Effect of Prostaglandin F$_{2\alpha}$ on Growth of $Staphylococcus$ aureus Associated with Bovine Mastitis

Chloe A. Autran$^1$, Joe C. Dalton$^1$, Mark A. McGuire$^{1,2}$, Kevin G. Carnahan$^1$ and Amin Ahmadzadeh$^*$

$^1$Department of Animal and Veterinary Science, College of Agricultural and Life Sciences, University of Idaho, Moscow, Idaho, USA
$^2$Statistical Programs, College of Agricultural and Life Sciences, University of Idaho, Moscow, Idaho, USA

Abstract

$Staphylococcus$ aureus is a major infectious organism causing mastitis. Certain fatty acids have been shown to inhibit the growth of pathogens including $Staphylococcus$ aureus. An in vitro experiment was conducted to determine the effects of prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) on growth of $S$. aureus. Flasks containing tryptic soy broth (TSB) were inoculated with $S$. aureus Novel, and subsequently treated with PGF$_{2\alpha}$ (dinoprost tromethamine) at concentrations of 0, 0.3, 0.6, 1.2, and 2.4 mg/ml. Cultures were incubated at 37°C for 24 h and sampled every 3 h. The entire experiment was conducted three times in duplicates. Bacterial growth was assessed by counting colony forming units (CFU) in triplicates. Data were analyzed by repeated measures analysis of variance and reduced and full dummy variable regression models to determine the effect of PGF$_{2\alpha}$ concentrations on growth patterns of $S$. aureus over time. There was an effect of treatment and treatment by time interaction (P<0.05) on mean log CFU, indicating that bacterial growth over 24 h was different across treatments. At time of inoculation (0 h), mean log CFU values were not different among treatments; however at 24 h, mean log CFU for each PGF$_{2\alpha}$ treatment was different from control and decreased (P<0.05) with increasing concentrations of PGF$_{2\alpha}$. The predicted growth curve for each treatment was different (P<0.05) when compared with the control, and the rate of bacterial growth was less for 1.2 mg/ml PGF$_{2\alpha}$ when compared with the control and 0.6 mg/ml. The bacterial growth was completely inhibited during the 24 h period in 2.4 mg/ml PGF$_{2\alpha}$ treatment. Furthermore, a non-linear regression model was employed to estimate the dose response at the 24 h, and revealed that 1.2 mg/ml was the minimum inhibitory dose. These results provide evidence, for the first time, that PGF$_{2\alpha}$ in the form of dinoprost tromethamine, has bacteriostatic and bactericidal effects on $S$. aureus in vitro.

Keywords: Fatty acid, Mastitis, Prostaglandin $F_{2\alpha}$, $Staphylococcus$ aureus

Introduction

In dairy cattle, $Staphylococcus$ aureus ($S$. aureus) causes uterine [1] as well as intramammary infections [2]. $Staphylococcus$ aureus remains one of the most important causes of clinical mastitis, and the most frequently isolated pathogen in subclinical mastitis cases worldwide and was found to be the most contagious pathogen present on 43% of dairy operations in 17 of the top producing states in the US [3,4]. Contagious mastitis pathogens cause large economic losses to the dairy industry [5], mainly because of reduced milk production, increased involuntary culling rate, and discarded milk [6,7]. Treatments and immunizations against $S$. aureus mastitis have been studied for years [8]; however, despite the best possible antimicrobial treatments, bacteriological cure failures are common in $S$. aureus mastitis, and antimicrobial resistance is considered as one of the reasons for low cure rates [3,9,10,11]. Resistance to various antimicrobials is commonly seen in bovine $S$. aureus mastitis isolates and the intracellular survival of $S$. aureus is a significant factor contributing to the difficulty in clearing $S$. aureus infections following antibiotic therapy [12,13]. Over the years, there have been improvements in reducing contagious mastitis, nevertheless, treatment of $S$. aureus is quite challenging and a complex interaction between host and pathogen renders the complete eradication of $S$. aureus especially difficult. Finding an alternative treatment may potentially aid in eradicating mastitis infection caused by $S$. aureus pathogen. Extensive research has shown that various fatty acids have antimicrobial effects and may be used as an inhibitory agent against bacteria [14-16]. Hogan et al. [15] showed that long chain fatty acids (C12, C14, Polyene C18:2 and C18:3) have bacteriostatic and bactericidal effects on $S$. aureus and $Strep$. agalactiae Our laboratory [17] demonstrated that linoleic acid inhibited growth of two different mastitis strains of $S$. aureus. Interestingly, fatty acids can also be metabolized into various products which can also inhibit bacterial growth. For example, arachidonic acid, a fatty acid derived from linoleic acid [18] inhibited the growth of gram-positive bacteria such as $Streptococcus$ faecalis, $Staphylococcus$ epidermidis, $S$. aureus [19] and gram-negative H. pylori [20]. Moreover, arachidonic acid is a precursor to prostanooid synthesis when released from membrane phospholipids in response to various stimuli. One prostanooid synthesized from arachidonic acid is prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$), which is readily available for use in estrous synchronization in dairy cattle. Considering that both linoleic and arachidonic acid have been shown to inhibit bacterial growth, we hypothesized that PGF$_{2\alpha}$, synthesized from these fatty acids, may have similar antibacterial properties. To our knowledge there is no information on the effect of any prostaglandin on bacterial growth. The objective of this study was to determine the effects of PGF$_{2\alpha}$ using the commercially available form of dinoprost tromethamine on growth of $S$. aureus in vitro.

*Corresponding author: Amin Ahmadzadeh, University of Idaho, ID 83844-2330, Moscow, Idaho, USA, Tel: 208 885-7409; Fax: 208-885-6420; E-mail: amin@uidaho.edu

Received November 20, 2012; Accepted January 02, 2013; Published January 04, 2013


Copyright: © 2013 Autran CA, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Materials and Methods

Experimental Design and Treatment

The bacterial strain used was a clinical bovine mastitis isolate, *S. aureus* Novel [21]. Cultures were prepared by inoculating a single colony into 3 ml of tryptic soy broth (TSB) (EMD Chemicals Inc., Darmstadt, NJ) followed by overnight incubation at 37°C with shaking at 250 rpm. In order to obtain sufficient culture for the experiment, culture tubes containing 10 ml of fresh TSB were inoculated at 1:100 with 3 ml of overnight *S. aureus* culture, and once more inoculated overnight at 37°C with shaking at 250 rpm. Prostaglandin *F*<sub>2α</sub> in the form of dinoprost tromethamine (Lutalyse, Pfizer Animal Health, New York, NY) was added to flasks for a final concentration of 0.3, 0.6, 1.2, and 2.4 mg/ml (2 flasks/treatment). Lutalyse contains 0.9% to 1% benzyl alcohol as a preservative. Consequently, benzyl alcohol (Fischer Scientific, Pittsburgh, PA) was added to the flasks containing 0.3, 0.6, and 1.2 mg/ml of PGF<sub>2α</sub> mimicking the total concentration of benzyl alcohol in flasks containing the highest concentration of PGF<sub>2α</sub> (2.4 mg/ml). This resulted in each flask containing the same final concentration of benzyl alcohol (1%). Sterilized TSB was added to each flask containing the different concentrations of PGF<sub>2α</sub> to achieve a final concentration of 3% TSB in a final volume of 50 ml. Two different controls (2 flasks/each) were included in the experiment; the first contained bacteria and TSB alone and the second control contained bacteria, TSB and 1% benzyl alcohol. Flasks, which included both treatment and controls, were inoculated with the 10 ml overnight culture of *S. aureus* at a concentration of 1:100. Flasks were incubated at 37°C, shaken at 250 rpm for 24 h, and at 0 h, and every 3 h thereafter, 1 ml samples were taken from each flask to assess bacterial growth. The entire experiment was repeated three times, in duplicate, and each experiment started with a new subculture of the bacterium.

Bacteria Counts

To determine colony forming units (CFU), samples of 0.5 ml were taken from flasks for plating and centrifuged twice via a micro centrifuge (International Equipment Company, Needham, MA) to remove benzyl alcohol. The supernatant was removed and the cell pellet was re-suspended with 0.5 ml of fresh TSB. Serial dilutions were performed before samples were placed on agar plates (EMD Chemicals Inc., Darmstadt, NJ). Plate counts were done in triplicate per sample from each flask. Plates were incubated at least 12 h, or until colonies were apparent, at a constant temperature of 37°C [17].

Statistical Analyses

The number of live cells, as measured by log CFU, was determined by averaging the number of cells for the duplicate concentrations of both plates at each 3 h time point. Results for the three growth curves were also pooled by concentration, at each time point. A non-linear regression model was used (PROC NLMaxed procedures of SAS [22]) to determine a dose-response and minimum inhibitory concentration for log CFU at 24 h of bacterial growth. The estimation of the model was carried out using a non-linear iterative algorithm (Gauss Newton), where the fitted model took the form of Y = αe<sup>-βx + c</sup>.

where Y was the number of live cells (CFU/ml), x was the concentration of PGF<sub>2α</sub>, and α represented the intercept, β was the exponential rate of decay for CFU/ml, and c was the lower asymptote. A weighted least squares estimate was used to stabilize residual variance. The adequacy of fit was determined by the significance of the parameter estimates, the structure of correlation among estimated parameters, their corresponding magnitudes and signs, and the examination of the model residuals.

Results

Bacterial growth curves were evaluated in growth media containing PGF<sub>2α</sub> at concentrations of 0, 0.3, 0.6, 1.2, and 2.4 mg/ml with 0 mg/ml referring to the control with and without benzyl alcohol. Based on bacterial growth curves, PGF<sub>2α</sub> in the form of dinoprost tromethamine, has inhibitory effects on growth of *S. aureus*. Overall, growth of *S. aureus* decreased (P<0.05) with increasing concentrations of PGF<sub>2α</sub>, with 2.4 mg/ml of PGF<sub>2α</sub> being the most inhibitory. There was an effect of treatment and treatment by time interaction on log CFU/ml (P<0.05), providing evidence that bacterial growth of *S. aureus* over time was not similar among PGF<sub>2α</sub> treatments. Pre-planned contrasts were conducted to compare the mean log CFU/ml values between treatments at 0 and 24 h. At 0 h the mean log CFU/ml values were not different among treatments and control, and averaged 8.12 ± 0.02 log CFU/ml (Figure 1). At 24 h, log CFU/ml values for both controls (TSB and TSB with 1% benzyl alcohol) were not different, and therefore the data were pooled. Log CFU/ml values at 24 h for each PGF<sub>2α</sub> treatment took the form of

\[
y = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon_i
\]

where \(Y\) was the logarithmic value of the number of live cells (log CFU/ml), \(x\) represented time, \(\beta_0\) was the intercept (estimated log CFU/ml at time 0), \(\beta_1\) was the rate of increase for bacterial growth, \(\beta_2\) was the point of inflection (the maximum growth at a specific time), and \(\varepsilon_i\) represented the random error under the classical regression assumptions. The adequacy of fit was determined by the significance of the parameter estimates (declared at P<0.05), their corresponding magnitudes and signs, and the examination of the estimated residuals.

As an ancillary objective, a non-linear regression model was used to determine the dose-response and minimum inhibitory concentration for log CFU at 24 h of bacterial growth. The estimation of the model was carried out using a non-linear iterative algorithm (Gauss Newton), where the fitted model took the form of Y = αe<sup>-βx + c</sup>.

where Y was the number of live cells (CFU/ml), x was the concentration of PGF<sub>2α</sub>, and α represented the intercept, β was the exponential rate of decay for CFU/ml, and c was the lower asymptote. A weighted least squares estimate was used to stabilize residual variance. The adequacy of fit was determined by the significance of the parameter estimates, the structure of correlation among estimated parameters, their corresponding magnitudes and signs, and the examination of the model residuals.

![Figure 1: Growth of S. aureus as measured by log colony forming units (CFU)/mL, treated with PGF<sub>2α</sub> at 0 h (gray bars) and 24 h (black bars) for each concentration of PGF<sub>2α</sub> x a, b, c, d Bars designated by different letters are different (P<0.05) at 24 h.](image-url)
were different (P<0.05) from the pooled control (10.5 ± 0.04 log CFU/ml). At 24 h, mean log CFU/ml for 1.2 mg/ml (8.1 ± 0.04 log CFU/ml) and 2.4 mg/ml (1.8 ± 0.04 log CFU/ml) were different (P<0.05) when compared with each other and also with 0.3 (9.4 ± 0.04 log CFU/ml) and 0.6 (9.0 ± 0.04 log CFU/ml) mg/ml treatments (Figure 1). Interestingly S. aureus log CFU decreased for the 1.2 mg/ml PGF<sub>2α</sub> treatment and the greatest dose (2.4 mg/ml) completely inhibited the bacteria growth as log CFU/ml decreased from 8.12 ± 0.02 to 1.8 ± 0.04. The non-linear regression models were carried out to evaluate the effects of different PGF<sub>2α</sub> concentrations on the growth pattern of the bacteria over time. The parameter estimates of the reduced model for treatments 0, 0.3, 0.6, and 1.2 mg/ml of PGF<sub>2α</sub> were significant (P<0.05), indicating that the reduced model fit the data well for each of those treatments, and that all parameters are required (Table 1, Figure 2). However, the parameter estimates for 2.4 mg/ml PGF<sub>2α</sub> treatment were non-significant (P>0.05), indicating that the reduced model did not fit the data for 2.4 mg/ml PGF<sub>2α</sub> treatment (Table 1). The lack of fit at this specific concentration relates to the continuous decrease in bacterial growth associated with the bactericidal effects of 2.4 mg/ml PGF<sub>2α</sub> treatment. The declining growth pattern of S. aureus treated with 2.4 mg/ml PGF<sub>2α</sub> treatment differed from the rest of the treatments. Moreover, the rate of increase and decrease of log CFU, as measured by estimated log colony forming units (CFU), treated with PGF<sub>2α</sub> at concentrations of 0 (dimonds), 0.3 mg/ml (squares), 0.6 mg/ml (triangles), 1.2 mg/ml (circles), and 2.4 mg/ml (stars).

Figure 2: Full regression model* fits to growth of S. aureus, as measured by estimated log colony forming units (CFU), treated with PGF<sub>2α</sub> at concentrations of 0 (dimonds), 0.3 mg/ml (squares), 0.6 mg/ml (triangles), 1.2 mg/ml (circles), and 2.4 mg/ml (stars).

*Model: Y=β<sub>0</sub> + β<sub>1</sub>x + β<sub>2</sub>x<sup>2</sup> + ε, where β<sub>0</sub> represents y-intercept at time of inoculation; β<sub>1</sub> represents the rate at which bacterial growth increases; β<sub>2</sub> represents point of infection where growth reached its maximum at a specific time; ε represents random error.

Overall line of bacterial growth for each treatment differs (P<0.05) from control. The treatment 2.4 mg/ml was not included in contrasts. The rate of bacterial growth over time (β<sub>1</sub>) was less (P<0.05) for 1.2 mg/ml PGF<sub>2α</sub> treatment when compared with 0 and 0.6 mg/ml treatments.

### Table 1: Dose (mg/mL) Parameter Parameter Estimate Standard error P value<sup>a</sup>

| Dose (mg/mL) | Parameter | Parameter Estimate | Standard error | P value<sup>a</sup>
|--------------|-----------|--------------------|----------------|----------------
| 0            | β<sub>0</sub> | 8.543              | 0.126          | <0.0001        |
| 0            | β<sub>1</sub> | 0.249              | 0.024          | <0.0001        |
| 0            | β<sub>2</sub> | -0.007             | 0.001          | <0.0001        |
|              | Time to max<sup>x</sup> | 16.6              |                |                |
|              | Value at max<sup>x</sup> | 10.9              |                |                |
| 0.3          | β<sub>0</sub> | 8.358              | 0.130          | <0.0001        |
| 0.3          | β<sub>1</sub> | 0.242              | 0.025          | <0.0001        |
| 0.3          | β<sub>2</sub> | -0.008             | 0.001          | <0.0001        |
|              | Time to max<sup>x</sup> | 14.07             |                |                |
|              | Value at max<sup>x</sup> | 10.06             |                |                |
| 0.6          | β<sub>0</sub> | 8.285              | 0.139          | <0.0001        |
| 0.6          | β<sub>1</sub> | 0.211              | 0.027          | <0.0001        |
| 0.6          | β<sub>2</sub> | -0.007             | 0.001          | <0.0001        |
|              | Time to max<sup>x</sup> | 13.31             |                |                |
|              | Value at max<sup>x</sup> | 9.69              |                |                |
| 1.2          | β<sub>0</sub> | 8.280              | 0.181          | <0.0001        |
| 1.2          | β<sub>1</sub> | 0.091              | 0.036          | 0.0136         |
| 1.2          | β<sub>2</sub> | -0.004             | 0.001          | 0.0049         |
|              | Time to max<sup>x</sup> | 10.68             |                |                |
|              | Value at max<sup>x</sup> | 8.76              |                |                |
| 2.4          | β<sub>0</sub> | 8.690              | 0.549          | <0.0001        |
| 2.4          | β<sub>1</sub> | -0.188             | 0.107          | 0.1053         |
| 2.4          | β<sub>2</sub> | -0.005             | 0.004          | 0.2034         |
|              | Time to max<sup>x</sup> | -16.9             |                |                |
|              | Value at max<sup>x</sup> | 10.28             |                |                |

<sup>a</sup> Significance of parameter estimates represents appropriate fit of model (Y = β<sub>0</sub> + β<sub>1</sub>x + β<sub>2</sub>x<sup>2</sup> + ε) to data. β<sub>0</sub> represents y-intercept at time of inoculation; β<sub>1</sub> represents the rate of bacterial growth over time; β<sub>2</sub> represents point of infection where growth reached its maximum at a specific time; ε represents random error; ^Time to max = time (h) when maximal growth was achieved; | Value at max = amount of growth (estimated log CFU) at the time when maximal growth was achieved; ^For 2.4 mg/ml dose, the time to maximum growth and its value for this treatment should be interpreted with caution due to the lack of fit indicating that the reduced model did not fit the data for 2.4 mg/ml PGF<sub>2α</sub> treatment. The lack of fit at this specific concentration relates to the continuous decrease in bacterial growth associated with the bactericidal effects of 2.4 mg/ml PGF<sub>2α</sub> treatment.

Table 1: Parameter estimates, standard errors, and the associated P-values for the reduced regression models of S. aureus treated with various concentrations of PGF<sub>2α</sub>.

---

Our results provide evidence, for the first time, that PGF$_{2\alpha}$ in the form of dinoprostone tromethamine has inhibitory effects on growth of S. aureus. The antibacterial effects of fatty acids on pathogens have been studied for years and reviewed [16]. Interestingly, our preliminary results showed that PGF$_{2\alpha}$ has inhibitory effects on growth of Strep. uberis in vitro [23]. Growth of S. aureus and Strep. agalactiae was inhibited by long-chain fatty acids in vitro [24]. Similarly, Hezcko et al. [25] showed that both S. aureus and Strep. agalactiae were susceptible to four different fatty acids although S. aureus strains appeared to be generally less susceptible to all four fatty acids than streptococcal strains. Kelsey et al. [17] found that a variety of fatty acids and monoacylglycerols had inhibitory activity on the growth of two S. aureus strains, including the strain used for this study. The efficacy of fatty acids in specifically inhibiting growth of gram-positive bacteria has also been demonstrated [16,26], and coincides with the results of our present study. Arachidonic acid, a fatty acid originally derived from linoleic acid [18] has been shown to inhibit gram-positive bacteria such as Streplococcus faecalis and Staphylococcus epidermidis, and S. aureus [19]. Because both linoleic acid and arachidonic acid are precursors to PGF$_{2\alpha}$ it is plausible that PGF$_{2\alpha}$, produced from these fatty acids, have similar antibacterial properties. The results supported our hypothesis that commercially available PGF$_{2\alpha}$ (dinoprostone tromethamine) inhibited the growth of S. aureus Novel in vitro in a dose dependent manner (Figure 3), resembling the actions of linoleic acid on growth of S. aureus Novel as previously described [17]. Moreover, it has been shown [19] that the inhibitory effect of arachidonic acid on growth of S. aureus was dependent upon time and the concentration of arachidonic acid. In the current study, 1.2 mg/ml of PGF$_{2\alpha}$ appeared to have bacteriostatic effects, while 2.4 mg/ml had a bactericidal effect on S. aureus (Figure 3). Prostaglandins are involved in regulating numerous processes in the body, including reproductive function [27] and modulation of immune function [28,29]. To our knowledge, no research has investigated the role of PGF$_{2\alpha}$ as a regulator of bacterial growth. However, various studies have determined several immune functions related to PGF$_{2\alpha}$. Seals et al. [30] found that PGF$_{2\alpha}$ production was greater in dairy cows that did not develop uterine infections, compared with infected cows. Research with ewes [31] determined that PGF$_{2\alpha}$ aided the recovery of the uterus from infections independent of its effects on luteal progesterone production [31]. Thus, PGF$_{2\alpha}$ may act in multiple physiological processes to reduce the potential of bacterial infection. The mechanism by which PGF$_{2\alpha}$ affected the growth of S. aureus cannot be determined from the current study. Kabara et al. [16] reported that the presence and position of a double or triple bond was an important factor in inhibition of bacteria by long chain fatty acids (> C14), but had little or no effect in C11 fatty acids. Moreover, the inhibitory properties of fatty acids were also noted to be more distinct as the degree of unsaturation and chain length both increased [32,33]. Prostaglandin F$_{2\alpha}$ used in this study, contains two double bonds and consists of 24 carbons. These features may be important factors in its antibacterial properties. One potential mechanism of action centers on the ability of fatty acids to penetrate the plasma membrane of bacteria. In this scenario, it is hypothesized that the bacterial cell membrane may be disrupted as the hydrocarbon chains of fatty acids are inserted into the phospholipid bilayer, ultimately increasing the negative charge of the bacterial membrane surface [34]. Another proposed mechanism involves the hindering of bacterial growth via an interaction of the lipid at the cell membrane, resulting in a change in membrane permeability [33], or the disruption of transduction cascades leading to cell lysis [35]. It was also demonstrated [36] that the exposure to fatty acids can allow

**Parameter**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Asymptotic S.E.</th>
<th>95% Asymptotic Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>389.6</td>
<td>83.72</td>
<td>219.3 - 559.9</td>
</tr>
<tr>
<td>$\beta$</td>
<td>5.13</td>
<td>0.82</td>
<td>3.47 - 6.79</td>
</tr>
<tr>
<td>$c$</td>
<td>0.99</td>
<td>0.40</td>
<td>0.178 - 1.81</td>
</tr>
</tbody>
</table>

$\gamma=a e^{-5.13x} + c$; where $a$ is the intercept, $\beta$ represents the exponential rate of decay of CFU, and $c$ is the lower asymptote.

Significance of the parameter estimates determines adequacy of fit of the model.

95% asymptotic confidence interval on model parameters do not encompass 0, indicating significance of parameter estimates at $P<0.05$.

Table 3: Estimated parameter, standard errors (S.E.), and associated asymptotic confidence intervals for the non-linear regression model of S. aureus colony forming units (CFU) at 24 h, treated with different concentrations of PGF$_{2\alpha}$.

![Graph](image-url)
the bacteria to stay viable while disabling cell division and resulting in a bacteriostatic effect, similar to that observed in our study when \( S. aureus \) was treated with 1.2 mg/ml of PGF \(_{2\alpha} \). Lastly, it has been proposed [19] that the bactericidal effect of arachidonic acid (PGF \(_{2\alpha} \) precursor) is mediated through peroxidation of fatty acid catalyzed by bacterial iron and \( \text{H}_2\text{O}_2 \). Although the mechanisms(s) to how PGF \(_{2\alpha} \) inhibited bacterial growth are currently unknown, we speculate that similar membrane interactions and multi-stage mechanisms may be employed by PGF \(_{2\alpha} \) (dinoprost tromethamine) in limiting or preventing growth of \( S. aureus \). The exact mechanistic actions induced by PGF \(_{2\alpha} \) along with its practical application in vivo, still require further investigations.

Acknowledgments

This study was made possible by the support of Pfizer Animal Health, New York, NY, in providing dinoprost tromethamine (Lutalyse®), Idaho Dairymen's Association, NIH P20 RR15587 and the Idaho Agricultural Experiment Station. The authors would also like to thank Dr. W. J. Price for his help in statistical computations.

References
