Quantification of ventricular $\beta_2$-adrenoceptor density and ligand binding affinity in wild sockeye salmon

*Oncorhynchus nerka* smolts using a novel modification to the tritiated ligand technique

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A new, image-based, tritiated ligand technique for measuring cardiac $\beta_2$-adrenoceptor ($\beta_2$-AR) binding characteristics was developed and validated with adult rainbow trout *Oncorhynchus mykiss* hearts so that the tissue limitation of traditional receptor binding techniques could be overcome and measurements could be made in hearts nearly 14-times smaller than previously used. The myocardial cell-surface (functional) $\beta_2$-AR density of *O. nerka* smolts sampled at the headwaters of the Chilko River was 54.2 fmol mg protein$^{-1}$ and about half of that previously found in return migrating adults of the same population, but still more than twice that of adult hatchery *O. mykiss* (21.1 fmol mg protein$^{-1}$). This technique now opens the possibility of investigating cardiac receptor density in a much wider range of fish species and life stages.

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Sockeye salmon *Oncorhynchus nerka* (Walbaum 1792) show remarkable fidelity for their natal stream and their once-in-a-lifetime spawning (semelparity) results in genetically and geographically distinct populations of *O. nerka* within the Fraser River catchment (Beacham *et al.*, 2005). This genetic isolation would presumably allow strong selective pressures for adaptations that facilitate successful migration. Indeed, the hypothesis of intraspecific tailoring of the cardiorespiratory system to the upriver migration conditions was advanced for Fraser River *O. nerka* when it was found that aerobic and cardiac scope are positively associated with several migration difficulty indices (Eliason *et al.*, 2011, 2013). Among the Fraser River *O. nerka* populations, the Chilko population stand out with a ventricular cell surface $\beta_2$-adrenoceptor ($\beta_2$-AR) density ($B_{max}$) at least twice that of other *O. nerka* populations and even more when compared with another fish species from the same genus, rainbow trout *Oncorhynchus*...
mykiss (Walbaum 1792) (Gamperl et al., 1994, 1998; Olsson et al., 2000; Hanson et al., 2005; Eliason et al., 2011). Stimulation of the salmonid heart via $\beta_2$-AR can enhance maximum cardiac performance both during exercise and at high temperature (Farrell et al., 1986; Randall & Perry, 1992; Nielsen & Gesser, 2001), as well as protect cardiac performance under conditions of low oxygen, low pH and high temperature (Hanson et al., 2006; Hanson & Farrell, 2007). Furthermore, adult Chilko O. nerka are the only fish known to increase ventricular $B_{\text{max}}$ in response to increased acclimation temperature, nearly doubling their receptor density with a 8°C change (Eliason et al., 2011). In contrast, O. mykiss decrease ventricular $B_{\text{max}}$ in response to warm acclimation (Keen et al., 1993), whereas adult Nechako O. nerka (Eliason et al., 2011) and the African catfish Clarias gariepinus (Burchell 1822) (Hanson et al., 2005) do not alter their $B_{\text{max}}$ with temperature acclimation.

Thus far, ventricular $B_{\text{max}}$ has only been measured in adults and so it remains unknown if this extraordinary $B_{\text{max}}$ of Chilko O. nerka has a genotypic basis as well as phenotypic plasticity with temperature acclimation, in which case earlier developmental stages would have a similar high myocardial $\beta_2$-AR density as in the adults. As such, the objective of the current study was to quantify the myocardial $B_{\text{max}}$ of 1+ year-old Chilko O. nerka smolts. To work with the relatively small heart of a smolt (c. 15 mg here) when compared to adults (3.2 g; Eliason et al., 2011), however, the traditional ligand binding techniques used to measure ventricular $B_{\text{max}}$ in adult fish hearts had to be modified.

This study was conducted in accordance with guidelines of the Canadian Council of Animal Care, as administered by the University of British Columbia (Animal Care # A10-0002). All values are presented as mean±s.e. Wild, 1+ year-old Chilko O. nerka smolts ($n=161$; body mass ($M_B$) = 7.84±0.28 g; condition factor ($F_C$) = 0.740±0.003; relative ventricular mass ($M_{RV}$) = 0.194±0.003%) were captured on 8 May 2013 by dip-net at a government-run counting fence used to enumerate O. nerka passage as they leave Chilko Lake and enter the river. The counting fence was in place c. 1 km downstream from the Chilko Lake outflow between 22 April and 11 May 2013 when water temperatures ranged from 3 to 8°C. Before beginning their migration, smolts rear in Chilko Lake, a deep (339 m) glacial-fed lake with summer temperatures ranging from 4 to 9°C depending on depth (Desloges & Gilbert, 1998). Prior to sampling, smolts were held on-site for 4 days in a 5001 tank supplied with water (8.1±0.1°C) continuously pumped from the river. Water temperature in the holding tank was recorded each morning. Oncorhynchus mykiss were used to validate the assay techniques because previous studies have extensively studied ventricular $B_{\text{max}}$ in this species. Adult female O. mykiss ($n=9$; $M_B$ = 1.13±0.02 kg; $F_C$ = 1.51±0.03; $M_{RV}$ = 0.086±0.001) were purchased from Miracle Springs Inc. (http://miraclespringsinc.com/) and sampled on site as a reference group. The O. mykiss had been maintained in outdoor raceways (14°C) and fed to cessation twice daily with commercial trout pellets (EWOS Canada Ltd; www.ewos.com). All fishes were quickly euthanized by a blow to the head to measure fork length ($L_F$) and $M_B$, and excise the ventricle, which was rinsed in TES [N-[tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid] buffered saline (composition in mM: NaCl, 124.1; KCl, 2.5; MgSO$_4$−7H$_2$O, 0.9; CaCl$_2$−2H$_2$O, 2.5; d-glucose, 5.6; TES free acid, 3.9; TES Na salt, 6.1; pH 7.85 at 10°C) before freeze-clamping (O. mykiss) or freezing on an aluminum plate (O. nerka) using liquid nitrogen. The tissues were stored at −80°C until analysis.
The traditional tritiated ligand technique (tTLT) of Watson-Wright et al. (1989), as modified for fish hearts by Gamperl et al. (1994), is a well-established technique to measure ventricular cell-surface $\beta_2$-AR density ($B_{max}$) and binding affinity ($K_d$) in adult teleosts (Gamperl et al., 1994, 1998; Olsson et al., 2000; Hanson et al., 2005; Mendonca & Gamperl, 2009; Eliason et al., 2011). The ventricle of an O. nerka smolt, however, was at least 14-times smaller (14-63 ± 0.47 mg) than any heart previously tested (Olsson et al., 2000) and this prevented using tissue punches, the standardizing unit of the tTLT. While alternative methods to measure cardiac $B_{max}$ and $K_d$ with less tissue exist, e.g. the isolated sarcolemmal fraction technique used by Keen et al. (1993), questions remain about the usefulness of these techniques as they may not be an accurate measure of cell-surface (i.e. functional) receptors (Gamperl et al., 1994). The ventricle size limitation was overcome by developing a new, image-based, tritiated ligand technique (iTLT) in which tissue punches were replaced with 350 $\mu$m thick cross-sectional slices of the ventricle. Each smolt ventricle was sliced in its entirety from the apex to base with a McIlwain tissue chopper (www.fishersci.com/us/en/brands/I9C8LSKU/brinkmann-instruments-inc.html and www.tedpella.com). Calibrated pictures were taken of each cross-section using a Canon Rebel T2i (Canon Canada Inc.; www.canon.ca) adapted to fit a Nikon PB-5 bellows and Micro-Nikkor 55 mm f/2.8 macro lens (Nikon Canada Inc.; www.nikon.ca) before they were incubated with a ligand in wells of a tissue culture plate. All subsequent steps of the assay technique followed that of the tTLT and are described in detail elsewhere (Gamperl et al., 1994). Three to five slices from the centre of each ventricle were selected to maximize the tissue mass per heart (each heart yielded only five to seven slices) and 30–33 individual hearts were pooled for each assay (i.e. $n=5$ assay replicates using a total of 161 smolts). Pooling of ventricular tissue has been used previously to meet the tissue requirements of the tTLT [e.g. Olsson et al. (2000) using c. 150 g Trematomus bernacchii Boulenger 1902 and Hanson et al. (2005) using 300–700 g C. gariepinus]. The surface area (mm$^2$) of individual ventricular cross-sections was determined using image analysis software (ImageJ; http://imagej.nih.gov/ij/), and multiplied by 0.35 mm (slice thickness) to determine tissue volume. The radioactivity of each ventricular cross-section, incubated with 500 $\mu$l of several concentrations (0.05–3.5 nM) of the tritiated $\beta$-AR ligand [$^3$H] CGP 12177 (specific activity 37.7 Ci mol$^{-1}$; PerkinElmer Inc.; www.perkinelmer.com) in saline and counted with a liquid scintillation counter (LS 6500, Beckman Instruments; www.beckmancoulter.com), was then expressed per unit volume [disintegrations per minute (DPM) mm$^{-3}$] and used to generate a ligand-binding curve. The iTLT was validated against the tTLT in adult O. mykiss. To apply the iTLT to adult O. mykiss, ventricles were sectioned 350 $\mu$m thick as in the tTLT then, instead of having tissue punches taken, slices were cut into pieces similar in size to the O. nerka smolt ventricular cross-sections. These ventricular pieces were then treated in the same manner as the cross-sections obtained from the smolt hearts. Ventricular punches and pieces for the O. mykiss groups were taken from both the compact and spongy myocardium, whereas the compact myocardium was not well defined in the ventricular cross-sections of the O. nerka smolts.

Binding parameters were determined using a Scatchard plot as described by Zivin & Waud (1982). The $r^2$ values for CGP-binding curves ranged from 0.93 to 0.97. Protein content of representative punches for the tTLT (mg protein punch$^{-1}$) and representative ventricular cross-sections for the iTLT (protein mm$^{-3}$ of tissue)
were determined using the Better Bradford Protein Assay Kit (Bio Basic Inc.; store.biobasic.com) so that $B_{\text{max}}$ could be expressed in fmol mg protein$^{-1}$. $B_{\text{max}}$ and $K_d$ values were compared between the three test groups (iTLT and tTLT for $O$. mykiss, and iTLT for Chilko $O$. nerka smolts) using a one-way ANOVA (GraphPad Prism 6; www.graphpad.com/scientific-software/prism), followed by a Tukey’s post hoc test for multiple comparisons. The limit for statistical significance was set as $P < 0.05$.

Ventricular cell-surface $B_{\text{max}}$ for $O$. mykiss at 14$^\circ$ C [tTLT = 18.5 fmol mg protein$^{-1}$ and iTLT = 21.1 fmol mg protein$^{-1}$; Fig. 1(a)] was not significantly different. Additionally, $B_{\text{max}}$ for $O$. mykiss closely matched previous reports for $O$. mykiss acclimated to 14$^\circ$ C. Hanson et al. (2005) reported a $B_{\text{max}}$ of 26.4 fmol mg protein$^{-1}$ [included in Fig. 1(a) for comparison], and Gamperl et al. (1998) reported a $B_{\text{max}}$ of 24.0 fmol mg protein$^{-1}$. Thus, the new and the traditional methodologies used here to quantify myocardial $\beta_2$-AR density appear to be comparable.

Fig. 1. (a) Ventricular $\beta_2$-adrenoreceptor density ($B_{\text{max}}$) and (b) $[^3]$H] CGP-12177 dissociation constant ($K_d$) of 1+ year-old Chilko $O$. oncorynchus nerka smolts at 8$^\circ$ C measured using a novel image-based ligand binding assay (■). Measurements were also performed on adult $O$. mykiss acclimated to 14$^\circ$ C with both the image-based (■) and traditional tissue punch-based techniques (■) to validate the methodology. Values of adult $O$. mykiss (14$^\circ$ C; Hanson et al., 2005) and return migrating Chilko $O$. nerka (■; 13$^\circ$ C; Eliason et al., 2011) are included for visual comparison only, and are excluded from the statistical analysis. Values are means ± s.e. Dissimilar letters denote statistically significant differences at $P < 0.001$. © 2016 The Fisheries Society of the British Isles, Journal of Fish Biology 2016, 88, 2081–2087
ventricular $\beta_2$-AR density of Oncorhynchus nerka smolts

$B_{max}$ for the Chilko $O.\ nerka$ smolts (54.2 fmol mg protein$^{-1}$) was significantly higher than $O.\ mykiss$ [Fig. 1(a)]. $B_{max}$ for 8°C acclimated Chilko smolts was $\sim 60\%$ of the $B_{max}$ for adults acclimated to 13°C [78.5 fmol mg protein$^{-1}$; included in Fig. 1(a) for comparison] and just $\sim 30\%$ for adults acclimated to 19 and 21°C (123.3 and 128.2 fmol mg protein$^{-1}$; Eliason et al., 2011). $B_{max}$ for Chilko $O.\ nerka$ smolts was similar to $B_{max}$ for adult Nechako $O.\ nerka$ acclimated to 13–21°C (46.8–66.2 fmol mg protein$^{-1}$; Eliason et al., 2011) and for adult Stamp River $O.\ nerka$ sampled either at the riverside at 21°C (45.2 fmol mg protein$^{-1}$) or after being acclimated in captivity for 3 weeks to 20°C (47.5 fmol mg protein$^{-1}$; Olsson et al., 2000). Like the Chilko population, the Nechako $O.\ nerka$ are another long migrating Fraser River population, but the Stamp River $O.\ nerka$ have a much shorter river migration.

$K_d$ did not differ significantly between the techniques or the species [Fig. 1(b)]. This result is consistent with previous studies of $K_d$ for several Oncorhynchus spp., including $O.\ mykiss$ and $O.\ nerka$ (Gamperl et al., 1998; Olsson et al., 2000; Eliason et al., 2011). Numerically, the $K_d$ values (0.39–0.45 nM) are roughly twice those previously reported for other salmonids (0.13–0.27 nM; Olsson et al., 2000; Eliason et al., 2011) and closer to that of 0.36 nM for Stamp River $O.\ nerka$ sampled at riverside at 21°C, which then decreased to 0.20 nM after 3 weeks in captivity (Olsson et al., 2000). Therefore, differences in how fishes are handled and maintained before sampling may affect $K_d$. Also, small differences in saline composition (KCl was 3.1 mM v. 2.5 mM earlier) and pH (pH 7.85 at 10°C v. 7.85 at 15°C) could potentially have small effects on the dissociation constant of myocardial $\beta_2$-AR for the $\beta$-AR ligand CGP-12177. While it is known that $\beta$-AR ligand affinity for multiple ligands decreases at lower pH (Ijzerman et al., 1984; Modest & Butterworth, 1995; Ghanouni et al., 2000), the specific effects of such small variations in pH on CGP-12177 binding are unknown.

Beyond new information on the ventricular $\beta_2$-AR density for the smolt life stage of an interior population of $O.\ nerka$, these data support the hypothesis that a high ventricular $B_{max}$ is a trait of the Chilko population (Eliason et al., 2011). More work will be needed to resolve the phenotypic and genotypic contributions. This is because the present study used an acclimation temperature of 8°C for smolts, whereas $B_{max}$ in adults increased between acclimation temperatures of 13 and 19°C (Eliason et al., 2011). Thus, further work is needed with all life stages of Chilko population to test whether or not ventricular $B_{max}$ responds similarly to temperature as in the adults and, more importantly, how the heart responds to adrenergic stimulation. Future research could test the possibility that the high cell surface expression of myocardial $\beta_2$-ARs is phenotypically enhanced in Chilko adults specifically when they leave cool sea water for warmer water in the Fraser River during their summer spawning migration. Future work could also test if the high constitutive expression of myocardial $\beta_2$-AR in the Chilko $O.\ nerka$ population is related to juveniles rearing in a cold, alpine lake given that cold acclimation of $O.\ mykiss$ increases cell surface expression of myocardial $\beta_2$-ARs (Keen et al., 1993) to potentially offset the effect of cold temperature on calcium delivery for excitation contraction coupling (Shiels et al., 2003).

The success of the image-based modification to the tritiated ligand technique opens the door to investigations of ventricular cell surface $\beta_2$-AR densities in a much wider range of fishes and life stages. The technique is no longer limited by the previous need for a large ventricular size. Nevertheless, the iTLT has limitations. Notably, c. 30 fish were pooled for each assay replicate; therefore, large numbers of fish are needed. Also, the compact and spongy myocardium cannot be distinguished because entire
cross-sections of ventricle were used. Gamperl et al. (1998) showed $B_{max}$ was 14% higher in the spongy myocardium.

In conclusion, this study presents the first measurement of ventricular $\beta_2$-AR density and CGP binding affinity in a juvenile life stage of a fish, the 1+ year-old Chilko $O. \text{nerka}$ smolt. Smolt $B_{max}$ was elevated compared to adult $O. \text{mykiss}$, but did not approach values previously seen for adult Chilko $O. \text{nerka}$ acclimated to higher temperatures. It remains unclear exactly why Chilko smolts have a lower $B_{max}$ than their adult counterparts and further studies utilizing the image-based tritiated ligand technique can now be used to better elucidate the relationship between $B_{max}$ and temperature.

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