with skewed starting value and stochastic dose-window to estimate optimal efficacy dose (OED) of BP-C2 after IL-1β stimulation in Atlantic salmon.
Methods: 54 healthy smolt of Atlantic salmon between 50 and 100 g before habituated to salt water were included. The study was conducted as a one-dimensional, randomized between-patient three-level RSP designed trial with one interventional- and one response variable and odd outcomes. The interventional variable was intraperitoneal injected BPC2 with skewed starting dose of 0.10 mg/100 g related to the initial dose-window <0.02-0.5 mg/100 g. The response variable was the Ct-value of mRNA IL-1β expression 24 hours after injection.
Results: Skewed starting value of 0.10 mg/100 g was chosen in the first design-level with a dose-window of <0.0-0.20]. The three smolt obtained a reduction in Ct-value above 15%, and the dose-window adjusted with the lower boundary equals the previous dose. The five smolt at second design-level received 0.16 mg/100g with a dose-window [0.10-0.22]. Four smolt obtained above 15% and one of 0.5% reduction in cycle threshold (Ct)-value. Six smolt in the third design-level received 0.21 mg/100 g and one 0.16 mg/100 g. The mean Ct-value was reduced from 30.0 in the unstimulated situation to 25.0, 24.8 and 26.4 after BP-C2 stimulation of 0.10, 0.16 and 0.21 mg/100 g, respectively. The OED of BP-C2 related to IL-1β was estimated to 0.14 mg/100 g.
Conclusions: Skewed starting value in the initial dose-window made the K-adjustment factor and dose-window stochastic. The RSP-procedure works in accordance to the expectation and estimated OED of BP-C2 sufficiently.

Keywords: Response surface pathway design, Skewed starting value, Stochastic dose-window, IL-1β mRNA expression, BP-C2, Salmon

INTRODUCTION

Vaccination against diseases in farmed Atlantic salmon is an important undertaking both related to animal welfare and as a substantial source for economic loss for the producers.[1,2] Use of adjuvants or immune-stimulants is as a general rule necessary to obtain necessary vaccine efficacy.[3] The search for alternative adjuvant molecules or certain combinations of them as adjuvants is desirable in order to increase animal welfare without reducing the long-lasting disease protection level following vaccination. Most efficient vaccine adjuvants in use today are based on mineral oils.[3] Growth reduction and mild to severe adverse events are reported. Lack of available antiviral vaccines indicates the importance of identifying other adjuvant combinations and immune modulators for use in vaccines for farmed fish.[4-6]

A promising substance named BP-Cx1, consisting ligand polymers of benzene poly-carboxylic acids.[7] BP-Cx1 has been used in combination with cisplatin as cancer treatment (BP-C1) and molybdenum in anti-radiation (BP-C2). Controlled clinical studies of BP-C1 against Stage IV metastatic breast cancer have shown promising effect both in humans and in dogs.[8-12] The treatment has been found very well tolerated and only few and mainly mild AE has been reported.[13] A pancreatic cancer case study with BP-C2 shows promising, and interesting results related to strengthening of the immune system.[14] Pre-clinical studies and studies related to the mechanism-of-action of the substances, discovered strong immune stimulating effects of both BP-Cx-1 and BP-C2.[15,16] Similar immune stimulating effect has previously been reported in fish caused by humic acid.[17,18] The pre-clinical human based results show the best immune stimulating effect of BP-C2 closely followed by BP-Cx1 and maybe candidates as vaccine adjuvants[16]. However, optimal efficacy dose (OED) of BP-C2 related to immune stimulation of fish is required.

Response surface pathway (RSP) is based on combining classical up-and-down procedure with common response surface methodology.[19] The idea behind RSP is to capture and utilize the generated normative data during the study for faster and more accurately achieving valid scientific results. RSP represents interesting opportunities when optimizing the design for dose–response studies. Earlier RSP-studies have demonstrated that it is possible to obtain statistically stronger results with fewer included patients in less time.[12,13,19]

The procedure to escalate or de-escalate in the RSP design is based on the k-adjustment factor. In order to ensure that all doses to be included will fall inside the dose-window, the mid-value of the interval has to be used as a starting value. The k-adjustment factor depends on the starting dose, the upper and lower boundary in the predefined dose window and the number of levels within the design.[13,19]

The number of design levels used will influence the width of the step. If the dose window is known to be wide, the number of design levels may be increased. However, when the dose window is narrow, a smaller number of design levels may be sufficient.

The RSP design was developed for toxicity study in laboratory animals, but also in common dose-finding studies without requiring any prior probability distribution.[8,19] The procedure for escalating and de-escalating is based purely on patient outcomes and the demand for covering the predefined dose-window.[19]

In dose finding studies, a correct dose window can be difficult to set and a midway strategy is often chosen as starting point. If the dose window is uncertain and wide, but the clinical estimate is that the starting point is leaning under or above the middle point, the RSP method can be modified to get away from this midway approach. By introduction of a skewed starting point, both the k-factor and the dose-window can be adjusted between each design level and becomes stochastic. This gives us an opportunity to set the starting dose skewed whilst narrowing in the dose window underway in the RSP study without missing the unexpected high or low results. Until now only a mid-point starting value with a fixed k-factor for adjustments within the dose window to cover it evenly have been developed. By changing the dose window based on the clinical results from previous design level the k-factor goes from being a fixed constant to a stochastic variable. This enables us to maneuver with increasing certainty from one design level to the next by both adjusting the upper and lower limit whilst completely covering the decreasing dose-window.

The aim of this paper is to introduce RSP-design with skewed starting value and stochastic dose-window in order to estimate OED of BP-C2 in IL-1β stimulation of farmed Atlantic salmon.

METHODS

The study material consisted of healthy farmed Atlantic salmon between 50 and 100 g before habituated to salt water and classified as smolt. The total study sample consisted of 54 smolt stalled at the freshwater laboratory aquarium at the Norwegian University of Life Sciences. All the smolt was acclimated to the environment before study start. The study period was from May to October 2018 and approved by the Norwegian Animal Research Authority.

Study injection

BP-C2 is a combination of the benzene poly-carboxylic acid ligand with molybdenum and given as intraperitoneal injection (IP) in different doses in these studies. Assessment of IL1ß mRNA expression in head-kidney at different time post injection was done by euthanizing the fish at fixed time intervals as outlined in the different paragraphs below. About 30 mg of tissues were used for mRNA extraction. Tissues were homogenized in trizol. After homogenization, the samples were centrifuged. The supernatant was transferred to a new tube and 0.2 ml chloroform was added. The aqueous phase was then transferred to a gDNA eliminator spin column provided with the RNeasy kit and centrifuged. From this step onwards, the protocol provided with the RNeasy kit was followed. The concentration of the ribonucleic acid (RNA) was determined by spectrophotometry using a nanodrop ND1000. 500 ng RNA was used for cDNA synthesis using a transcriptor first strand cDNA synthesis kit following the protocol provided by the manufacturer. Quantitative polymerase chain reaction (PCR) was performed in 96 well plates using the Light-Cycler 480 or 96 systems. Each reaction contained 3µl cDNA, 10 pmol gene-specific primers for IL1ß-F

CGTCACATTGCCAACCTCAT and IL1ß-R ACTGTGATGTACTGCTGAAC and 10µl Light-Cycler 480 SYBR green I master mix. The final volume was adjusted to 20µl using RNase free water. The data obtained were analyzed by the ΔΔCT relative quantification method using β-actin as reference gene.

Main variable

The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the Ct level the greater the amount of target nucleic acid in the sample.[20] In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal.

The first pilot study consists of 18 smolt received IP injected BP-C2 in the dose of 0.10 mg/100 g. The smolt was randomly divided in three groups of six smolt each. IL-1β was measured in the spleen by real time PCR at the time of injection in the first group, eight hours after the injection in the second group and 24 hours after the injection in the third group. The mean Ct-level at the time of injection was 30.1 (95% CI: 29.9-31.3). This was reduced to 29.3 (95% CI: 28.7-29.6) eight hours after the injection and further to 24.8 (95% CI: 24.3-25.3.3) 24 hours after the injection (Figure 1A). This indicates that the measurement of IL-1β has to be performed 24 hours after injection in the main study.
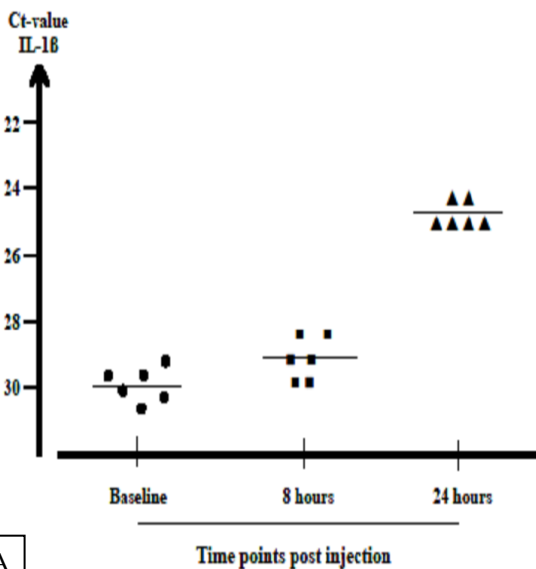
Figure 1A: Pilot study 1: The development in Ct-value of IL-1β after a single BPC2 injection of 0.10 mg/100g fish during 24 hours.
Six smolt were measured at baseline ●, 8 hours ▲ and 24 hours ■ after injection. The results expressed by mean as a horizontal line and the dispersion by a vertical line with upper and lower boundary.
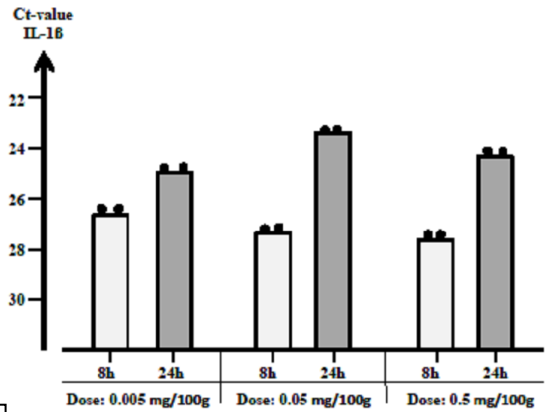
Figure 1B: Pilot study 2: The mean Ct-value of IL-1β measured after 8 and 24 hours after a single BPC2 injection of 0.005 mg/100g, 0.05 mg/100g and 0.5 mg/100g.
Two smolt were measured at each sampling within each dose. The results marked by ● and the columns indicate the mean values.

The second pilot study consists of 12 smolt randomly divided in three groups of four smolt each. The first group received IP injected BP-C2 in the dose of 0.005 mg/100 g, the second 0.05 mg/100 g and the third in the dose of 0.5 mg/100 g. Two smolt in each group were measured eight hours after the injection and two after 24 hours. The results support the conclusion from the first pilot study to perform the measurement in the main study 24 hours after injection (Figure 1B). The effect of the BP-C2 injection seemed to increase with increasing dose, but reduces again to 0.5 mg/100 g. The result indicates a maximum effect between 0.05 and 0.5 mg/100g but most probably closest to 0.05 mg/100 g (Figure 1B).

The main study consists of 24 smolt of which nine used as unstimulated controls. The material of 15 fish was divided in three design-levels with three, five and seven smolt, respectively. Three unstimulated controls were used at each design-level.

Ethics approval was given by the Norwegian Animal Research Authority and the studies conducted in the Faculty of Veterinary Medicine at Norwegian University of Life Sciences.

Design of the main study

The study was conducted as a one-dimensional, randomized between-patient three-level RSP designed trial with one interventional and one response variable and odd number of outcome.[21] RSP design is an adaptive design and the methodology has previously been presented within and between patients with one interventional and one response variable.[13] Development of the methodology in laboratory animals and simulations demonstrated that allocation of equal number of subjects to each level is not an optimal solution.[19] By starting with

a low number of patients at first design level and increase this number with increased level, the sample size reduces without reduction in accuracy.

Dose adjustment procedure in RSP design

Let m denote the starting dose, m_i the dose at design level i, and k the dose adjustment factor. The dose at design level i given by equation 1). Let D_u denote the upper limit of the interventional variable and n=the number of design levels, then D_u given as the sum of a geometric series in equation 2).[22]

1) $$m_i = m_{i-1} \pm \frac{m}{k^{i-1}} : i = 1, 2 \dots n$$

2) $$D_U = \frac{m(k^n - 1)}{(k^n - k^{n-1})}$$

With known upper limit of the interventional variable, the starting value m and design level n, the k-adjustment factor calculates from equation 2).

Escalation and de-escalation procedure

The response variable is multinomial with unequal number of categories denoted as 2c+1. Of these possible 2c+1 response value, c gives escalation, c de-escalation and 1 remaining unchanged value of the interventional variable for the patients in the next design level. The predefined window of the interventional variable is denoted as D_U=the upper and D_L=the lower limit. It may be convenient to use the mid value of the predefined dose window as the starting value denoted as m. To ensure coverage of the dose window, a dose adjustment procedure was established.[21]

Randomized between-patient RSP design

In order to optimize the RSP model, the number of smolt is reduced to a minimum in the first design level and increases with increasing level.[21] The recommended increase procedure is to start with three smolt at level 1, increasing to 5, 7, 9, and so on at level 2 and upward (Table 1).

Table 1: Randomization of the IP injected BP-C2 dose for the next design level based upon the outcome in the previous level.

Design level	Dose	Randomization	Dose used in the next design level
Design level 1 (n=3)	0.10 mg/100 g	None	a_1 smolt randomizes to A_1
			a_2 smolt randomizes to A_2
			a_3 smolt randomizes to A_3
			b_1 smolt randomizes to B_1
Design level 2 (n=5)	A_1	$a_1 : a_2 : a_3$	b_2 smolt randomizes to B_2
	A_2		b_3 smolt randomizes to B_3
	A_3		b_4 smolt randomizes to B_4
			b_5 smolt randomizes to B_5
			c_1 smolt randomizes to C_1
Design level 3 (n=7)	B_1	$b_1 : b_2 : b_3 : b_4 : b_5$	c_2 smolt randomizes to C_2
	B_2		c_3 smolt randomizes to C_3
	B_3		c_4 smolt randomizes to C_4
	B_4		c_5 smolt randomizes to C_5
	B_5		c_6 smolt randomizes to C_6
			c_7 smolt randomizes to C_7

Table 2: The dose change from one design level to the next based on the obtained results.

Dose on the first design level	% response increase design level 1	Dose on the design level 2	% response increase design level 2	Dose on Design level 3 (mg/100 g)
m (0.10 mg/100g)	Classification 5 ≥ 15	M + m/k (0.16 mg/100 g)	≥ 15	M + m/k + m/k^2 (0.20)
			<15-7.5	m + m/k + m/k^3 (0.19)
			<-7.5-7.5>	m + m/k (0.16)
			-7.50– -15>	m + m/k – m/k^3 (0.14)
			$\leq$-15	m + m/k – m/k^2 (0.12)
	Classification 4 <15–7.5	M + m/k^2 (0.14 mg/100 g)	≥ 15	m + m/k^2 +m/k^3 (0.16)
			< 15-7.5	m + m/k^2+m/k^4 (0.15)
			<-7.5–7.5>	m + m/k^2 (0.14)
			-7.50– -15>	m + m/k^2 – m/k^4 (0.13)
			$\leq$-15	m + m/k^2 – m/k^3 (0.12)

Continued.

Dose on the first design level	% response increase design level 1	Dose on the design level 2	% response increase design level 2	Dose on Design level 3 (mg/100 g)
			≥ 15	$m + m/k$ (0.21)
			< 15–7.5	$m + m/k^2$ (0.17)
	Classification 3 <-7.5–7.5>	m (0.10 mg/100 g)	<-7.5–7.5>	m (0.16)
			-7.50– -15>	$m - m/k^2$ (0.15)
			$\leq$-15	$m - m/k$ (0.11)
			$\geq$15	$m - m/k^2 + m/k^3$ (0.09)
	Classification 2 {-7.50–-15>	$m-m/k^2$ (0.06 mg/100 g)	<15–7.5	$m - m/k^2 + m/k^4$ (0.08)
			<-7.5–7.5>	$m - m/k2$ (0.06)
			-7.50–-15>	$m - m/k^2 – m/k^4$ (0.05)
			$\leq$-15	$m - m/k^2 – m/k^3$ (0.04)
			≥ 15	$m - m/k + m/k^2$ (0.08)
	Classification 1 $\leq$-15	m-m/k (0.04 mg/100 g)	< 15–7.5	$m - m/k + m/k^3$ (0.06)
			<-7.5–7.5>	$m - m/k$ (0.04)
			-7.50– -15>	$m - m/k – m/k^3$ (0.01)
			$\leq$-15	$m - m/k – m/k^2$ (0.00)

m=10mg/100g indicate the chosen starting dose on the first design level with dose-window [0.0–0.20] mg/100 g.

Performance of the main study

The interventional variable was the IP injected BPC2 dose and the response variable was the Ct- value of IL-1β recorded 24 hours after the injection. In previously published RSP studies the mid-value strategy has been used to determine the starting value of the intervention variable.[13,19,21] A mid-value strategy gives a start value in this study of 0.25 mg/100 g with a dose-window <0.00-0.5]. Based on the results obtained in the second pilot study, the MED of BPC2 related to IL-1β is most probably closest to 0.05 mg/100g. A starting dose of 0.10 mg/100 g with starting dose-window <0.00-0.20] was chosen. A skewed value strategy was introduced.

The response variable in the study was percent reduction in the Ct-value of IL-1β and categorized as ≥15% increase, <15–7.5% increase, ±7.5% change, 7.5-15>% reduction and ≥15% reduction. Three smolt allocated to the first design level received 0.10 mg/100 g. Based on the results obtained from the first design level, five new smolt were subsequently included to the second design level. The injected BPC2 dose was separately determined (Table 2). Seven smolt were included in the third design level with doses based on results obtained from the second design level.

Dose-window adjustment procedure

The initial dose-window in this case was <0.0-0.5] mg/100 g and by using a mid-value strategy the starting dose would have been 0.25 mg. The skewed strategy using 0.10 mg/100 g with a dose-window <0.0-0.20] was chosen. In case the results from at all the three smolt in the first design level recommend a maximum increase or maximum reduction in the dose, the dose-window for the remaining study will be changed.

- In case the recommendation is maximum dose increase, the lower level of the adjusted dose-window will be chosen equal to the previous starting value *m* with the new starting value equal to the maximum dose recommended for design level 2 in the last design model. Based on the mid-value strategy in the RSP design, an adjustment of the dose window will occur.

- In case the recommendation is maximum dose reduction, the upper level of the adjusted dose-window will be chosen equal to the previous starting value *m* with the new starting value equal to the dose recommended for design level 2 in the last design model. Consequently, an adjustment of the dose window will occur in the same way as described above. In case the lower level of the adjusted dose-window is lower than the lower level of the basic window, the lower boundary in the basic window may be chosen.

In the present study it is chosen a starting value below the mid-value of the basic dose window. In case the chosen start value is above the mid-value, the suggested procedure has been turned around.

- In case the recommendation is maximum dose reduced, the upper level of the adjusted dose-window will be chosen equal to the previous starting value *m* with the new starting value equal to the minimum dose recommended for design level 2 in the last design model. Based on the mid-value strategy in the RSP design, an adjustment of the dose window will occur.

- In case the recommendation is maximum dose increase, the lower level of the adjusted dose-window will be chosen equal to the previous starting value *m* with the new starting value equal to the dose recommended for design level 2 in the last design model. Consequently, an adjustment of the dose window will occur in the same way as described above. In case the upper level of the adjusted dose-window is above the upper level of the basic window, the upper boundary in the basic window may be chosen.

Assuming that at all the three smolt from the first design level in the present example recommending a maximum dose increase of 0.16 ml/100 g (Table 2). Consequently, the new adjusted dose-window will be (0.10-0.22) mg/100 g (Table 3). In according to the RSP-design, five smolt will be included in the second design level receiving 0.16 mg/100 g. In case the results from all smolt in this second design level recommend a maximum dose increase, the lower limit of the next adjusted dose-window will be 0.16 mg/100 g and the dose-window (0.16-0.26). The seven smolt included in the third design level will receive the recommended maximum dose equals 0.21 mg/100 g.

This procedure may continue as long as the new starting value is equal or below the mid-value of the initial dose-window. In this example it is possible to perform adjustment of the dose-window three times (Table 4). The *k*-factor increases and the range of the dose-window reduce with the number of adjustments.

Statistical analysis

Continuously distributed variables are expressed as mean values, standard deviation (SD) in brackets, and 95% confidence interval.[23] Let X denotes the BPC2 dose and the sample space expressed as $\Omega = \{D_L \leq x \leq D_U\}$. Let μ represent OED and assume μ covers by Ω. The dose is ordinal in interventional variables. The dose response follows a quadratic function and not monotonically over the sample space. However, it is monotonically increasing from D_L and up to OED. Isotonic regression is the suggested model for analyzing the material in this monotonically increasing part of the sample space.[24] Polynomial regression analysis was used in order to describe the dose-response curve.[25]

RESULTS

Three smolt were given 0.10 mg/100 g. All smolt obtained a reduction in Ct-value above 15% compared to the mean of the controls. The chosen start value was found too small and used as the lower boundary of the adjusted dose window (Table 3). In accordance with the RSP-design all the five smolt included in the second design level were given 0.16 ml/100g (Figure 2).

Four of these fish obtained a reduction in Ct-value above 15% compared to the mean of the controls and one smolt obtained a reduction of only 0.5%. The randomization of seven smolt to the third design level was then 0.16 and 0.21 mg/100 g in ratio 1:4. Six smolt were randomized to 0.21 mg/100 g and one smolt to 0.16 ml/100 g (Figure 2). Of the six smolt allocated to 0.21 mg/100 g, one obtained a reduction in Ct-value above 15% whilst four obtained a reduction between 7.5% and 15% and one a reduction of 7%. The smolt allocated to 0.16 ml/100 g in the third design level obtained a reduction in Ct-vale above 15% compared to the mean of the controls.

Table 3: Corrected change in the dose from the second design level to the next based on the obtained results.

% response increase design level 2	Dose on the design level 3	% response increase design level 3	Dose on design level 4 (mg/100 g)
m (0.16 mg/100g)			
Classification 5 ≥15	m + m/k (0.21 mg/100g)	≥15%	m + m/k + m/k² (0.22)
		<15–7.5	m + m/k + m/k³ (0.21)
		<-7.5–7.5>	m + m/k (0.21)
		{-7.50–-15>	m + m/k – m/k³ (0.20)
		≤-15	m + m/k – m/k² (0.19)
Classification 4 <15–7.5}	m + m/k² (0.17 mg/100g)	≥15	m + m/k² +m/k³ (0.18)
		<15–7.5	m + m/k²+m/k⁴ (0.17)
		<-7.5–7.5>	m + m/k² (0.17)
		-7.50–-15>	m + m/k² – m/k⁴ (0.17)
		≤-15	m + m/k² – m/k³ (0.16)
Classification 3 <-7.5–7.5>	m (0.16 mg/100g)	≥15	m +m/k (0.21)
		<15–7.5	m + m/k² (0.17)
		<-7.5–7.5>	m (0.16)
		{-7.50–-15>	m - m/k² (0.15)
		≤-15	m - m/k (0.11)
Classification 2 -7.50–-15>	m - m/k² (0.15 mg/100g)	≥15	m - m/k² + m/k³ (0.16)
		<15–7.5	m - m/k² + m/k⁴ (0.15)
		<-7.5–7.5>	m - m/k2 (0.15)
		{-7.50–-15>	m - m/k² – m/k⁴ (0.15)
		≤-15	m - m/k² – m/k³ (0.14)
Classification 1 ≤-15	m - m/k (0.11mg/100g)	≥15	m - m/k + m/k² (0.12)
		<15–7.5}	m - m/k + m/k³ (0.12)
		<-7.5–7.5>	m - m/k (0.11)
		{-7.50–-15>	m - m/k – m/k³ (0.11)
		≤-15	m - m/k – m/k² (0.10)

m=0.16 mg/100 g represent the new starting dose with adjusted dose-window [0.10-0.22] mg/100g.
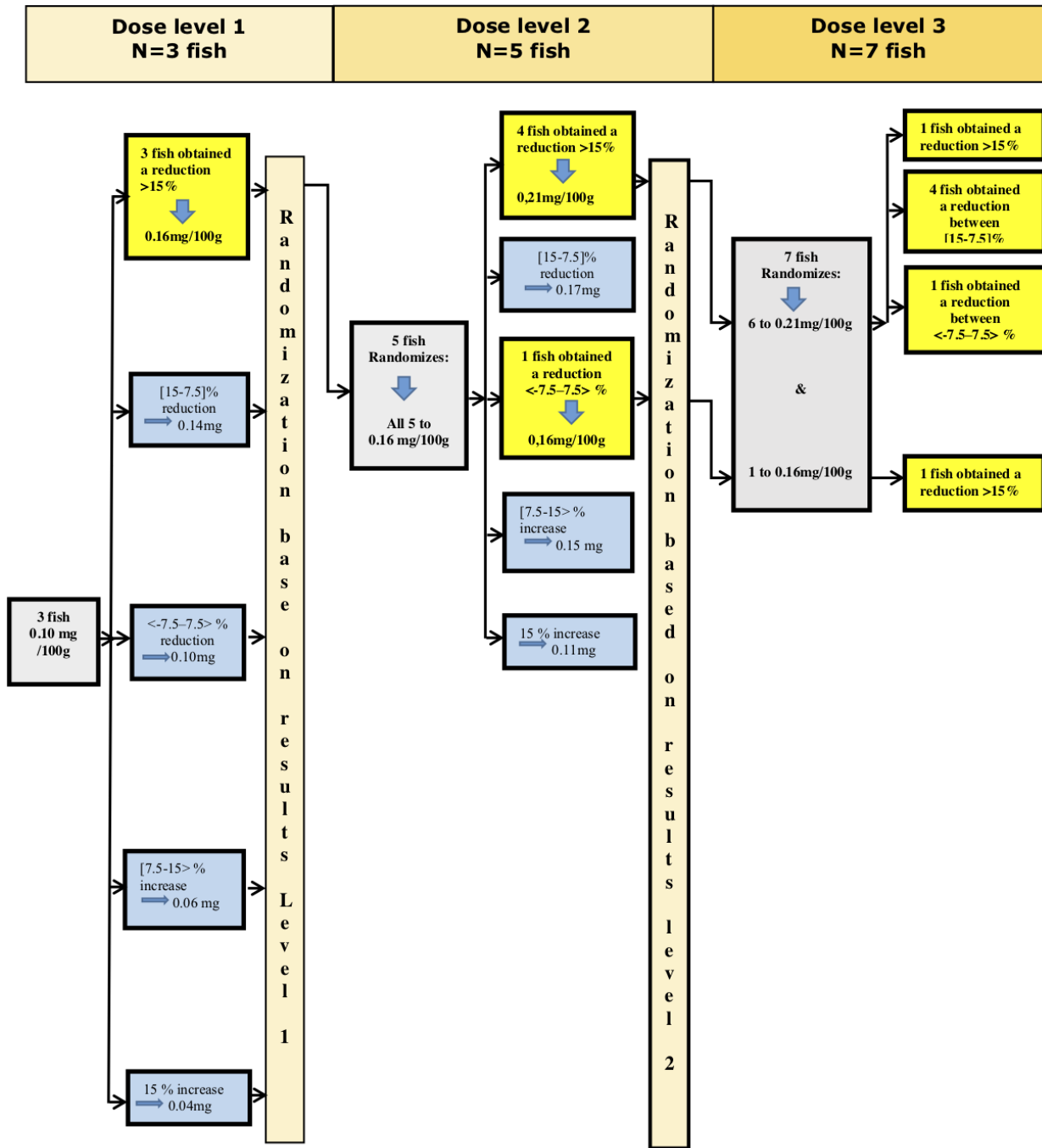
Figure 2: The results obtained in accordance with the response surface pathway design.
The grey box within each design level gives the number of smolt receiving the given doses of BPC2 and the yellow boxes the obtained reduction in Ct-value. The blue boxes give the unused alternative doses within each design level.

Table 4: The development in adjusted *k*-factor and dose-window based on the skewness of the starting-value related to the origin dose-window.

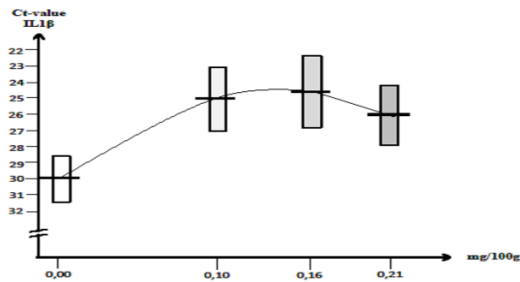|	Start values	*k*-factor	Dose window	Window range
Midway strategy	m=0.25 mg/100g	1.62	0.00–0.50	0.50
Chosen skewness	m=0.10 mg/100g	1.62	0.00–0.20	0.20
1st adjusted skewness	m=0.16 mg/100g	3.45	0.10–0.22	0.12
2nd adjusted skewness	m=0.21 mg/100g	5.00	0.16–0.26	0.10
3rd adjusted skewness	m=0.25 mg/100g	7.20	0.21–0.29	0.08

Figure 3: The development in Ct-value of IL-1β with increasing single BPC2 injection.

The results are expressed by mean values as the horizontal line and the columns 95% confidence intervals. The dotted line combining the mean values indicate the predicted Ct-value of IL-1β as a function of the dose.

The OED of BPC2 related to IL-1β was estimated to 0.14 mg/100g [95% CI; 0.125-0.155]. The mean Ct-value in the unstimulated smolt was 30.0 [95% CI: 31.4-28.6]. With a stimulation of 0.10 mg/100g the mean Ct-value reduces significantly ($p<0.01$) to 25.0 [95% CI: 23.0-26.9] and further to 24.8 [95% CI: 22.6-27.0] with 0.16 ml/100g (Figure 3). By increasing the BPC2 dose to 0.21 mg/100g the mean CT-value was 26.4 [95% CI: 24.7-28.1]. This reduction compared to the controls was also significant ($p<0.01$), but substantially less than the reduction after the BPC2 stimulation of 0.16 ml/100 g (Figure 3). The difference in Ct-value between 0.16 mg/100 g and 21 mg/100 g was however not found significant ($p=0.08$).

DISCUSSION

Despite an initially wide and uncertain dose window the study design was with sufficient accuracy able to predict OED of BPC2 related to IL-1β in Atlantic salmon. The study design worked well, presented the opportunity to reinvestigate obtained results and demonstrated an efficient pathway toward the area of clinical interest. The additional RSP procedure introduced in this paper opens up the possibility to accurately narrow the dose window from one design level to the next even with a skewed starting dose away from the mid-value strategy.

Injection of BPC2 in smolt was new to the species and two pilot studies performed to investigate sampling time and dose-window. Sampling after 24 hours gave significantly better IL-1β response compared to 8 hours and placebo. The results from the dose pilot study indicated a maximum effect between 0.05 and 0.5 mg/100 g, but most probably closest to 0.05 mg/100 g. The Ct-response followed a quadratic function in the BPC2 dose. This results in adapting the RSP design with a skewed starting dose away from the earlier used mid-value strategy. Such knowledge would have been important in a Bayesian designed dose-response study in order to choose a possible distribution over the parameter space in question.[26-28] However, the RSP-strategy was chosen, but with a skewed starting dose base on the same knowledge. Which of these two types of designs gives the

most accurate results with the lowest number of smolt should be investigated. The RSP-design with skewed starting value estimated in this situation OED of BPC2 with sufficient accuracy using only 15 smolt.

One of the strengths with the RSP procedure is the independency of such chosen distribution. Instead of using a Bayesian approach, it was decided to develop a lower skewed dose-window as a variable within the overall broader dose-window ranging from 0 to 0.5. This opens up the possibility to utilize the RSP methodology within a smaller skewed dose-window whilst at the same time treating the dose-window as RSP variable within the initial and larger dose-window. In this it was possible to maneuver accurately within a smaller dose-window that will become narrower and change up or down along the overall larger dose-window from one design level to the next during the trial.

Instead of starting with a window range of 0.50 mg/100 g (0 to 0.5) with a mid-start value of 0.25 mg/100 g, a skewed start value of 0.1 mg/100 g using the same 1.62 *k*-adjustment factor was chosen. This gave a window range of 0.2 mg/100 g for the first design level. Based upon the results from the first design level, the next adjusted skewness in the second design level reduces the dose-window from 0.1 to 0.22 mg/100 g, giving a window range of 0.12 mg/100 g and a dose of 0.16 mg/100 g to be used. In case all the five smolt in the second design level resulted in the recommending maximum dose increase for the third design-level, the new dose-window would have been [0.16-0.26] mg/100 g leading to a further reduction in window range to 0.10 mg/100 g. If performance of a fourth design level was needed, the window range would be further reduced to 0.08 mg/100 g going from 0.21 to 0.29 mg/100 g and an injection dose of 0.25 mg/100 g. This is equal to the starting value in the RSP-design with mid-value strategy and the initial dose-window <0.0-0.5] mg/100 g. In case the results from the first design level in the present study had recommended maximum dose reduction, the procedure described in 2) would have been used. This would have given a dose of 0.04 mg/100 g and an upper boundary of the adjusted dose window of 0.10 mg/100 g and a window range of 0.10 mg/100 g. By choosing a skewed starting dose too low or too high above the mid-value this RSP procedure will move the parameter in question against the mid-value with decreasing adjusted dose-window range. In case the chosen start dose is too high above or too much below the mid-value, the procedure will move the dose against the upper and lower boundary of the initial dose-window, respectively.

The aim of dose-finding studies is often to estimate a maximum tolerated dose of a substance, but also OED might be of interest. Usually, a dose-response function f (dose) is monotonically increasing in toxicity score with increasing dose. In some cases, it follows a quadratic function in efficacy in which f (dose) increase with the dose until a maximum level and then decreases. Most of the known design used in dose-finding study assumes a

monotonically increase in toxicity score and less usable for estimation of OEM in which f (dose) follows a quadratic function. By inclusion of this new procedure and skewed starting dose, RSP-design may be used in both situations. Three design levels are recommended used in a between-patient RSP design with 3+5+7 patients.[19] One weakness with this new RSP procedure is that in some situation a fourth design level may be needed, resulting in a sample increase of 9 subjects. However, the previous RSP studies have used the mid-value approach with fixed k-factor to determine dose in the next design level.[13,19,21,29,30] The present study demonstrates that the RSP method can be used with a skewed starting point whilst still taking into account the possibility of results outside the set dose window.

The induced mRNA expression of IL-1β is interesting in light of the potential use of BPC2 as an immunomodulating compound in fish vaccine formulations. IL-1β is a pro-inflammatory cytokine, also in salmonid fish and compounds that induce expression of pro-inflammatory cytokines are potential candidates as vaccine adjuvants or immunomodulators, also shown for compounds like bacterial flagellins.[31] The potential effect of BPC2 as an immune modulator would have to be tested *in vivo* in combination with bacterial or viral antigens.

CONCLUSION

The present study demonstrates that the RSP method can be used with a skewed starting point whilst still taking into account the possibility of results outside the set dose window. Inclusion of skewed starting value related to the initial dose-window made the k-adjustment factor and the dose-window stochastic. The RSP-procedure works in accordance with the expectation and estimated OED of BPC2 sufficiently.

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